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*

fermentum ATCC 11739, *Lactobacillus salivarius* ATCC 11741, *L. reuteri* ATCC 23272, *Lactobacillus coryniformis* ssp. *coryniformis* ATCC 25602, *L. rhamnosus* ATCC 53103, *L. acidophilus* BCRC 14065 (a locally isolated strain), and *P. acidilactici* ATCC 8081. Moreover, the following pathogenic strains were acquired: *Escherichia coli* HER1255, *Enterococcus faecalis* ATCC 29212, *Salmonella typhi* ATCC 19430, *Salmonella typhimurium* ATCC 13311, *Salmonella typhimurium* ATCC 14028, and *Staphylococcus aureus* ssp. *aureus* ATCC 25923.

The probiotics *B. infantis*, *B. longum*, and *B. bifidum* were cultured in de Man, Rogosa, and Sharpe (MRS) medium (Oxoid, Basingstoke, UK) plus 0.05% cysteine at 37°C under anaerobic conditions. All other probiotics were incubated in MRS medium without cysteine at 37°C under anaerobic conditions. All pathogenic strains were cultured in tryptic soy broth (TSB) at 37°C under aerobic conditions.

Antibacterial Activity of Antibacterial Proteins and Probiotics

To evaluate the antibacterial activities of apo-bLf and bLf hydrolysate on selected probiotic strains, a broth inhibitory assay was conducted under anaerobic conditions (Chen et al., 2004). Probiotic strains were first incubated in MRS broth (plus cysteine if needed) for 48 h. Then, the bacterial broth was adjusted to 1.5×10^8 cfu/mL. Apo-bLf and bLf hydrolysate were serially diluted in MRS broth (plus cysteine if needed) to 128, 64, 32, 16, 8, 4, 2, 1, and 0.5 mg/mL. Then, 100 μ L of the serially diluted antimicrobial solution and 100 μ L of the bacterial solution were combined in a 96-well plate, and the plate was incubated at 37°C. The absorbance of each well was measured after 0, 6, 12, 24, 36, and 48 h using light at a wavelength of 600 nm. The MIC of each agent was determined as the lowest concentration of the antimicrobial agent at which no change in absorbance was observed within a well at 48 h. All tests were performed as true triplicates.

The antibacterial activity test of apo-bLf and bLf hydrolysate toward selected pathogenic strains was conducted using a broth inhibitory assay. Briefly, pathogenic strains were incubated in TSB for 24 h. Then, the bacterial broth was adjusted to 1.5×10^8 cfu/mL. Apo-bLf and bLf hydrolysate were serially diluted in TSB to 128, 64, 32, 16, 8, 4, 2, 1, and 0.5 mg/mL. Then, 100 μ L of the serially diluted antimicrobial solution and 100 μ L of the bacterial solution were combined in a 96-well plate, and the plate was incubated at 37°C. The absorbance of each well was measured at 0, 6, 12, and 24 h using light at a wavelength of 600 nm. The MIC of the antimicrobial agents were determined as described above.

The antibacterial activity of the supernatants produced by culturing each probiotic strain was determined as previously reported (Olivares et al., 2006) with some modifications. Briefly, *Lactobacillus* and *Pediococcus* were grown in MRS broth and *Bifidobacterium* was grown in MRS broth plus 0.05% cysteine under anaerobic conditions for 48 h. Then, culture density was adjusted to 6×10^8 cfu/mL and the cultures were centrifuged at $6,000 \times g$ for 10 min at 4°C. Supernatants were collected, the pH was adjusted to pH 7.2, and the adjusted solutions were filtered through 0.22- μ m membranes (Millipore, Bedford, MA). The pathogenic strains were grown in TSB for 24 h, and the culture was adjusted to a density of 1.5×10^8 cfu/mL. Then, 100 μ L of supernatant and 100 μ L of bacterial solution were combined in a 96-well plate. The MIC of the supernatants were determined as described above.

Test of Synergistic Effects

The antibacterial activities of apo-bLf or bLf hydrolysate in combination with supernatants produced by selected probiotics were determined using the broth microdilution checkerboard method (Chen et al., 2004). First, pathogenic strains were incubated in TSB at 37°C for 24 h under aerobic conditions and adjusted to a culture density of 6×10^8 cfu/mL. Next, apo-bLf or bLf hydrolysate and a supernatant (produced by a probiotic strain) were serially diluted to the desired concentration in TSB. Separate aliquots (50 μ L) of bLf hydrolysate or apo-bLf were mixed with supernatant (100 μ L) and a pathogen solution (50 μ L) in wells of a 96-well plate in accordance with the configuration sheet. The plates were incubated at 37°C for 18 h, and the absorbance of each well was measured at various times using light at a wavelength of 600 nm. The MIC were determined as described above. All tests were performed as true triplicates. The interaction between apo-bLf or bLf hydrolysate and each antimicrobial supernatant was evaluated by determining the fractional inhibitory concentration (FIC) index, as described previously (Chen et al., 2004). The interactions between the antibacterial protein and antimicrobial supernatant were classified as synergistic, partially synergistic, indifferent, or antagonistic.

RESULTS

Effects of apo-bLf and bLf Hydrolysate on the Growth of Probiotics

The effects of apo-bLf and bLf hydrolysate on the growth of probiotics were determined by measuring optical cell density. Apo-bLf inhibited the growth of *L.*

Table 1. Effects of apo-bovine lactoferrin on the growth of probiotic bacteria from 6 to 48 h

Target bacterial strain	MIC of lactoferrin (mg/mL)				
	6 h	12 h	24 h	36 h	48 h
<i>Lactobacillus acidophilus</i> ATCC 4356	>64 ¹	16	16	8	8
<i>Lactobacillus rhamnosus</i> ATCC 7469	>64	>64	>64	>64	>64
<i>Lactobacillus salivarius</i> ATCC 11741	>64	32	32	32	32
<i>Lactobacillus reuteri</i> ATCC 23272	>64	>64	>64	>64	>64
<i>Lactobacillus rhamnosus</i> ATCC 53103	16	8	8	4	1
<i>Lactobacillus fermentum</i> ATCC 11739	>64	>64	>64	>64	>64
<i>Lactobacillus coryniformis</i> ATCC 25602	>64	>64	>64	>64	>64
<i>L. acidophilus</i> BCRC 14065	>64	>64	>64	>64	>64
<i>Bifidobacterium infantis</i> ATCC 15697	>64	>64	>64	>64	>64
<i>Bifidobacterium longum</i> ATCC 15707	>64	>64	>64	4	4
<i>Bifidobacterium bifidum</i> ATCC 29521	>64	>64	>64	>64	>64
<i>Pediococcus acidilactici</i> ATCC 8081	>64	>64	>64	>64	>64
<i>Bifidobacterium lactis</i> BCRC 17394	>64	>64	8	4	2

¹Growth of probiotic bacteria was not affected by lactoferrin.

acidophilus ATCC 4356, *L. salivarius*, *L. rhamnosus*, *B. longum*, and *B. lactis* (Table 1). However, apo-bLf did not inhibit the growth of *L. rhamnosus*, *L. reuteri*, *L. fermentum*, *L. coryniformis*, *L. acidophilus* BCRC 14065, *B. infantis*, *B. bifidum*, or *P. acidilactici*. These data indicate that the latter series of probiotics is resistant to apo-bLf. In contrast, probiotic bacterial strains resistant to apo-bLf also resisted bLf hydrolysate (Table 2).

Effects of bLf, bLf Hydrolysate, or Probiotics on the Growth of Pathogens

The above data show that some probiotic strains can resist the growth-inhibition effect exerted by apo-bLf or bLf hydrolysate. Next, we determined the effects of apo-bLf, bLf hydrolysate, and probiotic strains on the growth of foodborne pathogens. Apo-bLf inhibited the

growth of all selected pathogens except that of *Ent. faecalis* (Table 3), which was somewhat resistant to bLf. As expected, bLf hydrolysate also inhibited the growth of all pathogens and possessed a stronger antibacterial activity than apo-bLf because a lower MIC was observed for bLf hydrolysate against *E. coli*, *Staph. aureus* ATCC 25923, and 1 strain of *S. typhimurium* (Table 4).

Some probiotic culture supernatants contain compounds that exhibit inhibitory activities against pathogens (Talarico et al., 1988; Arqués et al., 2004). Therefore, supernatants from cultures of these selected probiotic strains were assessed for antibacterial activity. Supernatants produced by cultures of *L. fermentum* and *B. lactis* inhibited the growth of *E. coli*, *Ent. faecalis*, *S. typhi*, and 2 strains of *S. typhimurium* (Table 5). Moreover, supernatants produced by cultures of *B. longum* inhibited the growth of *E. coli*, *S. typhi*, and *Staph. aureus*.

Table 2. Effects of bovine lactoferrin (bLf) hydrolysate on the growth of probiotic bacteria

Target bacterial strain	MIC of bLf hydrolysate (mg/mL)				
	6 h	12 h	24 h	36 h	48 h
<i>Lactobacillus acidophilus</i> ATCC 4356	>64 ¹	16	16	8	8
<i>Lactobacillus rhamnosus</i> ATCC 7469	>64	>64	>64	>64	>64
<i>Lactobacillus salivarius</i> ATCC 11741	>64	32	32	32	32
<i>Lactobacillus reuteri</i> ATCC 23272	>64	>64	>64	>64	>64
<i>L. rhamnosus</i> ATCC 53103	16	8	8	4	1
<i>L. acidophilus</i> BCRC 14065	>64	>64	>64	>64	>64
<i>Lactobacillus fermentum</i> ATCC 11739	>64	>64	>64	>64	>64
<i>Lactobacillus coryniformis</i> ATCC 25602	>64	>64	>64	>64	>64
<i>Bifidobacterium infantis</i> ATCC 15697	>64	>64	>64	>64	>64
<i>Bifidobacterium longum</i> ATCC 15707	>64	>64	4	2	2
<i>Bifidobacterium bifidum</i> ATCC 29521	>64	>64	>64	>64	>64
<i>Pediococcus acidilactici</i> ATCC 8081	>64	>64	>64	>64	>64
<i>Bifidobacterium lactis</i> BCRC 17394	>64	>64	8	4	2

¹Growth of probiotic bacteria was not affected by bLf.

Table 3. Effects of bovine lactoferrin on the growth of pathogens over 24 h

Target bacterial strain	MIC of lactoferrin (mg/mL)		
	6 h	12 h	24 h
<i>Enterococcus faecalis</i> ATCC 29212	>64 ¹	>64	32
<i>Escherichia coli</i> HER 1255	16	8	8
<i>Salmonella typhimurium</i> ATCC 13311	64	32	16
<i>S. typhimurium</i> ATCC 14028	64	8	4
<i>Salmonella typhi</i> ATCC 19430	>64	4	1
<i>Staphylococcus aureus</i> ATCC 25923	>64	8	8

¹Growth of probiotic bacteria was not affected by lactoferrin.

Effects of a Combination of bLf or bLf Hydrolysate with Selected Probiotics on Pathogen Growth

The data shown above demonstrated that bLf and bLf hydrolysate inhibited the growth of almost all of the selected foodborne pathogens. Furthermore, supernatants produced by cultures of *L. fermentum*, *B. lactis*, and *B. longum* exhibited antibacterial activity against some pathogens. Thus, the antibacterial effects of the combination of bLf or bLf hydrolysate with the above-described supernatants were studied further and expressed as the FIC index (Table 6; Chen et al., 2004). Intriguingly, synergistic or partially synergistic growth-inhibiting effects were found for almost all combinations of apo-bLf and supernatants from *L. fermentum*, *B. longum*, or *B. lactis* against *E. coli*, *S. typhi*, and 2 strains of *S. typhimurium*. However, *Staph. aureus* was relatively resistant to the combination of apo-bLf and supernatants from *B. lactis* or *L. fermentum*. Moreover, 1 strain of *S. typhimurium* was resistant to the combination of apo-bLf and *B. longum*. The combination of bLf hydrolysate with any of the 3 supernatants led to synergistic or partially synergistic effects on growth inhibition (Table 7). Collectively, these results indicate that the combination of apo-bLf or bLf hydrolysate with specific probiotics enhances antibacterial activity compared with apo-bLf or bLf hydrolysate alone.

DISCUSSION

In the current study, we evaluated 13 probiotic strains of different origin for their ability to resist bLf-

related proteins. These 13 strains included 7 probiotics widely used in the market (Table 8). Interestingly, 8 strains—*L. rhamnosus*, *L. reuteri*, *B. infantis*, *B. bifidum*, *P. acidilactici*, *L. fermentum*, *L. coryniformis*, and *L. acidophilus* (a locally isolated strain)—were resistant to the antibacterial activity of apo-bLf and bLf hydrolysate. Further, commercially used strains, including *B. bifidum*, *B. longum* ssp. *infantis*, *L. acidophilus* BCRC 14065 (a local strain isolated in Taiwan), *L. reuteri*, and *L. rhamnosus*, were resistant to apo-bLf and bLf hydrolysate. Table 9 lists the characteristics of the probiotics tested in this study. Most of the probiotic strains were good candidates as live supplements to Lf-containing products because they were resistant to apo-bLf. Most importantly, this study showed that most of the probiotics tested were resistant to the bactericidal activity of bLf hydrolysate (Tomita et al., 1991; Bellamy et al., 1992a; Chen et al., 2004). Neither apo-bLf nor its hydrolysate could inhibit the growth of specific probiotic strains; therefore, Lf in milk may help to maintain specific probiotics in the intestinal tract, as indicated previously (Artym and Zimecki, 2005; Coppa et al., 2006). Importantly, the present findings merit further investigation into the effects of co-administering Lf-related products and specific probiotic strains.

This study also evaluated the possible benefit of combining apo-bLf or bLf hydrolysate with specific probiotics to inhibit the growth of foodborne pathogens. Foodborne pathogens were selected because they may encounter Lf-related products or specific probiotics in the gastrointestinal tract. As expected, apo-Lf or bLf

Table 4. Effects of bovine lactoferrin hydrolysate on the growth of pathogens over 24 h

Target bacterial strain	MIC of hydrolysate (mg/mL)		
	6 h	12 h	24 h
<i>Enterococcus faecalis</i> ATCC 29212	>64 ¹	>64	32
<i>Escherichia coli</i> HER 1255	16	4	4
<i>Salmonella typhimurium</i> ATCC 13311	64	32	8
<i>S. typhimurium</i> ATCC 14028	64	8	4
<i>Salmonella typhi</i> ATCC 19430	>64	4	1
<i>Staphylococcus aureus</i> ATCC 25923	8	4	4

¹Growth of probiotic bacteria was not affected by lactoferrin.

Table 5. Antibacterial activity for supernatants produced by probiotic bacterial strains¹

Probiotic	Culture pH	<i>Escherichia coli</i> HER1255	<i>Enterococcus faecalis</i> ATCC 29212	<i>Salmonella typhi</i> ATCC 19430	<i>Salmonella typhimurium</i> ATCC 13311	<i>S. typhimurium</i> ATCC 14028	<i>Staphylococcus aureus</i> ATCC 25923
<i>Lactobacillus acidophilus</i> ATCC 4356	5.1	—	—	—	—	—	—
<i>Lactobacillus rhamnosus</i> ATCC 7469	5.4	—	—	—	—	—	—
<i>Lactobacillus fermentum</i> ATCC 11739	5.4	+	+	+	+	+	—
<i>Lactobacillus salivarius</i> ATCC 11741	4.9	—	—	—	—	—	—
<i>Lactobacillus reuteri</i> ATCC 23272	5.2	—	—	—	—	—	—
<i>Lactobacillus coryniformis</i> ATCC 25602	5.2	—	—	—	—	—	—
<i>L. rhamnosus</i> ATCC 53103	4.8	—	—	—	—	—	—
<i>L. acidophilus</i> BCRC 14065	5	—	—	—	—	—	—
<i>Pediococcus acidilactici</i> ATCC 8081	5.3	—	—	—	—	—	—
<i>Bifidobacterium infantis</i> ATCC 15697	5	—	—	—	—	—	—
<i>Bifidobacterium longum</i> ATCC 15707	5.2	+	—	+	—	—	+
<i>Bifidobacterium bifidum</i> ATCC 29521	5.3	—	—	—	—	—	—
<i>Bifidobacterium lactis</i> BCRC 17394	5.5	+	+	+	+	+	—

¹Where — indicates that bacterial growth was not inhibited (i.e., the probiotic did not have antibacterial activity) and + indicates that bacterial growth was inhibited (proiotic had antibacterial activity).

hydrolysate alone could inhibit growth of all selected foodborne pathogens. Furthermore, supernatants produced by cultures of *L. fermentum*, *B. lactis*, and *B. longum* showed antibacterial activity against most of the tested foodborne pathogens. Therefore, for possible clinical application, we determined the antimicrobial potential of the combination of supernatants with apo-bLf or bLf hydrolysate. Apo-bLf or bLf hydrolysate plus supernatant led to synergistic or partially synergistic effects on the growth inhibition of selected pathogens. Of note among these probiotics, only *L. fermentum* was resistant to apo-bLf and bLf hydrolysate. Thus, in terms of efficacy, *L. fermentum* is likely the best candidate as a live supplement to apo-bLf or bLf

hydrolysate. As an alternative strategy, supernatants produced by *B. lactis* and *B. longum* could be harvested and then administered with apo-bLf or its hydrolysate to yield improved antibacterial activities. In agreement with the present report, bLf (10–20% iron-saturated) plus the *L. reuteri* supernatant showed additional inhibition of pathogens (Tian et al., 2010). Collectively, these data support the use of combination therapy to combat pathogens.

Supernatants produced from the other 10 probiotic strains showed no antibacterial activities. However, these strains may still be applied as live supplements with apo-bLf or bLf hydrolysate because apo-bLf or bLf hydrolysate alone possess antibacterial activity against selected foodborne pathogens, and both inhibit the growth of a wide spectrum of bacteria (Chierici, 2001; Farnaud and Evans, 2003; Chen et al., 2004). Thus, although apo-bLf or bLf hydrolysate is supplemented with bLf-resistant probiotics in vivo, the growth of pathogens would be inhibited, whereas the growth of bLf-resistant probiotic strains would be maintained, which may indirectly select probiotics as the major population in the gastrointestinal tract. Furthermore, this dominant population of probiotics could exert an antimicrobial effect and promote health by competing for nutrients and adhesion sites, altering microbial metabolism, or stimulating immunity (de Vrese and Schrezenmeir, 2008; Oelschlaeger, 2010). Importantly, both apo-bLf and certain probiotics possess various health-promoting effects (Yamauchi et al., 2006; de Vrese and Schrezenmeir, 2008; Tomita et al., 2009; Oelschlaeger, 2010; Wang and Tian, 2010). Therefore, these

Table 6. Fractional inhibitory concentration (FIC) index for the interaction between various supernatants (from probiotics) and apo-bovine lactoferrin (bLf) against pathogens

Probiotic supernatant	FIC index ¹					Interaction with apo-bLf
	<i>Escherichia coli</i> HER1255	<i>Salmonella typhi</i> ATCC19430	<i>Salmonella typhimurium</i> ATCC13311	<i>S.typhimurium</i> ATCC14028	<i>Staphylococcus aureus</i> ATCC25923	
<i>Lactobacillus fermentum</i> ATCC11739	0.63	0.19	0.19	0.38	— ²	Synergy to partial synergy
<i>Bifidobacterium longum</i> ATCC15707	0.25	0.38	—	0.63	0.63	Synergy to partial synergy
<i>Bifidobacterium lactis</i> BCRC 17394	0.38	0.63	0.13	0.38	—	Synergy to partial synergy

¹Synergistic effects were defined as follows: synergy = FIC index ≤ 0.5 ; partial synergy = $0.5 < \text{FIC index} < 1$.

²The growth of the pathogen was not affected by the probiotic.

Table 7. Fractional inhibitory concentration (FIC) index for the interaction between various supernatants (from probiotics) and bovine lactoferrin (bLf) hydrolysate against pathogens

Probiotic supernatant	FIC index ¹					Interaction with bLf hydrolysate
	<i>Escherichia coli</i> HER1255	<i>Salmonella typhi</i> ATCC19430	<i>Salmonella typhimurium</i> ATCC13311	<i>S. typhimurium</i> ATCC14028	<i>Staphylococcus aureus</i> ATCC25923	
<i>Lactobacillus fermentum</i> ATCC11739	0.63	0.75	0.5	0.63	— ²	Synergy to partial synergy
<i>Bifidobacterium longum</i> ATCC15707	0.31	0.75	—	0.75	0.25	Synergy to partial synergy
<i>Bifidobacterium lactis</i> BCRC 17394	0.75	0.75	0.56	0.75	—	Partial synergy

¹Synergistic effects were defined as follows: synergy = FIC index ≤ 0.5 ; partial synergy = $0.5 < \text{FIC index} < 1$.

²The growth of the pathogen was not affected by the probiotic.

benefits may be retained and even augmented when apo-bLf or bLf hydrolysate is combined with specific bLf-resistant probiotics in vivo. Some products available on the market include both Lf and specific probiotic strains as ingredients. Furthermore, previous in vivo reports showed that a bifidobacteria-predominant microflora was present in half of the studied infants after treatment with an Lf-enriched formula for 3 mo (Roberts et al., 1992). Furthermore, Lf in breast milk has been suggested to play a major role in maintaining a major population of probiotics such as bifidobacteria and lactobacilli in the gastrointestinal tract of infants (Artym and Zimecki, 2005; Coppa et al., 2006). However, appropriate probiotics should be selected for supplementation with apo-bLf related products because the growth of *L. acidophilus*, *L. salivarius*, *L. rhamnosus*, *B. longum*, and *B. lactis* are inhibited by both apo-bLf and bLf hydrolysate, as shown in the current study. The effects of apo-bLf or bLf hydrolysate on the growth of probiotics can be classified into 2 groups: inhibited and unaffected (Table 9). As described in the Introduction, several short antimicrobial peptides can be isolated from bLf hydrolysate (Dionysius and Milne, 1997; Hoek et al., 1997). Thus, it is of interest to elucidate the interaction between bLf-related derivatives and specific probiotic strains.

Based on this study, *L. fermentum*, *B. lactis*, and *B. longum*, as well as their supernatants, possess antibacterial activities under our incubation conditions. How-

ever, before conducting the antibacterial experiments, all supernatants were adjusted to a pH of 7. This adjustment may have diminished the antibacterial effects exerted by probiotics under acid conditions because the secretion of probiotics is drastically affected by incubation conditions. Thus, it is important to elucidate the antibacterial activity of these supernatants under different conditions, and this will be pursued in our next study. For example, probiotic supernatants produced by *L. reuteri* contain reuterin, which inhibits the growth of pathogens (Talarico et al., 1988; Arqués et al., 2004), but the supernatants produced by *L. reuteri* cultures under our conditions did not show antibacterial activity. This was expected because glycerol was not added in our experiments, and addition of glycerol induces *L. reuteri* to secrete reuterin (Talarico et al., 1988; Arqués et al., 2004). At present, we have no knowledge of the inhibitory compounds produced by the probiotics studied. These antimicrobial compounds will be analyzed in a subsequent study in our laboratory. Moreover, whether *L. fermentum*, *B. longum*, and *B. lactis* BCRC 17394 secrete the same antibacterial components in vivo as in vitro should be further elucidated.

CONCLUSIONS

This study provided additional evidence that most of the evaluated probiotics, including several strains currently on the market, are resistant to the antibacterial

Table 8. Effects of lactoferrin (Lf) or its hydrolysate on the growth of probiotics used in yogurt in Taiwan

Probiotic in yogurt	Strain tested	Interaction with Lf or its hydrolysate
<i>Bifidobacterium animalis</i> ssp. <i>lactis</i>	BCRC 17394	Inhibited
<i>Bifidobacterium bifidum</i>	ATCC 29521	Unaffected
<i>Bifidobacterium longum</i> ssp. <i>infantis</i>	ATCC 15697	Unaffected
<i>B. longum</i> ssp. <i>longum</i>	ATCC 15707	Inhibited
<i>Lactobacillus acidophilus</i>	ATCC 4356	Inhibited
<i>L. acidophilus</i>	BCRC 14065	Unaffected
<i>Lactobacillus reuteri</i>	ATCC 23272	Unaffected
<i>Lactobacillus rhamnosus</i>	ATCC 7469	Unaffected
<i>L. rhamnosus</i>	ATCC 53103	Inhibited

Table 9. The characteristics of probiotics in this study

Probiotic	Source ¹	Growth status [incubated with bovine lactoferrin (bLf) derivatives]	
		Apo-bLf	Pepsin hydrolysate
<i>Lactobacillus acidophilus</i> ATCC 4356	Human	Inhibited	Inhibited
<i>Lactobacillus rhamnosus</i> ATCC 7469	Not available	Unaffected	Unaffected
<i>Lactobacillus salivarius</i> ATCC 11741	Saliva	Inhibited	Inhibited
<i>Lactobacillus reuteri</i> ATCC 23272	Human feces	Unaffected	Unaffected
<i>L. rhamnosus</i> ATCC 53103	Human feces	Inhibited	Inhibited
<i>Lactobacillus fermentum</i> ATCC 11739	Saliva	Unaffected	Unaffected
<i>Lactobacillus coryniformis</i> ATCC 25602	Silage	Unaffected	Unaffected
<i>L. acidophilus</i> BCRC 14065	Commercial culture	Unaffected	Unaffected
<i>Bifidobacterium infantis</i> ATCC 15697	Infant intestine	Unaffected	Unaffected
<i>Bifidobacterium longum</i> ATCC 15707	Adult intestine	Inhibited	Inhibited
<i>Bifidobacterium bifidum</i> ATCC 29521	Infant feces	Unaffected	Unaffected
<i>Pediococcus acidilactici</i> ATCC 8081	Fermented milk	Unaffected	Unaffected
<i>Bifidobacterium lactis</i> BCRC 17394	Not available	Inhibited	Inhibited

¹Provided by the Food Industry Research and Development Institute (Hsinchu, Taiwan).

activity of apo-bLf and bLf hydrolysate. Furthermore, synergistic or partially synergistic growth-inhibition effects were observed against important foodborne pathogens by combining apo-bLf or bLf hydrolysate with specific supernatants produced by 3 different probiotics. Notably, the growth of 5 probiotic strains was inhibited by both apo-bLf and bLf hydrolysate. Thus, it is important to select proper combinations for clinical studies.

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REFERENCES

- Agostoni, C., I. Axelsson, O. Goulet, B. Koletzko, K. F. Michaelsen, J. W. Puntis, J. Rigo, R. Shamir, H. Szajewska, and D. Turck. 2004. Prebiotic oligosaccharides in dietetic products for infants: A commentary by the ESPGHAN Committee on Nutrition. *J. Pediatr. Gastroenterol. Nutr.* 39:465–473.
- Aguila, A., A. G. Herrera, D. Morrison, B. Cosgrove, A. Perojo, I. Montesinos, J. Perez, G. Sierra, C. G. Gemmell, and J. H. Brock. 2001. Bacteriostatic activity of human lactoferrin against *Staphylococcus aureus* is a function of its iron-binding properties and is not influenced by antibiotic resistance. *FEMS Immunol. Med. Microbiol.* 31:145–152.
- Arqués, J. L., J. Fernandez, P. Gaya, M. Nunez, E. Rodriguez, and M. Medina. 2004. Antimicrobial activity of reuterin in combination with nisin against food-borne pathogens. *Int. J. Food Microbiol.* 95:225–229.
- Artym, J., and M. Zimecki. 2005. Rola laktoferryiny w prawidłowym rozwoju noworodka (The role of lactoferrin in the proper development of newborns). *Postepy Hig. Med. Dosw.* 59:421–432. (In Polish)
- Baveye, S., E. Ellass, J. Mazurier, G. Spik, and D. Legrand. 1999. Lactoferrin: A multifunctional glycoprotein involved in the modulation of the inflammatory process. *Clin. Chem. Lab. Med.* 37:281–286.
- Bellamy, W., M. Takase, H. Wakabayashi, K. Kawase, and M. Tomita. 1992a. Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. *J. Appl. Bacteriol.* 73:472–479.
- Bellamy, W., M. Takase, K. Yamauchi, H. Wakabayashi, K. Kawase, and M. Tomita. 1992b. Identification of the bactericidal domain of lactoferrin. *Biochim. Biophys. Acta* 1121:130–136.
- Brock, J. H. 1980. Lactoferrin in human milk: Its role in iron absorption and protection against enteric infection in the newborn infant. *Arch. Dis. Child.* 55:417–421.
- Bullen, J. J. 1975. Iron-binding proteins in milk and resistance to *Escherichia coli* infection in infants. *Postgrad. Med. J.* 51(Suppl. 3):67–70.
- Chen, P. W., S. P. Ho, C. L. Shyu, and F. C. Mao. 2004. Effects of bovine lactoferrin hydrolysate on the in vitro antimicrobial susceptibility of *Escherichia coli* strains isolated from baby pigs. *Am. J. Vet. Res.* 65:131–137.
- Chierici, R. 2001. Antimicrobial actions of lactoferrin. *Adv. Nutr. Res.* 10:247–269.
- Collins, M. D., and G. R. Gibson. 1999. Probiotics, prebiotics, and synbiotics: Approaches for modulating the microbial ecology of the gut. *Am. J. Clin. Nutr.* 69:1052S–1057S.
- Coppa, G. V., L. Zampini, T. Galeazzi, and O. Gabrielli. 2006. Prebiotics in human milk: A review. *Dig. Liver Dis.* 38(Suppl. 2):S291–S294.
- de Vrese, M., and J. Schrezenmeir. 2008. Probiotics, prebiotics, and synbiotics. *Adv. Biochem. Eng. Biotechnol.* 111:1–66.
- Dionysius, D. A., and J. M. Milne. 1997. Antibacterial peptides of bovine lactoferrin: Purification and characterization. *J. Dairy Sci.* 80:667–674.
- Farnaud, S., and R. W. Evans. 2003. Lactoferrin—A multifunctional protein with antimicrobial properties. *Mol. Immunol.* 40:395–405.
- Gaggia, F., P. Mattarelli, and B. Biavati. 2010. Probiotics and prebiotics in animal feeding for safe food production. *Int. J. Food Microbiol.* 141(Suppl. 1):S15–S28.
- Griffiths, E. A., L. C. Duffy, F. L. Schanbacher, D. Dryja, A. Leavens, R. L. Neiswander, H. Qiao, D. Di Rienzo, and P. Ogra. 2003. In vitro growth responses of bifidobacteria and enteropathogens to bovine and human lactoferrin. *Dig. Dis. Sci.* 48:1324–1332.
- Haney, E. F., K. Nazmi, J. G. Bolscher, and H. J. Vogel. 2012. Influence of specific amino acid side-chains on the antimicrobial activity and structure of bovine lactoferrampin. *Biochem. Cell Biol.* 90:362–377.

- Hoek, K. S., J. M. Milne, P. A. Grieve, D. A. Dionysius, and R. Smith. 1997. Antibacterial activity in bovine lactoferrin-derived peptides. *Antimicrob. Agents Chemother.* 41:54–59.
- Kim, W. S., M. Ohashi, T. Tanaka, H. Kumura, G. Y. Kim, I. K. Kwon, J. S. Goh, and K. Shimazaki. 2004. Growth-promoting effects of lactoferrin on *L. acidophilus* and *Bifidobacterium* spp. *Biomaterials* 17:279–283.
- Kuwata, H., T. T. Yip, M. Tomita, and T. W. Hutchens. 1998. Direct evidence of the generation in human stomach of an antimicrobial peptide domain (lactoferricin) from ingested lactoferrin. *Biochim. Biophys. Acta* 1429:129–141.
- Legrand, D., and J. Mazurier. 2010. A critical review of the roles of host lactoferrin in immunity. *Biomaterials* 23:365–376.
- Levy, P. F., and M. Viljoen. 1995. Lactoferrin: A general review. *Haematologica* 80:252–267.
- Makino, Y., and S. Nishimura. 1992. High-performance liquid chromatographic separation of human apolactoferrin and monoferric and diferric lactoferrins. *J. Chromatogr.* 579:346–349.
- Oelschlaeger, T. A. 2010. Mechanisms of probiotic actions—A review. *Int. J. Med. Microbiol.* 300:57–62.
- Olivares, M., M. P. Diaz-Ropero, R. Martin, J. M. Rodriguez, and J. Xaus. 2006. Antimicrobial potential of four *Lactobacillus* strains isolated from breast milk. *J. Appl. Microbiol.* 101:72–79.
- Orsi, N. 2004. The antimicrobial activity of lactoferrin: Current status and perspectives. *Biomaterials* 17:189–196.
- Petschow, B. W., R. D. Talbott, and R. P. Batema. 1999. Ability of lactoferrin to promote the growth of *Bifidobacterium* spp. in vitro is independent of receptor binding capacity and iron saturation level. *J. Med. Microbiol.* 48:541–549.
- Resta, O., M. P. Foschino-Barbaro, P. Bonfitto, S. Talamo, G. Legari, G. De Pergola, A. Minenna, and R. Giorgino. 2000. Prevalence and mechanisms of diurnal hypercapnia in a sample of morbidly obese subjects with obstructive sleep apnoea. *Respir. Med.* 94:240–246.
- Roberfroid, M. B. 1998. Probiotics and synbiotics: Concepts and nutritional properties. *Br. J. Nutr.* 80:S197–S202.
- Roberts, A. K., R. Chierici, G. Sawatzki, M. J. Hill, S. Volpato, and V. Vigi. 1992. Supplementation of an adapted formula with bovine lactoferrin: 1. Effect on the infant faecal flora. *Acta Paediatr.* 81:119–124.
- Schrezenmeir, J., and M. de Vrese. 2001. Probiotics, prebiotics, and synbiotics—Approaching a definition. *Am. J. Clin. Nutr.* 73(Suppl):361S–364S.
- Strøm, M. B., O. Rekdal, and J. S. Svendsen. 2000. Antibacterial activity of 15-residue lactoferricin derivatives. *J. Pept. Res.* 56:265–274.
- Talarico, T. L., I. A. Casas, T. C. Chung, and W. J. Dobrogosz. 1988. Production and isolation of reuterin, a growth inhibitor produced by *Lactobacillus reuteri*. *Antimicrob. Agents Chemother.* 32:1854–1858.
- Teitelbaum, J. E., and W. A. Walker. 2002. Nutritional impact of pre- and probiotics as protective gastrointestinal organisms. *Annu. Rev. Nutr.* 22:107–138.
- Tian, H., I. S. Maddox, L. R. Ferguson, and Q. Shu. 2010. Influence of bovine lactoferrin on selected probiotic bacteria and intestinal pathogens. *Biomaterials* 23:593–596.
- Tomita, M., W. Bellamy, M. Takase, K. Yamauchi, H. Wakabayashi, and K. Kawase. 1991. Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. *J. Dairy Sci.* 74:4137–4142.
- Tomita, M., H. Wakabayashi, K. Shin, K. Yamauchi, T. Yaeshima, and K. Iwatsuki. 2009. Twenty-five years of research on bovine lactoferrin applications. *Biochimie* 91:52–57.
- Troost, F. J., J. Steijns, W. H. Saris, and R. J. Brummer. 2001. Gastric digestion of bovine lactoferrin in vivo in adults. *J. Nutr.* 131:2101–2104.
- van der Kraan, M. I., J. Groenink, K. Nazmi, E. C. Veerman, J. G. Bolscher, and A. V. Nieuw Amerongen. 2004. Lactoferrampin: A novel antimicrobial peptide in the N1-domain of bovine lactoferrin. *Peptides* 25:177–183.
- Wang, J., and Z. Tian. 2010. Recent advances in lactoferrin research and development during the past two years (2007–2009): In lieu of a preface of the Special Issue Lactoferrin. *Biomaterials* 23:355–357.
- Ward, P. P., S. Uribe-Luna, and O. M. Conneely. 2002. Lactoferrin and host defense. *Biochem. Cell Biol.* 80:95–102.
- Yamauchi, K., H. Wakabayashi, K. Shin, and M. Takase. 2006. Bovine lactoferrin: Benefits and mechanism of action against infections. *Biochem. Cell Biol.* 84:291–296.