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Antimicrobial potential for the combination of bovine lactoferrin or its hydrolysate with lactoferrin-resistant probiotics against foodborne pathogens

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ABSTRACT

Previous reports have shown that several probiotic strains can resist the antibacterial activity of bovine lactoferrin (bLf), but the results are inconsistent. Moreover, a portion of orally administered apo-bLf is digested in vivo by pepsin to yield bLf hydrolysate, which produces stronger antibacterial activity than that observed with apo-bLf. However, whether bLf hydrolysate affects the growth of probiotic strains is unclear. Therefore, various probiotic strains in Taiwan were collected and evaluated for activity against apo-bLf and bLf hydrolysate in vitro. Thirteen probiotic strains were evaluated, and the growth of *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus salivarius* ATCC 11741, *Lactobacillus rhamnosus* ATCC 53103, *Bifidobacterium longum* ATCC 15707, and *Bifidobacterium lactis* BCRC 17394 were inhibited by both apo-bLf and bLf hydrolysate. The growth of 8 strains were not affected by apo-bLf and bLf hydrolysate, including *L. rhamnosus* ATCC 7469, *Lactobacillus reuteri* ATCC 23272, *Lactobacillus fermentum* ATCC 11739, *Lactobacillus coryniformis* ATCC 25602, *L. acidophilus* BCRC 14065, *Bifidobacterium infantis* ATCC 15697, *Bifidobacterium bifidum* ATCC 29521, and *Pediococcus acidilactici* ATCC 8081. However, apo-bLf and its hydrolysate inhibited the growth of foodborne pathogens, including *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Enterococcus faecalis*. Moreover, the supernatants produced by *L. fermentum*, *B. lactis*, and *B. longum* inhibited the growth of most pathogens. Importantly, a combination of apo-bLf or bLf hydrolysate with the supernatants of cultures of the organisms described above showed synergistic or partially synergistic effects against the growth of most of the selected pathogens. In conclusion, several probiotic strains are resistant to apo-bLf and bLf hydrolysate, warranting clinical studies to evaluate the antimicrobial potential for the combination of apo-bLf or its hydrolysate with specific probiotics.

Key words: lactoferrin, probiotic, lactic acid bacteria, bifidobacteria

INTRODUCTION

Lactoferrin (**Lf**) is an iron-binding glycoprotein mainly present in milk but also secreted in most external mammalian fluids (Baveye et al., 1999). At present, many physiological roles have been ascribed to Lf (Farnaud and Evans, 2003; Wang and Tian, 2010), including immunomodulatory and antimicrobial properties (Levay and Viljoen, 1995; Baveye et al., 1999; Chierici, 2001; Legrand and Mazurier, 2010). Thus, Lf is thought to play an important role in innate immunity. Interestingly, Lf possesses both bacteriostatic and bactericidal activities against various pathogens (Farnaud and Evans, 2003; Orsi, 2004). For example, the bacteriostatic activity of Lf is mainly attributed to its ability to bind iron and thereby deprive bacteria of free iron (Bullen, 1975; Brock, 1980). Furthermore, after pepsin digestion of apo-Lf, another short peptide, lactoferricin, can be obtained and purified from Lf hydrolysate. Interestingly, bovine lactoferricin (residues 17 to 41 of mature bovine Lf, **bLf**) and human lactoferricin possess bactericidal but not bacteriostatic activities (Tomita et al., 1991; Bellamy et al., 1992b). Multiple antimicrobial subfragments have been isolated from pepsin Lf hydrolysate, corresponding to residues 1 to 16 and residues 43 to 48 of bLf (Dionysius and Milne, 1997). Chymosin hydrolysate of bLf also generates antimicrobial subfragments (residues 1 to 10 and 11 to 26) with different antimicrobial activities (Hoek et al., 1997). Furthermore, several antimicrobial domains of bLf have been identified, including residues 17 to 31 (Strøm et al., 2000) and residues 268 to 284 (designated lactoferrampin; van der Kraan et al., 2004; Haney et al., 2012). Thus, both Lf and Lf hydrolysate have the potential to be applied clinically because of their antibacterial abilities. Indeed, Lf-related proteins have been used to treat several infections (Levay and Viljoen, 1995; Aguila et al., 2001).

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The human gastrointestinal tract is sterile at birth but the microecology in the intestine develops rapidly when infants are exposed to the environment. The gastrointestinal microecology is known to significantly influence health (Resta et al., 2000). For example, probiotic bacterial strains of bifidobacteria and lactobacilli represent a dominant group of microflora in animal and human intestines. These probiotics show efficacy in hosts because they decrease gastrointestinal infections, inhibit the growth of pathogens, and modulate the mucosal physiology and immunology of intestines (Schrezenmeir and de Vrese, 2001; Teitelbaum and Walker, 2002; Agostoni et al., 2004; Gaggia et al., 2010). Thus, it is important to maintain these probiotics as the predominant population in hosts. Several factors are involved in maintaining specific probiotic bacteria in hosts (Roberfroid, 1998; Collins and Gibson, 1999; Coppa et al., 2006). For example, Lf is an antibacterial protein that can inhibit the growth of a wide range of pathogens, and Lf in breast milk has been suggested to promote a predominance of specific probiotics in infant intestines (Artym and Zimecki, 2005; Coppa et al., 2006). The growth of *Lactobacillus acidophilus* is stimulated by bovine holo-Lf (the iron-saturated form of Lf) but not by apo-Lf (the iron-free form of Lf) in in vitro studies (Kim et al., 2004). In addition, both holo-bLf and apo-bLf (in mature milk) stimulated the growth of *Bifidobacterium breve*, *Bifidobacterium infantis*, and *Bifidobacterium bifidum* in vitro (Petschow et al., 1999). However, bLf (10–20% iron saturated) shows no growth-promoting or growth-inhibiting effects on probiotic bacteria such as *L. acidophilus*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Pediococcus acidilactici*, and *Bifidobacterium lactis* (Tian et al., 2010). Moreover, holo-bLf, holo-human lactoferrin (**hLf**), apo-bLf, and apo-hLf did not stimulate the growth of *B. bifidum*, *B. infantis*, or *L. acidophilus*, but moderate growth inhibition of these bacteria was observed in the presence of Lf (Griffiths et al., 2003). In contrast, another in vitro study indicated growth-promoting activity of bLf and hLf on *B. infantis* and *B. bifidum*, independent of the iron saturation level of Lf (Petschow et al., 1999).

As indicated above, a portion of orally administered apo-bLf is digested in vivo to yield bLf hydrolysate by passage through the gastrointestinal tract (Kuwata et al., 1998; Troost et al., 2001), and apo-bLf hydrolysate will encounter some probiotics in the gastrointestinal tract in vivo. However, whether bLf hydrolysate influences the growth of probiotics remains unclear.

Previous reports of the effects of apo-bLf and bLf hydrolysate on the growth of probiotic strains are inconsistent. Therefore, to confirm and dissect the resistance of probiotics to apo-bLf or bLf hydrolysate, various probiotic strains in Taiwan were collected and evaluated for their resistance to apo-bLf and bLf hydrolysate. In addition, for possible clinical applications, we investigated the antibacterial potential of a combination of apo-bLf, or its hydrolysate, with specific probiotic strains.

We selected apo-bLf and bLf hydrolysate for several reasons. First, Lf found in external secretions (especially in milk) is almost entirely in its iron-free form, and approximately 95% of milk Lf is in the monoferric or apo-Lf state (Makino and Nishimura, 1992). This implies that the natural form of Lf is apo-Lf. Moreover, holo-Lf may not be practical for clinical use because only apo-Lf shows efficacy (Ward et al., 2002). Finally, only apo-bLf, not human Lf or short Lf-related peptides, can be obtained in large quantities and at affordable prices.

This study provides new in vitro evidence of the effect of apo-bLf or bLf hydrolysate on the growth of various probiotic strains. It also provides useful data on the antibacterial potential for the combination of bLf or its hydrolysate with probiotics against foodborne pathogens.

MATERIALS AND METHODS

Proteins and Peptides

Apo-bLf was purchased Glanbia (Monroe, WI) and stored at 4°C. The purity of apo-bLf was >95%, and its iron content was <15 mg/100 g of protein. Bovine Lf hydrolysate was prepared as described previously (Tomita et al., 1991; Chen et al., 2004). Briefly, apo-bLf was dissolved in distilled water at 5% (wt:vol) and the pH was adjusted to 2 to 3. Porcine pepsin was added to prepare a final concentration of Lf of 3% (wt/wt), and the digestion was conducted at 37°C for 4 h. The reaction was terminated by heating the solution at 80°C for 15 min, followed by neutralization to pH 7.0 by the addition of 1 *N* NaOH. Remaining insoluble peptides were removed by centrifugation at 15,000 × *g*, and the supernatant was immediately used in experiments or stored at −20°C until use.

Bacterial Strains and Growth Conditions

Probiotic bacterial strains and foodborne pathogens were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The selected probiotic strains were *B. longum* ssp. *infantis* ATCC 15697, *B. longum* ssp. *longum* ATCC 15707, *Bifidobacterium animalis* ssp. *lactis* BCRC 17394 (a locally isolated strain), *B. bifidum* ATCC 29521, *L. acidophilus* ATCC 4356, *L. rhamnosus* ATCC 7469, *Lactobacillus*