Activated lactoferrin. Part 1: A novel antimicrobial formulation *

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ABSTRACT

Activated Lactoferrin (ALF) is a novel formulation of commercial (normal) lactoferrin for enhancing antimicrobial function by using a specific molecular-milieu optimization process. This all-natural ALF formulation acts as a powerful deterrent to pathogenic bacteria that may be present in a biofilm. ALF is the first intervention technology with 'microbial blocking action' that detaches and prevents attachment of microorganisms to bio-surfaces and also inhibits growth multiplication of pathogens in a variety of applications. ALF technology is currently under commercialization.

INTRODUCTION

Lactoferrin (LF) is an iron-binding glycoprotein present in milk and many exocrine secretions that bathe the mucosal surface. The term 'LF' though implies an iron-binding component in milk, this molecule co-ordinately binds to various metal ions and occurs in divergent biological milieu including saliva, tears, seminal fluids, mucins, and the secondary granules of neutrophils. LF has a multifunctional role in a variety of physiological pathways and is considered a major component of the innate defense in mammals (1). The ability of LF to bind two Fe^{+3} ions with high affinity in co-operation with two HCO_3^- ions is an essential characteristic that contributes to its major structure-functional properties including antimicrobial activity (2).

LACTOFERRIN – ENIGMA AND MIND BENDERS

LF co-exists with an array of molecules in different mucosal secretions with varying milieu conditions. These substrates and/or physio-chemical conditions exert a specific effect on the structural reorganization of LF molecule and thereby define its multifunctional properties.

Although antimicrobial activity of LF against mucosal pathogens has been widely reported in milk, saliva and other exocrine secretions, a significant volume of research has also elucidated dysfunctional forms of LF during the pathogenesis of several infections. The aggregation of LF complexes in milk during mastitis in cows (3) and formation of dimeric and tetrameric LF forms during chronic recurrent parotitis in humans (4) indicate the functional impact of citrate/bicarbonate ratios and elevated calcium levels in milieu, respectively. These altered physico-chemical conditions lead to LF dysfunctionality, and subsequent diminished antimicrobial activity (5,6).

In recent years, the importance of structure-function relationship in LF molecule and factors that could enhance its antimicrobial activity have been reported (7-10). The highly cationic nature of the N-terminus region of LF (11) and the role of protein surface charge to facilitate LF binding to glycosaminoglycans and microbial surfaces have been elucidated (12, 13). Subsequently, Naidu and co-workers have identified, isolated and characterized LF-binding microbial targets in a variety of Gram-positive and Gram-negative bacteria (9,14-17). Specific high-affinity interaction of LF with pore-forming outer membrane proteins (OMPs) of *Escherichia coli*, in particular, has unraveled a molecular mechanism for antimicrobial activity, which seems to be well conserved in Gram-negative enterics (18-20).

These findings together with the LF-mediated outer membrane (OM) damage in Gram-negative bacteria reported by Ellison et al. (21) has provided explanation to certain antimicrobial effects such as antibiotic potentiation (10), release of lipopolysaccharides (LPS) and alterations in microbial OM permeation.

The following observations on structure-function relationship of LF with bacterial pathogens helped in unraveling the enigma behind dysfunctional LF and led to a novel approach for '*molecular activation*' of LF, accordingly:

- Citrate:bicarbonate ratio and basic pH of the milieu exert a specific influence on LF activity.
- LF could block microbial attachment factors such as fimbriae and other adhesins (putative receptors).
- Neutralization of cationic activity with sodium chloride could facilitate

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specific interactions of LF with microbial surface.
- Immobilization of LF to mucosal surfaces containing sulfated glycans such as heparan sulfate or its closely related analogs including galactose-rich polysaccharide (a water-soluble fraction from agar) and carrageenans via cationic N-terminus region of LF could enhance the microbial blocking activity.

ACTIVATED LACTOFERRIN (ALF) – MICROBIAL BLOCKING ACTIVITY

Microbial attachment to epithelial mucosa or biosurfaces is the initial step in the pathogenesis of many infections as well as in food contamination. Bio-interactions involving ligands/adhesins with specific receptors, and non-specific binding mechanisms (electrostatic, hydrophobic, and van der Waals forces) contribute to adhesion/colonization of microorganisms. Several mucosal pathogens harbor specific cell surface appendages such as fimbria and/or adhesins that facilitate anchorage to host tissue matrix components (i.e fibronectin, collagens and mucins) (1). Any possible blockade of pathogen-host tissue interaction could potentially lead to the prevention (prophylactic) or reduction (therapeutic) of microbial pathogenesis.

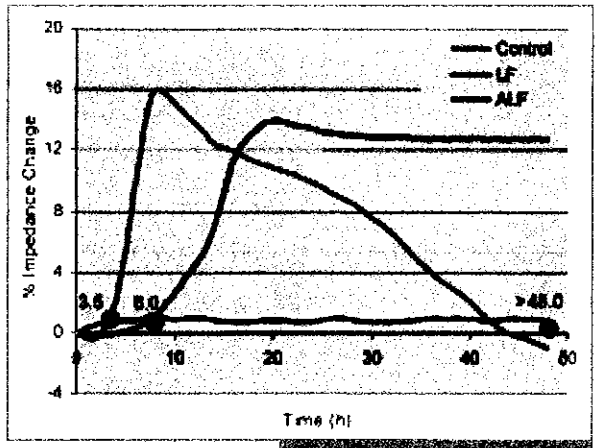
ALF represents the true definition of a "Microbial Blocking Agent (MBA)" that interferes in adhesion-colonization, detaches microorganisms from biological surfaces and inhibits microbial growth/multiplication.

ALF – MICROBIAL GROWTH-BLOCKING (STASIS) EFFECTS

The ability of iron to alternate between two valency states $Fe^{+2} \leftrightarrow Fe^{+3}$, is the most important biological property, evident in many pathways of bio-energy synthesis, including the electron transport system that forms ATP by phosphorylation. Thus, iron-deprivation leads to cellular conservation of energy and inhibition of cell multiplication. Accordingly, various iron-binding proteins have evolved in the animal physiological system to sequester iron from the milieu.

The reversible transistion of LF between iron-deficient (apo-form) to iron-sufficient (holo-form) states is the key mechanism for microbial growth-blocking activity. Furthermore, the pathogenesis of bovine mastitis, the udder disease in the LF rich milk environment, emphasizes the critical role for citrate/bicarbonate ratios in limiting apo/holo transistion of LF (22). The LF binding to microbial surface results in increased microbial growth-blocking activity (9). This interaction could be enhanced by reduction or neutralization of the net surface charge (milieu near pI) on the LF molecule. The above function limiting factors, milieu and molecular, inhibitory and augmentory, are controlled in the ALF formulation to optimize microbial growth-blocking activity.

Studies were performed to validate the enhanced stasis activity of ALF compared to its counterpart, the normal LF. Bacteriostasis experiments were performed using a Bactometer® Microbial Monitoring System Model-128 (bioMerieux Vitek, Hazelwood, Mo.). Microbial metabolism causes electrical charge alterations in cultivation media due to breakdown of nutrients. Based on this principle, Bactometer® was used to monitor bacterial growth in the presence of LF and ALF by measuring impedance signals in the cultivation media. Sterile tryptic soy broth (1 mL) was inoculated with a 4-log density of bacteria and the growth curves were graphically plotted as percent changes of impedance signals



versus incubation time (37°C for 48 hours). The amount of time required to cause a series of significant deviation from baseline impedance value was defined as the 'impedance detection time' (IDT). Difference in IDT values between growth control and LF or ALF test samples was considered as the 'stasis' (growth-blocking) time.

A typical growth curve of *E. coli* O157:H7 with an IDT reading at 3.5 h is depicted in Figure 1. The presence of normal LF (1% final concentration) extended the IDT to 8.0 h and caused a stasis of 4.5 h. Under similar conditions, ALF elicited a total bacteriostasis of >42.4 h, i.e. the *E. coli* showed no cell proliferation, and an IDT value was not recorded over a 48-h incubation.

The comparative stasis effects of LF and ALF formulations at 1% concentrations against a variety of Gram-negative and Gram-positive bacterial pathogens is shown in Table I. Evidently, ALF demonstrated an enhanced bacteriostasis effect against all the test strains compared to the normal LF.

ALF – MICROBIAL ADHESION-BLOCKING EFFECTS

Specific binding of ALF to OMPs of Gram-negative bacteria could cause an instant collapse of bacterial outer membrane barrier function. This leads to the inhibition of various cellular functions and de-regulation of adhesin and fimbrial synthesis on bacterial surfaces. ALF-OMP interaction also cures out plasmids by outer membrane diffusion resulting in a loss of colonization factor antigens and certain enterotoxins in *E. coli*.

Binding to tissue matrix components such as collagens, mucins and fibronectin is a pivotal anchor mechanism for many bacteria. ALF also binds to the same anchor sites on tissue surfaces with a higher affinity than bacteria. Thus, ALF could block tissue interaction of bacteria by competitive binding-inhibition. In binding-displacement studies, ALF at low molar concentration could detach tissue bound bacteria either dead (debris) or alive (23). These two mechanisms of adhesion-blockade activity, when combined, makes, ALF a potent microbial blocking agent.

Adhesion-blocking experiments were performed to elucidate the enhanced functionality of ALF on biosurfaces. Accordingly, the bacterial interactions were monitored and visualized on collagen matrices in the presence of LF and ALF

preparations. *E. coli* O157:H7 (ATCC43895) strain isolated from a raw hamburger implicated in hemolytic colitis outbreak, known to produce shiga-like toxin (SLT)-I and SLT-II was made competent and transformed with a pGLO plasmid DNA. The green-fluorescent *E. coli* (pGLO) cells grown in Luria-Bertani broth containing arabinose (6 mg/mL) and ampicillin (50 μg/mL) were tested for attachment to collagen surface using Cell Environments™ 24-well plates. Collagen wells were treated with 2% solutions of LF or ALF for 1 h at room temperature and aspirated. The treated surfaces were inoculated with a 4-log density of green-fluorescent *E. coli* (pGLO) and incubated at 37°C for 18 h. Bacterial growth (*E. coli* total cell mass) was microscopically measured as cellular intensity of green fluorescence. After growth measurements, wells were aspirated, washed twice with 0.9% saline, and microscopically re-examined for bacterial attachment (*E. coli* adherent cell mass). Captured images were analyzed using the SigmaScan Pro version 5.0 (SPSS Science, Chicago, IL, USA).

As shown in Figure 2, *E. coli* growth in the absence of LF (control) showed a thick biofilm formation on the collagen surface, revealed as a dense green fluorescence (91% fluorescence density/cm^2). In the presence of normal LF, the *E. coli* attachment was read as 70% fluorescence density/cm^2, indicating a 23% adhesion-blockade compared to control. Finally, ALF elicited a 99% blockade of *E. coli* attachment to collagen matrices (1% fluorescence density/cm^2) compared to control, suggesting a potent microbial blocking activity.

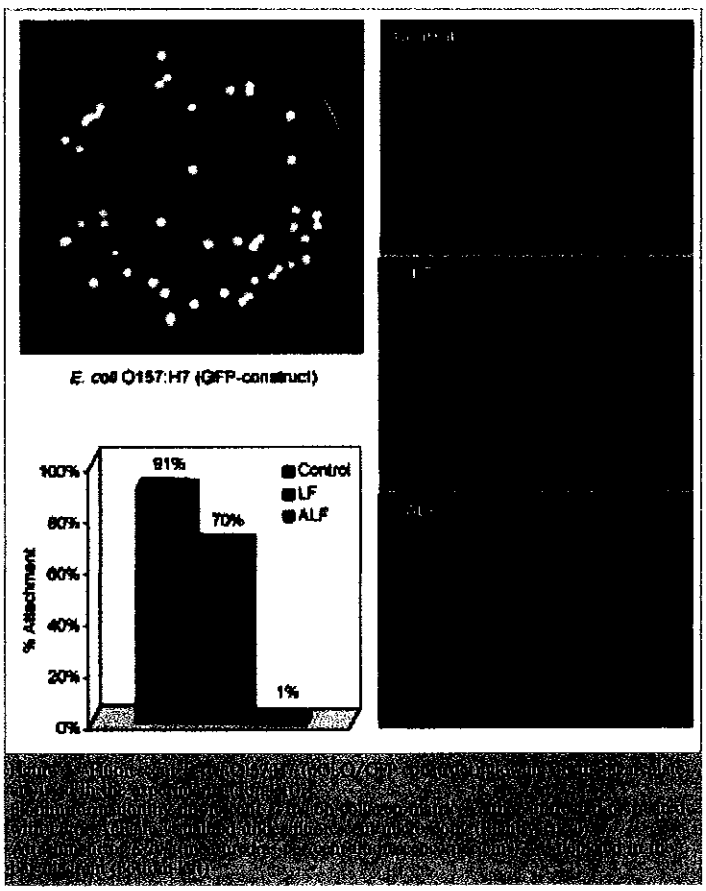
Table I - Comparative stasis effects of LF and ALF against Gram-negative and Gram-positive bacterial pathogen

Bacterial pathogen	Impedence Detection Time (Stasis in hours)		
	Control	LF	ALF
Escherichia coli ATCC433895	3.5	8.0 (4.5)	>48.0 (>44.5)
Salmonella typhimurium ATCC14028	5.4	6.2 (0.8)	>48.0 (>42.6)
Enterobacter cloacae ATCC23355	7.4	7.4 (0.0)	37.3 (29.9)
Proteus vulgaris ATCC13315	8.2	9.0 (0.8)	>48.0 (>39.8)
Pseudomonas aeruginosa ATCC27853	9.4	15.0 (5.6)	>48.0 (>38.6)
Klebsiella pneumoniae ATCC13883	6.6	9.4 (2.8)	>48.0 (>41.4)
Serratia marcescens ATCC8100	7.0	14.2 (7.2)	>48.0 (>41.0)
Staphylococcus aureus ATCC25923	7.4	9.8 (2.5)	>48.0 (>40.6)
Staphylococcus epidermidis ATCC12228	9.4	13.4 (4.0)	>48.0 (>38.6)
Streptococcus pyogenes ATCC19615	11.0	15.4 (4.4)	34.8 (23.8)
Streptococcus faecalis ATCC29212	5.4	6.6 (1.2)	15.6 (10.2)

IDT value difference between control and LF or ALF groups was considered 'stasis'

ALF – PATENT AND SCOPE OF APPLICATIONS

ALF technology is patent protected [Naidu. A.S. (Inventor) *Immobilized Lactoferrin Antimicrobial Agents and the Use Thereof*", US Patent 6,172,040 B1]. In 2001, a joint venture has been established between DMV International, a division of Campina, Holland, and National Beef, a division of Farmland USA, to commercialize the ALF formulation.



ALF invention specifically caters to applications that demand microbial detachment or stasis benefits. ALF falls under the obvious technical category of potential agents for biofilm control. Biofilm control is critical for a wide range of human health applications including:

- *Food protection* – reduction of pathogens (food safety) and prevention of spoilage organisms (shelf life and keeping quality);
- *Oral health care* – plaque control with dentifrices, mouth rinses, oral films, denture cleansing, mouth fresheners etc.;
- *Wound Care* – wound closure, wound management, moist healing and biological dressings;
- *Supplements* – mouth fresheners for oral hygiene and halitosis (gums, chews); tablets or chews for intestinal health and/or treatment of GI disorders;
- *Diary applications* – nutraceuticals, sports drinks and other consumer health products.

In conclusion, ALF is an all-natural broad-spectrum antimicrobial formulation. Its enhanced antimicrobial activity, when taken into consideration its impressive list of multifunctional benefits undoubtedly makes ALF as one of the rapidly emerging nutraceutical technologies. ALF applications in various areas of health and nutrition industry will be discussed in an article entitled

'*Activated Lactoferrin (Part II)*' in the forthcoming issue of AGRO FOOD INDUSTRY *hi-tech*.

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