

# Antibacterial Effect of Bovine Lactoferrin Against Udder Pathogens

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**Kutila T, Pyörälä S, Saloniemi H, Kaartinen L: Antibacterial effect of bovine lactoferrin against udder pathogens. Acta vet. scand. 2003, 44, 35-42.** – The antibacterial effect of lactoferrin (Lf) was tested on isolates of *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), and coagulase-negative staphylococci (CNS) as well as on *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Klebsiella pneumoniae* (*K. pneumoniae*), originally isolated from bovine mastitis. Concentrations of Lf used were 0.67 mg/ml, 1.67 mg/ml, and 2.67 mg/ml. Growth of udder pathogens was monitored by turbidometry either in broth culture or in whey prepared from normal milk. We focused on 3 different growth variables: lag time, slope, and maximum absorbance of bacterial growth curves. Growth inhibition was seen in the broth but hardly at all in whey. The isolates of *E. coli* and CNS did not grow sufficiently well in whey to draw any conclusions. The most effective inhibitory activity of Lf was seen against *E. coli* and *P. aeruginosa*. All 5 *E. coli* isolates had similar growth patterns. Inhibition of growth by Lf was concentration-dependent. The concentration of 0.67 mg/ml in broth and whey was generally too low for a significant inhibitory effect.

**mastitis pathogens; growth inhibition.**

## Introduction

Lactoferrin (Lf) is an iron-binding glycoprotein found in milk, other external secretions, and the granules of neutrophilic polymorphonuclear leukocytes (Baggiolini *et al.* 1970, Masson *et al.* 1966, 1969). Lf has been shown to be bacteriostatic *in vitro*, and this inhibitory activity is believed to be the result of the powerful iron-chelating ability of Lf, making iron unavailable to bacteria (Reiter & Oram 1967, Weinberg 1978). Lactoferrin has a broad-spectrum antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Streptococcus mutans* and *Candida albicans*, among others (Arnold *et al.* 1980, Lonnerdal & Iyer 1995). Lf has also been shown to enhance the activity of some antimicrobials *in vitro* (Sanchez & Watts 1999, Diarra *et al.* 2002).

Evidence from a number of studies indicates that the antimicrobial activity of Lf is more complex than simple Fe chelation. Lf has bactericidal activity and can kill susceptible bacteria by a mechanism distinct from sequestering of Fe (Arnold *et al.* 1980, Dalmastrì *et al.* 1988, Ellison *et al.* 1988, 1990). Bellamy *et al.* (1992) established that the antimicrobial domain is near the N-terminus of Lf in a region distinct from its iron-binding sites. Apo-Lf (iron-free Lf) was shown to increase bacterial cell membrane permeability and directly damage the outer membrane of Gram-negative bacteria (Ellison *et al.* 1988, 1990).

Normal bovine milk contains low concentrations of Lf, approximately 0.1 mg/ml or less, but in dry udder secretion Lf concentration is markedly higher and can reach a level of 20

mg/ml or higher (Schanbacher et al. 1997, Welty 1976). During the dry period, the udder is very resistant to coliform infections, mostly due to the high Lf content of the secretion (Oliver & Bushe 1987). In mastitic cows, Lf concentrations of the milk have been shown to increase dramatically and can range from 0.3 mg/ml to 2.3 mg/ml (Harmon et al. 1976, Kawai et al. 1999).

Lf may have therapeutic potential in mastitis (Diarra et al. 2002, Lohuis et al. 1995). It could partly replace the use of antimicrobials, which cause problems due to residues in milk and the risk for emergence of resistance. Studies on the *in vitro* susceptibility of udder pathogens to Lf are, however, scant. The aim of this study was to determine the antibacterial activity of Lf against bovine udder pathogens *in vitro*.

## Materials and methods

### *Lactoferrin*

Bovine Lf was purified from cheese whey or concentrated cheese whey by the expanded bed absorption chromatography method (Isomäki 1999). Iron content of native Lf was approximately 8%-15% (Isomäki 1999). Lf was stored frozen at -20°C and sterile-filtered (32 mm Acrodisc PT Syringe Filters 0.8/0.2 µm, Gelman Laboratory REF: 4658) before use. The final concentration of Lf in the product was 35.5 mg/ml (Isomäki 1999). Purity of Lf was tested in SDS-page (sodium dodecyl sulphate polyacrylamide gel electrophoresis) (Isomäki 1999). Apo-Lf was prepared from Lf by citrate dialyzing (Dionysius et al. 1993), and its iron content was approximately 4%.

### *Growth media*

Two growth media were used for bacterial cultures: the commercial Iso Sensitest-Broth (ISB CM473, Oxoid Ltd., Basingstoke, Hampshire, England), and whey. Whey was prepared from 3 liters of fresh raw milk obtained from the uni-

versity dairy herd by high-speed centrifugation of defatted milk (32600 g for 60 min at 4°C). Aliquots of 40-ml whey were sterile-filtered and frozen immediately after preparation for later use.

### *Bacterial isolates and the preparation of inoculum*

Five isolates of *E. coli*, *S. aureus*, and coagulase negative-staphylococci (CNS), 2 isolates of *P. aeruginosa* and 2 isolates of *K. pneumoniae* originally isolated from subclinical or clinical cases of bovine mastitis were used. These isolates were received from the mastitis laboratory of the Faculty of Veterinary Medicine and the National Veterinary and Food Research Institute, Helsinki. One of the *S. aureus* isolates was the reference isolate M60 kindly provided by Dr. A. J. Guidry (Immunology and Disease Resistance Laboratory USDA, Beltsville, USA). During the experiment, bacteria were maintained on blood agar plates at 8°C. To adapt the bacterial isolates to grow in whey, they were grown overnight at 37°C in a growth medium consisting of 2/3 Iso Sensitest-Broth and 1/3 sterile whey. The cultures were tested using Gram-staining for purity. Bacteria were harvested by centrifugation (5000 g for 10 min) and washed twice between centrifugations using sterile saline (0.9% NaCl, 20°C). A suspension containing approximately 10<sup>9</sup> colony-forming units (CFU) in 0.9% NaCl was prepared according to the McFarland standard (bioMérieux sa, 69280 Marcy l'Etoile, France) by spectrophotometry (550 nm, Hitachi U-2000, Hitachi, Ltd., Tokyo, Japan). Bacterial suspension was diluted to the final concentration of 1.5×10<sup>3</sup> CFU/ml used in each well.

### *Analysis of bacterial growth by turbidometry*

Bacterial growth was measured using turbidometry (Bioscreen instrument, Labsystems, Helsinki, Finland). The instrument is a fully au-

Table 1. Effect of Lf on bacterial growth in ISB culture, measured by lag time and maximum absorbance.

Bacteria	Lag time (h)			Maximum absorbance		
	Mean	Min-max	SD	Mean	Min-max	SD
<i>Escherichia coli</i> (n=5)						
Control	3.8	3.0-4.0	0.45	0.91	0.70-1.05	0.15
0.67 mg/ml Lf	6.6	5-9	1.52	0.67 *	0.57-0.73	0.06
1.67 mg/ml Lf	9.0	5-15	4.1	0.52 *	0.25-0.70	0.18
2.67 mg/ml Lf	10.6	6.0-20.0	6.0	0.42 *	0.11-0.68	0.24
<i>Staphylococcus aureus</i> (n=5)						
Control	4.8	4.0-5.0	0.45	0.60	0.49-0.65	0.07
0.67 mg/ml Lf	7.2	5.0-11	2.4	0.55	0.42-0.69	0.11
1.67 mg/ml Lf	8.4 *	6.0-12	2.2	0.52	0.40-0.66	0.10
2.67 mg/ml Lf	8.4 *	6.0-12	2.2	0.49 *	0.40-0.64	0.10
<i>Coagulase-negative staphylococci</i> (n=5)						
Control	6.4	5.0-10	2.2	0.56	0.53-0.60	0.03
0.67 mg/ml Lf	12.2	6.0-20	7.2	0.36	0.10-0.56	0.23
1.67 mg/ml Lf	12.2	6.0-20	2.2	0.34	0.11-0.52	0.21
2.67 mg/ml Lf	12.4	7.0-20	7.0	0.34	0.10-0.55	0.21

Lag time = time from the beginning of incubation until the time-point when the absorbance began to increase; maximum absorbance = highest absorbance value measured during the 20-h incubation period.

Statistically significant difference when compared with negative control: \*  $p \leq 0.05$

tomated analyzing system for measuring bacterial growth using the vertical light bath with wide band absorption principle; 200 individual samples can be run simultaneously. Each well contained 100  $\mu$ l of ISB broth or 150  $\mu$ l of whey as the growth medium, 50  $\mu$ l of bacterial suspension, and 50  $\mu$ l of Lf concentrate. Physiological saline was added to bring the final volume to 300  $\mu$ l: 50  $\mu$ l and 100  $\mu$ l in ISB and whey wells, respectively. Tested amounts of Lf were 200  $\mu$ g (the final Lf concentration in the well was 0.67 mg/ml), 500  $\mu$ g (1.67 mg/ml), and 800  $\mu$ g (2.67 mg/ml). Control wells contained all components except Lf, which was replaced by 50  $\mu$ l of 0.9% saline. Five parallel wells were used. Wells between whey and ISB wells were filled with 0.9% NaCl to prevent cross-contamination. The wells on 2 100-well plates were

covered and preincubated in the Bioscreen instrument for 30 min at 37°C. The change in turbidity was monitored automatically every hour for 20 h at 37°C. The plates were shaken 10 min before each measurement. Two *E. coli* and 2 *S. aureus* isolates were also tested with Apo-Lf. The lag time (time from beginning of incubation until the time-point when absorbance began to increase), slope (slope of the growth curve in logarithmic growth phase), and maximum absorbance (highest absorbance value measured during the 20-h period) were used as variables describing the bacterial growth. After the 20-h incubation period, bacterial survival and bactericidal effect of Lf in the wells were confirmed by culturing aliquots of 10 ml on blood agar plates and incubating the plates overnight at 37°C.

### Statistical methods

The effect of different Lf concentrations on lag time, slope, and maximum absorbance was tested by repeated measures analysis of variance with concentration as a within factor. The significance of concentration was evaluated by Greenhouse-Geisser adjusted p-values. Concentrations of 0.67, 1.67, and 2.67 mg/ml of Lf were further compared with a negative control.

### Results

Results of growth inhibition by Lf in ISB for *E. coli*, *S. aureus*, and CNS are shown in Table 1. The best inhibitory activity of Lf in the ISB was seen against *E. coli* and *P. aeruginosa* (data not shown for the latter). The typical growth curves of *E. coli* in the ISB broth with different concentrations of Lf are shown in Fig. 1. The inhibitory effect of Lf for *E. coli* was concentration-dependent (Fig. 2), and variation between

the 5 isolates of *E. coli* was small. None of the isolates was totally resistant to Lf. The effect of Lf on the maximum absorbance and the slope of *E. coli* in ISB was statistically significant ( $p = 0.025$  and  $p < 0.001$ , respectively), whereas the effect on the lag time was not significant ( $p = 0.065$ ).

The growth of 2 isolates of *P. aeruginosa* was clearly inhibited in ISB. In contrast, the growth of 2 *K. pneumoniae* isolates was hardly inhibited at all. In whey, *K. pneumoniae* showed variable susceptibility and the results were contradictory (data not shown).

The isolates of CNS and *S. aureus* in ISB showed more variation to Lf than *E. coli*. Lf had significant effects on lag time ( $p = 0.014$ ) and maximum absorbance ( $p = 0.014$ ) of *S. aureus*, while no significant effects were seen for CNS (Table 1). As regards the slope, a statistically significant difference was present between Lf

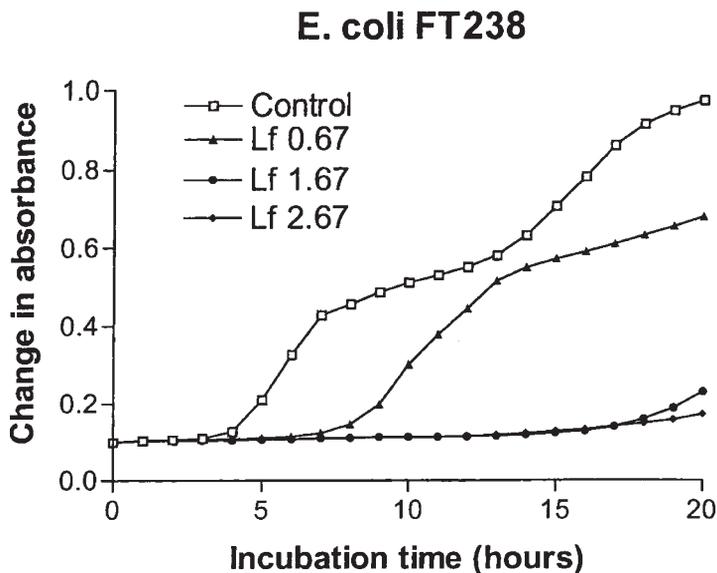


Figure 1. Growth curves of *E. coli* FT238 in Iso Sensitest-Broth (ISB) with concentrations of 0.67, 1.67, and 2.67 mg/ml lactoferrin (Lf) and without Lf.

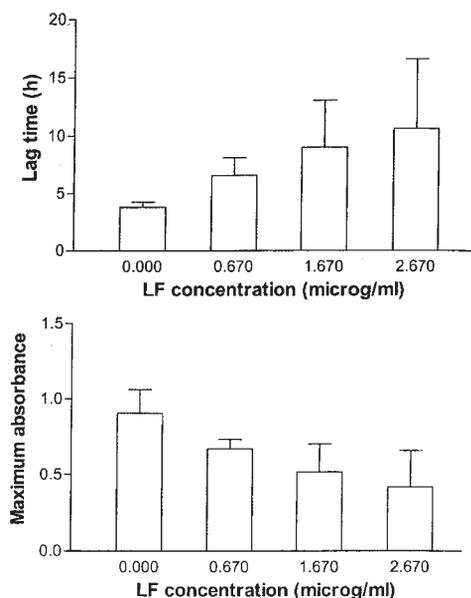


Figure 2. Mean and SD of lag time and maximum absorbance of five *E. coli* isolates in ISB with concentrations of 0.67, 1.67, and 2.67 mg/ml Lf and without Lf.

concentration and the control in the growth of *S. aureus* ( $p = 0.001$ ) and CNS ( $p = 0.002$ ). The growth of four CNS isolates was somewhat in-

hibited by Lf, but one was totally resistant. Three isolates of *S. aureus* were more susceptible to Lf than the 2 other isolates at 0.67 mg/ml of Lf. The results for *S. aureus* in whey are presented in Table 2. Lf concentration had a significant inhibitory effect on lag time ( $p = 0.015$ ), maximum absorbance ( $p = 0.011$ ), and slope ( $p = 0.027$ ) of *S. aureus* in whey. The initial absorbancies in whey wells were higher than in ISB cultures ( $\sim 1.0$ ) because whey is more turbid than ISB. The growth of *E. coli*, *P. aeruginosa*, and CNS isolates was so poor in normal whey that it was not possible to draw any conclusions about the effect of Lf in that medium.

Two isolates of *E. coli* and 2 isolates of *S. aureus* were also tested with Apo-Lf in ISB. The results were similar to those of native Lf (data not shown).

## Discussion

Our results demonstrate that bovine Lf *in vitro* is bacteriostatic towards some udder pathogens. The most interesting finding was the clear inhibitory activity of Lf against *E. coli*, which is in agreement with many previous studies (Dionysius *et al.* 1993, Nonnecke & Smith 1984, Rainard 1986). Unfortunately, the most

Table 2. Effect of Lf on the growth of *S. aureus* in whey culture, measured by lag time and maximum absorbance. The initial absorbancies in whey wells were higher than in ISB cultures ( $\sim 1.0$ ) because whey is more turbid than ISB. For explanations see Table 1.

Bacteria	Lag time (h)			Maximum absorbance		
	Mean	Min-max	SD	Mean	Min-max	SD
<i>Staphylococcus aureus</i> (n=5)						
Control	10.6	8.0-13	1.82	1.66	1.12-2.22	0.48
0.67 mg/ml Lf	14.2	8.0-20	5.6	1.23 *	0.95-1.59	0.27
1.67 mg/ml Lf	16.8 *	10-20	4.6	1.05 *	0.87-1.28	0.16
2.67 mg/ml Lf	20 **			- *		

- = no bacterial growth

Statistically significant difference when compared with negative control:

\*  $p < 0.05$     \*\*  $p < 0.01$

important target pathogen, *E. coli*, was in our study unable to grow in whey prepared from bulk milk with low somatic cell count. Rainard (1986) tested the *in vitro* susceptibility to Lf of 35 *E. coli* isolates, which had originally been isolated from clinical or subclinical bovine mastitis. He found that most of the isolates were completely inhibited by 0.1 mg/ml Apo-Lf at the end of the 16-h incubation period. A few isolates partially resisted the bacteriostatic action of Lf, but none was totally resistant. Nonnecke & Smith (1984) reported bacteriostatic, but not bactericidal, activity of bovine Lf against Gram-negative mastitis-causing bacteria *E. coli* and *Kl. pneumoniae*. Dionysius et al. (1993) reported that an Lf concentration of 1.0 mg/ml inhibited growth of all 19 isolates of enterotoxigenic *E. coli* isolated from porcine enteritis. The degree of inhibition was strain-dependent. Bacterial killing occurred at relatively high initial concentrations of bacteria ( $5 \times 10^3$  CFU/ml), but bacteriostatic effects were seen even at higher concentrations. Contradictory results have also been found; Sanchez & Watts (1999) did not see effect of Lf alone at concentrations from 0.5 to 3 mg/ml on three *E. coli* strains isolated from bovine mastitis. Dionysius et al. (1993) found no significant differences in the activity between native (32% Fe) and Apo-(<1% Fe) Lf. Bhimani et al. (1999) pointed out that Apo- and Fe-saturated forms of bovine Lf were equally effective against experimental *S. aureus* in *in vivo* infections in mice; bovine Lf with different degrees of iron saturation (9%-97%) was found to be similar. We conducted limited testing using Apo-Lf, the results being comparable with those of native Lf. However, because Apo-Lf is not a realistic candidate for potential use in cows, we focused on native Lf. We used whey as a growth medium because it simulates the environment of the milk compartment of the cow udder. Whey from milk of

healthy cows is known to inhibit the growth of a number of bacterial species (Maisi et al. 1984). Whey prepared from milk of mastitic cows may have been a better medium for our studies, but it would have been difficult to standardize the medium and compare our results with those of other authors.

The mechanism by which Lf inhibits bacterial growth has not been fully elucidated. Early studies attributed such effects to the acquisition of essential Fe from the environment, but more recent findings have implicated wider cell interactions. Lf damages the outer membrane of bacteria, with a concomitant release of LPS from Gram-negative bacteria. The ultrastructural alterations caused by Lf to the bacteria enhance the activity of some antimicrobial agents (Sanchez & Watts 1999, Diarra et al. 2002), and one approach could be to combine Lf with antibiotics in treating infections. We decided to test Lf alone to avoid the problems related to the use antibiotics and to see the real net effect of Lf against several bacteria species. Diarra et al. (2002) demonstrated a synergistic effect between Lf and penicillin against three *S. aureus* strains tested. Lf alone showed a weak inhibitory activity which agrees with our results. Lf can also bind LPS and at least partly block its detrimental effects (Appelmeik et al. 1994). Zhang et al. (1999) demonstrated *in vitro* and in an experimental mouse model that the *E. coli* endotoxin-neutralizing capability of human Lf was derived from a 33-mer synthetic peptide, lactoferricin. Lf or lactoferricin could potentially be used for the treatment of endotoxin-induced septic shock. Gram-negative bacteria, mainly *E. coli*, cause severe mastitis in lactating cows, which may result in endotoxin shock and death. Lf could be a potentially useful treatment for this condition, but its efficacy should be tested using *in vivo* studies.

## Conclusions

The antibacterial effects of Lf could only be demonstrated in the ISB medium, as bacterial growth in whey was weak and variable. The best inhibitory activity of Lf was seen against Gram-negative *E. coli* and *P. aeruginosa*. The variation in the susceptibility of the 5 isolates of *E. coli* to Lf was small, and none of the isolates was totally resistant. The response of *S. aureus* and CNS isolates to Lf was more variable. Our findings confirm that bovine Lf *in vitro* is antibacterial towards some major pathogens.

## Acknowledgements

We thank our cooperators Riitta Keiski, Liisa Myllykoski, Kimmo Vahtola, Carita Berg, and Päivi Anttila at the University of Oulu, Finland, who provided the Lf. We also thank Arto Ketola, M. Sc. (Soc.), for statistical analysis and Ilkka Saastamoinen (technician) and Susann Sunqvist (laboratory assistant) for their technical support.

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## Sammanfattning

*Antibakteriell effekt av bovint laktoferrin på juverpatogener.*

Den antibakteriella effekten av laktoferrin (Lf) testades på isolat av *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*) och koagulas-negativa stafylokocker (KNS), samt på *Pseudomonas aeruginosa* (*P. aeruginosa*) och *Klebsiella pneumoniae* (*K. pneumoniae*) som ursprungligen isolerats från bovin mastit. Lf-koncentrationer på 0.67 mg/ml, 1.67 mg/ml och 2.67 mg/ml användes. Tillväxten av juverpatogener övervakades med turbidometri antingen i buljongkultur eller i vassle framställd ur normal mjölk. Vi fokuserade på 3 olika tillväxtvariabler: retardationstid, lutning och maximal absorbans för bakteriernas tillväxtkurvor. En tillväxthämning observerades i buljong men knappt alls i vassle. Tillväxten av *E. coli* och KNS-isolaten i vassle var inte tillräcklig för att kunna dra några slutsatser. Den effektivaste hämmande Lf-aktiviteten observerades emot gram-negativa bakterier, förutom *K. pneumoniae*. Alla fem *E. coli*-isolat uppvisade liknande tillväxtmönster. Den tillväxthämmande effekten av Lf var beroende av koncentrationen. Generellt sett var 0.67 mg/ml en för låg koncentration i buljongkultur och vassle för att uppnå en signifikant hämmande effekt.

(Received January 10, 2003; accepted January 12, 2003).

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