*International Food Research Journal 16: 261-276 (2009)*

# Antimicrobial effects of probiotics against selected pathogenic and spoilage bacteria in cheese-based dips

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**Abstract:** Spot-on lawn, spot and streak, co-culturing techniques and HPLC analysis were used for the detection and screening of inhibitory activity produced by probiotic bacteria against spoilage and pathogenic bacteria. After incubation, plates were examined for either zones of inhibition and for other effects of suppression around the wells/spots and streaks or cfu/g of target bacteria. The spore-formers were inhibited by the probiotic organisms to a greater extent than the non-spore formers. The inhibitory effect of all probiotic bacteria was weakest against *E. coli* and strongest against *B. cereus*. *S. aureus* was inhibited to a greater extend by *B. animalis* and by *L. rhamnosus*. The order of probiotic bacteria in terms of level of inhibition on agar plates (and zone of inhibition in mm) was *L. rhamnosus* (21)> *L. acidophilus* (19) = *L. casei* (19) > *L. paracasei subsp. paracasei* (18) > *B. animalis* (15) > *P. freudenreichii subsp. shermanii* (11). Though co-culturing of target organisms with probiotic bacteria in reconstituted skim milk showed inhibitory effect against target organisms; however, in French onion dip, there was no additive effect in controlling the bacterial population except that for *B. cereus*. Varying quantities of organic acids (acetic, lactic, formic, propionic, butyric, benzoic and phenyllactic) that were responsible for the inhibition were detected.

**Key words: Probiotics, antimicrobial property, spoilage organisms, cheese-based dips**

## Introduction

With increasing awareness of processing loss of nutritional value and the possible health risk of foods preserved with certain chemical preservatives, consumer interests in foods that are natural, fresh and healthy are increasing. Despite improved manufacturing conditions and implementation of effective legislative control on food processing procedures, such as hazard analysis and critical control points (HACCP) in the food industries, the number of food borne illness still remains a concern. The contamination and growth of psychrotrophic pathogenic and spoilage microorganisms in refrigerated foods is a major risk in food industry. These microorganisms include several groups of bacteria, yeasts and moulds. The use of naturally produced anti-microbial agents without any adverse effects on human health to inhibit the proliferation of pathogenic microorganisms in food is a more congenial option to overcome the problems associated with food contamination.

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Anti-microbial metabolites such as organic acids, short chain fatty acids, hydrogen peroxide, reuterin, diacetyl, bacteriocins and bacteriocin-like inhibitory substances are some of the metabolic products of these bacteria suggested to have potential antimicrobial effects (Holzapfel *et al.*, 1995; Ouwehand, 1998; Cleveland *et al.*, 2001; Shah and Dave, 2002). Dieleveux *et al.* (1998) attributed phenyllactic acid to the inhibition of various pathogenic bacteria such as *Listeria monocytogenes, Staphylococcus aureus, Escherichia coli* and *Aeromonas hydrophila*. Phenyllactic acid is reported to be one of the most abundant aromatic acids to which anti-microbial properties have been attributed and occur in several honeys.

There has been some interest in using cheese-based dips as a delivery vehicle for probiotic bacteria as they provide buffering effects due to ingredients used and prebiotics. However, little is known about the survival of probiotic bacteria in dips during their shelf life. Ingredients used to improve the texture, safety (pH) and organoleptic qualities of dips such as organic acids (acetic acid, lactic acid and citric acid) and, oil and gums may affect the survival of probiotic bacteria and other pathogens and spoilage bacteria in dips. The potential of improving the microenvironment of the dip in order to improve the shelf life of the product by inhibiting spoilage bacteria with the help of probiotic bacteria is worthy of investigation. *Echerichia coli, Salmonella typhimurium, Staphylococcus aureus, Bacillus cereus,* and spoilage organisms such as *Bacillus stearothermophilus,* and *Pseudomonas aerogenosa* are commons contaminants in cheese based dips.

The aim of this study was to determine anti-microbial properties of *Lactobacillus acidophilus*, *Bifidobacterium animalis*, *Lactobacillus paracasei* subsp. *paracasei, Lactobacillus rhamnosus* and *Propionibacterium freudenreichii* subsp. *shermanii* against selected pathogenic and spoilage organisms in cheese-based dips and to identify the anti-microbial substances produced by these bacteria against selected pathogenic and spoilage bacteria.

## Materials and Methods

### Microorganisms and their maintenance

*Probiotic bacterial cultures*

Cultures of *Lactobacillus acidophilus* (LAC1), *Bifidobacterium animalis* (BLC1), *Lactobacillus paracasei* subsp. *paracasei* (LCS1) and *Propionibacterium freudenreichii* subsp. *shermanii* (Pb10360) were obtained from DSM (DSM Food Specialties Australia Pty. Ltd., Werribee, Australia). *L. acidophilus* (LA5), *B. animalis* (Bb12), *L. paracasei* subsp. *paracasei* (LC01) and *P. freudenreichii* subsp. *shermanii* (PS1) were received from Chr. Hansen (Chr. Hansen Pty. Ltd., Bayswater, Australia). *L. acidophilus* (LA-74), *B. animalis* (BF-420) and *L. rhamnosus* (LC 705) were obtained from Danisco Cultor (Danisco Australia Pty. Ltd., Moorabbin, Australia). *L. rhamnosus* LBA was obtained from Rhodia (Rhodia Australia Pty. Ltd., Nottinghill, Australia) and *P. freudenreichii* subsp. *shermanii* P was obtained from Bronson and Jocobs Pty. Ltd. (Bronson and Jocobs Pty. Ltd., Dingley, Melbourne, Australia). *L. rhamnosus* strain LR1254 was received from Culture Collection of Victoria University (Werribee, Australia). Yakult drink and Valiaa yoghurt were purchased from the supermarket. *L. casei* Shirota strain (YLC) and *L. rhamnosus* (LGG) were isolated from Yakult drink and Valiaa yoghurt using the method described by Tharmaraj and Shah (2003, 2004 and

grown in reconstituted skim milk (RSM) and stored at –37°C. The starter cultures other than YLC and LGG were in freeze-dried direct vat set (DVS) or frozen DVS form. The storage and maintenance of the cultures were carried out as per the recommendation of the manufacturers. Before use, all organisms were tested for purity using Gram stain and sugar fermentation pattern (Tharmaraj and Shah, 2004).

*Pathogenic and spoilage organisms*

Pathogenic organisms including *Echerichia coli, Salmonella typhimurium, Staphylococcus aureus, Bacillus cereus,* and spoilage organisms such as *Bacillus stearothermophilus,* and *Pseudomonas aerogenosa* were obtained from Victoria University Culture Collection (Werribee, Australia).

*Culture media and incubation conditions*

Bacterial cultures were maintained at -37°C. *L. acidophilu*s, *B. animalis, L. casei*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus* were grown in 11% non-fat dry milk supplemented with 1% glucose and 0.3% yeast extract (RSM). L-cysteine.hydrochloride (0.05%) was added for growing *B. animalis*. *P. freudenreichii* subsp. *shermanii* was grown in sodium lactate broth (NaLa broth) (Tharmaraj and Shah, 2003). Pathogenic and spoilage organisms were maintained in filter-sterilised glycerol-nutrient broth. The overnight grown cultures were then transferred into 2 mL cryogenic vials (Iwaki Glass, Canada) and stored at -37°C. Working cultures were made from the frozen stock cultures. To make working cultures, 1 mL of frozen cultures was inoculated in 9 mL aliquot of suitable broth media such as MRS broth for *L. acidophilu*s, *L. casei*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus,* MRS broth + L-cysteine hydrochloride (0.05%) for *B. animalis*, and NaLa broth for *P. freudenreichii* subsp. *shermanii.* Incubation was carried out anaerobically at 37°C for 18 h for all cultures except for *P. freudenreichii* subsp. *shermanii*, which was incubated for 24 h at 30°C. MRS agar was used for LAB (*L. acidophilus*, *L. casei*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus*), MRS agar + L-cysteine.hydrochloride (0.05%) for *B. animalis*, NaLa agar was used for *P. freudenreichii* subsp. *shermanii,* nutrient agar for pathogenic bacteria, unless otherwise stated. The plates were incubated anaerobically for 48 h at 37°C for *L. acidophilu*s, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus*. Aerobic and anaerobic incubations for 48 h at 37°C were carried out for spoilage bacteria, and anaerobic incubations at 33°C for 72 h for *P. freudenreichii* subsp. *shermanii*.

*Determination of anti-microbial activity*

Spot-on lawn technique described by Tagg *et al.* (1976) was used with some modification for the preliminary detection and screening of inhibitory activity produced by selected probiotic bacteria. The diameter (mm) of the well and the surrounding zones of inhibition was measured. A 0.8% agar media was used to increase the migration of inhibitory substances, instead of 1% agar suggested by Tagg *et al.* (1976). Twenty-five milliliters of 0.8% suitable agar medium were poured into sterile petri-plates. Wells were cut in the solidified agar using a sterile metal borer (7.0 mm diameter), and the bottom of the wells was sealed with 0.8% agar. Fifty micro-litres of an active culture of producing organisms (strains of *L. acidophilu*s, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii*) were then filled into the wells. The plates were

left at room temperature for 2 h to allow migration and settling of the test cultures, and then incubated for three hours at 37°C. After the initial growth, the remaining depth of the well was sealed with 1% agar. Finally the spotted plates were overlaid with ~ 10 mL of 0.8% agar seeded with 1% indicator organisms (approximately $1x10^7$ cfu.$mL^{-1}$ of pathogenic bacteria and incubated at suitable incubation conditions as mentioned above. After incubation, plates were examined for zones of inhibition and for other effects of suppression around the wells (Dave and Shah, 1997).

The nature of inhibitory substance produced by the organisms in the initial screening was determined in liquid media by the well diffusion technique (Tagg *et al*., 1976). A suitable agar (0.8%) medium held at 45°C was inoculated with 1% of active culture of the target organism. About 25 mL of the seeded agar was poured in to a sterile Petri dish and wells were cut in the solidified agar as before. Cell-free extract of overnight grown (16 - 18 h) cultures producer organisms, was collected from active broth of producer organisms by centrifuging (4000 x g, 12 min, 4°C) and the supernatant filter-sterilised using 0.45 $\mu$m acrodisc (Gelman Sciences, Ann Arbor, MI, USA) membranes. The supernatant was divided into two portions: untreated (A) and neutralised (B) to pH 6 with 5M NaOH. Wells were filled with 200 $\mu$L of the above treated- and untreated- supernatant. The agar plates were then left for 18 h at 4° C for probiotic, pathogenic and spoilage bacteria for diffusion of the test material into the inoculated agar. The plates were then incubated anaerobically for *L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* using Oxoid anaerobic system BR038B (Unipath Ltd., Hampshire, England) or aerobically for pathogenic and spoilage bacteria.

*Anti-microbial effect of probiotic bacteria on spoilage and pathogenic bacteria*

The inhibitory effect of *L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* on pathogenic and spoilage bacteria were studied. Effect of spot-on lawn method, well diffusion method and co-culturing of probiotic bacteria with pathogenic and spoilage organisms were used in this study. Two strains of *L. acidophilus*, 2 strains of *B. animalis*, 1 strain of *L. casei*, 2 strains of *L. paracasei* subsp. *paracasei*, 4 strains of *L. rhamnosus* and 3 strains of *P. freudenreichii* subsp. *shermanii* were used as producer organisms. Two Gram negative pathogenic bacteria (*E. coli* and *S. typhimurium*), 1 Gram negative spoilage bacterium (*P. aeroginosa*) 2 Gram positive pathogenic bacteria (*S. aureus* and *B. cereus*), and 1 Gram positive spoilage bacterium (*B. stearothermophilus*) and 2 spore formers (*B. cereus* and *B. stearothermophilus*) were used as indicator organisms. Zones of inhibitions were measured. Finally the effect of probiotic bacteria on pathogenic and spoilage bacteria in dips was determined.

*Bacterial inocula*

Before a new working culture was prepared from the frozen stock cultures, all cultures were propagated twice before use, and sub-cultured into a suitable broth weekly for a maximum of 10 subcultures. For routine culturing of Lactobacilli, *B. animalis*, *P. freudenreichii* subsp. *shermanii* and pathogenic and spoilage bacteria, MRS broth, MRS broth supplemented with 0.05% L-cysteine.hydrochloride, sodium lactate broth and nutrient broth, respectively, was used.

*Spot on lawn assay for bacteria*

Spot-on lawn assay was used to study the inhibitory effect of *L. acidophilus*, *B. animalis, L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* on pathogenic and spoilage bacteria. Two strains of *L. acidophilus*, 2 strains of *B. animalis*, 1 strain of *L. casei*, 2 strains of *L. paracasei* subsp. *paracasei*, 4 strains of *L. rhamnosus* and 3 strains of *P. freudenreichii* subsp. *shermanii* were used as producer organisms. One strain each of *E. coli*, *S. typhimurium*, *S. aureus*, *B. cereus, B. stearothermophilus* and *P. aeroginosa* were used as indicator organisms. Zones of inhibition were measured and recorded.

*Well diffusion assay for bacteria*

Well diffusion method was used to identify the nature of inhibitory substances. Since strains of *P. freudenreichii* subsp. *shermanii* did not show notable inhibitory effect, they were not included in the study. Supernatant of two strains of *L. acidophilus,* 2 strains of *B. animalis*, 1 strain of *L. casei*, 2 strains of *L. paracasei* subsp. *paracasei*, and 4 strains of *L. rhamnosus* were tested. One strain each of *E. coli*, *S. typhimurium*, *S. aureus, B. cereus*, *B. stearothermophilus* and *P. aeroginosa* were used as indicator organisms. Zones of inhibition were measured and recorded.

*Effect of co-culturing probiotic bacteria with pathogenic and spoilage bacteria*

This method was used to study the effect of probiotic bacteria on the population of pathogenic and spoilage bacteria in RSM. A 9 mL aliquot of RSM was inoculated with 1 mL overnight culture of probiotic bacteria and 0.1 mL of pathogenic or spoilage bacteria. Inoculated RSM medium was mixed well and incubated at 37$^{o}$ C for 24 h. Following incubation, the population of spoilage and pathogenic bacteria was counted on nutrient agar.

*Effect of probiotic bacteria on the inoculated pathogenic and spoilage bacteria in French onion dip*

French onion dip was obtained from the production line of Poseidon and Black Swan Pty. Ltd. (Clayton, Victoria, Australia). Two kilogram lots of dips were inoculated with pathogenic and spoilage bacteria (at $10^8$ cfu $g^{-1}$) and with probiotic bacteria cultures (at $10^8$ cfu $g^{-1}$). Inoculated dips were packed in 100 g lots in plastic containers and sealed and stored at 4$^{o}$C. After 24 h of storage, 10 g of inoculated dip was mixed with 90 mL of 0.015% peptone water and the population of pathogenic and spoilage bacteria were determined using nutrient agar.

*Analysis of supernatant of probiotic cultures for organic acids*

Supernatant of each strain of *L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* was analysed for the end products of fermentation using high performance liquid chromatography (HPLC; Varian Australia Pty. Ltd., Mulgrave, Australia). Supernatant of overnight cultures of lactobacilli and *Bifidobacterium* that were grown in MRS broth and *Propionibacterium* that was grown in sodium lactate broth at suitable growth conditions using 1% inoculum was subjected to HPLC analysis using the method described by Dubey and Mistry (1996 a,b). Briefly, 100 µL of 15.8 M $HNO_3$ and 14.9 mL of 0.009 M $H_2SO_4$ were added to 1.5 mL of overnight-grown cultures and centrifuged at 4$^{o}$C at 4000 x g for 15 min using a bench top centrifuge (Sorvall RT7, Newton, CT, USA). The supernatant was filtered using 0.22 µm Millipore filters and 2 ml aliquot were stored at –20$^{o}$C until

analysed. The HPLC system consisted of a Varian 9012 solvent delivery system, Varian 9100 auto-sampler, Varian 9050 variable wavelength UV/V turnable absorbance detector and a 730 data module. An Aminex HPX-87H column (300 mm x 7.8 mm, Bio-Rad Laboratories, Richmond, CA, USA) and a guard column with disposable cartridges H+ (Bio-Rad Laboratories) maintained at 65°C were used for the analysis of organic acid. The degassed mobile phase of 0.009 M $H_2SO_4$, filtered through a 0.45 µm membrane filter was used at a flow rate of 0.3ml/min. The wavelength of detection was optimised at 220 nM and the sample injection was 50 µL. The standard solutions of acetic acid, lactic acid, formic acid, propionic acid, butyric acid, phenyl lactic acid and benzoic acid (Sigma Chemical Co., St. Louis, MO, USA) were prepared in mobile phase solution to establish elution times and standard curves.

All the experiments were carried out twice and all analyses were carried out in duplicate. The results presented are average of four determinations.

## Results and Discussion

*Effect of probiotic bacteria on pathogenic and spoilage bacteria*

Antagonism of probiotic bacteria on spoilage and pathogenic bacteria can be affected by many factors including bacterial load, growth conditions and resistance of the pathogen to the inhibitory substances. In this experiment, various broth media (nutrient broth, MRS broth and sodium lactate broth), different growth conditions (aerobic and anaerobic) and various agar media were evaluated using spot-on-lawn assay and well diffusion assay to assess the anti-microbial properties of the probiotic bacteria.

In a preliminary spot-on-lawn assay, the indicator organisms were tested for antagonistic effects by probiotic bacteria under aerobic conditions. In this experiment, the indicator organisms (*E. coli, S. typhimurium*, *P. aeroginosa*, *S. aureus, B. cereus*, *B. stearothermophilus*) were inoculated individually in nutrient agar and were overlaid on a plain nutrient agar plate (control) or over plates with producer organisms (*L. acidophilus, B. animalis, L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* or *P. freudenreichii* subsp. *shermanii*) that were grown with nutrient broth. The plates were then incubated aerobically at 37°C. The pathogen and spoilage organisms showed prolific growth on the control plates and those with *L. acidophilus, B. animalis* and *P. freudenreichii*. Plates with *L. paracasei* subsp. *paracasei* and *L. rhamnosus* showed limited growth of *E. coli, S. typhimurium*, and *S. aureus* (data not shown). The poor tolerance of *L. acidophilus* and *B. animalis* to aerobic conditions may have affected their establishment and therefore the ability to show inhibitory effects. This clearly indicates that aerobic condition affects some probiotic bacteria more than the other, in producing inhibitory substances. However, due to their relative ability to tolerate aerobic condition, *L. casei*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus* were able to establish and show some inhibitory action against *E. coli, S. typhimurium*, and *S. aureus.* The growth media (nutrient broth/ agar) might not have provided enough nutrient (especially sugar) to probiotic bacteria, thereby affecting their growth and ability to produce sufficient quantity of organic acids that inhibit pathogenic and spoilage bacteria. Further, when sugar was not available and other growth conditions are favourable, *L. casei*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus* might have produced inhibitory substances that were not

originated from sugar to produce above observed inhibition.

When this preliminary experiment was repeated by replacing the producer growth media from nutrient broth to MRS broth, *L. casei*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus* showed clear inhibitory effects against *E. coli, S. typhimurium*, *S. aureus* and *P. aerogenosa* and slight inhibitory effect against *B. cereus* and *B. stearothermophilus*. This suggests that sugar (in MRS media) may have influenced the production of inhibitory substances. In this preliminary experiment, *P. freudenreichii* subsp. *shermanii* strain P showed inhibition against *B. stearothermophilus* and on *B. cereus*. The inhibition zone produced against *B. stearothermophilus* and *B. cereus* was very sharp and with a definite margin without a diffusion zone. Though not initially planned in the experiment, the plates were kept in the refrigerator (4$^{o}$C) for 2 weeks. After 2 weeks of storage in the refrigerator, the zone of inhibition produced on *B. stearothermophilus* did not disappear but that of *B. cereus* started to shrink. The thermophilic nature of *B. stearothermophilus* would have prevented it from growing at 4$^{o}$C thus not affecting the zone of inhibition. One possible explanation for reduction in the zone of inhibition exhibited by *B. cereus* is that, either the inhibitory substance produced by *P. freudenreichii* subsp. *shermanii* strain P might not be stable at 4$^{o}$C or *P. freudenreichii* subsp. *shermanii* strain P might not have been active at 4$^{o}$C. Another reason may be that the psychrotrophic bacteria, *B. cereus* might have developed resistance to the substance in the absence of its continuing production and have started to grow. This indicates that storage temperature also affects the anti-microbial effect of probiotic organisms.

When the producer organisms were grown in suitable media (MRS broth for *L. acidophilus, L. casei*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus*, MRS broth + 0.05% of L-cysteine.hydrochloride for *B. animalis* and NaLa broth for *P. freudenreichii* subsp. *shermanii*) with the overlay of indicator organisms (*E. coli, S. typhimurium, P. aeroginosa*, *S. aureus, B. cereus*, *B. stearothermophilus*) inoculated in nutrient agar and incubated anaerobically at 37$^{o}$ C, all producer organisms produced inhibitory zones except the aerobic bacteria, *P. aeroginosa* and *B. stearothermophilus* (Table 1). *P. aeroginosa* and *B. stearothermophilus* did not grow at all under anaerobic incubation. But, following the 72 h anaerobic incubation, a further aerobic incubation for 24 h at 37$^{o}$C was employed, plates with *P. aeroginosa* and *B. stearothermophilus* also showed zones of inhibition that were larger than the zones produced on *E. coli, S. typhimurium*, and *S. aureus* and *B. cereus* in the anaerobic phase (Table 1). This indicates that these two bacteria were controlled by anaerobic conditions alone (such as under modified or vacuum packed conditions) and may not require any additional control by probiotic organisms. It is also suggested that the inhibitory substances produced and diffused during the initial anaerobic phase of this experiment may have caused the greater inhibition zones against these two bacteria during the aerobic phase. The proliferation of the probiotic bacteria during the anaerobic phase in the absence of competition from an actively growing indicator organism may also have contributed to the greater inhibitory effect during the aerobic phase. Therefore for products that are stored aerobically such as surface application, actively growing cultures with their metabolites are essential to produce successful results.

Under anaerobic conditions, all producer organisms produced considerable inhibition zones against all pathogenic bacteria (Table 1). On an average, among all the probiotic and spoilage bacterial interactions, the spore-formers were inhibited by the probiotic organisms to a greater extent (average zone of inhibition, 19 mm) than the non-spore formers (average zone of inhibition, 14 mm). Also, the Gram positive bacteria were inhibited more (average zone of inhibition, 18 mm) than the Gram negative bacteria (average zone of inhibition, 14 mm). However, this discriminatory inhibition between Gram positive and Gram negative bacteria was not prominent with *B. animalis* (average zone of inhibition, 15.7 mm). *P. freudenreichii* subsp. *shermanii* inhibited only the Gram positive bacteria (*S. aureus* and *B. stearothermophilus*). Though *B. cereus* was inhibited in the preliminary experiment, when incubated anaerobically, the zone of inhibition was not prominent. This might be due to the growth rate might have been faster for *B. cereus* than *P. freudenreichii* subsp. *shermanii* under current incubation conditions.

The order of probiotic bacteria in terms of level of inhibition (and zone of inhibition in mm) was *L. rhamnosus* (21)> *L. acidophilus* (19) = *L. casei* (19) > *L. paracasei* subsp. *paracasei* (18) > *B. animalis* (15) > *P. freudenreichii* subsp. *shermanii* (11). There was a considerable difference between probiotic strains in the ability to inhibit pathogenic and spoilage bacteria. Out of the *L. acidophilus* strains, LAC1 inhibited both Gram positive and Gram negative bacteria better than *L. acidophilus* LA5. The strain BB12 was better for both types of pathogenic bacteria among the *B. animalis* strains tested. The *L. casei* strain YLC was the best among the 3 *L. casei* and *L. paracasei* subsp. *paracasei* strains tested followed by *L. paracasei* subsp. *paracasei* strain LCS1. The difference in the activity between the strains of *L. casei* and *L. paracasei* subsp. *paracasei* was not very prominent. Out of the *L. rhamnosus* strains, LR1524 and GG were found to be more inhibitory for both types of pathogenic and spoilage bacteria, though the other two strains also showed considerable amount of inhibitory effects (Table 1).

The effect of co-culturing probiotic bacteria with pathogenic and spoilage bacteria in RSM media is shown in Table 2. Compared to the control (without any probiotic bacteria) treatment, all four pathogenic and spoilage bacteria were inhibited by all probiotic strains tested to varying degrees. On an average, the probiotic bacteria have reduced the population of spoilage and pathogenic bacteria by 2.8 log units, a level that was less than that found in the control. *B. cereus* was inhibited to a greater degree by all probiotic bacteria and strains than other pathogenic bacteria. On an average, the inhibitory effect of all probiotic bacteria and strains was the weakest against *E. coli*. *S. aureus* was inhibited to a greater degree by *B. animalis* and *L. rhamnosus* than the other probiotic bacteria. The level of reduction in the population of pathogenic bacteria by probiotic bacteria was greatest on *B. cereus* (by 3.6 log units), followed by *S. typhimurium* (by 3.2 log units), *S. aureus* (2.6 units) and *E. coli* (by 1.6 units). *P. freudenreichii* subsp. *shermanii* strain P and *L. acidophilus* showed considerable inhibition against *B. cereus* but not against any other pathogenic bacteria. The results (Table 2) indicate that, out of the probiotic bacteria tested, *B. animalis* and *L. rhamnosus* were most effective against

**Table 1.** Diameter of zone of inhibition (mm) produced by probiotic bacteria (*L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *Shermanii*) on pathogenic and spoilage bacteria (*E. coli*, *S. typhimurium*, *S. aureus*, *P. aeroginosa*, *B. cereus* and *B. stearothermophilus*) using spot on lawn assay

| Producer organism | Anaerobic incubation at 37°C for 72 h | | | | | | Anaerobic incubation at 37°C for 72 h followed by aerobic incubation at 37°C 24 h | |
|---|---|---|---|---|---|---|---|---|
| | *E. coli* | *S. typhymurium* | *S. aureus* | *B. cereus* | *B. stearothermophilus*[#] | *P. aeruginosa*[#] | *B. stearothermophilus* | *P. aeruginosa* |
| *L. acidophilus* | | | | | | | | |
| LA5 | 13 | 14 | 15 | 15 | NG | NG | 19 | 25 |
| LAC1 | 17 | 14 | 18 | 20 | NG | NG | 33 | 27 |
| *B. animalis* | | | | | | | | |
| BB12 | 15 | 14 | 14 | 16 | NG | NG | 24 | 21 |
| BLC1 | 14 | 12 | 12 | 14 | NG | NG | 15 | 21 |
| *L. casei and L. paracasei subsp. paracasei* | | | | | | | | |
| LC01 | 13 | 14 | 14 | 16 | NG | NG | 30 | 14 |
| LCS1 | 15 | 12 | 15 | 17 | NG | NG | 28 | 29 |
| YLC | 14 | 13 | 14 | 16 | NG | NG | 33 | 25 |
| *L. rhamnosus* | | | | | | | | |
| LC705 | 11 | 13 | 13 | 17 | NG | NG | 36 | 29 |
| LBA | 12 | 12 | 15 | 20 | NG | NG | 33 | 26 |
| LGG | 15 | 14 | 23 | 17 | NG | NG | 34 | 25 |
| LR1524 | 15 | 15 | 21 | 19 | NG | NG | 38 | 35 |
| *P. fredenreichii subsp. shermanii* | | | | | | | | |
| P | 0 | 0 | 12 | 0 | NG | NG | 15 | 0 |
| PS1 | 0 | 0 | 15 | 0 | NG | NG | 12 | 0 |
| PB 10360 | 0 | 0 | 12 | 0 | NG | NG | 0 | 0 |

**Table 2.** Effect of co-culturing with probiotic bacteria in reconstituted skim milk media on log population of pathogenic and spoilage bacteria

| Probiotic bacteria/Strain | Pathogenic bacteria | | | |
|---|---|---|---|---|
| | *E. coli* | *S. typhimurium* | *S. aureus* | *B. cereus* |
| *L. acidophilus* | | | | |
| LA5 | 7.0 | 7.5 | 6.6 | 5.6 |
| *B. animalis* | | | | |
| BB12 | 7.0 | 4.7 | 4.7 | 4.0 |
| *L. paracasei* subsp. *paracasei* | | | | |
| LC01 | 7.0 | 5.9 | 5.3 | 4.0 |
| LCS1 | 7.0 | 5.7 | 5.8 | 4.0 |
| *L. rhamnosus* | | | | |
| LC705 | 7.6 | 4.0 | 5.0 | 4.6 |
| LBA | 7.0 | 4.0 | 5.3 | 4.3 |
| *P. freudenreichii* subsp. *shermanii* | | | | |
| P | 7.6 | 7.3 | 6.6 | 4.7 |
| PS1 | 8.0 | 7.7 | 8.6 | 5.7 |
| Control (no probiotic) | 8.9 | 9.1 | 8.6 | 8.2 |

Therefore biological control using probiotic bacteria can only be one of the hurdles in the food safety program. Reducing the chances of contamination by controlling critical control points of the production is vital.

Table 3 shows the zone of inhibition (with or without the zone of diffusion) using well-diffusion assay. In general, these results were similar to those found in spot-on-lawn assay, described earlier (Table 1). All probiotic bacterial strains produced a clear zone of inhibition and a less clear zone of diffusion, except *L. rhamnosus* strains LC705 and LBA, which did not show a zone of diffusion. This suggests that part of the inhibitory substance/s produced by these strains may have disappeared or easily destroyed during extraction of the supernatant or produced lesser quantity of anti-microbial compounds that can diffuse.

The proportion of organic acids produced by each bacterium on the overnight-cultured supernatant of the probiotic bacteria using HPLC analysis is given in Table 4. HPLC analysis shows that the production of acetic acid is the lowest in these 2 strains of *L. casei* compared to the other probiotic bacteria. However, *L. rhamnosus* strain GG showed a zone of diffusion. *L. rhamnosus* strain GG produced formic acid as indicated by HPLC analysis (Table 4). The reduced quantity of acetic acid could explain the lack of zone of diffusion.

To find out whether the inhibitory effect were due to acid or any other substances, a part of the supernatant was nutralised to pH 6.0 and used. The nutralised supernatant did not produce any zone of inhibition, indicating that the inhibitory substance was a single or a group of organic acids and/or acid derivatives and/or a bacteriocin that is not active at pH 6.0. Though *L. acidophilus* is well documented for its production of hydrogen peroxide, this

**Table 3.** Diameter of zone of inhibition (mm) produced by probiotic bacteria; *L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus* on pathogenic and spoilage bacteria; *E. coli*, *S. typhimurium*, *P. aeruginosa*, *S. aureus*, *B. cereus* and *B. stearothermophilus* in well diffusion assay

| Producer organism / Indicator organism<br>Strain | *E. coli* | *S. typhimurium* | *P. aeruginosa* | *S. aureus* | *B. cereus* | *B. stearothermophilus* |
|---|---|---|---|---|---|---|
| *L. acidophilus* | | | | | | |
| LA5 | 15 (23)[#] | 13 (23) | 10 (25) | 14 (23) | 12 | 10 (18) |
| LAC1 | 11 (23) | 10 (20) | 13 (27) | 12 (21) | 28 | 12 (20) |
| *B. animalis* | | | | | | |
| BB12 | 11 (24) | 13 | 13 (21) | 15 (24) | 11 | 15 (21) |
| BLC1 | 15 (25) | 11 (23) | 10 (20) | 0 | 10 | 12 (22) |
| *L. casei* and *L. paracasei* subsp. *paracasei* | | | | | | |
| LCO1 | 12 (21) | 14 (24) | 15 (25) | 10 (23) | 15 | 15 |
| LCS1 | 11 (23) | 13 (22) | 14 (29) | 17 (25) | 13 | 14 (24) |
| YLC | 15 (24) | 15 (27) | 15 (25) | 13 (27) | 15 | 11 (27) |
| *L. rhamnosus* | | | | | | |
| LC705 | 9 | 12 | 26 | 12 | 18 | 0 |
| LBA | 14 | 0 | 27 | 11 | 18 | 11 |
| LGG | 14 (24) | 14 (25) | 16 (34) | 15 (24) | 15 | 15 (28) |

[#] Values in parenthesis denote zones of inhibition plus zone of diffusion

**Table 4.** Concentration of organic acids in µg $g^{-1}$ and in mM (parenthesis) in overnight-grown culture supernatant of probiotic bacteria; *L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus*, and *P. freudenreichii* subsp. *shermanii*

| Probiotic bacteria/ Strain | pH | Organic acid µg $g^{-1}$ and (mM) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Acetic acid pKa = 4.76 | Formic acid pKa = 3.75 | Lactic acid pKa = 3.86 | Propionic acid pKa = 4.87 | Benzoic acid pKa = 4.20 | Butyric acid pKa = 4.82 | Phenyllactic acid pKa = 3.46 |
| *L. acidophilus* | | | | | | | | |
| LA5 | 4.27 | 2820.2 (47.0) | | 7088.1 (78.8) | | 394.5 (3.2) | | 277.3 (1.6) |
| LAC1 | 3.90 | 2691.5 (44.9) | | 11383.5 (126.5) | | 246.1 (2.0) | | 335.3 (2.0) |
| *B. animalis* | | | | | | | | |
| BB12 | 4.15 | 3024.9 (50.4) | 18.7 (0.41) | 2552.3 (30.9) | | 142.1 (1.2) | 68.8 (0.7) | 159.5 (0.9) |
| BLC1 | 3.82 | 3392.9 (56.6) | 879.3 (19.1) | 2862.6 (31.8) | | 95.8 (0.8) | 79.4 (0.9) | |
| *L. casei* and *L. paracasei* subsp. *Paracasei* | | | | | | | | |
| LCO1 | 3.94 | 1614.9 (26.9) | | 7984.1 (88.7) | | 77.1 (0.6) | 28.8 (0.3) | 121.9 (0.7) |
| LCS1 | 3.87 | 1817.1 (30.3) | | 9356.4 (103.9) | | 57.7 (0.5) | 17.7 (0.2) | 181.3 (1.1) |
| YLC | 3.73 | 1582.2 (26.4) | | 9546.8 (106.1) | | 87.0 (0.7) | 38.0 (0.4) | 177.4 (1.1) |
| *L. rhamnosus* | | | | | | | | |
| LC705 | 4.01 | 1582.9 (26.4) | | 6935.1 (77.1) | | 41.09 (0.3) | 41.3 (0.4) | 207.9 (1.2) |
| LBA | 3.74 | 1586.3 (26.4) | | 9388.7 (104.3) | | 101.3 (0.8) | 42.7 (0.4) | 302.3 (1.8) |
| LGG | 3.86 | 1670.7 (27.9 | 72.3 (1.6) | 8859.7 (98.4) | | 33.3 (0.3) | 14.9 (0.1) | 290.4 (1.7) |
| LR1524 | 3.92 | 1763.9 (29.4) | | 9203.7 (102.3) | | 209.6 (1.7) | 67.2 (0.7) | 683.4 (4.1) |
| *P. freudenreichii* subsp. *Shermanii* | | | | | | | | |
| P | 5.97 | 2435.9 (40.6) | 1336.2 (29.1) | 2156.4 (23.9) | 1902.9 (25.7) | 281.5 (2.3) | 109.9 (1.2) | 128.3 (0.7) |
| PS1 | 6.27 | 1892.9 (31.5) | 947.7 (20.6) | 2965.8 (32.9) | 1800.3 (24.3) | 272.8 (2.3) | 97.5 (1.1) | 73.0 (0.4) |
| PB10360 | 6.20 | 2298.7 (38.3) | 863.5 (18.8) | 1118.9 (12.4) | 2600.0 (35.1) | 218.9 (1.8) | 78.6 (0.8) | 32.9 (0.2) |

**Table 5.** Effect of co-culturing with probiotic bacteria in French onion dip on log population of pathogenic and spoilage bacteria inoculated at a rate of log 8 cfu $g^{-1}$

| Probiotic bacteria/Strain | Pathogenic bacteria | | | |
|---|---|---|---|---|
| | *E. coli* | *S. typhimurium* | *S. aureus* | *B. cereus* |
| *L. acidophilus* | | | | |
| LA5 | 4.6 | 4.5 | 4.5 | 2.3 |
| *B. animalis* | | | | |
| BB12 | 4.7 | 4.6 | 4.7 | <2 |
| *L. casei* and *L. paracasei* subsp. *paracasei* | | | | |
| LC01 | 4.8 | 4.8 | 4.7 | 2.5 |
| LCS1 | 4.8 | 4.8 | 4.8 | <2 |
| YLC | 4.8 | 4.7 | 4.8 | <2 |
| *L. rhamnosus* | | | | |
| LC705 | 4.9 | 4.8 | 4.7 | <2 |
| LBA | 4.7 | 4.7 | 4.5 | 2.0 |
| LGG | 4.7 | 4.6 | 4.5 | <2 |
| LR1524 | 4.8 | 4.7 | 4.8 | 2.9 |
| Control (no probiotic) | 5.0 | 5.0 | 5.0 | 4.0 |

effect was not very prominent in the above experiments.

Organic acids such as formic acid, acetic acid, lactic acid, propionic acid, benzoic acid and free fatty acids are produced from sugars- (Ray and Sandine, 2000), amino acid-(Gummalla and Broadbent, 2001) and/or lipid- (Magnusson, 2003) metabolism in bacterial cells. These acids that are responsible for the inhibition of pathogenic and spoilage organisms can be produced by *L. acidophilus, B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei, L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* in varying quantities. Lactic acid bacteria are found to produce large quantities of lactic acid, which reduces the pH of the media of the environment to hostile levels for other microorganisms (Eklund, 1989). In addition to the pH effect, there are other modes through which the acids inhibit pathogenic and spoilage microorganisms. Un-dissociated forms of weak organic acids diffuse through the pathogenic bacterial cell membrane. These diffused acids dissociate inside the cell to a degree depending on the intracellular pH. The $H^+$ ions released during the dissociation are reported to acidify the cytoplasm to cause collapse of the electrochemical proton gradient, resulting in bacteriostasis and eventual death of the susceptible bacteria (Axelsson, 1998; Piard and Desmazeaud, 1991; Eklund, 1989). When large proportion of the acid is in un-dissociated form, at a pH value that is below the $pK_a$ value of the organic acids, the inhibitory effect is more pronounced (Axelsson, 1998; Piard and Desmazeaud, 1991). The $Pk_a$ values of formic acid, acetic acid, lactic acid, propionic acid, benzoic acid and phenyllactic acid are 3.75, 4.76, 3.86, 4.87, 4.20 and 3.46, respectively. The smaller molecular structure and the lipophilic characteristics of organic acids can also contribute to the anti-microbial action.

Lipophilic, smaller un-dissociated molecules can diffuse faster into the cell to effect more damage. Meat Net Newsletter (September 2003) stated that formic acid had the best bactericidal effect on pathogenic bacteria and though the effectiveness depended on the pH, the un-dissociated form of formic acid was the strongest inhibitor compared to acetic acid, propionic acid and hydrochloric acid (Dibner and Buttin, 2002). Dibner and Buttin (2002) also found that at pH 4.0, formic acid reduced the *E. coli* population by 4 log units while lactic acid and hydrochloric acid reduced only by 1.5 log and 0.5 log units, respectively. All the bacteria tested produced large quantities of lactic acid except *Propionibacterium*. On an average, the strains of *L. acidophilus, B. animalis, L. casei*, *L. paracasei* subsp. *paracasei, L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* produced 103, 57, 106, 97, 94 and 23 mM lactic acid and 46.00, 53.70, 26.20, 28.75, 27.60 and 36.80 mM acetic acid, 2.7, 1.0, 0.7, 0.5, 0.8 and 2.3 mM $L^{-1}$ benzoic acid and 1.35, 0.97, 0.92, 1.08, 2.50 and 0.50 mM of phenyl lactic acid, respectively (Table 4).

Except *L. acidophilus*, all other strains were found to produce smaller quantities of butyric acid. *P. freudenreichii* subsp. *shermanii* produced around 29.0 mM propionic acid. *B. animalis*, *P. freudenreichii* subsp. *shermanii* and *L. rhamnosus* strain GG produced formic acid (9.8, 22.8, 1.5 mM). The ability to reduce pH by producing large quantities of lactic acid or acetic acid along with the ability to produce smaller and lipophilic organic acids such as formic acid and, perhaps in larger quantities in more favourable conditions may be attributed to the ability of these bacteria to inhibit pathogenic bacteria. The findings of Dibner and Buttin (2002) were in agreement with this. *L. acidophilus* and *P. freudenreichii* subsp. *shermanii* produced relatively large quantities of benzoic acid while *L. casei*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus* produced very little benzoic acid. All of the strains tested produced considerable amounts of phenyllactic acid. But *L. acidophilus* strain LAC1 (2.03 mM) and *L. rhamnosus* strain LR1524 (4.14 mM) produced the largest quantities of phenyl lactic acid. Though, strains of *P. freudenreichii* subsp. *shermanii* had the ability to produce all 7 organic acids (Table 4), which are potential microbial inhibitors, they did not show inhibition of spoilage and pathogenic bacteria using spot-on-lawn assay (Table 1). This can be attributed to slow growth rate and slower production of inhibitory substances. The inability of *P. freudenreichii* subsp. *shermanii* to produce large quantities of lactic acid may also have contributed to this effect. However, *P. freudenreichii* subsp. *shermanii* strain P inhibited *B. cereus* (Table 3).

Table 5 shows the effect of co-culturing probiotic bacteria on the log population of pathogenic and spoilage bacteria in French onion dip. There had been a reduction of the bacterial population (from log 8 to log 5), even in the control treatment. This might have been caused by the acidity of the dip (pH 4.3). Though, the probiotic strains have also reduced the log population of the pathogenic bacteria in the dips, this was not significantly greater than that achieved in the control treatment. However, *B. cereus* has been controlled by all the probiotic bacteria to much lower level (from log 8 to around log 2) than that achieved in the control treatment (Table 5). This indicates that the lactic, acetic and citric acid present in the product may have acted as a preservative. It also appears that the probiotic bacteria also produce these acids along with others indicated in Table 4 as metabolic by-products which play a key role

in the inhibition of pathogenic and spoilage bacteria. *B. cereus* was indicated to be very sensitive to acid level than the other pathogens tested in this study and therefore, the additional acidity generated in the presence of probiotic bacteria may have contributed to the greater inhibition of *B. cereus* compared to the other pathogens.

## Conclusions

All the probiotic bacteria tested possess varying degrees of inhibition towards spoilage and pathogenic bacteria. Spore formers and Gram positive bacteria were affected more than Gram negative bacteria. Organic acids such as lactic, formic, acetic, propionic, benzoic and phenyllactic acids, produced by the bacteria appeared to play an important role in inhibiting pathogenic bacteria. In dips, probiotic bacteria played a limited role in inhibiting pathogenic bacteria such as *E. coli*, *S. typhimurium* and *S. aureus*. However, they showed considerable inhibitory effect against *P. aeroginosa*, but slight inhibition against *B. cereus* in dips. Since, acetic, citric and lactic acids are components of dips, the natural pH of the dip is acidic (4.3-4.4). Therefore, inclusion of probiotic bacteria in frozen or freeze-dried form (commercially available forms) in the dip does not appear to add to the inherent inhibitory properties of dips.

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