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Inhibition effect of camel milk immune proteins against some mastitis-causing bacteria

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ABSTRACT

Immunoglobulin G (IgG), immunoglobulin M (IgM) and lactoferrin (LF) were isolated from camel milk using gel filtration and ion exchange chromatography. The isolated proteins were tested for purity using polyacrylamide gel electrophoresis. The IgG and IgM activities were tested using ELISA technique. The inhibitory effect of IgG, IgM and LF was examined alone or in combination against some isolates of mastitis-causing pathogens. Results revealed a positive bacteriostatic effect for LF, IgG and IgM against *Streptococcus pyogenes, Escherichia coli, Staphylococcus aureus,* and *Enterobacter aerogenes*, when they tested alone. The inhibition rate was markedly varied between LF and antibodies. When IgG or IgM was added to LF, the inhibition rate was enhanced. From these results it can be concluded that the inhibition effect of such immune proteins may explain the pronounced use of camel milk as an antimicrobial agent in many different areas of the world.

KEYWORDS

Camel milk;
Immunoglobulins;
Lactoferrin;
Pathogenic bacteria;
Mastitis.

INTRODUCTION

Milk considers the sole fluid for thousands of mammals' neonates as it provides the complete nutritional requirements of each corresponding species. Meanwhile, it contains components that provide critical nutritive elements, immunological protection, and biologically active substances to both neonates and adults[24]. Meanwhile, as do other biological secretions such as saliva, tears, bronchial, nasal and pancreatic fluid, milk contains minor protective proteins. These are antibodies (immunoglobulins) and non- antibody-components, i.e., complements, lysozyme, lactoferrin, lactoperoxidase, xanthine oxidase and leucocytes. While, the antibodies are directed against specific antigens, the non-antibodies protective proteins augment and complement the antibody mechanism. The concentration of protective proteins varies according to species. It is recognized that the concentration of the nutrients varies according to the needs of the offspring and depends on such factors as maturity at birth, rate of growth, digestive system and environment. However, it is not known what determines the variation in the concentration of the non-antibody protective proteins. The composition of milk of different species responds to the special needs of the newborn; for example human milk is rich in lactoferrin and lysozyme while in bovine milk lactoperoxidase and xanthine oxidase are the main protective proteins[23]. Camel milk is characterized by higher contents of immunoglobulins, lysozyme and lactoferrin[9,10]. Although, several researchers have studied the antimicrobial action of lactoferrin and immunoglobulins from

milk of different species against different microorganismis [22,5,6,21,27,1,9,19,26], no attempts have been made to study the inhibition effect of camel immune proteins against mastitis-causing bacteria.

MATERIALS AND METHODS

Milk samples

Camel milk was obtained from farms at El-Alamin and Bourg El-Arab areas around Alexandria, cow's milk from the herd of Faculty of Agriculture, Alexandria University, Egypt.

Animals

Pure strains of rabbits were obtained from the farm of Faculty of Agriculture, Alexandria University, Egypt.

Chemicals

All chemicals used in gel electrophoresis were from Bio-Rad (Richmond, CA 94804, USA). Polyvalent antiserum of goat anti-rabbit- IgG labeled with horse radish peroxidase; 3,3'-diaminobenzidine, O-phenyl endiamine, H_2O_2 (30%), Tween 20, Sephacryl S-300, CM-cellex, Freund's adjuvants were from Sigma (St Louis, MO 63178,USA).

Organisms and media

Strains of *Streptococcus pyogenes, Escherichia coli, Staphylococcus aureus,* and *Enterobacter aerogenes* have been isolated from bovine subclinical mastitic animals from the herd of faculty of Agriculture, Alexandria University. Isolates were identified as described by[25]. Bacterial isolates were maintained on nutrient agar slants at 4^0C. Before, experiments, they were grown overnight at 37^0C in BHI broth.

Inhibition assay test

For growth inhibition, 4 ml of BHI broth were incubated with 100µl of stock broth culture. 50µl of purified protein solutions (1mg/ml) which sterilized using 0.22µm filter membrane (Millipore) were added and incubated at 37^0C for 24 hours. At interval times portions were taken for colony counts which were determined using standard plate technique.

Preparation of immune proteins

Immunoglobulins G and M as well as lactoferrin were isolated from camel milk by gel filtration (Sephacryl S-300) and cation exchange (CM-cellex) chromatography as described[7].

Polyacrylamide gel electrophoresis(PAGE)

Homogeneity of isolated lactoferrin and immunoglobulin G and M was analysed by alkaline native PAGE. Protein solution was diluted 1: 3 (v/v) with buffer 0.05 M Tris-HCl, pH 6.8; then mixed in the ratio 1:1 (v/v) with sample buffer 0.5 M Tris-HCl, pH 6.8 containing glycerol (7.5%), bromophenol blue (0.5%) and subjected to electrophoresis[13]. The running buffer consisted of 0.192M glycine and 0.025M Tris. Runs were carried out at 150 V until the end of electrophoresis. Electrophoresis was performed using Mini-Protean II cell (Bio-Rad) and protein bands were localized in the gels using Coomassie blue R-250 (0.1%).

Antisera production (immunization)

Polyvalent antisera to camel milk proteins were prepared according to the procedure described by[3]. Rabbits were first immunized, with 0.5 ml of antigen (5 mg/ml sterile NaCl, 0.9%) in suspension with 0.5 ml complete Freund's adjuvant by intramuscular injection in several sites at week 1. In weeks 3 and 5, each animal was injected intradermaly with a booster dose 0.5 ml of antigen (1mg/ml) in suspension with 0.5 ml incomplete Freund's adjuvant. The sera were tested for antibody production before the third immunization. The animals were bled about 14 days after the last immunization. Blood was taken from rabbits and the antiserum titre was measured.Antisera were stored at -30^0C until used.

Enzyme linked immunosorbent assay (ELISA)

Antibody activity was measured by ELISA which was performed as described by[15] using ninety-six wells; round bottom, microtitre plates (Falcon Laboratory ware, CA 93030, USA). Plates were coated with 50µl per well of 20 µg/ml of cow milk casein. 50 µl sample were added. Polyclonal antiserum of rabbit anti-camel IgG was added to each well. 50 µl of polyvalent antiserum of goat anti-rabbit- IgG labeled with horse radish peroxidase were added. The reaction was developed with O-phenylendiamine-H_2O_2. Absorbance was measured at 490 nm in a Titertek Multiskan spectrophotometer.

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RESULTS AND DISCUSSION

The inhibition effects of camel milk lactoferrin and immunoglobulin G and M (1mgml) on some pathogenic bacteria as *Streptococcus pyogenes*, *Escherichia coli*, *Staphylococcus aureus*, and *Enterobacter aerogenes* are shown in figures (1-4), respectively. Results revealed

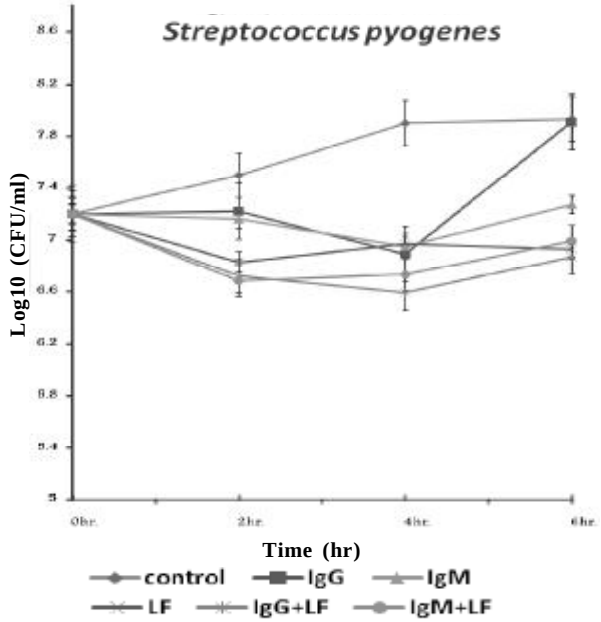

Figure 1: Inhibition effect of camel milk immunoglobulin G, immunoglobulin M and lactoferrin on *Streptococcus pyogenes.* (Mean Values± SEM)

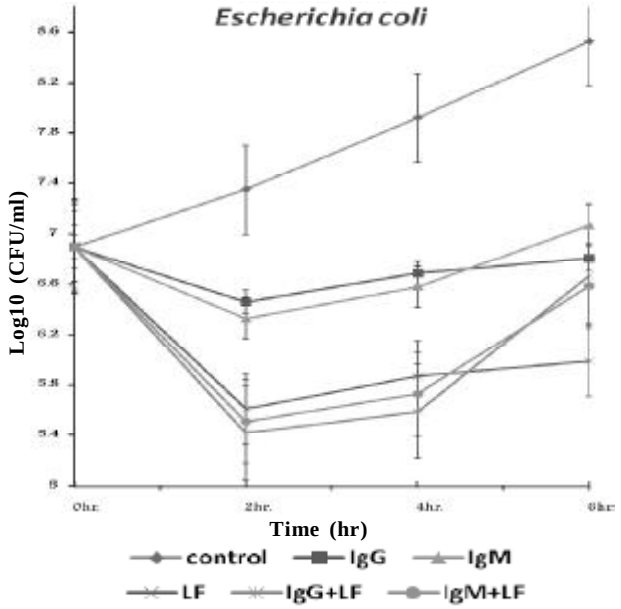

Figure 2: Inhibition effect of camel milk immunoglobulin G, immunoglobulin M and lactoferrin on *Escherichia coli.*(Mean Values± SEM)

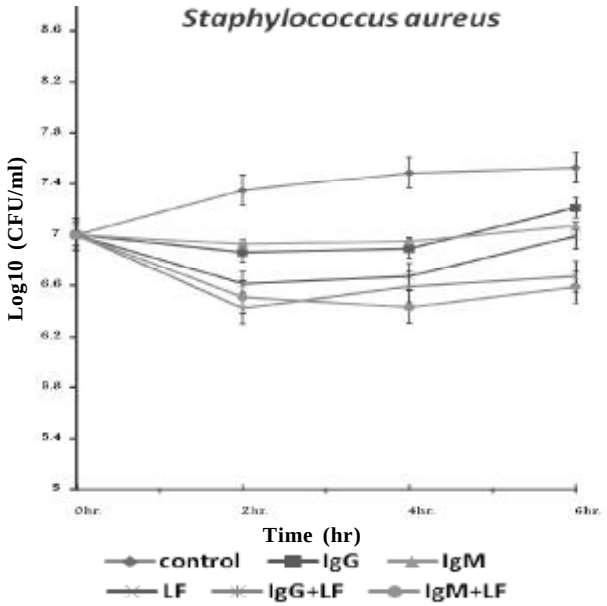

Figure 3: Inhibition effect of camel milk immunoglobulin G, immunoglobulin M and lactoferrin on *Staphylococcus aureus.*(Mean Values± SEM)

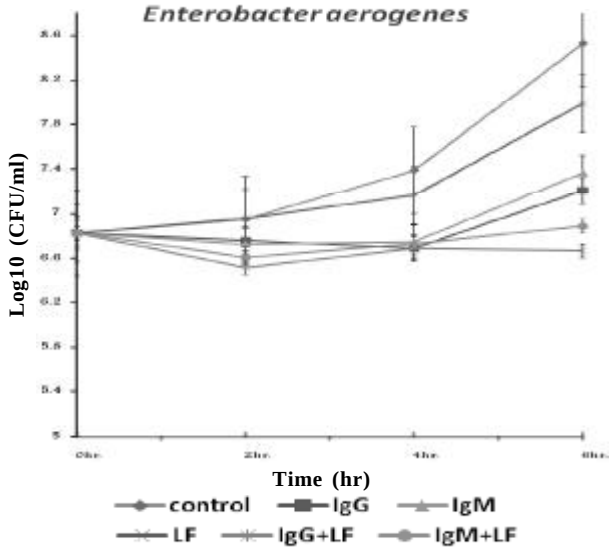

Figure 4: Inhibition effect of camel milk immunoglobulin G, immunoglobulin M and lactoferrin on *Enterobacter aerogenes* (Mean Values± SEM)

that lactoferrin had a pronounced effect on the growth of all tested strains and none of them was able to resist the bacteriostatic effect of lactoferrin. The bacterial growth was markedly reduced in the first 4 hours of incubation comparing with control. The maximum growth inhibition of lactoferrin was recorded with *E.coli* than any other strain. This result agrees with early reported data by[3], who found that purified lactoferrin from cow milk was able to inhibit *E.coli.* The highest inhibition of

lactoferrin against *E.coli* rather than other strains may due to that lactoferrin is a glycoprotein which is able to bind two metal binding cations, preferably ferric ions at specific binding sites.Therefore it competes with other bacteria for iron present in the media. Since *E.coli* is the highest strain for iron requirement in the growth, therefore it was the most affected by lactoferrin[16], It was reported that the inhibition effect of lactoferrin against mastitic isolates of *Staphylococcus aureus* but there was no effect against *Streptococcus agalactia* and *Streptococcus uberis*. The study attributed that to the variation in iron requirements among different isolates. It was reported that the concentrations of lactoferrin in cow, human and camel milks were increased in subclinical mastitis[17,12,2]. Meanwhile it was found that antimicrobial activity of lactoferrin may be different in Gram-negative and Gram-positive bacteria due to the differences in the cell membrane structure. However, previous studies in cattle and humans showed bacterial isolates (both Gram-positive and Gram-negative) inhibited by *in vitro* addition of lactoferrin[20,11,18]. Lactoferrin can act as either a bacteriostatic and/or bactericidal agent[12,18]. This difference in the activity may, in part, explain the wide range of lactoferrin action. This may due to the presence of lactoferrin-binding proteins or lactoferrin receptors on the surface of the microorganisms may partially explain the resistance of these isolates to lactoferrin[14].

Al-Majali et al.[2] studied the antibacterial effects of camel lactoferrin against some selected isolated bacteria from subclinical mastitic milk. All tested bacterial isolates were resistant to the camel lactoferrin except *Staphylococcus aureus* (20 isolates), 2 Strepto cocuss agalactiae[2], and 12 Streptococci other than Streptococcus agalactiae (growth was not inhibited at 50 mg/mL lactoferrin concentration). Lactoferrin failed to inhibit any of the *A.pyogens*, and *Escherichia coli* isolates. The most sensitive isolate to lactoferrin was one of the *Staphylococcus aureus* isolate with an MIC value of 0.006 mg/mL. Lactoferrin isolated from camels milk was able to inhibit growth of only 20% of the *Staph. aureus* isolate

The concentration of lactoferrin in camel milk might be associated with the pathogenicity of the bacterial species that present in the mammary gland[2]. In cattle, the high levels of lactoferrin were observed in milk infected with *S.aureus*[28]. A low lactoferrin concentration in milk with *E.coli* may lead to rapid growth of the bacteria and exaggeration of the clinical disease. On the contrary, lactoferrin was significantly increased in cows experimentally infected with *E.coli*[14].

Concerning immunoglobulinG and M, results revealed that all strains were inhibited by both types of immunoglobulins. *Streptococcus pyogenes, Escherichia coli and Staphylococcus aureus were* more affected by the action of both types of immunoglobulins than *Enterobacter aerogenes*. Immunoglobulin M was more effective than IgG on *Streptococcus pyogenes* and *Escherichia coli* than *Staphylococcus aureus and Enterobacter aerogenes*. The synersegtic inhibition effect between immunoglobulin G or M and lactoferrin were noticed. In all cases the inhibition effect was enhanced by the synergestic effect between lactoferrin and IgG or IgM. It was more pronounced in case of *Staphylococcus aureus and Escherichia coli*. Results revealed that IgG with lactoferrin was more effective than IgM with lactoferrin on all bacterial isolates except *Streptococcus pyogenes*[6]. Who studied the effect of purified immunoglobulin G1 and lactoferrin from buffalo milk on some Gram-negative and Gram-positive bacteria causing mastitis in cattle. They it found that there was a pronounced variation between lactoferrin and IgG1 in their inhibition effect. Lactoferrin was more effective on Gram-negative than Gram-positive bacterial isolates. IgG1 was effective on both types of bacteria. The presence of IgG1 with lactoferrin resulted in enhancement of its bacterial inhibition[6].

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