Effects of bovine lactoferrin hydrolysate on the in vitro antimicrobial susceptibility of *Escherichia coli* strains isolated from baby pigs

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Objective—To determine the antibacterial activity of bovine lactoferrin hydrolysate (bLf-lysate) alone or in combination with other antimicrobials against antimicrobial-resistant *Escherichia coli* strains isolated from baby pigs.

Sample Population—3 clinical strains of *E coli* were isolated from baby pigs with severe diarrhea and designated as strains 9061, 9062, and 9065.

Procedure—The broth microdilution checkerboard and fractional inhibitory (or bactericidal) concentration index were used to evaluate the antibacterial effect elicited by bLf-lysate in combination with kanamycin, gentamicin, cephalothin, cefamandole, penicillin G, ampicillin, tetracycline, erythromycin, or rifampicin against the 3 strains of *E coli*.

Results—The 3 strains of *E coli* were susceptible to gentamicin and rifampicin but highly resistant to most of the other antimicrobials tested, except for strain 9061 that was also susceptible to cephalothin but intermediately inhibited by kanamycin and cefamandole. Synergistic growth-inhibitory activity was observed between bLf-lysate and gentamicin against 1 strain of *E coli* (strain 9062); synergistic bactericidal activity was found between bLf-lysate and rifampicin against all 3 strains of *E coli*. Moreover, partial synergy was observed between bLf-lysate and kanamycin, gentamicin, cephalothin, or cefamandole against the strains of *E coli*, but this partial synergistic activity was mostly seen against only 1 of the strains. Little interaction between bLf-lysate and tetracycline, ampicillin, penicillin G, or erythromycin was observed against the clinical strains of *E coli*.

Conclusions and Clinical Relevance—A combination of bLf-lysate and certain antimicrobials may prove clinically effective against antimicrobial-resistant strains of *E coli*. (*Am J Vet Res* 2004;65:131–137)

Many strains of bacteria are resistant to antimicrobials used in clinical or field settings. To deal with this problem, many antibacterial peptides have been examined for their efficacy against bacterial infection. Lactoferrin is an antibacterial protein found in most biological fluids of mammals.[1] It is especially abundant in milk and azurophile granules of neutrophils.[2,3] During inflammation, neutrophils release lactoferrin into the microenvironment to enhance the local concentration of that protein.[4] Lactoferrin is active in inhibiting the growth of many pathogenic bacteria.[2,5] The antibacterial mechanism for lactoferrin is believed to be its ability to decrease the availability of free iron for bacteria,[6,7] bind directly to the surface of bacteria, and damage the outer membrane of gram-negative bacteria.[8-10] The antibacterial activity of lactoferrin has also been attributed to a peptide called lactoferricin. Lactoferricin is derived from pepsin digestion of lactoferrin. Antibacterial activity is greater for lactoferricin than for native lactoferrin. Lactoferricin does not bind to iron but does inhibit the growth of a broad spectrum of bacteria.[11-13]

Lactoferrin and lactoferricin are natural products that can be derived from mammals. They are considered to be good candidates that could be developed into drugs to cope with bacterial infection. In addition, lactoferrin and lactoferricin have been evaluated for their antibacterial efficiency when used alone as well as their synergistic effect when used in combination with several antimicrobials against bacteria. It was documented in 1 study[14] that human lactoferrin and **bovine lactoferrin (bLf)** could increase the susceptibility of salmonellae to cefuroxime, erythromycin, ampicillin, ciprofloxacin, chloramphenicol, and rifampicin. Human lactoferrin can increase the susceptibility of clinical isolates of *Staphylococcus epidermidis* to vancomycin[15] as well as that of clinical isolates of *Pseudomonas aeruginosa* to rifampicin, chloramphenicol, and doxycycline.[16,17] Bovine lactoferrin can enhance the activity of novobiocin and cephapirin against clinical isolates of *Escherichia coli*.[18] Moreover, synergistic effects have been observed for a combination of **bovine lactoferricin (bLfcin)** or short lactoferricin fragments (12 residues) with erythromycin against standard strains of *E coli*.[19,20] In 1 study,[21] investigators found that bLf or bLfcin increased the susceptibility of several clinical isolates of *S aureus* to penicillin G. Moreover, electron microscopy revealed that penicillin G at amounts less than the minimal inhibitory concentration can induce ultrastructural damage in *S aureus* and this damage was further enhanced by lactoferrin.[21] Therefore, a combination of lactoferrin or lactoferricin with certain antimicrobials can elicit strong damaging effects against bacteria.

However, lactoferrin and lactoferricin may not be practical for clinical use because only apo-lactoferrin has potential antibacterial activity[22] and naturally derived lactoferrin needs to be unbound from iron

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before it is useful. Because lactoferricin is derived from lactoferrin hydrolysate (the product of pepsin digestion of native lactoferrin), it needs to be purified before use. In contrast, lactoferrin hydrolysate (a crude lactoferricin mixture) can inhibit the growth of a broad spectrum of bacterial strains and is reportedly[13] 8-fold more potent than native lactoferrin against several bacterial strains. A portion of native lactoferrin is digested to yield lactoferrin hydrolysate in vivo after passing through the stomach.[23,24] Thus, lactoferrin hydrolysate could be derived in vivo and in vitro.

In the study reported here, we investigated the potential for practical use of **bLf hydrolysate (bLf-lysate)** alone and in combination with several antimicrobials against antimicrobial-resistant strains of *E coli*. Thus, we selected several antimicrobials that could inhibit synthesis of the cell wall, proteins, or nucleic acids of bacteria.[25] Results of this study could provide an alternative approach to deal with clinical bacterial infections induced by antimicrobial-resistant bacteria.

Materials and Methods

Sample population—*Escherichia coli* (**American Tissue Culture Collection [ATCC]** 25922)[a] was purchased. Three strains of *E coli* were isolated from baby pigs with severe diarrhea and designated as strains 9061, 9062, and 9065. These *E coli* strains were highly resistant to antimicrobials as determined by use of the disk-agar diffusion method.[26] The bacterial strains were stored at –70°C before use, grown in Luria-Bertani medium (10 g of bactotryptone/L, 5 g of yeast extract/L, and 5 g of NaCl/L) at 37°C, and diluted with a 1% growth broth[b] (pH, 6.8).

Antimicrobials—Kanamycin,[c] gentamicin,[d] cephalothin,[e] cefamandole,[f] ampicillin,[g] penicillin G,[h] tetracycline,[i] rifampicin,[j] and erythromycin[k] were purchased from a commercial supplier. A stock solution of rifampicin was prepared by use of methanol, whereas a stock solution of erythromycin was prepared by use of 95% ethanol and stock solutions of cephalothin and ampicillin were prepared by use of phosphate buffer (0.1 mol/L; pH, 6.0). Stock solutions of the other antimicrobials were prepared by use of distilled water. The 1% growth broth[b] (pH, 6.8) was used as the working solution for all antimicrobials, as described elsewhere.[19] Concentration of the working solutions for kanamycin, cephalothin, ampicillin, and penicillin G was 256 µg/mL, whereas it was 128 µg/mL for tetracycline and cefamandole, 8 µg/mL for gentamicin and erythromycin, and 16 µg/mL for rifampicin.

Peptides—Bovine lactoferrin[l] was purchased from a commercial source. Bovine lactoferrin hydrolysate was prepared by pepsin digestion of bLf, as described elswhere.[13] Briefly, lactoferrin was dissolved in distilled water at 5% (wt:vol), and pH was adjusted to 2 or 3. Porcine pepsin[m] was added to make a final concentration of lactoferrin of 3% (wt:wt). The reaction was conducted at 37°C for 4 hours; it was terminated by heating at 80°C for 15 minutes 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 used immediately in experiments or stored at –20°C until subsequent use.

Determination of antimicrobial susceptibility—The **minimum inhibitory concentration (MIC)** or **minimum bactericidal concentration (MBC)** of each antimicrobial was determined by use of the microdilution technique described in other studies.[19,27] Briefly, each antimicrobial or bLf-lysate was serially diluted to the desired concentrations. The *E coli* strains were also diluted to 2×10^6 **colony-forming units (CFUs)**/mL in 1% growth broth[b] (pH, 6.8). Then, 100 µL of the serially diluted antimicrobial and 100 µL of the bacterial solution were combined in a 96-well microplate,[n] which was followed by incubation at 37°C for 18 hours. Absorbance of each well was measured at various time points by use of light at a wavelength of 600 nm. All antimicrobials were tested at concentrations close to those that could be achieved in vivo.

The MIC was determined as the lowest concentration of the antimicrobial at which there was no change in absorbance within a well between 0 and 18 hours. The MBC was determined as the lowest concentration at which there was no growth of bacteria after further subculturing of a well. All tests were performed in triplicate.

For comparison, the MICs for *E coli* (strain ATCC 25922) were also established for each antimicrobial. Furthermore, the clinical *E coli* strains were also tested for their antimicrobial susceptibility by use of the standardized disk-agar diffusion method.

Test of synergistic effects—The antibacterial activities of bLf-lysate in combination with each of the selected antimicrobials were determined by use of the broth microdilution checkerboard method, as described elsewhere.[28] Briefly, bLf-lysate and each antimicrobial were serially diluted to the desired concentration in 1% growth broth[b] (pH, 6.8). The *E coli* strains were also diluted to 2×10^6 CFUs/mL in the same broth. Separate aliquots (50 µL) of bLf-lysate were mixed with each antimicrobial (50 µL) and bacterial solution (100 µL) in wells of a 96-well microplate[n] in accordance with the configuration sheet. Microplates were incubated at 37°C for 18 hours, and absorbance of each well was measured at various time points by use of light at a wavelength of 600 nm. The MIC and MBC were determined by use of the aforementioned procedures. All tests were performed in triplicate. All antimicrobials were tested at concentrations close to those that could be achieved in vivo.

The interaction of bLf-lysate with each antimicrobial was evaluated by determining the **fractional inhibitory concentration (FIC)** index or **fractional bactericidal concentration (FBC)** index.[28] The indices were calculated by use of the MIC (or MBC) for each antimicrobial alone and each antimicrobial in combination with bLf-lysate. The equation used was as follows:

$$\text{FIC index} = (A/MICA) + (B/MICB) = FICA + FICB$$

where A is the lowest MIC for a well of drug A, MICA is the MIC for drug A alone, B is the lowest MIC for a well of drug B, and MICB is the MIC for drug B alone; FICA and FICB represent the FIC index for drugs A and B, respectively. The FBC index is calculated in the same manner as the FIC index, where the MBC of each antimicrobial was used instead of the MIC. Synergistic effects were defined when the FIC index (or FBC index) was ≤ 0.5, a partial synergistic effect was defined when 0.5 < FIC index (or FBC index) < 1, an indifferent effect was defined when 1 ≤ FIC index (or FBC index) ≤ 4, and an antagonistic effect was defined when the FIC index (or FBC index) was > 4.

Results

The MIC and MBC of each antimicrobial against *E coli* strains were determined by use of the microdilution method (**Table 1**). Two clinical *E coli* strains were susceptible to gentamicin and rifampicin but highly resistant to most of the other antimicrobials. The exception was *E coli* strain 9061, which was suscepti-

Table 1—Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of various antimicrobials and bovine lactoferrin hydrolysate (bLf-lysate) against strains of *Escherichia coli*

	E coli 9061†		*E coli* 9062†		*E coli* 9065†		*E coli* ATCC 25922	
Agents (µg/mL)*	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Lactoferrin hydrolysate	750	1,500	750	1,500	750	1,500	1,000	2,000
Kanamycin	32	64	64	> 64	64	> 64	0.5	0.5
Gentamicin	1	2	1	2	1	2	0.062	0.5
Cephalothin	4	8	64	> 64	64	> 64	16	16
Cefamandole	16	16	32	> 32	32	> 32	1	1
Tetracycline	> 32	> 32	> 32	> 32	> 32	> 32	1	1
Ampicillin	> 64	> 64	> 64	> 64	> 64	> 64	1	1
Penicillin G	> 64	> 64	> 64	> 64	> 64	> 64	16	16
Rifampicin	1	4	1	2	1	2	2	2
Erythromycin	> 2	> 2	> 2	> 2	> 2	> 2	> 2	> 2

*The achievable systemic concentration of each antimicrobial was as follows: kanamycin, 64 µg/mL; gentamicin, 16 µg/mL; cephalothin, 32 µg/mL; cefamandole, 32 µg/mL; tetracycline, 16 µg/mL; ampicillin, 32 µg/mL; penicillin G, 64 µg/mL; rifampicin, 4 µg/mL; and erythromycin, 2 µg/mL. Erythromycin is not considered to be active against *E coli* strains. †Clinical strains of *E coli* isolated from baby pigs with diarrhea.
ATCC = American Tissue Culture Collection.

Table 2—Fractional inhibitory concentration (FIC) index for the interaction between various antimicrobials and bLf-lysate against strains of *E coli*

	FIC index			
Antimicrobial	***E coli* 9061***	***E coli* 9062***	***E coli* 9065***	**Interaction with bLf-lysate†**
Kanamycin	0.70	1.02	1.02	Partial synergy to indifferent effect
Gentamicin	0.56	0.50	0.73	Synergy to partial synergy
Cephalothin	0.79	0.63	0.67	Partial synergy
Cefamandole	1.0	0.63	0.63	Partial synergy to indifferent effect
Rifampicin	0.75	0.58	0.58	Partial synergy

*Synergistic effects were defined as follows: synergy, FIC index ≤ 0.5; partial synergy, 0.5 < FIC index < 1; indifferent effect, 1 ≤ FIC index ≤ 4. †Clinical strains of *E coli* isolated from baby pigs with diarrhea.

Table 3—Fractional bactericidal concentration (FBC) index for the interaction of various antimicrobials with bLf-lysate against strains of *E coli*

	FBC index			
Antimicrobial	***E coli* 9061***	***E coli* 9062***	***E coli* 9065***	**Interaction with bLf-lysate†**
Kanamycin	0.77	0.92	0.83	Partial synergy
Gentamicin	0.54	1.0	0.50	Synergy to indifferent effect
Cephalothin	0.83	1.13	0.63	Partial synergy to indifferent effect
Cefamandole	0.75	0.75	0.46	Synergy to partial synergy
Rifampicin	0.38	0.42	0.29	Synergy

See Table 2 for key.

ble to cephalothin and intermediately susceptible to kanamycin and cefamandole. The MICs of each antimicrobial for *E coli* ATCC 25922 as a control strain were acceptable because this strain was susceptible to kanamycin, gentamicin, cephalothin, cefamandole, tetracycline, ampicillin, and rifampicin but resistant to erythromycin. The antimicrobial susceptibility of clinical *E coli* strains was confirmed by use of the standardized disk-agar diffusion method. In addition, the MICs and MBCs for the antimicrobial-resistant strains were consistent with results of the test of synergistic effects.

We did not detect resistance to bLf-lysate among the clinical *E coli* strains. The clinical and control *E coli* strains were all inhibited and killed by bLf-lysate (Table 1).

The interaction between bLf-lysate and the antimicrobials against the bacterial strains was expressed as the FIC index and FBC index (**Table 2 and 3**). Synergistic growth-inhibiting effects were found for a combination of only bLf-lysate and gentamicin against 1 strain of *E coli* (strain 9062). In most of the other combinations, partial synergy was observed against the clinical *E coli* strains for bLf-lysate in combination with kanamycin, gentamicin, cephalothin, cefamandole, or rifampicin. However, this partial synergy was not always consistently expressed against the 3 clinical strains of *E coli* because several indifferent interactions were observed for the combinations of bLf-lysate and kanamycin or bLf-lysate and cefamandole. Only partial synergy in growth-inhibiting activity was found for combinations of bLf-lysate and certain antimicrobials against the clinical *E coli* strains, which indicated that the susceptibility of the bacteria to the antimicrobials was increased by the addition of bLf-lysate.

Synergy of bactericidal activity was found for the combination of bLf-lysate and rifampicin against all 3 *E coli* strains. Synergy was also evident for the combinations of bLf-lysate and gentamicin or bLf-lysate and cefamandole against 1 strain of *E coli* (strain 9065). However, most of the other combinations of bLf-lysate and antimicrobials had partial synergy or an indifferent effect for bactericidal activity against the *E coli* strains tested. Moreover, this partial synergy was not always consistent among the 3 *E coli* strains.

The FIC index (or FBC index) of the combinations of tetracycline, ampicillin, penicillin G, or erythromycin with bLf-lysate could not be determined because the MICs of the 4 antimicrobials against the *E coli* strains were more than the achievable systemic

Table 4—Minimum bactericidal concentration (MBC) of antimicrobials after addition of various concentrations of bLf-lysate against 3 antimicrobial-resistant strains of *E coli*

	bLf-lysate (μg/mL)										
Antimicrobial	**0**	**62.5**	**93.7**	**125**	**187.5**	**250**	**375**	**500**	**750***	**1,000**	**1,500†**
Ampicillin (64 μg/mL)	—	—	—	—	—	—	—	—	≥ 64	≥ 64	0
Penicillin G (64 μg/mL)	—	—	—	—	—	—	—	—	≥ 64	≥ 64	0
Tetracycline (32 μg/mL)	—	—	—	—	—	—	—	—	≥ 32	≥ 32	0
Erythromycin (2 μg/mL)	—	—	—	—	—	—	—	—	≥ 2	≥ 2	0

*The MIC of bLf-lysate for antimicrobial-resistant strains of *E coli*. †The MBC of bLf-lysate for antimicrobial-resistant strains of *E coli*.
— = No bactericidal effect of the combination of the concentration of bLf-lysate with the antimicrobial.

Table 5—Growth-inhibiting effect (ie, MIC) for antimicrobial agents used alone or in combination with bLf-lysate against 3 clinical strains of *E coli* strains

	MIC (μg/mL)				
Antimicrobial	**Antimicrobial alone**	**bLf-lysate alone**	**Antimicrobial plus bLf-lysate**	**Interaction***	***E coli* strain†**
Kanamycin	32	750	1 and 500	Partial synergy	9061
Gentamicin	1	750	0.25 and 187.5	Synergy	9062
Cephalothin	64	750	32 and 93.75	Partial synergy	9062
Cefamandole	32	750	4 and 375	Partial synergy	9062, 9065
Rifampicin	1	750	0.25 and 250	Partial synergy	9062, 9065

*Synergistic effects were defined on the basis of the FIC index as follows: synergy, FIC index ≤ 0.5; partial synergy, 0.5 < FIC index < 1; indifferent effect, 1 ≤ FIC index ≤ 4. †Clinical strains of *E coli* isolated from baby pigs with diarrhea.

Table 6—Bactericidal effect (ie, MBC) for antimicrobials used alone or in combination with bLf-lysate against 3 clinical strains of *E coli*

	MBC(μg/mL)				
Antimicrobial	**Antimicrobial alone**	**bLf-lysate alone**	**Antimicrobial plus bLf-lysate**	**Interaction***	***E coli* strain†**
Kanamycin	64	1,500	1 and 750	Partial synergy	9061
Gentamicin	2	1,500	0.50 and 375	Synergy	9065
Cephalothin	> 64	1,500	8 and 750	Partial synergy	9065
Cefamandole	> 32	1,500	4 and 500	Synergy	9065
Rifampicin	2	1,500	0.25 and 750	Synergy	9065

See Table 5 for key.

concentration of each agent. This implied that there was no interaction of tetracycline, ampicillin, penicillin G, or erythromycin with bLf-lysate in clinically attainable concentrations for these antimicrobial-resistant strains of *E coli*. Although the indices of these 4 antimicrobials could not be determined, tetracycline inhibited the growth of *E coli* isolate 9065 for 16 hours when bLf-lysate (187.5 to 500 μg/mL) was added. This inhibition was not seen when the same concentrations of tetracycline and bLf-lysate were used separately (data not shown). Moreover, ampicillin, penicillin G, tetracycline, and erythromycin, when used in combination with bLf-lysate (750 and 1,000 μg/mL), also had bactericidal activity at their highest achievable systemic concentration against the *E coli* strains (**Table 4**). However, this activity was not consistent among the triplicate tests.

The most effective combination of bLf-lysate and antimicrobials against *E coli* strains was determined (**Table 5 and 6**). The MIC and MBC for kanamycin, gentamicin, cephalothin, cefamandole, or rifampicin when used alone were 2- to 64-fold higher than when these antimicrobials were used in combination with bLf-lysate. Moreover, the MIC and MBC of bLf-lysate when used alone were also 2- to 8-fold higher than when used in combination with the various antimicrobials.

Discussion

Analysis of results of the study reported here indicated that there would be some benefit for use of a combination of bLf-lysate with certain antimicrobials against antimicrobial-resistant bacteria. However, the synergistic growth-inhibiting effect was found only for a combination of bLf-lysate and gentamicin against 1 antimicrobial-resistant strain of *E coli*, and the synergistic bactericidal effect was found only for a combination of bLf-lysate with rifampicin against all 3 clinical *E coli* strains. Nevertheless, analysis of our results indicated that bLf-lysate could enhance the susceptibility of antimicrobial-resistant *E coli* to several antimicrobials, as expressed by the partial synergy observed for the combinations of bLf-lysate and kanamycin, gentamicin, cephalothin, or cefamandole against the growth of clinical *E coli* strains. However, this partial synergistic effect by the combination of bLf-lysate and certain antimicrobials may not be sufficiently practical

(effective) to be used clinically because the interactions for those were not always consistently effective against the 3 clinical strains of *E coli*.

In 1 study,[19] investigators detected partial synergy by the combination of pure bLfcin and gentamicin, vancomycin, or penicillin G as well as synergy for a combination of bLfcin and erythromycin against *E coli* (strain ATCC 25922). In another study,[20] investigators compared the interaction of 5 short (6 to 18 residues) peptides with several antimicrobials against the same standard *E coli* (strain ATCC 25922) and detected synergy between 1 of their short peptides (bLfcin fragment; 12 residues) and erythromycin or rifampicin. In the study reported here, we documented similar partial synergy between bLf-lysate and kanamycin, cephalothin, cefamandole, or gentamicin against antimicrobial-resistant *E coli* as evaluated by the FIC and FBC indices. Similar synergy was also observed between bLf-lysate and gentamicin against an antimicrobial-resistant strain of *E coli* (by use of the FIC index) and between bLf-lysate and rifampicin against 3 antimicrobial-resistant strains of *E coli* (by use of the FBC index). Therefore, the crude bLfcin mixture (ie, bLf-lysate) was as effective as purified bLfcin (ie, pure lactoferricin) when used in combination with certain antimicrobials against the *E coli* strains. However, little interaction against the clinical *E coli* strains in this study was observed between bLf-lysate and erythromycin, tetracycline, ampicillin, or penicillin G. Synergy was considered lacking for the aforementioned combinations because the MICs of the 4 antimicrobials were much higher than the achievable systemic concentration for the antimicrobials alone or in combination with bLf-lysate. In another study,[20] the FIC index for the combination of short bLfcin fragments (12 residues) with ampicillin, vancomycin, or tetracycline ranged from 0.5 to 1.0 against a standard strain of *E coli*. By use of our definition for an interaction between peptides and antimicrobials, those results would be considered a partial synergy; however, researchers in that other study[20] did not mention a partial synergistic action. They defined the synergistic action as FIC index < 0.5 and indifference as $1 <$ FIC index ≤ 4. Thus, we suggest that differences in target bacterial strains may be responsible for the susceptibility of these strains for the interaction between bLf-lysate (or bLfcin) and antimicrobials against the bacteria. This may explain the inconsistency of synergy or partial synergy observed in our study against the 3 antimicrobial-resistant strains of *E coli* by the combination of bLf-lysate with the same antimicrobials.

Antimicrobial susceptibility of bacterial strains may also be responsible for some of the interactions between peptides and antimicrobials. In the study reported here, the MICs of 3 clinical strains of *E coli* for tetracycline, ampicillin, penicillin G, and erythromycin were much higher than the systemically achievable concentration of each antimicrobial and little interaction was found between bLf-lysate and these antimicrobials. In contrast, the 3 *E coli* strains had intermediate susceptibility to kanamycin, cefamandole, and cephalothin or their growth was inhibited at the highest systemically achievable concentration of these antimicrobials. The combination of bLf-lysate and kanamycin, cephalothin, or cefamandole resulted in partial synergy against the antimicrobial-resistant strains of *E coli*. In all other situations, the clinical strains of *E coli* were susceptible to rifampicin and gentamicin. Coincidentally, the synergistic antibacterial effect against the *E coli* strains was found for combinations of bLf-lysate and rifampicin or gentamicin.

The antibacterial mechanism of bLfcin or bLf-lysate may support our proposed interaction between peptides and antimicrobials. Bovine lactoferricin binds to the lipopolysaccharides on the surface of *E coli*, leading to a disruption of normal permeability functions of the cytoplasmic membrane.[29,30] Furthermore, tryptophan and arginine residues of bLfcin are important for antibacterial activity.[31,32] The indole ring of tryptophan may interact with hydrophobic and hydrophilic constituents of the membrane structure because of its amphipathic property.[33] When bLfcin binds to the membrane structure, the integrity of the membrane would be perturbed by an effect referred to as positive membrane curvature.[34-36] Therefore, the permeability of antimicrobial-resistant *E coli* strains to certain antimicrobials may be increased by bLf-lysate. Investigators in 1 study[21] used electron microscopy to document that alterations of morphologic characteristics and ultrastructure of *S aureus* exerted by penicillin G were enhanced by bLf. We speculate that all of the antimicrobials tested in the study reported here were able to enter the antimicrobial-resistant *E coli* strains in greater amounts than typical because all strains were inhibited and killed by the bLf-lysate. Thus, use of bLf-lysate in combination with the potent antimicrobials (ie, gentamicin and rifampicin) or intermediately potent antimicrobials (ie, kanamycin, cephalothin, and cefamandole) for the clinical strains of *E coli* may have allowed the antimicrobials to more easily exert their effects inside or at the surface of *E coli*. Although the access of ampicillin, penicillin G, tetracycline, and erythromycin to clinical strains of *E coli* may be increased after the addition of bLf-lysate, this may not be sufficiently effective to overcome all of the antimicrobial resistance possibly controlled by various mechanisms in the clinical strains of *E coli*. The effectiveness of combinations of polycationic peptides or other antimicrobial peptides with antimicrobials against bacteria has been documented. The synergistic reaction was believed to be attributable to the peptides facilitating the entrance of antimicrobials into bacterial cells.[20,37,38] However, it has also been suggested[20] that increased access of the antimicrobials into bacteria, as facilitated by the peptides, may not be adequate for the synergistic effect to be revealed and a second effect of the peptides may also be involved. Taken together, we propose that the specific types of antimicrobials (ie, susceptibility of various pathogens), differential target bacterial strains, and antibacterial peptides (ie, antibacterial mechanisms) would all affect the interaction between peptides and antimicrobials.

Other studies[19,20] did not reveal synergistic effects between bLfcin or short bLfcin fragments and several

antimicrobials against the standard gram-positive bacteria, *S aureus* (ATCC 25923). This appears to imply that bLfcin may not increase the susceptibility of *S aureus* to antimicrobials. However, the investigators in 1 of those studies[20] stated that the low MIC of *S aureus* makes it technically difficult to detect synergy. We suggest that differential target bacterial strains and the susceptibility of bacterial strains to antimicrobials may affect some of the interaction between peptides and antimicrobials. Therefore, this kind of synergistic study may be needed to determine the interaction between bLfcin and antimicrobials against antimicrobial-resistant, gram-positive bacteria. In another study,[21] it was reported that a strain of *S aureus* that produced high amounts of β-lactamase was completely inhibited when exposed to a combination of bLfcin and penicillin G (at concentrations less than the MIC of each agent). Nevertheless, we reported here the results of the effectiveness of the interaction between bLf-lysate and antimicrobials against gram-negative bacteria (ie, antimicrobial-resistant strains of *E coli*).

We detected partial synergy of growth-inhibiting activity for a combination of bLf-lysate and gentamicin, cephalothin, cefamandole, or rifampicin against antimicrobial-resistant strains of *E coli*, albeit this may not be sufficiently effective for clinical use. However, synergistic bactericidal activity was detected for the combination of bLf-lysate and rifampicin against the antimicrobial-resistant strains of *E coli*.

[a]CCRC 11509, Food Industry Research and Development Institute, Hsinchu, Taiwan.
[b]Bacto Peptone broth (1% [pH, 6.8]), Difco, Detroit, Mich.
[c]K 4000, Sigma Chemical Co, St Louis, Mo.
[d]G 3632, Sigma Chemical Co, St Louis, Mo.
[e]C 4520, Sigma Chemical Co, St Louis, Mo.
[f]C 7270, Sigma Chemical Co, St Louis, Mo.
[g]A 9518, Sigma Chemical Co, St Louis, Mo.
[h]P 3032, Sigma Chemical Co, St Louis, Mo.
[i]T 3383, Sigma Chemical Co, St Louis, Mo.
[j]R 8883, Sigma Chemical Co, St Louis, Mo.
[k]E 6376, Sigma Chemical Co, St Louis, Mo.
[l]Lactoferrin-100, TATUA, Morrinsville, New Zealand.
[m]P 7000, Sigma Chemical Co, St Louis, Mo.
[n]Falcon 3072, Becton, Dickinson & Co, Franklin Lakes, Md.

References

1. Masson PL, Heremans JF, Prignot JJ, et al. An iron-binding protein common to many external secretions. *Clin Chim Acta* 1966; 14:735–739.
2. Bullen JJ, Rogers HJ, Leigh L. Iron-binding proteins in milk and resistance to *Escherichia coli* infection in infants. *Br Med J* 1972;1:69–75.
3. Wright DG, Gallin JI. Secretory responses of human neutrophils: exocytosis of specific (secondary) granules by human neutrophils during adherence in vitro and during exudation in vivo. *J Immunol* 1979;123:285–294.
4. Masson PL, Heremans JF, Schonne E. Lactoferrin, an iron-binding protein in neutrophilic leukocytes. *J Exp Med* 1969; 130:643–658.
5. Dionysius DA, Grieve PA, Milne JM. Forms of lactoferrin: their antibacterial effect on enterotoxigenic *Escherichia coli*. *J Dairy Sci* 1993; 76:2597–2606.
6. Bullen JJ. The significance of iron in infection. *Rev Infect Dis* 1981;3:1127–1138.
7. Reiter B. The biological significance of lactoferrin. *Int J Tissue React* 1983;15:87–96.
8. Arnold RR, Cole MF, McGhee JR. A bactericidal effect for human lactoferrin. *Science* 1977;197:263–265.
9. Ellison RT. The effects of lactoferrin on gram-negative bacteria. *Adv Exp Med Biol* 1994;357:71–90.
10. Yamauchi K, Tomita M, Giehl TJ, et al. Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment. *Infect Immun* 1993;61:719–728.
11. Bellamy W, Takase M, Yamauchi K, et al. Identification of the bactericidal domain of lactoferrin. *Biochim Biophys Acta* 1992; 1121:130–136.
12. Tomita M, Takase M, Wakabayashi H, et al. Antimicrobial peptides of lactoferrin. *Adv Exp Med Biol* 1994;357:209–218.
13. Tomita M, Bellamy W, Takase M, et al. Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. *J Dairy Sci* 1991;74:4137–4142.
14. Naidu AS, Arnold RR. Lactoferrin interaction with salmonellae potentiates antibiotic susceptibility in vitro. *Diagn Microbiol Infect Dis* 1994;20:69–75.
15. Leitch EC, Willcox MD. Lactoferrin increases the susceptibility of *Staphylococcus epidermidis* biofilms to lysozyme and vancomycin. *Curr Eye Res* 1999;19:12–19.
16. Alkawash M, Head M, Alshami I, et al. The effect of human lactoferrin on the MICs of doxycycline and rifampicin for *Burkholderia cepacia* and *Pseudomonas aeruginosa* strains. *J Antimicrob Chemother* 1999;44:385–387.
17. Fowler CE, Soothill JS, Oakes L. MICs of rifampicin and chloramphenicol for mucoid *Pseudomonas aeruginosa* strains are lower when human lactoferrin is present. *J Antimicrob Chemother* 1997;40:877–879.
18. Sanchez MS, Watts JL. Enhancement of the activity of novobiocin against *Escherichia coli* by lactoferrin. *J Dairy Sci* 1999; 82:494–499.
19. Vorland LH, Osbakk SA, Perstolen T, et al. Interference of the antimicrobial peptide lactoferrin B with action of various antibiotics against *Escherichia coli* and *Staphylococcus aureus*. *Scand J Infect Dis* 1999;31:173–177.
20. Ulvatne H, Karoliussen S, Stiberg T, et al. Short antibacterial peptides and erythromycin act synergically against *Escherichia coli*. *J Antimicrob Chemother* 2001;48:203–208.
21. Diarra MS, Petitclerc D, Lacasse P. Effect of lactoferrin in combination with penicillin on the morphology and the physiology of *Staphylococcus aureus* isolated from bovine mastitis. *J Dairy Sci* 2002; 85:1141–1149.
22. Ward PP, Uribe-Luna S, Conneely OM. Lactoferrin and host defense. *Biochem Cell Biol* 2002;80:95–102.
23. Kuwata H, Yip TT, Tomita M, et al. Direct evidence of the generation in human stomach of an antimicrobial peptide domain (lactoferricin) from ingested lactoferrin. *Biochim Biophys Acta* 1998; 1429:129–141.
24. Troost FJ, Steijns J, Saris WH, et al. Gastric digestion of bovine lactoferrin in vivo in adults. *J Nutr* 2001;131:2101–2104.
25. Tortora GJ, Funke BR, Case CL. Antimicrobial drugs. In: Tortora GJ, Funke BR, Case CL, eds. *Microbiology*. Redwood, Calif: The Benjamin/Cummings Publishing Co, 1992;496–498.
26. Quinn PJ, Carter ME, Markey BK, et al. Antimicrobial agents. In: Quinn PJ, Carter ME, Markey BK, et al, eds. *Clinical veterinary microbiology*. London: Wolfe Publishing, 1994;95–102.
27. Kang JH, Lee MK, Kim KL, et al. Structure-biological activity relationships of 11-residue highly basic peptide segment of bovine lactoferrin. *Int J Pept Protein Res* 1996;48:357–363.
28. Moody JA. Synergism testing. Broth microdilution checkerboard and broth macrodilution methods. In: Isenberg HD, ed. *Clinical microbiology procedures handbook*. Washington, DC: American Society for Microbiology, 1999;5.18.1–5.18.14.
29. Bellamy WR, Wakabayashi H, Takase M, et al. Role of cell-binding in the antibacterial mechanism of lactoferricin *Br J Appl Bacteriol* 1993;75:478–484.
30. Elass-Rochard E, Roseanu A, Legrand D, et al. Lactoferrin-lipopolysaccharide interaction: involvement of the 28-34 loop region of human lactoferrin in the high-affinity binding to *Escherichia coli* O55B5 lipopolysaccharide. *Biochem J* 1995;312:839–845.
31. Vogel HJ, Schibli DJ, Jing W, et al. Towards a structure-function analysis of bovine lactoferricin and related tryptophan- and arginine-containing peptides. *Biochem Cell Biol* 2002;80:49–63.

32. Chen PW, Shyu CL, Mao FC. Antibacterial activity of short hydrophobic and basic-rich peptides. *Am J Vet Res* 2003;64:1088–1092.

33. Schiffer M, Chang CH, Stevens FJ. The functions of tryptophan residues in membrane proteins. *Protein Eng* 1992;5:213–214.

34. Epand RF, Epand RM, Formaggio F, et al. Analogs of the antimicrobial peptide trichogin having opposite membrane properties. *Eur J Biochem* 2001;268:703–712.

35. Matsuzaki K, Sugishita K, Ishibe N, et al. Relationship of membrane curvature to the formation of pores by magainin 2. *Biochemistry* 1998;34:11856–11863.

36. Schibli DJ, Hwang PM, Vogel HJ. The structure of the antimicrobial active center of lactoferricin B bound to sodium dodecyl sulfate micelles. *FEBS Lett* 1999;446:213–217.

37. Giacometti A, Cirioni O, Del Prete MS, et al. Combination studies between polycationic peptides and clinically used antibiotics against gram-positive and gram-negative bacteria. *Peptides* 2000; 21:1155–1160.

38. Mangoni ML, Rinaldi AC, Di Giulio A, et al. Structure-function relationships of temporins, small antimicrobial peptides from amphibian skin. *Eur J Biochem* 2000;267:1447–1454.