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Anti-Bacterial Activity of Bovine Milk Lactoferrin Against Some Mastitis Causative Pathogens with Special Regard to Mycoplasmas

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Abstract: From a private farm a total number of 36 milk samples was collected from clinically mastitic cows. The bacteriological examination showed isolation of *Mycoplasma bovis*, *S. aureus*, *E. coli*, *P. aeruginosa* and *S. agalactiae* as single or mixed infection. Lactoferrin concentrations (LFC) in the clinical mastitic cow's milk were higher (1.4 to 3.2 mg/ml) than normal milk. The average LFC in milk from infected cows with *M.bovis* or *S. aureus* was obviously high either in single or mixed infection. The total number of 36 clinically mastitic milk samples was positive to *M. bovis* antibodies by using ELISA test while only 38.9% of the examined samples were positive for *M. bovis* by cultivation method. The most interesting finding in this assay was the antibacterial effect of lactoferrin (Lf) on *M. bovis*, *M. bovigentalium* as well as on *P. aeruginosa* and *S. agalactiae*, originally isolated from bovine mastitis. Inhibitory activity of Lf was seen in all tested strains. The most effective inhibitory activity of Lf was seen against *P. aeruginosa* and *S. agalactiae*, followed by inhibitory activity of Lf on the two *Mycoplasma* strains. Inhibition of growth by Lf was concentration-dependent. The concentration of 1 mg/ml was generally too low for a marked inhibitory effect.

Key words: Milk · Lactoferrin · Mastitis · Bacteria · Mycoplasmas

INTRODUCTION

Milk is a vital nutritional source for the offspring of all mammals, including humans. In addition to its nutritional value, it is a rich source of proteins including lactoferrin(Lf). Lactoferrin is a truly multifunctional protein that has been studied extensively over the past decades [1]. Lactoferrin is an iron-binding glycoprotein synthesized by specific granules in polymorphonuclear leukocytes [2] and glandular epithelial cells 3]. ince acteria equired ron or rowth, Lf can inhibit bacteria by chelating iron under certain conditions [4-6]. In addition to its iron-binding function, it has been reported that Lf may directly kill certain bacterial strains [7-10] or may weaken bacterial resistance by adhesion to the surface of bacteria [11]. Lf in milk seems to play a key role in the defense mechanisms in the mammary gland of dairy cows. However, the influence of Lf in preventing infection has not yet been fully studied.

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 [12,13]. During the dry period, the udder is very resistant to coliform infections, mostly due to the high Lf content of the secretion [14]. In mastitic cows, Lf concentrations of the milk have been shown to increase dramatically and can range from 0.3 to 2.3 mg/ml [15, 16].

Lf may have therapeutic potential in mastitis [17, 18]. It could partly replace the use of antimicrobials, which cause problems due to residues in milk and the risk for emergence of resistance.

Mycoplasmal bovine mastitis is potentially a highly contagious disease that can cause severe economic problems in affected herds. The purchase of replacement heifers and cows are frequently the origin of Mycoplasmal mastitis. Detection of *Mycoplasma* infected cows by culture of milk is the only technique used for routine diagnosis at present. However, this process is rather laborious and time consuming whereas the final results are available only after several days [19]. Studies on the *in vitro* susceptibility of udder pathogens to Lf are, however, scant.

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The aim of this study was to determine the antibacterial activity of Lf against bovine udder pathogens *in vitro*, especially that have high antibiotic resistance.

MATERIALS AND METHODS

Milk Samples: From a private farm, a total number of 36 milk samples was collected from clinically mastitic cows showed signs of inflammation as hotness, redness, swelling and painful sensation on palpation. Three composite milk samples of all four quarters were collected, cooled and maintained at approximately 4°C.

Isolation and Identification of Bacteria: The samples were transported to the laboratory, one sample directed to mycoplasmas examination, the second milk sample was used for whey preparation for ELISA test and the third milk sample was pre-incubated at 37°C / 24h then 50 μL was streaked onto blood agar (with 8% sheep blood), MacConkey agar, Manitol salt agar, Edward's media for detection of bacteria. Colonies were described for their morphological characters appearance and haemolytic activity. Suspected colonies were picked up and examined microscopically in Gram stained films before being transferred into semisolid or slope agar to be subjected for further identification. Pure cultures of the isolates were identified according to Cruickshank *et al.* [20] and Quinn *et al.* [21]. Isolation and identification of *Mycoplasmas* from milk samples were done according to Ruhnke and Rosendal [22].

Preparation of the Whey from the Tested Milk Samples for ELISA, [23]: Whey samples were prepared by addition of 5.0 μL of a 10 % rennet solution/ ml milk and incubation in water bath at 32°C for 15 to 30 minutes for casein precipitation. Casein was removed together with the fat by centrifugation in eppendorff 3200 rpm table centrifuge and then the whey was collected and frozen at-20°C until used for detection of Lf concentration and *Mycoplasma* antibodies by using ELISA test.

Preparation of Mycoplasma Antigen: One ml of an active growing approximately 10^7cfu/ml estimated according to Rodwell and Whitcomb [24] culture of *M. bovis* was first transferred into 9ml of broth (supplemented with horse serum, yeast extract DNA, Penicillin G sodium and thallium acetate). After 72 hr incubation, the contents were aseptically poured into 90 ml brain heart infusion broth and then after incubation period of 72 hr the content flasks (approximately 100 ml) were transferred to the final propagation flask containing 900ml of broth. After 72hr incubation, the *Mycoplasma* cells were harvested by centrifugation at 18000 rpm in cooling centrifuge for 1hr. Sterility checks were routinely carried out with each passage of the growth to eliminate the possibility of bacterial contamination. After two thorough washing in sterile PBS, cells were centrifugation resuspended in 15ml of PBS [22]. This stock suspension was sonicated (Bandelin electronic UW2070 Sonicator) at 20kc/min for 10 successive times on ice and one minute interval [25].

ELISA Test for Detection of *M. bovis* Antibodies: ELISA test was carried out to estimate the *M. bovis* antibodies in milk whey of the clinical milk samples under the study according to Hajkova and Jurmanova [26].

ELISA Test for Detection of Lactoferrin Concentration: lactoferrin concentrations were detected by using anti-bovine lactoferrin (obtained from Organon Teknika Corp. West Chester, U.S.A.) according to Rejman *et al.* [27].

The Bacteriological Properties of Lactoferrin [28]: Organisms and Growth Condition: The tested organisms (*Mycoplasma bovis* (*M. bovis*), *Mycoplasma bovigentalium(M. bovigentalium)* isolated from other milk samples), *P. aeruginosa* and *S. agalactiae* isolates were kept in soft agar. To obtain suspensions for the study the isolates were grown on their specific media then one colony was placed in 10 ml Muller Hinton Broth (MHB) and incubated for 16- 18 hr at 37°C.

Bovine Lactoferrin (Sigma Chemical Co. St. Louis, MO): Solution of Lf (43.5 mg / ml) was prepared from frozen stock at -20 °C. in aliquots of 0.3 ml per vial.

Effect of Lactoferrin on Growth of the Tested Organisms:

- Bacteria were grown in MHB and a final concentration of 1 X 10^5-10^6 Cfu/ml was used.
- A volume of 0.1 ml was added to each well in a tissue culture plate.
- Lactoferrin was thawed and diluted to contain final concentrations of 1and 3 mg / ml, each well-received 0.5 ml of the appropriate dilution of lactoferrin.
- The control consisted of bacteria without lactoferrin.
- The mixture was incubated at 37°C.
- Aliquots were removed after 1, 3, 6, 12, 24 hours and serially diluted, then plated overnight at 37°C on Muller Hinton Agar (MHA) to be counted.

RESULTS

Pathogens Isolated from Cow's Milk with Clinical Mastitis: The average LFC in milk from dairy cows with clinical mastitis ranged between 1.4 to 3.2 mg/ml, was significantly higher than that from normal cows (0.53mg/ml). The average LFC in milk infected with *M.bovis* or *S. aureus* was significantly high either in single or mixed infection (Table 1).

Detection of *M. Bovis* by Culture Method and ELISA Test in the Tested Milk Samples: The total number of 36 milk samples collected from clinically mastitic cows was positive for *M. bovis* antibodies by using ELISA test (Table 2).

The Study of Antibacterial Activity of Lactoferrin on the Tested Pathogens Isolated from Mastitic Cows: There was severe inhibition in the growth of *M. bovis,* especially within 3-6 hr incubation with Lf (Fig. 1). The same result was noted in *M.bovigentalium,* especially after 6hr incubation (Fig. 2). Moreover, Lf had severe bacteriostatic effect on *P. aeruginosa* (Fig. 3) and *S. agalactiae* after 12hr incubation. The concentration of 1 mg/ml was generally too low for a significant inhibitory effect as the inhibition of growth by Lf was concentration-dependent (Table 3).

Table 1: Relationship between lactoferrin (Lf) and bacteriological findings in milk samples collected from the examined cows.

Isolated bacteria	Total no. of clinical milk samples (36)	%	Average Lf concentration (mg/ml)
A- single infection:			
S. aureus	5	13.90	2.4
E. coli	5	13.90	2.5
M. bovis	2	5.56	2.7
S. agalactiae	1	2.78	2.2
P. aeruginosa	1	2.78	1.6
B- mixed infection:			
S. aureus + *E. coli*	1	2.78	1.4
S. aureus + *M. bovis*	2	5.56	2.5
S. aureus + *P. aeruginosa*	1	2.78	3.2
CNS + *E. coli*	1	2.78	2.2
E. coli +*M. bovis*	1	2.78	2.5
S. aureus +*E. coli* +*M. bovis*	8	22.22	2.5
S. aureus +*P. aeruginosa* +*M. bovis*	1	2.78	2.6
Negative for bacteria	7	19.40	2.3

Table 2: Comparison between culture method and ELISA test

Status	Isolation	ELISA
Positive	14 (38.9%)	36 (100%)
Negative	22 (61%)	_
Total milk samples	36	36

Table 3: Antibacterial activity of Lf (3mg\ ml) on the tested bacterial strains within 24hr incubation (cfu / ml)

	Incubation period			
	Count			
Microorganisms	3hr	6hr	12hr	24hr
M. bovis	5,800	40,000	450,000	800,000
Control of M. bovis (without Lf)	27,000	80,000	690,000	uncountable
M.bovigentalium	7,900	8,000	90,000	180,000
Control of M.bovigentalium (without Lf)	22,000	25,000	170,000	uncountable
P. aeruginosa	100,000	20,000	1,000	10,000
S. agalactiae	Mild inhibition	Moderate inhibition	Severe inhibition	Moderate inhibition

N.B. *S. agalactiae* couldn't be counted as the colonies are very small but the inhibition was very clear.

Fig. 1: *M. bovis* with Lf (3mg/ml) after 6hr incubation (left side) in contrast to *M. bovis* control without Lf (right side)

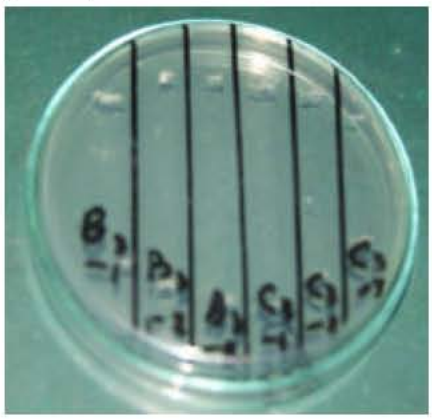

Fig. 2: *M. bovigentalium* with Lf (3mg/ml) after 6hr incubation (left side) in contrast to *M.bovigentalium* control without Lf (right side)

Fig. 3: *P. aeruginosa* with Lf (3mg/ml) after 6hr incubation (right side) in contrast to *P. aeruginosa* control without Lf (left side)

DISCUSSION

Lactoferrin (Lf), an iron-binding glycoprotein, is well known to act as a general antibacterial molecule during infections of mammary gland [29]. One of the first antimicrobial properties discovered for Lf was sequestering of iron from bacterial pathogens, thus inhibiting bacterial growth, [4, 11]. This was believed to be the sole antimicrobial action of Lf for a long time and was supported by several studies demonstrating that only apo-lactoferrin possessed antibacterial activity and that this activity was reduced upon iron saturation [7, 30].

In the present study, the average Lf concentration (LFC) in milk from dairy cows with clinical mastitis was significantly higher than that of normal cows. The average LFC in milk from quarters infected with *M.bovis* or *S. aureus* was significantly high either in single or mixed infection. These results were in accordance with Kawai *et al*. [16] and Hagiwara *et al*. [31] who demonstrated that, LFC in milk of cows with clinical mastitis can range from 0.3 to 2.3 mg/ml; these concentrations are generally higher than in normal cows or those with subclinical mastitis. More over, Kawai *et al*. [16] determined LFC in the milk from dairy cows with clinical mastitis to evaluate the relationship between LFC in milk and the non-specific defensive capability of the udder. Also he mentioned that the mean LFC in milk from quarters infected with *M. bovis* or *S. aureus* was significantly higher than that of quarters infected with CNS. Hagiwara *et al*. [31] said that the mean LFC in milk from quarters infected with *S. aureus* or *streptococci* were significantly higher than in milk from quarters infected with CNS and *Corynebacterium bovis*. While Asfour [32] recorded significantly higher LFC in clinically mastitic cows and buffaloes (2.79 and 3.88 mg/ml, respectively) and generally the infected quarters with *S. intermedius* had mean LFC (3.08 mg/ml) greater than that infected with *E. coli, coagulase negative staphylococci* (CNS), *S. agalactiae* and *S. aureus* (2.79, 2.65, 2.52 and 1.93 mg/ml, respectively).

Thus, variations in Lf concentration in normal lactating and mastitic cows and its well-documented antibacterial activity make it a candidate marker for genetic selection towards reduced susceptibility in dairy animals. The gene encoding for bovine Lf is organized into 17 exons and polymorphisms within bovine Lf gene have been reported to be associated with susceptibility/ resistance to mastitis and even with some economically important production traits [33-36].

In this investigation *M.bovis* was isolated either as single cause of clinical mastitis or mixed with other contagious bacteria (*S. aureus* and *S. agalactiae*) or mixed with environmental bacteria (*E. coli,* CNS and *P. aeruginosa*).These results agreed with Ghadersohi *et al.* [37] who reported the isolation 43 and 62% in herds positive for *M. bovis* in Victoria and North Queensland, respectively. Moreover, *M. bovis* was detected in 76% of cows of which 19% had *M. bovis* alone as a single infection, 17% had *M. bovis* mixed with other major mastitis pathogens and 40% had *M. bovis* mixed with non-major mastitis pathogens. On the other hand, *M. bovis* was detected in 8.2% of quarter milk samples collected from clinical mastitic cases in Northern Greece. Diagnosis of *Mycoplasma* infection in milk has developed primary up on microbiological culture of udder secretions. Although the reliability of this method is reasonably satisfactory, it is time consuming. Incubated plates must be at least 2-3 days before colonies may be observed and shouldn't be considered as negative before 7 days of incubation. Determination of *Mycoplasma* species has required further study which may be biochemical or immunological work [38] so we decided to compare between the cultural results and the results of detection of Mycoplasmas antibodies by ELISA test.

In this work, the total number of 36 milk samples collected from clinically mastitic cows was positive to *M. bovis* antibodies by using ELISA test. Correspondingly, the cultivation results revealed that, only14 (38.9%) cases were *M. bovis* positive. Tola *et al.* [39] explained the higher results of ELISA than the isolation results as the bacteriological isolation of *M. bovis* is the gold test for diagnosis, but discontinues shedding of *Mycoplasma*, progressive acidification of milk may cause reduced viability of *Mycoplasma*. Also, high percentage of bacterial flora may hinder the growth of *Mycoplasma* in selective agar plates and making the identification of its colonies difficult.

Our results demonstrated that bovine Lf has *in vitro* bacteriostatic property towards some udder pathogens. The most interesting finding was the clear inhibitory activity of Lf against *P. aeruginosa*, which is in agreement with many previous studies. Kutila *et al.* [40] tested the antibacterial effect of Lf on isolates of *E. coli, S. aureus* and *coagulase-negative staphylococci* (CNS) as well as on *P. aeruginosa* and *K. pneumoniae*, originally isolated from bovine mastitis and demonstrated that the most effective inhibitory activity of Lf was seen against *E. coli* and *P. aeruginosa*. The inhibition of bacterial growth by Lf was concentration-dependent. Odeh and Quinn [41] and Singh *et al.* [42] reported that, the biofilm formation, which represents a colonial organization of bacterial cells, was a well studied phenomenon, especially for *P. aeruginosa* where it has been proposed to occur in patients suffering from cystic fibrosis. Through biofilm formation, bacteria also become highly resistant to host cell defense mechanisms and antibiotic treatment. However, interestingly, Lf inhibits biofilm formation of *P. aeruginosa* at concentrations lower than that those needed to kill the bacteria or prevent its regular growth. Asfour [32] recommended that 3mg/ml Lf alone has bactericidal effect on *E.coli* and electron microscopic examination showed sever obvious effect on *E. coli* cells, explained as severe irregularity and lysis of the bacterial cell wall, cell fragments, strong vaculation and shrinkage of the cytoplasm of the bacterial cell, complete destruction and loss of the bacterial flagella, these results showed complete change in the morphology of the bacterial cell as a result of strong effect of Lf alone on the *E. coli.* Moreover, Lf has bacteriostatic effect on *S.aureus* isolated from mastitic milk and has synergistic effect for penicillin and cephaprin sodium. Administration of Lf hydrolysate into mammary glands of cows with sub-clinical mastitis caused reduction in the number of bacteria shed in milk [43]. Further Lf has been found to reduce the invasion of mammary epithelial cells in culture by *S. aureus*, one of the common etiological agents for mastitis in cattle [44].

Nonnecke and Smith [45], reported bacteriostatic, but not bactericidal, activity of bovine Lf against Gram-negative mastitis-causing bacteria *E. coli* and *K. pneumoniae*. Diarra *et al.* [17] 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 (5×10^3 cfu/ml), but bacteriostatic effects were seen even at higher concentrations. Contradictory results have also been found; Schanbacher *et al.* [12] didn't see effect of Lf alone at concentrations from 0.5 to 3 mg/ml on three E. coli strains isolated from bovine mastitis.

The mechanism by which Lf inhibits bacterial growth has not been fully elucidated. Early studies attributed such effects to the acquisition of essential iron 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 ultrastuctural alterations caused by Lf to the bacteria enhance the activity of some antimicrobial agents and one approach could be to combine Lf with antibiotics in treating infections [17, 28].

Crystal structure studies of Lf have demonstrated that the protein has large cationic patches on its surface [46], facilitating direct interaction with anionic Lipid A, a component of the lipopolysaccharide (LPS) of Gram-negative bacteria [47, 48]. Such interaction can damage the bacterial membrane, altering the outer membrane permeability and resulting in the release of LPS. By damaging the bacterial membrane, Lf is able to increase the antibacterial effect of commercial drugs [49]. This effect was easily inhibited by divalent cations like Mg2 and Ca2; leading to hypothesize that Lf could work as a cation chelator like EDTA, which also is known to induce LPS release from bacterial membranes [50, 51]. Direct binding of Ca2 by Lf has recently been confirmed, strengthening the cation chelator hypothesis [52] and thus also explaining the broad antibacterial spectrum of Lf [53, 54]. It was later demonstrated that Lf is also able to kill *Streptococcus mutans* through an iron-independent mechanism [55], an effect hypothesized to result from direct interaction of Lf with the bacterial cell surface [56, 57]. On the other hand, the importance of iron for bacterial growth, in combination with the iron sequestering ability of host components like Lf have stimulated bacterial strains to develop strategies to overcome iron depletion. Under iron-restricted conditions, a number of Gram-negative bacterial pathogens have developed mechanisms for acquiring iron from iron-saturated Lf [4, 11]. In this concern *Streptococcus pneumoniae* has been specifically demonstrated to recognize and bind human Lf, using a surface receptor homologous to pneumococcal surface protein A and it has been suggested that *S. pneumoniae* may use this receptor to overcome iron limitation at mucosal surfaces [58].

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.* [17] 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 Appelmelk *et al.*[47] and Zhang *et al.* [59]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.

In our work, we noticed that, Lf alone has great bacteriostatic effect on the two strains of *Mycoplasma* which considered as highly contagious organism for dairy farms. As Lf causes severe inhibition in the growth of of *M. bovis* especially within 3-6 hr incubation with Lf. The same result was noted in *M.bovigentalium* especially after 6hr incubation.

Minion *et al.* [60] mentioned that, like all mycoplasmas, *M. hyopneumoniae* must acquire essential nutrients from its host because it lacks biosynthetic pathways due to the limited coding capacity of its genome. One of these nutrients, iron, is an essential metal for almost all living systems. Iron serves as a cofactor or as a prosthetic group for essential enzymes that are involved in basic cellular functions [61]. Free iron catalyses the formation of free oxygen radicals and as such is highly toxic to cells. Therefore, iron is normally bound to specific proteins in the host such as transferrin and Lf to prevent its precipitation in aqueous systems and to protect the cell from damaging UV light and lipid peroxidation reactions. Transferrin, Lf and lactotransferrin have iron-binding constants in the range of ~1020 and transferrin is never fully saturated under normal conditions, giving the host additional iron-sequestration capabilities during bacterial infections. In this way, iron is both sequestered for use by the host and use by invading pathogens is prevented as part of the innate immune defense [62, 63].

Unlike other bacteria, *Mycoplasmas* have minimal gene coding capacity [64] and lack cell walls, so it is thought that their interactions with the host are less complex, including the acquisition of essential nutrients. Pathogenic mycoplasmas pose interesting questions concerning iron metabolism because they lack a host-free lifestyle, they have a single membrane and few studies have been performed on iron requirements. While it has not been unequivocally shown that iron is essential for growth, Bauminger *et al.* [65] showed that iron was stored in *M. capricolum* and Tryon and Baseman [66] tested the in vitro binding of human iron-sequestering Lf and transferrin by the mucosal-surface pathogens, *M. pneumoniae* and *M. genitalium* and shown that

M. pneumoniae can take up human Lf also it has a highly specific receptor for recognizing and binding Lf, but not the closely related transferring. No significant binding of Lf or transferrin by *M. genitalium* was observed. *Mycoplasmas* also contain enzymes that have been shown in other systems to contain iron, such as dehydrogenases [67].Whether *Mycoplasmas* contain complex iron-acquisition mechanisms is not known. Iron deprivation in other bacterial systems not only triggers the genes required for its acquisition but also in many pathogens is a signal for controlling virulence factor expression [68-71]. Thus, virulence factors can sometimes be identified by their response to low-iron growth conditions. In these studies, 2, 29-dipyridyl was used to chelate iron in growing *M. hyopneumoniae* cultures and experiments using microarrays showed that iron deprivation *in vitro* leads to transcriptional changes. The previous literatures may explain why the count of *Mycoplasma* strains under the present study was increased after 12hr incubation.

In conclusion, lactoferrin (Lf) has antibacterial effect of on *M. bovis*, *M. bovigentalium* as well as on *P. aeruginosa* and *S. agalactiae*, isolated from bovine mastitis. The most effective inhibitory activity of Lf was seen against *P. aeruginosa* and *S. agalactiae*, followed by inhibitory activity of Lf on the two *Mycoplasma* strains. Inhibition of bacterial growth by Lf was concentration-dependent.

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