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Influence of bovine lactoferrin on selected probiotic bacteria and intestinal pathogens

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Abstract This study investigated the effects of bovine lactoferrin (BLf) on the growth of different groups of bacteria in vitro. BLf showed a significant inhibitory effect on the growth of selected pathogens but not probiotics. BLf, in combination with probiotics, has the potential to influence the composition of the gut microflora via inhibition of intestinal pathogens with no significant effect on probiotic bacteria.

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Introduction

Lactoferrin (Lf) is an iron-binding glycoprotein that is secreted in most mammalian external fluids. It acts as a first line defense agent against infections in the body (Levay and Viljoen 1995). It has been suggested that lactoferrin in breast milk plays a major role to maintain a predominance of bifidobacteria and lactobacilli in the infant intestinal system (Artym and Zimecki 2005; Coppa et al. 2006). This evidence suggests that the presence of Lf in the human gastrointestinal (GI) tract may contribute to re-structure the composition of the intestinal microflora. The bacteriostatic or bactericidal activity of lactoferrin is exhibited against a number of G^+ and G^- bacteria (Bullen 1976; Murdock et al. 2007). However, the reason why numbers of lactic acid bacteria, such as bifidobacteria and lactobacilli, in the digestive system remain high during breast-feeding of infants and in lactoferrin-supplemented animal model systems remains unclear (Artym and Zimecki 2005; Coppa et al. 2006; Tang et al. 2009). The effects of BLf on the growth of lactic acid probiotic bacteria appear not to have been studied. In the present study, the effects of BLf on the growth of different bacteria were tested, as well as the effect on pathogen growth of a combination of BLf and a selected probiotic bacteria strain.

Materials and methods

Bovine lactoferrin

Bovine lactoferrin (NZMP7100) was kindly supplied by Fonterra Co-operative Group, New Zealand. In this product the lactoferrin content is >90% of protein, and iron saturation is 10–20%. The pH value (5% solution at 20°C) is 5.5–6.5. Bacterial analysis is negative.

Bacteria strains

Probiotic bacteria *Lactobacillus acidophilus* DPC201, *Lactobacillus plantarum* DPC206, *Pediococcus acidilactici* DPC209, *Lactobacillus reuteri* DPC16, *Bifidobacterium lactis* HN019 and *Lactobacillus rhamnosus* HN001 strains and pathogen strains *Listeria monocytogenes* Scott-A ATCC49594, *Staphloccocus aureus* ATCC 25932, *Salmonella enterica* serovar Typhimurium ATCC 1772 and *Escherichia coli* O157:H7 strain 2988 were provided by Bioactives Research New Zealand (BRNZ), Auckland, New Zealand.

Culture media and conditions

The probiotic bacteria were grown in deMan-Rogosa-Sharpe (MRS) (Difco, Michigan, USA) broth containing cysteine (0.5 g/l) or on MRS agar plates for 18 h in an anaerobic jar at 37°C (Clayson Incubator, New Zealand). The pathogens were cultured in Brain Heart Infusion (BHI) (BBL, USA) broth or on BHI agar plates aerobically at 37°C for 24 h. Bovine Lactoferrin was filter-sterilized and added to autoclaved media as required.

Spectrophotometric turbidity bioassay

Bacterial cultures were diluted tenfold and 25 µl were transferred into a 96-well culture plate containing 225 µl of lactoferrin-supplemented medium. The growth response of each bacterial strain was monitored by determining the optical density at 620 nm using an ELISA plate reader (Model Multiskan EX, Thermo Electron Corporation) (Rosendale et al. 2008). Double strength bacterial culture medium was used in the experiments where cell-free supernatants of probiotic bacteria were tested for their inhibitory effects on the growth of pathogens and probiotics.

Bacteria standard drop plate count assay

After incubation, each bacterial culture was diluted in series using peptone saline water in 96-well culture. Appropriate concentrations were placed on agar plates using the standard drop plate count method (Chen et al. 2003). The viable colonies of bacteria were counted and reported as Log CFU/ml.

Statistical analysis

Data were processed using Excel spreadsheets. Results were expressed as means ± SD, and the significance of differences was determined using ANOVA.

Results and discussion

Effects of bovine lactoferrin on the growth of some pathogens and probiotics

The effects of bovine lactoferrin on the growth of pathogens, including G^+ and G^- strains, were determined by measuring the cell optical density. Figure 1a shows that bovine lactoferrin inhibited the growth of these pathogens in a dose-dependent manner. No significant inhibition was observed on the growth of probiotic strains (Fig. 1b).

As a comparison, bovine serum albumen (BSA), at the same concentrations as those used for lactoferrin, exhibited no inhibitory effects on the growth of either the pathogens or the probiotics. Figure 2 shows the colonies of *Escherichia coli* O157:H7 on BHI agar plates after it was incubated in the BLf- and BSA-supplemented growth medium at 37°C for 24 h.

Effects of polymyxin on growth of pathogens and probiotics

Polymyxin B is an antibiotic that is used for controlling G^- bacteria, but which has less effect on G^+ bacteria. Polymyxins are cationic proteins that bind to the bacterial cell membrane and alter its structure making it more permeable (Cardoso et al. 2007). In this study, it was hypothesized that the mechanism of action of the cationic protein lactoferrin against bacteria may be similar to that of polymyxin B. Thus, the effect of polymyxin on the growth of the selected pathogens and probiotics was studied. Figure 3a shows that

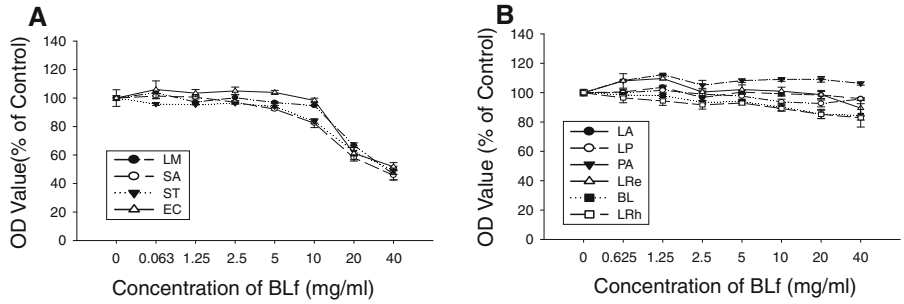

Fig. 1 **a** Dose responses of bovine lactoferrin (BLf) on growth of pathogens *Listeria monocytogenes* (LM), *Staphlococcus aureus* (SA), *Salmonella typhimurium* (ST) and *Escherichia coli* (EC). **b** Dose responses of bovine lactoferrin on probiotics *L. acidophilus* (LA), *L. plantarum* (LP), *P. acidilactici* (PA), *L. reuteri* (LRe), *B. lactis* (BL) and *L. rhamnosus* (LRh)

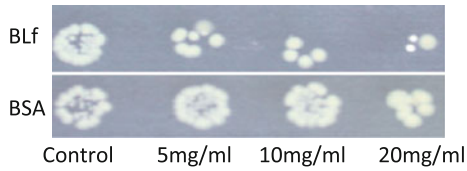

Fig. 2 The effects of BLf and BSA on the growth of EC tested by the standard drop plate count method

polymyxin B strongly suppressed the growth of the G^- bacteria ST and EC. A concentration of 60 μg/ml was required before significant inhibition of the G^+ bacteria LM and SA was observed. In contrast, Fig. 3b shows that polymyxin B did not inhibit any probiotics, even at the highest concentration tested.

Effects of bovine lactoferrin in combination with a probiotic cell-free supernatant on the growth of pathogens and probiotics

It is known that the probiotic supernatant contains compounds such as reuterin in *L. reuteri* culture that are inhibitory to the pathogens (Arques et al. 2004; Talarico et al. 1988). In the present study, the supernatant produced by *L. reuteri* DPC16 was examined for its effect on the growth of the pathogens, in the presence and absence of bovine lactoferrin. The results, shown in Fig. 4, reveal that the combination of lactoferrin (10 mg/ml) and *L. reuteri* DPC16 supernatant at a concentration of 0.125 (v/v) showed additional inhibition of the pathogens (Fig. 4a), but no effect on the probiotic bacteria (Fig. 4b), during growth for 24 h at 37°C.

In summary, the results demonstrated that Lf inhibited the growth of all tested G^+ and G^- pathogens but not the probiotics, at the concentrations tested. In contrast, BSA, tested as a protein control, inhibited neither the pathogens nor the probiotics. It is concluded, therefore, that lactoferrin is a special protein that possesses antibacterial capabilities to some bacteria, but not all. We propose that the special cell structures or metabolic substances of probiotics may protect the cells from the activities of cationic proteins

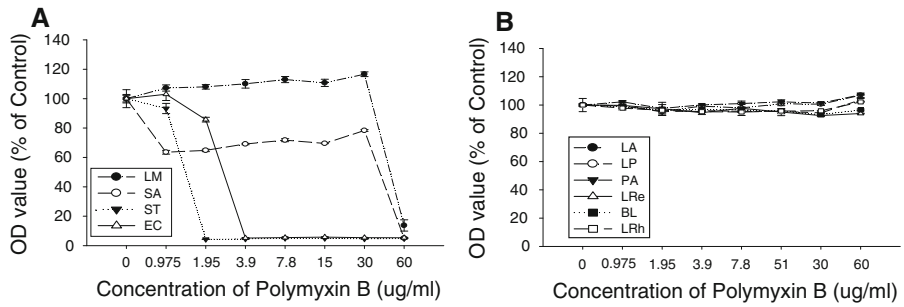

Fig. 3 **a** Dose response of polymyxin B against pathogens. **b** Dose response of polymyxin B against the probiotic bacteria. Symbols as for Fig. 1b

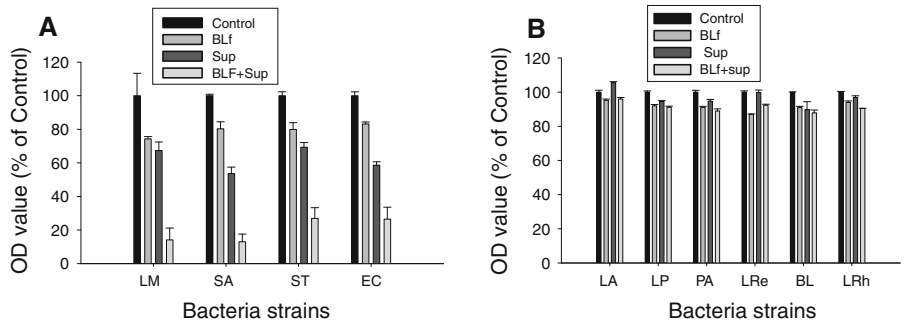

Fig. 4 **a** The effect of a combination of BLf and *L. reuteri* DPC16 supernatant on growth of pathogens. **b** The effect of a combination of BLf and *L. reuteri* DPC16 supernatant on growth of probiotic bacteria

such as lactoferrin and polymyxin B. Our results also show that a probiotic cell-free supernatant can augment the antibacterial activity of lactoferrin against pathogens without affecting the growth of the probiotics. These data provide more support to the suggestion that consumption of bovine lactoferrin with probiotics may contribute to a beneficial microflora balance in the GI tract.

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