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Effect of bovine lactoferrin against *Carnobacterium viridans*

Anas A. Al-Nabulsi, Richard A. Holley*

Department of Food Science, University of Manitoba, Winnipeg, R3T 2N2 Canada

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Abstract

Lactoferrin (LF) alone (8 mg/ml) was able to kill 4 $\log_{10}$ CFU/ml of *C. viridans* at 4, 10 and 30 °C in all purpose tween (APT) or Lauria broth (LB). In the presence of 2.5% NaCl in each broth system, LF activity shifted from a bactericidal to bacteriostatic effect at all tested temperatures. The addition of sodium bicarbonate (SB) up to 0.16 M did not alter the activity of LF in LB broth containing 2.5% NaCl. However, when 5 mg/ml sodium hexametaphosphate (SHMP) was used with 2.5% NaCl, LF regained some of its bactericidal activity at 30 °C in APT but not in LB broth. This reversal did not occur in APT at 4 or 10 °C. Except for the observation with SHMP at 30 °C, neither sodium lactate (SL) nor SHMP enhanced the bactericidal activity of LF in LB or APT broth containing < 2.5% NaCl. It was suggested that 2.5% NaCl in LB and APT broths increased media osmolarity and prevented LF access to binding sites on the contracted cell membrane. This is the first report showing a bactericidal effect of unmodified LF at neutral pH and refrigeration temperatures.

Keywords: Lactoferrin; *Carnobacterium*; Bactericidal action; Natural antimicrobial

1. Introduction

Interest has arisen recently in the possible use of lactoferrin (LF) as a natural antimicrobial agent in food. LF is the main iron-glycoprotein present in the milk of various mammals and it exerts an antimicrobial effect against a wide range of Gram-negative and Gram-positive bacteria, fungi, and parasites (Shimazaki, 2000). In addition, LF has antioxidant, antiviral, anti-inflammatory, immune-modulating, anti-cancer effects and can promote the growth of probiotic bacteria like *Bifidobacterium* (Aguila and Brock, 2001).

LF has been shown to be bacteriostatic due to its ability to bind iron and limit its availability in the growth environment. LF can be bactericidal by binding to the surface of Gram-negative bacteria and causing release of lipopolysaccaride (LPS) (Ellison et al., 1988). In addition, LF is reported to have bactericidal effects against Gram-positive bacteria by binding to lipomannan, which is present on the surface of *Micrococcus luteus* or binding to proteins on the surface of *Clostridium perfringens* (De Lillo et al., 1997; Tomito et al., 1998).

The antibacterial activity of LF is dependent upon its concentration, the degree of iron saturation of the molecule as well as its interaction with mineral media constituents. Payne et al. (1990) found that 46 mg/ml of LF (52% iron-saturated) reduced the viability of *Listeria monocytogenes* in UHT milk 16% when compared with control samples, but a bacteriostatic effect was achieved when 30 mg/ml of 18% iron-saturated LF was used. When EDTA was added to UHT milk the activity of LF against *Escherichia coli* O157:H7 or *L. monocytogenes* was not enhanced (Murdock and Matthews, 2002). Salamah and Al-Obaidi (1995) reported that $\leqslant 60$ mM Ca^{2+} did not affect the bactericidal activity of human LF against *Yersinia pseudotuberculosis* but 3–32 mM Mg^{2+} decreased activity in a manner inversely related to its concentration. High concentrations of divalent cations in growth media can protect bacteria from LF by causing the latter to change its tertiary structure, forming tetramers that adversely affect LF biofunctionality and by increasing

*Corresponding author. Tel.: +204-474-9601; fax: +204-474-7630
E-mail address: rick_holley@umanitoba.ca (R.A. Holley).

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the stability of the cell membrane (Shimazaki, 2000). Ellison et al. (1988) reported that bicarbonate could restore the ability of human LF to release LPS from *E. coli* CL99-2 and *Salmonella* Typhimurium SL696 grown in Hanks balanced salt solution (HBSS) containing high concentrations of calcium and magnesium. Naidu (2001) found that the addition of 0.01 M sodium bicarbonate enhanced the ability of immobilized LF to detach *E. coli* O157:H7 from surfaces of beef carcasses. This suggested that bicarbonate could act as stabilizing agent for LF and serve as a companion anion to chelate metal ions. Ellison and Giehl (1991) reported that the combination of LF and lysozyme was bactericidal against *Vibrio cholerae*, *S.* Typhimurium and *E. coli* while each protein alone produced a bacteriostatic effect. Some work has been done to study the effect of monovalent cations on the activity of LF, but interactions are not well understood. Bortner et al. (1989) found that NaCl did not eliminate the antimicrobial effect of LF against *Legionella pneumophila*, but the concentrations used were not mentioned. In addition, 2 and 5 M NaCl did not cause the dissociation of human LF or bovine LF from the surfaces of *Cl. perfringens* and *Shigella flexneri*, respectively (Tigyi et al., 1992; Tomito et al., 1998).

The activity of LF was temperature dependent against *L. pneumophila* (Bortner et al., 1986) and *Y. pseudotuberculosis* (Salamah and Al-Obaidi, 1995). The antibacterial activity of LF at 37 and 42 °C was lost when the treated samples were incubated at 1–25 °C. Bortner et al. (1989) found that human LF was unable to bind to the surface of *L. pneumophila* at 4 °C. Further, Nibbering et al. (2001) reported that expression of bactericidal effects by human LF required bacterial cells to be metabolically active. Murdock and Matthews (2002) reported that after 7 d in UHT milk at 4 °C there was no difference in numbers of inoculated *L. monocytogenes* between untreated control samples and those treated with LF at neutral pH.

The objectives of the present study were to investigate the antimicrobial activity of bovine LF against a cured meat spoilage organism, *Carnobacterium viridans* and determine how this activity is affected by growth media (Lauria broth, LB, or all purpose tween broth, APT), which contained different cation concentrations. The influence of temperature, NaCl concentration and the presence of compounds capable of chelating cations in reaction mixtures were studied to better understand factors governing LF action.

2. Materials and methods

2.1. Preparation of culture and test media

Carnobacterium viridans MPL-11 was originally isolated from spoiled cured meat (Holley et al., 2002) and was kept frozen in glycerol. It was maintained on APT agar slants at 4 °C and transferred monthly to maintain viability. Working cultures were prepared by growth in 10 ml APT broth incubated at 30 °C for 2 d. For tests, 100 μl was transferred to 100 ml APT broth and incubated at 30 °C until the absorbance ($A_{620\,nm}$) reached 0.045, which corresponded to 7.4 $\log_{10}$ CFU/ml. This was approximately the mid-exponential phase of growth. The culture was added to test media (APT or LB) to give a final concentration of approximately 4 $\log_{10}$ CFU/ml.

Bovine LF (Bioferrin, 2000) was obtained from Glanbia Nutritionals (Glanbia Ingredients Inc., Monroe, WI). Stock solutions of LF were prepared to give final concentrations of 8, 16 and 32 mg/ml by dissolving LF in distilled water and filter sterilizing (0.22 μm syringe filter, Fisher Scientific, Fairlawn, New Jersey) prior to addition to sterilized growth media.

Stock solutions of sodium bicarbonate, SB (J.T Baker, Phillipsburg, New Jersey) were prepared to give final concentrations of 0.005–0.16 M at 2-fold increments. Sodium hexametaphosphate, SHMP (Sigma, St. Louis, Missouri) was prepared to give final concentrations of 1, 3 and 5 mg/ml by dissolution in distilled water and sterilization at 121 °C for 15 min. Sodium lactate, SL (Sigma) stock solutions were prepared to give final concentrations of 1%, 3% and 5% (w/v) by dissolution in distilled water and sterilization by filtration (0.22 μm, Fisher Scientific) before addition to heat-sterilized growth media.

Double strength LB containing 10 g/l tryptone (Difco, division of Becton Dickinson, Sparks, Maryland), 5 g/l yeast extract (Difco), 1 g/l glucose (Mallinckrodt, Paris, KY) and 5 g/l NaCl (Fisher Scientific) and double strength APT (46.2 g/l) broth (Difco) were used to assess the activity of LF. To investigate the effect NaCl on the activity of LF, double strength broths above were prepared to yield 0.5%, 1.0%, 1.5%, 2.0% and 2.5% NaCl at testing. Standard formulations for cured meat products often include 2.5% NaCl. The pH of each growth medium was measured with an Accumet Basic pH meter (Fisher Scientific). Calcium, magnesium, sodium, potassium and iron content in both LB and APT broth containing 0.5 and 2.5% NaCl were determined by Inductively Coupled Plasma -Optical Emission Spectrometry, (ICP-OES; Varian Liberty 200, Varian Canada Inc., Mississauga, ON).

2.2. Antimicrobial assay

Antimicrobial assays were carried out following the procedure outlined by Davidson and Parish (1989). Microcentrifuge tubes (1.5 ml flat top microcentrifuge tubes, Fisher Scientific) used contained the following: 250 μl growth medium, 250 μl distilled water, 250 μl SHMP, 250 μl SL, or 250 μl SB, 500 μl LF and 500 μl culture diluted in same growth medium and then the