Antibacterial activity of Lactoferrin and Lactoferricin against oral Streptococci

ANCA ROSEANU[1], PAULA FLORIAN[1], MARIA CONDEI[2],   DANA CRISTEA[2] MARIA DAMIAN[2]
[1]*Institute of Biochemistry of Romanian Academy, Splaiul Independentei 296, 060031 Bucharest, Romania*
[2]*National Institute of Research-Development for Microbiology and Immunology I. Cantacuzino, Splaiul Independentei 103, Bucharest, Romania*
*Corresponding author: Anca Roseanu; Institute of Biochemistry of Romanian Academy
 Splaiul Independentei 296, 060031 Bucharest, Romania, Tel/Fax +40 21 2239068/69*
Email: roseanua@yahoo.com

Abstract

Lactoferrin (Lf) is an iron-binding glycoprotein which exhibits antibacterial activity against a broad range of Gram-negative and Gram-positive bacteria. The mechanisms of this property are complex and involve iron sequestration as well as direct interaction with bacteria. Lactoferricin (LFcin), a Lf-derived peptide released by pepsin digestion of lactoferrin, is a more potent antibacterial compound than the native protein.
In this study the antibacterial capacity of iron–free Lf, either human or bovine, and bovine Lfcin against the growth of some oral streptococci was investigated. Both human and bovine Lf, and bovine Lfcin were found to exert bacteriostatic effects which were dependent on their concentration and the streptococcal species tested. Even within species, strains exhibited different susceptibilities to the action of Lfs and Lfcin. The most sensitive streptococcal species were S. parasanguinis, S. gordoni and S. plurianimalium. Bovine Lfcin was also bactericidal against S. plurianimalium strain 5.1 at 9mg/ml. These findings are encouraging for the use of Lf/Lfcin as compounds with antimicrobial activity against oral streptococci.

Keywords: lactoferrin, lactoferricin, streptococci, antibacterial activity

Introduction

Lactoferrin (Lf) is an-iron binding glycoprotein involved in a large spectrum of biological properties including antibacterial activity against Gram-negative and Gram-positive bacteria [1]. Initially iron chelation was considered as a major mechanism for its antimicrobial action. Now it is well established that iron-independent mechanisms such as direct interaction with bacteria leading to membrane destabilization, modulation of bacteria motility, aggregation or endocytosis into host cells, inhibition of adherence and biofilm formation are also responsible for the antimicrobial property of Lf. In addition, recently it has been suggested that the proteolytic activity of Lf could be used to degrade bacterial virulence factors [2-5]

Lactoferricin (Lfcin), a peptide derived from the N-terminal region of Lf, was reported to be a more potent antimicrobial agent than the native molecule [6, 7]. In this case, the antibacterial effects take place through the depolarization of the bacterial membrane, followed by membrane penetration and metabolic injury [8, 9].

The oral bacterial plaque is the major aetiological factor of the periodontal diseases and has also an important role in caries formation and inflammation of oral mucosa [10-12]. Oral cavities are inhabited by both commensal and pathogenic bacterial species. In some

conditions bacteria belonging to the indigenous or resident oral microorganisms can led to infectious dental diseases. Among them, streptococci species such as *S. mitis, S. gordoni, S. mutans, S. salivarius* are often associated with a high risk of caries development, abscesses, periodontal infectious or endocarditis if present in high number [13,14]. Due to their antimicrobial capacity against oral pathogens, Lf and Lfcin may have beneficial effects in prevention, treatment and combination therapy of oral diseases [15, 16].

The aim of the current study was to assess the *in vitro* effect of iron-free Lf, human (Apo-HLf) or bovine (Apo-BLf), and bovine Lfcin (BLfcin) on the growth of some oral streptococcal species.

Material and methods

Proteins

HLf and BLcin were purchased from Sigma Chemicals (St Louis, MO, USA). BLf was kindly provided by Dr. Rex Humphrey (Tatua Dairy Cooperative, New Zealand). Lfs, either human or bovine, were rendered iron-free by dialysis against citric acid as previously described [17]. The purity of proteins was checked by SDS-PAGE and was higher than 90%. Contaminating endotoxin (lipopolysaccharide) was removed from all samples using Detoxi-gel (Pierce Chemical, Co), and the endotoxin content was less than 3 EU/ml (250ngLPS/mg protein or peptide) as measured by the Limulus Amoebocyte Lysate (LAL) assay (Sigma Chemicals, St Louis, MO, USA).

Streptococcal strains

Microbiological samples were collected from the gingival sulcus, oral mucosa and mucosal aspect of the dentures from subjects (healthy, partially and totally edentulous) of the Faculty of Dentistry of University of Medicine "Carol Davila", Bucharest and the streptococcal strains isolated and identified as previously described [18]. Briefly, the samples were inoculated in Todd-Hewitt broth, plated onto Columbia agar medium supplemented with 7% defibrinated sheep blood and incubated in 10% CO_2 atmosphere. After 24-48 hours at 37 °C the culture plates were examined for morphology of colonies and each type of colony was sub-cultured in non-selective medium to obtain pure culture. Subsequently the strains were identified by Gram-staining, catalase production, biochemical tests using API galleries, Rapid ID and Vitek identification cards.

Antibacterial activity

The antibacterial capacity of different Lfs and BLfcin was evaluated by using a micro-dilution method. Briefly, test bacteria (10 µl of a 1/20 dilution of 0.5 MacFarland bacterial suspension) and different concentrations of Apo-HLf, Apo-BLf or BLfcin were incubated in 96- well plates, in growth medium (BHI), 100 µl total volume, for 24h at 37^0C. Inoculated growth medium without LFs/LFcin, and blank growth medium were used as controls. Optical readings were taken at 450/630 nm at the time of inoculation and after 24 h of incubation. The efficacy of the action of Lf/Lfcin samples against oral streptococci was evaluated by comparing the O.D values. To differentiate between bactericidal and bacteriostatic actions of lactoferrin/lactoferricin samples, the whole culture from each well was spread on Columbia agar supplemented with 7% defibrinated sheep blood. The presence of isolated colonies or the development of a semi-confluent culture was interpreted as a bacteriostatic effect. No bacterial growth was recorded as bactericidal activity.

Results and discussion

The antibacterial potential of Lf samples was evaluated on the growth of *Streptococcus mitis/ oralis* (26 strains), *Streptococcus parasanguinis* (14 strains),

Streptococcus gordoni (9 strains), *Streptococcus plurianimalium* (7strains), *Streptococcus anginosus* (4 strains), *Streptococcus cristatus* (1strain) and *Streptococcus alactolyticus* (1 strain) previously isolated from the oral cavity and identified as previously described [18]. The effect of Apo-HLf was investigated by incubation of the streptococcal strains with different concentrations of protein, between 0 and 5mg/ml. As shown in **Table 1,** the bacteriostatic activity is dependent on the lactoferrin concentration and the species tested. Thus, for 27 strains (43.5%) the effective concentrations ranged from 0.2 to 3.0 mg/ml, while no significant reduction in the bacterial growth was observed for 35 strains at 5 mg/ml. Even within the species, strains exhibit different susceptibility, ranging from sensitive to fully resistant.

Table 1. Antibacteriostatic activity of Apo-HLf and Apo-BLf

Species (number of tested strains)	Apo-HLf mg/ml (nr. sensitive strains)						Apo-BLf mg/ml (nr. sensitive strains)			
	>5	3	1.5	0.6	0.3	0.2	>5	3	0.6	0.3
S. mitis/oralis (26)	>5 (18)	3 (2)		0.6 (3)	0.3 (1)	0.2 (2)	>5 (23)		0.6 (1)	0.3 (2)
S. parasanguinis (14)	>5 (6)	3 (1)	1.5 (1)	0.6 (4)	0.3 (1)	0.2 (1)	>5 (4)	3 (1)		0.3 (9)
S. gordonii (9)	>5 (5)		1.5 (1)	0.6 (1)	0.3 (1)	0.2 (1)	>5 (7)		0.6 (1)	0.3 (1)
S. plurianimalium (7)	>5 (4)	3 (2)				0.2 (1)	>5 (6)			0.3 (1)
S. anginosus (4)	>5 (2)	3 (1)		0.6 (1)			>5 (3)		0.6 (1)	
S. cristatus (1)	>5 (0)	3 (1)								
S. alactolyticus (1)	>5 (0)			0.6 (1)						

Apo-BLf had a lower inhibitory activity than the human protein. Only 17 out of 60 strains showed a susceptibility to protein at a concentration of 0.3, 0.6 or 3.0 mg/ml of (**Table 1**), while all the other were total resistant at more than 5 mg/ml. These data were consistent with the results obtained when the treated strains were cutured on solid growth medium and the resulting colonies counted after 24 h of incubation.

The antibacterial activity of Lf has been investigated against a wide variety of bacteria [1, 19]. It was reported that a concentration of iron-free Lf between 0.5 to 1.0 mg/ml was nedeed to inhibit the growth of *S. mutans* and *S. gordoni*[20]. In comparison, our data showed that a much higher concentrations of Lf exhibited bacteriostatic activity. These differences may be the results of different growth conditions and exposure time of bacterial strains to protein in the growth media.

No inhibition was observed in the presence of iron-saturated BLf or HLf (data not shown). It is likely that the bacteriostatic effect was the result of iron sequestrion by Apo-Lf, either human or bovine. Because many periodontal pathogens require iron for growth, the iron-binding ability of Lf may contribute to its clinical significance in the treatment of oral disease.

Bovine lactoferricin exhibited a bacteriostatic activity against 25 oral streptococcal strains, the most sensitive species being *S. parasanguinis* and *S. plurianimalium*. As with the native protein, the effect was found to be concentration-and species-dependent. Thus,

lactoferricin was bacteriostatic over a concentration range of 2.0 to 9.0 mg/ml for *S. plurianimalium* (strain 24.11) and *S. parasanguinis/sanguinis* (strain 22.3) and bactericidal at 9.0 mg/ for *S. parasanguinis* (strain 5.1) (**Fig.1**). Again the results were in agreement with those obtained by growing the treated strains on solid medium. All the other strains exhibited a high resistance to the action of lactoferricin.
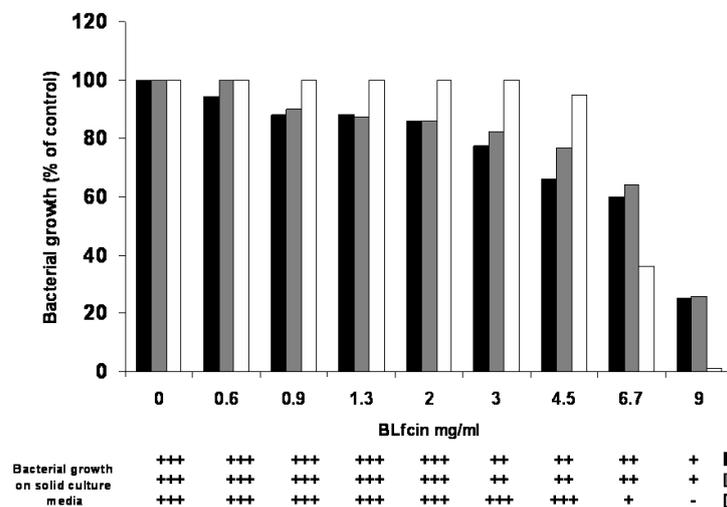

	0	0.6	0.9	1.3	2	3	4.5	6.7	9	
Bacterial growth	+++	+++	+++	+++	+++	++	++	++	+	■
on solid culture	+++	+++	+++	+++	+++	++	++	++	+	▨
media	+++	+++	+++	+++	+++	+++	+++	+	-	□

Figure 1. Effect of BLfcin on bacterial growth of *S. plurianimalium* (■), *S. parasanguinis/sanguinis* (▨) *and S. parasanguinis* (□)
        - no culture; + isolated colonies; + + semi-confluent culture; +++ confluent culture

It has been shown that oral bacteria such as *S. gordoni, S. mitis, S. oralis, S. parasanguinis, S. sanguinis, S. salivarius* are among the first group of bacteria reported to colonize the dental plaque. These species act as anchors for subsequent attachment for other bacterial species involved in biofilm establishment, a colonial organization through which bacteria become highly resistant to host defence mechanisms and the action of antibiotics [21]. The ability of Lf to interfere with surface attachment of *S. gordoni, S mutans* and other oral bacteria has been previously reported [21, 22]. Lf and/or the Lf-derived peptide Lfcin, as a consequence of their capacity to reduce bacterial growth, as demonstrated here by us and others, and to inhibit bacterial adhesion and biofilm formation, should be considered as useful antimicrobial therapeutic agents.

Acknowledgements.

References

1. S. FARNAUD, R.W. EVANS, Lactoferrin-a multifunctional protein with antimicrobial properties. *Mol. Immunol.*, 40, 395-405 (2003).
2. P. P. WARD, E.PAZ, O.M.CONNELY, Multifuctional roles of lactoferrin: a critical overview. *Cell. Mol. Life Sci.*, 62 (22), 2500-2508 (2005).
3. P.VALENTI, G.ANTONINI, Lactoferrin:an important host defence against microbial and viral attack. *Cell. Mol. Life Sci.*, 62 (22), 2576-2587 (2005).
4. D. LEGRAND, A. PIERCE, E. ELASS, C. MARILLER, J. MAZURIER, Lactoferrin Structure and Functions. *Adv. Exp. Med. Biol.*, 606, 163-194 (2008).
5. J. HAVARD, R.E.W.HANCOCK, Antimicrobial properties of lactoferrin. *Biochimie*, 91,

19-29 (2009).

6. W. BELLAMY, M. TAKASE, K. YAMAUCHI, H. WAKABAYASHI, K.KAWAZE, M. TOMITA, Identification of the bactericidal domain of lactoferrin. *Biochim. Biophys. Acta,* 1121, 130-136 (1992).
7. W. BELLAMY, M. TAKASE, H. WAKABAYASHI, K.KAWAZE, M. TOMITA, antimicrobial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. *J. Appl. Bacteriol.*, 73, 472-479 (1992).
8. K. YAMAUCHI, M.TOMITA, T. J. GIEHL, R.T. ELLISON, Antibacterial activity of lactoferrin and a pepsin–derived lactoferrin peptide fragment. *Infect. Immun.*, 61, 719-728, (1993).
9. J. L. GIFFORD, H.N. HUNTER, H. J. VOGEL, Lactoferricin; a lactoferrin –derived peptide with antimicrobial, antiviral, antitumor and immunological properties. *Cell. Mol. Life Sci.*, 62 (22), 2588-2598 (2005).
10. T. R. GLASS, J. W. BULLARD, C. S. HADLEY, E.W. MIX, R.S. CONRAD, Partial spectrum of microorganisms found in denture and possible disease implications. *J. Am. Osteopath. Assoc.,* 101 (2), 92-94 (2001).
11. D. BEIGHTON, The complex oral microflora of high-risk individuals and groups and its role in the caries process. *Comm. Dent. Oral Epidemiol.*, 33, 248-255 (2005).
12. M. AVILA, D.M. OJCIUS, O. YILMAZ, The oral Microbiota: Living with a PermanentGuest. *Cell. Biol.*, 28 (8), 1-7 (1009).
13. B. J. PASTER, S. K. BOCHES, J.L. GALVIN, R. E. ERICSON, C. N. LEVANSON, F.E. DEWHIRST, Bacterial diversity in human subgingival plaque. *J. Bacteriol.*, 183, 3770-3783 (2001).
14. R. R. B. RUSSELL, *The Molecular Biology of Cariogenic Bacteria*. In: Molecular OralMicrobiology, A. H. ROGERS, ed., Caister Academic Press, Great Britain, 2008, pp109-133.
15. H. WAKABAYASHI, K. YAMAUKI, T. KOBAYASHI, T. YAESHIMA, KIWATSUKI, H. YOSHIE, Inhibitory effects of lactoferrin on growth and biofilm formationof *Porphyromonas gingivalis* and *Prevotella intermedia. Antimicrob. Agents Chemother.*, 53,3308-3316 (2009).
16. H. WAKABAYASHI, I. KONDO, T. KOBAYASHI, K. YAMAUKI, T. TOIDA, K.IWATSUKI, H. YOSHIE, Periodontitis, periodontopathic bacteria and lactoferrin. *Biometals,*23, 419-424 92010).
17. P. L. MASSON, J. F. HEREMANS, Metal-combining properties of human lactoferrin(red milk protein). *Eur. J. Biochem.,* 6, 579-584 (1968).
18. M. DAMIAN, I. BABIUC, M. MALITA, M. PAUNA, Phenotypical identification ofbacterial species isolated from the oral Cavity. *Rev. Rom. Med. Dent.*, XII (3),185-197 (2009).
19. E.D. WEINBERG, The development of awareness of iron-withholding defence. *Perspect.Biol. Med.,* 36, 215-221 (1993).
20. S.Y. ARSLAN, K.P. LEUNG, C.D. WU, The effect of lactoferrin on oral bacterialattachment. *Oral Microbiol. Immunol.*, 24, 411-416 (2009)
21. P. I. DIAZ, N.I. CHALMERS, C. KONG, C. I. MILBURN, R. J. Jr. PALMER, P.E.KOLENBRANDER, Molecular characterization of subject-specific oral microflora duringinitial colonization of enamel. *Appl. Environ. Microbiol.*, 72, 2837-2848 (2006).
22. F. BERLLUTI, M. AJELLO, P. BOSSO, Both lactoferrin and iron influence aggregation and biofilm formation in *S. mutans. Biometals,* 17, 271-278 (2004).