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Synergistic Killing of Pathogenic *Escherichia coli* Using Camel Lactoferrin from Different Saudi Camel Clans and Various Antibiotics

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Abstract

Current study aimed to analyze the synergistic killing of pathogenic *Escherichia coli* using camel lactoferrin from different Saudi camel clans and various antibiotics. Methods: using multiple microbiological and protein analysis techniques, the results were shown that the purified camel lactoferrins (cLfs) from different Saudi camel have strong antimicrobial potentials against two strains of *E. coli*. Although all cLfs were superior relative to human or bovine lactoferrins (hLf or bLf), there was no noticeable difference in the antimicrobial potentials of cLfs from different camel clans. The effects of antibiotics and cLfs were synergistic, indicating the superiority of using cLf-antibiotic combinations against *E. coli* growth. Since these combinations possessed distinguished synergy profiles, it is likely that they can be used to enhance the low efficacy of antibiotics, as well as to control the problems associated with bacterial resistance. Furthermore, these combinations can reduce the cost of cure of bacterial infections, especially in the developing countries. The analysis of the molecular mechanisms of lactoferrin action revealed that expression of several *E. coli* proteins was affected by the treatment with these antibacterial factors. Several proteins of different molecular weights interacting with cLf-biotin were found. Scanning and transmission electron microscopy analysis revealed the presence of noticeable morphological changes associated with the treatment of *E. coli* strains by antibiotic carbenicillin or cLf alone, and in combination. Camel lactoferrin has superior potential killing of *E. coli* over bovine and human lactoferrin, and this potential can be further synergistically enhanced of cLF is combined with antibiotics.

Keywords Camel clans · Lactoferrins · Antibiotics · Synergy · *Escherichia coli* · Antimicrobial

1 Introduction

Although six enterotoxigenic *Escherichia coli* (ETEC) strains are recognized to cause diarrhea in children of the developing world, as well as in travelers to those areas [1], there is currently no preventive vaccine for these infections. The global burden of infection with these pathogenic *E. coli*

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strains ranges from 280 to 400 million of yearly episodes of diarrhea in children younger than 5, consequently causing 300,000–500,000 [2, 3], or possibly as many as one million, deaths per year in adolescents, adults, and children [4]. However, non-pathogenic *E. coli* strains constitute an important part of the normal intestinal microbiota of healthy mammals and birds [5–8].

ETEC, which is a Gram-negative, non-sporulating, facultative anaerobic, rod-shaped bacterium belonging to the *Enterobacteriaceae* family, attaches to specific receptors on the surface of enterocytes in the intestinal lumen via hair-like fimbriae (colonization factor antigens). ETEC then produces toxins [heat stable toxin (ST) and heat labile toxin (LT)], which stimulate the lining of the intestines to secrete excessive fluid, producing diarrhea [9].

While antibiotics are capable of shortening the span of a diarrheal infection, particularly if administered early, ETEC is often capable of resisting the effects of common antibiotics, including ampicillin. Because resistance to antibiotics is growing on a global level, the option of treating ETEC with an antibiotic should be considered very carefully. Therefore, the safe unconventional medicine may represent an alternative way to control this highly communicable pathogen [4]. For this and other reasons, many patients are looking for some alternative medicines instead of antibiotics. In Arab and other developing regions, camel milk is on top of the list of alternative medicines favored by patients. Furthermore, it serves as an important nutritional source in many countries. One of the illustrative examples is given by the fact that hepatitis C virus (HCV)-infected patients in Egypt and some other regions worldwide consume large quantities of camel milk as both an alternative and a supportive medicine [10].

The total worldwide camel population is estimated to be 24.1 million heads (http://www.fao.org/), with most of them being found in Africa. The greatest dairy camel population is present in the Northeast African countries, such as Somalia, Ethiopia, and Sudan [11, 12]. About 90% of the camels are one-humped (*Camelus dromedarius*), while 10% are two-humped (*Camelus bactrianus*) [13]. There is a continuous increase in the total number of camels used for milk production, mainly *Camelus dromedarius* [14], and this is accompanied by the noticeable increase in the annual camel milk production (http://www.fao.org/). In fact, in 2010 alone, about 6 million camels produced > 3 million tons of milk [15]. Camels represent an important protein source for many humans across the world [16], especially for those populations living in the arid lands of the world [17]. Furthermore, a need for the various camel milk products has been in constant raise in the past several years (http://www.fao.org/), and camel milk is gaining more attention as a healthy food [18]. While, the total population of dromedary in Arab world is estimated to be around 1.6 million heads within the Arabian Peninsula. The highest lactoferrin concentration present in human milk (~ 1.7 g/L) followed by camel (55–888 mg/L) and bovine (300–500 mg/L) [11, 12, 19].

The majority of the protecting and preventing properties of milk has been traditionally attributed to antibodies, lactoferrin and lactoperoxidase, as well as some complex carbohydrates, nucleosides, and nucleotides found in milk have also been recognized as bioactive elements [20–30]. After the Food and Drug Administration (FDA) considered lactoferrin as a Generally Recognized as Safe (GRAS) substance [31], numerous clinical trials on the potential treatment of infectious diseases have been performed with intact molecule or artificial peptides derived from this protein [32, 33].

In order to determine the origin of the protective potential of camel milk, we screened and analyzed five specific camel milk proteins for anti-HCV activity: amylase, lactoferrin (cLf), immunoglobulin G (cIgG), α-lactalbumin, and casein. Although amylase, cLf, cIgG, and α-lactalbumin are considered to be minor proteins in camel milk, casein comprises 80% (v/v) of the whole protein content. cLf is a single-chain, iron-binding glycoprotein of 80-kDa that was found to contain 5.6% carbohydrates in milk collected 15–30 days after parturition and 6.2% in colostral milk [34]. First results from our studies showed that cLf displayed significant activity against HCV cell entry into both human naïve leukocytes and HepG2 and Huh7.5 cell lines [10, 35–42]. cIgG was found to differentially bind HCV in vivo, and its core and envelope peptides in vitro [41]. On the other hand, α-lactalbumin, casein, and amylase failed to exert any anti-viral activity in the same tissue culture system. Furthermore, no cytotoxic effects on naïve leukocytes or hepatoma cell-lines were found for camel amylase, cLf, cIgG, or camel α-lactalbumin [39]. On the other hand, camel casein was shown to efficiently induce apoptosis in hepatoma cells [39].

Systematic analysis of the antimicrobial activity of lactoferrins isolated from the different species against *Escherichia coli* 0157:H7 revealed that cLf was the most active, whereas alpaca and human lactoferrins were the least active against this bacteria [43]. We also showed earlier that cLf or hLf alone or in combination with several antibiotics (such as oxacillin or vancomycin) possessed remarkable antibacterial potential against methicillin-resistant *Staphylococcus aureus* (MRSA) [44]. Combining cLf or hLf with either vancomycin or oxacillin at sub-MIC (minimum inhibitory concentration) levels offers greater in vitro antibacterial activity against MRSA when compared to using either agent on its own [44].

One of the aims of the current study was to extend these analyses and to investigate the antibacterial activities of cLf against the most pathogenic *Enterobacteriaceae* strains, such as *E. coli* that causes dramatic healthcare problems worldwide.

2 Materials and Methods

In the current study, the potential anti-bacterial activities of camel lactoferrin isolated from milk of different Saudi camel clans, as well as human and bovine lactoferrins against *E. coli*, were evaluated via bacteriostatic/bactericidal pathways. Human and bovine lactoferrins were purchased from Sigma-Aldrich (Germany), whereas camel lactoferrins were isolated from the milk of different Saudi camel breeds (see below). The iron saturation of lactoferrins was at the level of ~ 10% and ~ 15% in the case of bLf and hLf, respectively as determined by spectrophotometry ($A_{280/466}$).

Camel milk collection, processing and lactoferrin purification, labelling methods were cited in Supplementary Materials.

2.1 Bacterial Lipopolysaccharide–Lactoferrin Interaction Analysis

Reactivity of the lipopolysaccharide (LPS) of *E. coli* purchased from Sigma Aldrich (USA) and extracted with phenol–chloroform Westfalia method was measured using ELISA. The biotinylated lactoferrins at concentration of 1 µg/mL in carbonate/bicarbonate pH 9.6 buffer were incubated at 37 °C for 2 h with coated bacterial LPS in Coaster polystyrene plates. Streptavidin-peroxidase was added followed by peroxidase substrate addition. The color was scanned at 490 nm as previously described [45]. Carbonate/bicarbonate pH 9.6 buffer was used as a blank.

2.2 Bacterial Cultures

Two enteric bacterial strains of *E. coli* were analyzed in this study, ATCC#25922 and ATCC#35218. Approximately 100 µL culture aliquots of the strains were added to tryptic soy yeast extract broth (TSB), incubated for 21 h at 37 °C and stored at − 80 °C after adding 20% glycerol for future use as seed stocks. All working cultures were obtained by transferring an aliquot of a seed stock culture on an inoculating loop into the vials containing 10 mL tryptic soy broth (TSB) that were then incubated for 16 h. 200–300 µL were then speeded on plates containing tryptic soy agar (TSA) and incubated overnight. 10 mL of TSB were inoculated and incubated at 37 °C for 16–20 h using one of the colonies. These cultures were used for the antimicrobial assays, which were carried out in microtiter plates [44, 46].

2.3 Bacterial Cell Lysis

Separate 5 mL from the aforementioned cultures were collected, centrifuged and the pellet resuspended in original volume of PBS (pH 7.3). Each culture was treated with the following antimicrobial agents: carbenicillin (CB), carbenicillin-camel lactoferrin (CB-cLf), chloramphenicol (C), chloramphenicol-camel lactoferrin (C-cLF), imipenem (IMI), imipenem-camel-lactoferrin (IMI-cLf), and then incubated at the aforementioned experimental conditions. 200 µL from each of these cultures were withdrawn at 0, 30, 60, and 150 min, then divided into 100 µL aliquots. One of these aliquots was centrifuged, and its corresponding supernatant proteins content was measured by Bradford assay kite (Thermo Fisher Scientific, USA). The second 100 µL was used for SDS-PAGE profiling, then stained detained [44, 46].

2.4 Bacterial Membrane Proteins Fraction Preparation

The cells of *E. coli* (ATCC#35218) were harvested via centrifugation of their cultures and washed with PBS, pH 7.4. The cells were then suspended in protease inhibitor solution (0.5 mg/mL herbimycin A, 0.1 mM sodium vanadate, 25 mg/mL leupeptin, 50 mg/mL aprotinin, 750 mg/mL benzamidine, and 1 mM phenylmethylsulfonyl fluoride in PBS pH 7.1), and ultrasonicated at 130 W and 20 kHz for 20 min. Centrifugation at 11,000×*g* for 10 min was performed to separate the membrane fraction (precipitate, MF) and the cytosolic fraction (supernatant). The precipitated pellet was washed with protease inhibitor solution in cold PBS. The pellets were suspended in a lysis buffer (protease inhibitor solution containing 1% Triton X-100 and 1% CHAPS) and the membrane-associated fraction was obtained by centrifugation at 10,000×*g* for 15 min and then stored at − 80 °C until further use [44, 46]. The prepared bacterial membrane proteins (BMP) were used for SDS-PAGE and ELISA as described in an earlier study [44, 47]. ELISA plates were coated with carbonate/bicarbonate pH 9.6 buffer as a blank, free cLf and outer membrane proteins extract of *E. coli* then exposed to cLf-biotin followed by streptavidin-peroxidase and developed with TMP substrate. The plates OD were read at 450 nm, the data were presented as mean ± SD of 8 replica.

2.5 Antimicrobial Activity Assays

Discs of 17 antimicrobial agents, including vancomycin (30 µg), gentamicin (10 µg), chloramphenicol (30 µg), fucidic acid (10 µg), cefepime (30 µg), oxacillin (1 µg), augmentin (30 µg), ampicillin (10 µg), cefoxitin (30 µg), cotrimoxazole (25 µg), cephalothin (30 µg), amikacin (30 µg), aztreonam (30 µg), ceftazidime (30 µg), piperacillin (100 µg), ciprofloxacin (5 µg), and imipenem (10 µg) were purchased from Mast Diagnostics (Merseyside, UK).

2.6 Agar Disc-Diffusion Assay

Wells of 5–6 mm diameter were made on plates with cultured bacterial strains, and 50 µL aliquots of different concentrations of Lf were added to each well, then incubated for 24 h at 37 °C. The clear zone areas were measured using a transparent ruler, and the corresponding diameters were expressed in millimeters to establish the MIC. In parallel, appropriate antibiotic and sterile water was used in separates wells as positive and negative control, respectively [44]. Susceptibility screening of *E. coli* strains ATCC#25922 and ATCC#35218 for cLf (cLf1, cLf2, cLf3, cLf4), hLf, and bLf, and the different antibiotics was performed using the agar disc diffusion technique on Mueller–Hinton (MH) agar. Plates were overlaid with a final inoculum of 2×10^6 CFU/mL of *E. coli* ATCC#25922 and ATCC#35218 and incubated at 37 °C for 24 h in cation-adjusted Mueller–Hinton (CAMH) broth supplemented with different concentrations (0.00, 0.125, 0.250, 0.50, 0.750, 1, 1.5, 2, 2.5, and 3 mg/mL) of different Lfs. Then, plates were examined for the presence of the inhibition zones. The corresponding diameter was reported in millimeters and interpreted using the Clinical and Laboratory Standards Institute (CLSI) zone diameter interpretative standards [48, 49]. Growth of these enterobacteriaceae was monitored spectrophotometrically (OD_{620nm}) after 1, 3, 6, 12, and 24 h incubation at 37 °C.

2.7 Determination of Minimum Inhibitory Concentrations (MICs)

The MICs of antibacterial agents against *E. coli* ATCC#25922 and ATCC#35218 were determined by broth microdilution. Here, a final inoculum of 2×10^6 CFU/mL of *E. coli* (two strains) was added to 96-well microtiter plates (Falcon, Frickenhausen, Germany) with 100 µL of CAMH broth containing antimicrobial agents in serial dilutions. Plates were incubated at 37 °C for 24 h. The MICs were determined by measuring the absorbance at 620 nm. The MIC was defined as the lowest concentration, at which growth was completely inhibited. All MIC determinations were performed in triplicates. Bacteria in CAMH broth (lactoferrins-free cultures) were used as a growth control. Due to the possibility of ferrochelating-dependent antibacterial effects of lactoferrins, the MICs for all lactoferrin used were determined after iron supplementation (addition of 50 µM ferrinitrilotriacetate (FeNTA)) at the time of inoculation [44].

2.8 Checkerboard Assay to Study Synergy

Combinations of lactoferrins of different origin with different antibiotics, such as cLf-(chloramphenicol, Cefepime, Imipenem), hLf-(chloramphenicol, Cefepime, Imipenem), and bLf-(chloramphenicol, Cefepime, Imipenem), were tested on *E. coli* (ATCC#25922 and ATCC#35218 strains) using microdilution checkerboard technique. In brief, a final inoculum of 2×10^6 CFU/mL of *E. coli* (both strains) was added to 96-well microtiter plates containing two-fold serial dilutions of each Lf and the antibiotic in an LB broth. The ranges of lactoferrins and antibiotics concentrations were chosen based on previously obtained MIC data for each of them alone.

Solutions containing Lfs and antibiotics were serially diluted twofold, and Lfs and antibiotics alone or in combinations were assayed at 1/2 MIC, 1/4 MIC, and 1/8 MIC. In all cases, the lowest concentration at which no visible growth occurred was recorded to be the MIC value of the individual and combined antibacterial agents. After incubation at 37 °C for 24 h, the combined effects of cLf, hLf, and bLf with each antibiotic were analyzed by calculation of FICIs, and assessed the antibacterial interactions as follows. First, the fractional inhibitory concentrations (FIC) of antibacterials A or B were calculated as:

$$FIC_{\text{antibacterial A}} = MIC_{\text{antibacterial A in combination}}/MIC_{\text{antibacterial A alone}} \text{ and}$$

$$FIC_{\text{antibacterial B}} = MIC_{\text{antibacterial B in combination}}/MIC_{\text{antibacterial B alone}}$$

Then, the FIC index was calculated as

$$FICI = FIC_{\text{antibacterial A}} + FIC_{\text{antibacterial B}},$$

and the values of this index were used to determine whether indifference, antagonism, or synergism occurred between the antibacterial agents. The antimicrobial combination was considered indifferent at $0.5 < FICI < 4$, antagonistic at $FICI \geq 4$, and synergistic at $FICI \leq 0.5$ [50].

2.9 Time-Kill Assessment of cLf, hLf, and bLf Alone and in Combinations with Various Antibiotics Against *E. coli*

Assays for the rate of killing *E. coli* (both strains) by cLf, hLf, or bLf alone and in combinations with various antibiotics, were carried out using the method described in a previous study [44]. An overnight culture was diluted with LB broth in a total volume of 30 mL containing an inoculum of 2×10^6 CFU/mL of *E. coli* (both strains), and cLf, hLf, or bLf alone was added to yield concentrations of ½×, 1×, and 2×MIC in LB broth. cLf, hLf, or bLf at concentrations of ¼ MIC or 1×MIC was combined with cefepime, imipenem, or chloramphenicol, at concentrations of 1/4 MIC for each antibiotic. The cultures were incubated at 37 °C with shaking at 150 rpm. Viable colony counts were performed at 0, 4, and 8 h. The experiments were repeated three times and the results were presented as mean and the standard error of the mean (SEM). Synergy was defined as $\geq 2 \log_{10}$ CFU/mL decrease from the original inoculum. Bactericidal activity

was defined as ≥ 3 $\log_{10}$ CFU/mL reduction (99.9% kill) from the count of the culture control without antimicrobials.

2.10 Determination of Minimum Bactericidal Concentration

All the MIC wells containing no-growth were used for spreading on MH agar plates and incubated for 24 h at 37 °C, and the colonies were then counted. If bacterial growth was detected, the Lf was identified as bacteriostatic; if it did not grow, the Lf was identified as bactericidal. Minimum bactericidal concentration (MBC) was defined as the concentration at which there was a ≥ 99.9% decrease in viable cells.

2.11 Scanning Electron Microscopy (SEM)

The bacterial strain (ATCC#35218) that was susceptible to the Lfs and/or antibiotics was prepared for SEM. Small agar pieces were cut out from the inhibition zone and fixed in 3% (v/v) glutaraldehyde (buffered with 0.1 M sodium phosphate buffer, pH 7.2) for an hour at room temperature and then washed four times in buffer. The pieces were additionally washed four times in the buffer after they were post-fixed in 1% (w/v) osmium tetroxide (OsO_4) for an hour and then washed four times in buffer again. They were dehydrated in a graded alcohol series. The last stages of dehydration were performed by using propylene oxide ($CH_3CH{\cdot}CH_2{\cdot}O$). The specimens were dried and mounted onto stubs using double-sided carbon tape, and then coated with a thin layer of gold by a Polaron SC 502 sputter coater. They were examined using a Jeol JSM 6060 LV Scanning Electron Microscope [44].

2.12 Transmission Electron Microscopy (TEM)

To trace the localization of antibacterial Lf immunoprobe in fimbrial filaments of bacterial strain or intracellularly, immunoelectron microscopy was used. Fresh culture suspension of a maximum of 24 h was washed three times in cold PBS and fixed in 1% glutaraldehyde at 4 °C for 60 min. The fixed bacterial cells were washed in a cacodylate buffer and then dehyderated in serially graded ethanol for 10 min each. The bacterial samples were embedded in the resin and let to polymerization in an ultraviolet irradiator at − 20 to − 30 °C for 2 days, then at room temperature for two more days. Ultrathin sections were obtained by the ultramicrotome, then mounted onto Formvar-coated nickel grids for 10 min, and the excess moisture was removed. For comparison purposes, some bacterial samples were doubly fixed with 2% glutaraldehyde, then with 1% osmium tetroxide, and then embedded in other resin such as Durcupan epoxy resin. The ultrathin sections on the grid were processed for immunostaining on drop manner starting with blocking with BSA at room temperature for 60 min, washed 5 times in PBS, then reacted with free Lf (direct staining) or Lf-Gold nanoparticels Lf-AuNPs or free Lf, then anti-Lf gold nanoparticels (indirect staining) or biotinylated Lf, then streptaviding-alkaline phosphatase probe (indirect intracellular staining, following with TMB substrate for 30 min) for 60 min at room temperature. The stained sections were fixed in 1% glutaraldehyde, then washed five times with PBS. The sections were stained with a mixture of alcian/osmium tetroxide for 10 min, and then finally stained in 4% urnyle acetate before being examined under transmission electron microscopy [45, 46, 51, 52].

2.13 Statistical Analysis

All experiments were done in triplicate and the results were presented as mean ± SD of triplicate. The experimental data were analyzed by Student's *t* test and McNemar's test. A *P*-value of < 0.05 was considered statistically significant.

3 Results

3.1 Purification and Labeling of Lactoferrins

The dual column separation method for Lf purification that includes chromatography on the CM-Sephadex and the Heparin-Sepharose fast flow column utilized in this study was very efficient. In fact, the CM-Sephadex step generated 50-70% purity, whereas the Heparin-Sepharose step produced lactoferrin samples with a purity of about 99% (Figure S1A and B). About 100 mg was collected in final purified form from one liter of each camel clan milk.

Part of the purified proteins was labeled with gold nanoparticles or biotin. To analyze and confirm the success of corresponding conjugation, aliquots of free and conjugated lactoferrin (Lf-AuNPs or Lf-biotin) were run on SDS-PAGE. Results of this analysis are shown in Figure S2, illustrating that in comparison with the unlabeled Lf, the bands corresponding to the gold-labeled and biotinylated lactoferrin were moved up by one kDa and 10 kDa, respectively. It is worth noting that the fraction of Lf-AuNPs was retained on the stacking gel (brown-colored line) due to the large size of gold nanoparticles, and its shift by one KDa may indicate a low conjugation efficiency. The localization of the Lf-biotin in the SDS-PAGE 10 kDa above free lactoferrin is in line with the addition of molecular mass of added NHS-biotin. Further support of successful biotinylation was retrieved using ELISA assay for cLf-biotin, which indicated the presence of a strong signal from the cLf-biotin conjugate in comparison with cLf alone (Figure S3). These results demonstrated that Lf was successfully biotinylated.

3.2 Analysis of the Bacterial Lipopolysaccharides and Their Interaction with Lactoferrins

Unfortunately, when LPS-containing blots were examined in a substrate solution containing 0.5 mg/mL diaminobenzidine and 0.01% H_2O_2 for color development [53], no lactoferrin probe was recognized, and no signal was revealed after several trials, likely due to the SDS interference. In fact, since SDS is an ionic detergent, it can interfere with the bacterial LPS in several ways: it might affect LPS structure [53], change its charge, or affect organization of lactoferrin binding sites. This explanation was supported by the results of control experiments, where native LPS preparations (without SDS) were used in ELISA. However, silver nitrate was able to stain LPS of *E. coli* ATCC#25922 and ATCC#35218 on SDS-PAGE (data not shown).

The efficiency of Lf interaction with bacterial LPS was analyzed by ELISA. The results of these analyses revealed that all lactoferrins significantly ($p < 0.01$) interacted with bacterial LPS (Fig. 1). Generally, cLf was more reactive ($p < 0.001$) against bacterial LPS in comparison with hLf and bLf. However, there were no noticeable differences between the reactivity of cLf sub-types isolated from different camel clans with bacterial LPS. These results reflect two interesting points: 1. cLf sub-types (cLf1, cLf2, cLf3, and cLf4) isolated from different camel clans did not have structural differences specifically within their glycosylation moieties, which are used in the LPS-Lf interactions; 2. SDS, which is an ionic detergent, can interfere with the bacterial LPS structure during SDS-PAGE/Western blotting and can thereby block the LPS-Lf interaction site(s) and/or induce some structural changes.

3.3 Fractionation and Analysis of Bacterial Membrane Proteins

The extracted bacterial cell membrane proteins (BMP) were resolved on SDS-PAGE (Fig. 2 panel 1), as well as whole cell lysate proteins (Fig. 2 panel 2). This analysis revealed that there are some differences between the profile of membrane proteins and profile of whole cell lysates. Furthermore, when BMP crude extract was used in ELISA plate coating and subsequently exposed to cLf-biotin, significant signals were recorded (Fig. 3). These observations, together with the results of the Lf-LPS interaction analysis (see Fig. 1), may indicate that an efficient interaction is taking place between lactoferrin and *E. coli*, which might contribute to the Lf antibacterial action exerted via both bactericidal and bacteriostatic effects.

Tables 1 and 2, as well as Figures S4–S10 and Fig. 5, demonstrate the effects of different anti-bacterial agents used in this study (such as antibiotics, lactoferrins, and combinations of antibiotics and cLf) on the released, appeared, disappeared, and/or highly expressed bacterial proteins. The kinetics of changes in these bacterial proteins were followed through sequential chronological SDS-PAGE profiling. Figures S4–S10 and Fig. 4, as well as Tables 1 and 2, show that the profile of *E. coli* proteins significantly changes over time (30–150 min) and depends on the antimicrobial agents used. These findings were unexpected, and clearly indicated that different antibacterial agents used alone or in combination have substantial effects on *E. coli*, that these effects change with time, and that the actions of these antibacterial agents might be synergistic. Our results support and agree with the previous reports, where the presence of multiple and

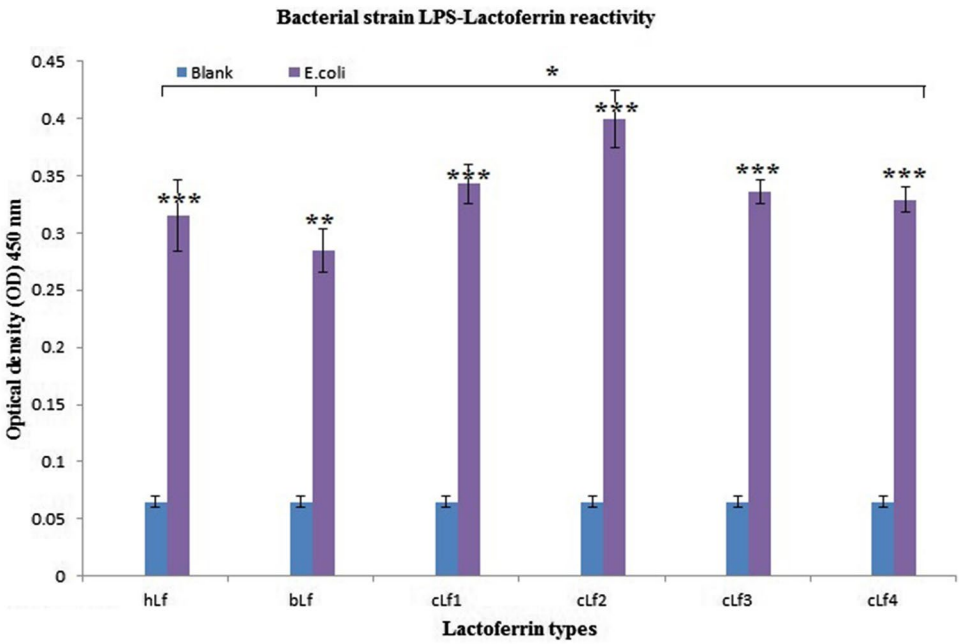


Fig. 1 Reactivity of the different preparations of lactoferrin with LPS of *E. coli* (ATCC#35218). The reactivity was measured using ELISA and proceeded as described in Materials and methods. The biotinylated lactoferrins were incubated with coated bacterial LPS in Coaster polystyrene plate. Streptavidin-peroxidase was added followed by the peroxidase substrate addition. The color was scanned at 450 nm. *, **, *** sign the significance $P < 0.05$, 0.001, respectively. All Lactoferrins seem significantly ($P < 0.001$) interact with LPS, while camel lactoferrin reactivity with LPS has more signal than bovine and human lactoferrin ($P < 0.05$)

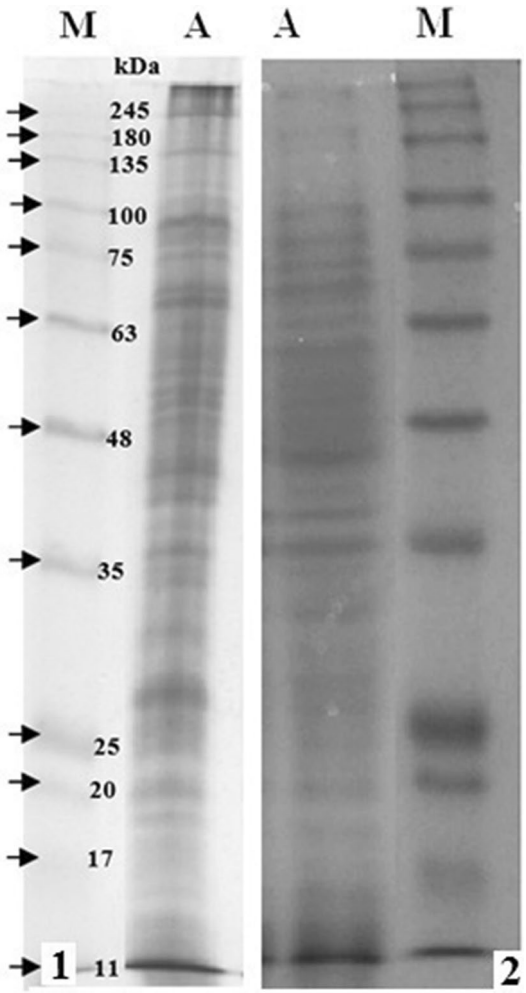


Fig. 2 12.5% reducing SDS-PAGE of the extracted membrane proteins (1A) and whole cell lysate proteins (2A) for *E. coli* (ATCC#35218). The M lane pointed the protein molecular weight marker. Panels (1) and (2) represent individual gels

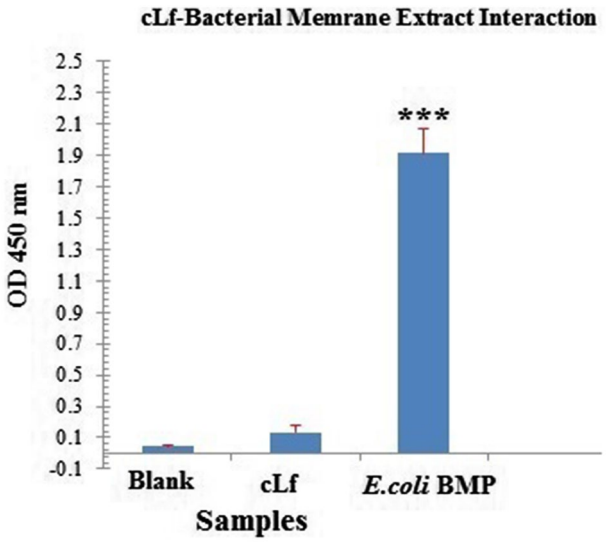


Fig. 3 Immunoassay evaluation of cLf-biotin interaction with *E. coli* (ATCC#35218) outer membrane proteins extract (BMP). ELISA plate was coated with free cLf and outer membrane proteins extract of *E. coli* then exposed to cLf-biotin followed by streptavidin—peroxidase and developed with TMP substrate. The plate OD was read at 450 nm. The data were presented as mean ± SD of 8 replicas, *** sign the significance $P < 0.001$, respectively. The signal of cLf-biotin with BMP was highly ($P < 0.001$) significant

different lactoferrin-binding proteins was found in different bacterial strains [54–62].

3.4 Bacterial Susceptibility Test

As expected, *E. coli* strains ATCC#25922 and ATCC#35218 were susceptible to several antibiotics, but showed some resistance against others (Table 3). The more resistant *E. coli* strain was ATCC#25922 that showed noticeable resistance against 8 antibiotics (see Table 3). The in vitro antibacterial activity of cLf (cLf1, cLf2, cLf3, and cLf4), hLf or bLf against *E. coli* strains ATCC#25922 and ATCC#35218 was assessed by measuring their inhibition zones. These strains were shown (via agar disc diffusion assay) to be efficiently inhibited by bLf, hLf, and cLf, each producing concentration-dependent inhibition zones (Table 4). All cLf types were able to completely inhibit growth of *E. coli* strains analyzed in this study at concentrations ranging from 0.125 to 3 mg/mL. hLf and bLf were able to inhibit growth of both strains of *E.coli* at concentrations ranging from 0.75 to 3 mg/mL (Table 4). These observations suggest that the inhibitory activity of cLf against *E. coli* (both strains) noticeably (sixfolds) exceeded the inhibitory potential of both hLf and bLf. However, the lactoferrins purified from different Saudi camel clans did not show any difference in their bacterial growth inhibitory potential (Table 4).

Therefore, our analysis revealed that both strains of *E. coli* used in this study were sensitive to hLf, bLf, and cLf. However, cLf showed significantly higher efficiency than lactoferrin of human or bovine origin. At the same time, there were no inter-differences between the cLf subtypes isolated from the milk of different Saudi camel clans. To the best of our knowledge, this is the first study of this kind.

3.5 Inhibitory Effects of cLf, hLf, and bLf on *E. coli*

The *E. coli* strains ATCC#25922 and ATCC#35218 were incubated with varied concentrations of bLf, hLf, and cLf to gauge the effects of Lf on bacterial growth. The growth of these strains was completely inhibited after 24 h incubation with 0.75–3 mg/mL of hLf and bLf, and 0.125–3 mg/mL of cLf (Table S1), while bacterial growth was significantly inhibited ($P < 0.005$) after 3–6 h of incubation with 0.125 and 0.75 mg/mL of different types of cLf, and 0.75 and

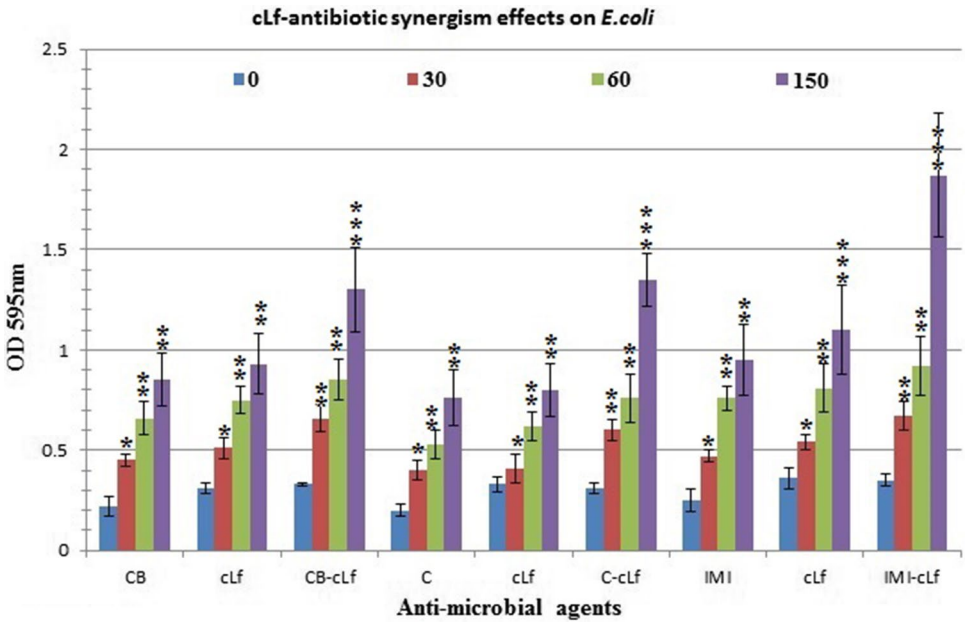


Fig. 4 The effects of different anti-bacterial agents used in this study (such as antibiotics, lactoferrins, and combinations of antibiotics and cLf) on the viability and proteins released from *E. coli* (ATCC#35218) after different time intervals (0, 30, 60, and 150 min).The antimicrobial used in this experiment and their abbreviation, Carbenicillin (CB), carbenicillin- camel lactoferrin (CB-cLf), chloramphenicol (C), chloramphenicol-camel lactoferrin (C-cLF), Imipenem (IMI), Imipenem-camel lactoferrin (IMI-cLf). Significance differences, *, **, *** ($P < 0.05$, $P < 0.001$)

Table 1 Effects of the different anti-bacterial agents on protein release during their bacteriostatic and/or bactericidal action

Anti-bacterial agents	Time of incubation			
	0 min	30 min	60 min	150 min
Effect of carbenicillin (CB) on *E. coli* (ATCC#25922)				
CB	0.22±0.05	0.45±0.03*	0.66±0.08*	0.85±0.13**
cLf	0.31±0.03	0.51±0.05*	0.75±0.07*	0.93±0.15**
CB-cLf	0.33±0.01	0.66±0.07*	0.85±0.1**	1.3±0.21**
Effect of chloramphenicol (C) on *E. coli*				
C	0.2±0.04	0.42±0.01*	0.66±0.04*	0.87±0.12**
cLf	0.3±0.01	0.54±0.02*	0.77±0.02*	0.91±0.1**
C-cLf	0.32±0.02	0.61±0.05*	0.84±0.11**	1.37±0.15**
Effect of imipenem (IMI) on *E. coli*				
IMI	0.25±0.06	0.47±0.03*	0.76±0.06*	0.95±0.18**
cLf	0.36±0.05	0.54±0.04*	0.81±0.12*	1.10±0.22**
IMI-cLf	0.35±0.03	0.67±0.07*	0.92±0.15**	1.87±0.31**

Plates were coated with released proteins from *E. coli* after treatment with antibacterial agents at different time intervals. All values were sum of triplicate ELISA experiments and their corresponding OD 595 nm reads are represented as mean ± SD. * and ** pointed the significance values, $p < 0.05$, 0.001 respectively

CB carbenicillin, *CB-cLf* carbenicillin-camel lactoferrin, *C* chloramphenicol, *C-Clf* chloramphenicol-camel lactoferrin, *IMI* imipenem, *IMI-cLf* imipenem-camel-lactoferrin

1 mg/mL of both hLf or bLf. On the other hand, no inhibitory effects on bacterial growth were detected using either hLf nor bLf at concentrations of 0.125 and 0.5 mg/mL, suggesting that different types of cLf are a much more effective inhibitor of the bacterial growth than hLf or bLf (Table S1). It is also worth noting that Table S1 illustrates that in the presence of lactoferrins, in general or cLf particularly, the optical density (OD) of culture decreases over time. These rather striking and interesting results suggest that in addition to the inhibition of bacterial growth, lactoferrins may be capable of inducing cell death via lysis. Since no difference was found between the different types of cLf (cLf1, cLf2, cLf3, or cLf4) in their inhibitory efficiency, we used only one type (cLf1) of cLf in the remaining part of this study.

These inhibitory activities of camel lactoferrin against *E.coli* are in agreement with our previous report [44], where the same proteins were used against MRSA, and where it was shown that the inhibitory potential of cLf was stronger than that of hLf and bLf. Although the bactericidal activity of cLF was analyzed in several earlier

Table 2 Proteins of *E. coli* (ATCC#25922) treated by various anti-bacterial on corresponding SDS-PAGE taken at different time intervals

Anti-bacterial agents	Time of incubation			
	0 min	30 min	60 min	150 min
Molecular weights (kDa) of *E. coli* proteins released by carbenicillin (CB)				
CB	0	38	38	38
cLf	0	55, 44, 35, 17	55, 44, 35, 17	55, 44, 35, 17
CB-cLf	0	38	36, 33	120, 49, 47, 38, 33
Molecular weights (kDa) of *E. coli* proteins released by chloramphenicol (C)				
C	0	56, 48, 35	56, 48, 35	56, 48, 35
C-cLf	0	70	70	70
Molecular weights (kDa) of *E. coli* proteins released by Imipenem (IMI)				
IMI	0	60, 52, 44, 42, 19	60,52,44,42,19	60, 52, 44, 42, 19
IMI-cLf	0	85, 63, 35, 25, 14, 12	85, 63, 35, 25, 14, 12	85, 63, 35, 25, 14, 12

All gels (SDS-PAGE) were stained with Coomassie blue stain, then scanned and analyzed manually. The corresponding gels are presented in Supplementary Materials as Figures S4–S10

CB carbenicillin, *CB-cLf* carbenicillin-camel lactoferrin, *C* chloramphenicol, *C-Clf* chloramphenicol-camel lactoferrin, *IMI* imipenem, *IMI-cLf* imipenem-camel-lactoferrin

Table 3 Testing of the susceptibility of *E. coli* strains ATCC#25922 and ATCC#35218 to a panel of 17 antibiotics by the agar disc diffusion method

Antimicrobial agent	Concentration of antimicrobial agent (µg/disc)	Average diameter of inhibition zone (± 1 mm)	
		E. coli ATCC#25922	*E. coli* ATCC#35218
Gentamicin (GM)	10	21	18
Vancomycin (VA)	30	R	10
Fucidic acid (FC)	10	13	12
Chloramphenicol (C)	30	18	17
Oxacillin (OX)	1	9	9
Cefepime (CPM)	30	R	17
Ampicillin (AP)	10	R	R
Augmentin (AUG)	30	R	10
Cefoxitin (FOX)	30	R	15
Cephalothin (KF)	30	25	20
Cotrimoxazole (TS)	25	R	17
Amikacin (AK)	30	23	18
Ceftazidime (CAZ)	30	R	9
Aztreonam (ATM)	30	R	R
Piperacillin (PRL)	100	30	10
Imipenem (IMI)	10	30	30
Ciprofloxacin (CIP)	5	30	21

R resistant (no inhibition zone)

studies, those analyses did not use cLf against enterobacteriaceae strains analyzed in this report. Furthermore, although those reports revealed the presence of the inhibitory activity of bLf and hLf, they did not determine the sensitivity concentrations, and no MIC data were reported. Therefore, this study may be the first report where the efficiency of camel lactoferrin against important enterobacteriaceae strains was evaluated.

3.6 The MIC Values of Antimicrobials

The MIC values determined for cLf, and hLf or bLf against *E. coli* (strains ATCC#25922 and ATCC#35218) were 125 and 750 µg/mL, respectively, confirming higher antibacterial activity of cLf. MIC values of chloramphenicol, cefepime, and imipenem against these enterobacteriaceae were 1.35, 1.73, and 1.88 µg/mL, respectively. The data

Table 4 Susceptibility of *E. coli* strains ATCC#25922 and ATCC#35218 to different lactoferrins at different concentrations

Agent	Concentration (mg/mL)	Average diameter of inhibition zone (±1 mm)	
		E. coli ATCC#25922	*E. coli* ATCC#35218
bLf	3	54	55
	2.5	47	49
	2	38	43
	1.5	29	38
	1	21	29
	0.75	9	11
	0.5	R	R
	0.25	R	R
	0.125	R	R
hLf	3	53	51
	2.5	48	48
	2	44	45
	1.5	40	39
	1	37	33
	0.75	25	27
	0.5	R	R
	0.25	R	R
	0.125	R	R
cLf1	3	54	56
	2.5	50	52
	2	48	49
	1.5	44	45
	1	40	41
	0.75	37	38
	0.5	35	33
	0.25	30	29
	0.125	25	23
cLf2	3	55	54
	2.5	50	51
	2	46	47
	1.5	39	40
	1	32	37
	0.75	27	30
	0.5	25	25
	0.25	23	22
	0.125	21	20
cLf3	3	56	58
	2.5	51	52
	2	47	48
	1.5	40	43
	1	36	38
	0.75	31	33
	0.5	27	28
	0.25	23	22
	0.125	20	18
cLf4	3	55	54
	2.5	51	50
	2	47	46
	1.5	42	41
	1	38	39
	0.75	33	36
	0.5	28	30
	0.25	25	24
	0.125	22	20

R resistant (no inhibition zone)

also revealed that the addition of iron (FeNTA) to cultures reversed inhibition. In fact, after the iron addition, the MICs for cLf and hLf or bLf against these strains were 2 and 3 mg/mL, respectively. These effects of iron load on lactoferrin action agreed with the results of our previous report, where the same effect was observed for the Lf activity against MRSA [44].

3.7 Synergistic Action of cLf, bLf, or hLf Combined with Antibiotics Against *E. coli*

We assessed the drug (Lfs and antibiotics) interactions in vitro by determining the fractional inhibitory concentration indexes (FICI) values of two agents (antibiotics and lactoferrin) combinations. Based on the standard protocol previously described and data shown in Table 5, when MICs were measured for a given antibiotic alone and in combination with three antibacterial proteins (cLf, hLf, or bLf), FICI values of 0.5 were found indicating that the MIC values were reduced to ¼ MIC for both reagents (antibiotic and protein) in the combination treatment. A FICI value of 0.37 indicated that values were reduced to 1/4 MIC for antibiotics and 1/8 MIC for Lf.

The synergy between the three antibiotics used in this study and cLf, hLf, or bLf was clear from their effects on *E. coli* (strains ATCC#25922 and ATCC#35218) which was evident from the corresponding FICI values ranging from 0.37 to 0.5 (Table 5). These in vitro results demonstrated, for first time, that different lactoferrins and antibiotics combinations show synergistic anti-enterobacteriaceae action. The synergy between them was further explored by examining the bactericide-induced changes in the morphology of bacterial cells, as described below.

Table 5 Effect of different lactoferrins in combination with antibiotics against *E. coli* (both strains)

Antimicrobial combination	FICI		Effect	
	E. coli ATCC#25922	*E. coli* ATCC#35218	*E. coli* ATCC#25922	*E. coli* ATCC #35218
cLf-C	0.37	0.37	Synergy	Synergy
hLf-C	0.5	0.5	Synergy	Synergy
bLf-C	0.5	0.5	Synergy	Synergy
cLf-CPM	–[a]	0.5	–	Synergy
hLf-CPM	–	0.5	–	Synergy
bLf-CPM	–	0.5	–	Synergy
cLf-IMI	0.37	0.37	Synergy	Synergy
hLf-IMI	0.5	0.5	Synergy	Synergy
bLf-IMI	0.5	0.5	Synergy	Synergy

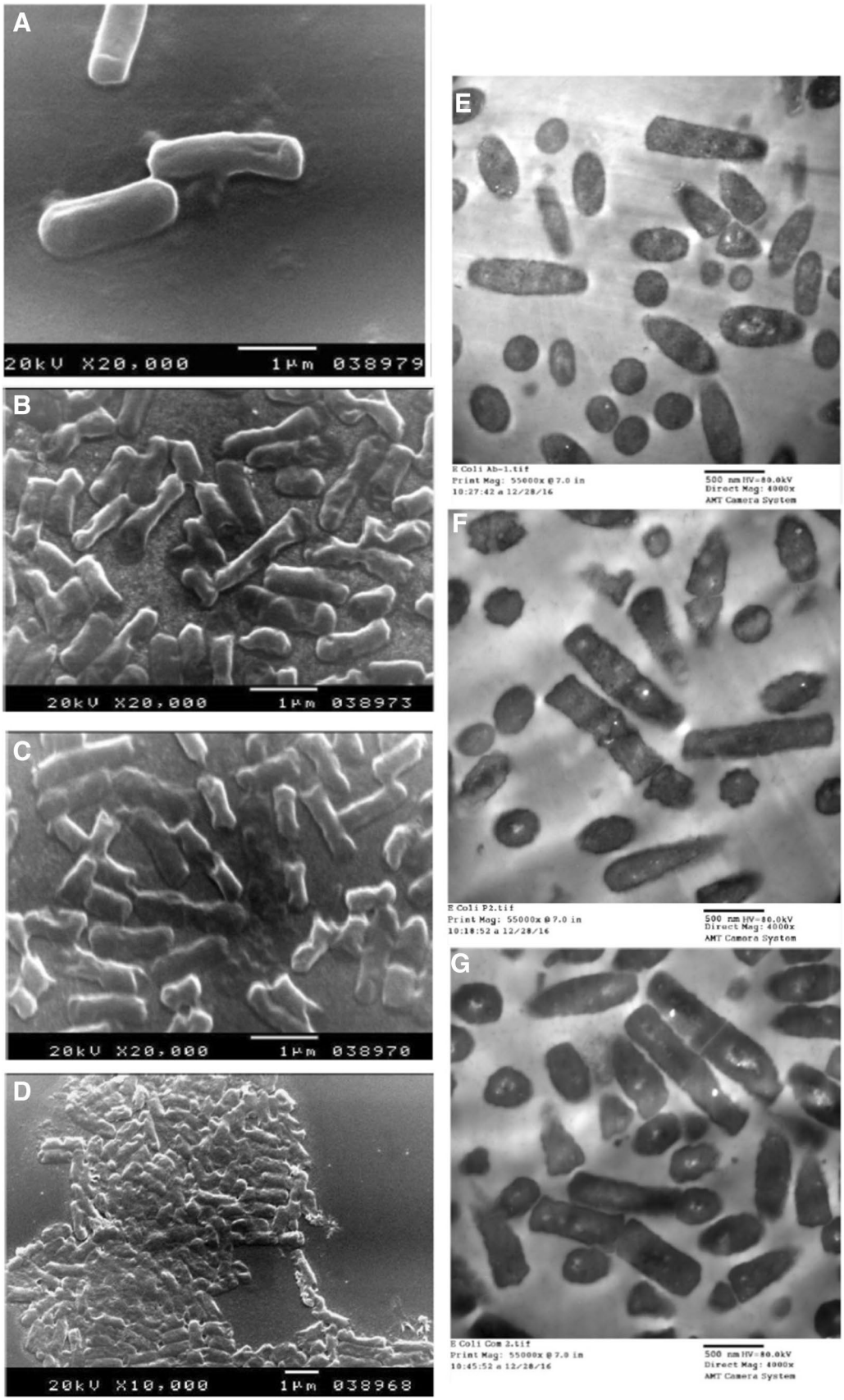
[a]Synergy was not studied as *E. coli* ATCC#25922 is resistant to cefepime (CPM)

3.8 Time-Kill Assay

Table 6 displays the results of the time-kill study as changes in the $\log_{10}$CFU/mL of *E. coli* strains ATCC#25922 and ATCC#35218 after 4 and 8 h incubation with cLf, hLf, or bLf alone, as well as in combinations with different antibiotics. The reduction in viable cell count was greatest after 8 h of incubation of these enterobacteriaceae with combinations of cLf with chloramphenicol ($-2.928\log_{10}$, and $-3.827\log_{10}$, respectively). In a similar way, the data presented in Table 6 show the trends of time-kill studies for hLf-chloramphenicol, bLf-chloramphenicol, cLf-cefepime, hLc-cefepime, bLf-cefepime, cLf-imipenem, hLf-imipenem, and bLf-imipenem combinations. These combinations were significantly more effective at reducing viable bacteria counts than cLf, hLf, or bLf alone. At concentrations of 1 × and 2 × MIC of cLf, hLf, or bLf alone, the substantial drop in analyzed bacteria population suggested that all lactoferrins display highly efficient bactericidal properties after incubation for 4 h,

Table 6 *In vitro* time-kill assessment of different lactoferrin forms alone and in combination with antibiotics against *E. coli* strains

Agent	Concentration	Log_{10}Kill					
		E. coli ATCC#25922			*E. coli* ATCC#35218		
		Incubation time with cLf, bLf or hLf alone and in combinations					
		0 h	4 h	8 h	0 h	4 h	8 h
cLf	½×MIC	2.230	3.432	4.551	2.188	3.211	4.750
	1×MIC	2.256	1.100	0.141	2.255	0.991	0.109
	2×MIC	2.341	0.112	−1.033	2.321	0431	−1.100
hLf	½×MIC	2.501	3.651	4.399	2.333	3.568	4.448
	1×MIC	2.286	1.298	0.326	2.267	1.213	0.104
	2×MIC	2.303	0.121	−1.901	2.234	0.101	−1.789
bLf	½×MIC	2.244	3.532	4.351	2.244	3.322	4.750
	1×MIC	2.251	1.121	0.130	2.265	0.891	0.118
	2×MIC	2.354	0.111	−1.120	2.254	0441	−1.120
cLf-C	¼ MIC	2.292	−1.345	**−2.928**	2.384	−2.390	**−3.827**
cLf-CPM	¼ MIC	2.393	−1.211	−1.511	2.374	−2.244	−3.133
cLf-IMI	¼ MIC	2.245	−1.442	−2.521	2.229	−2.428	−3.584
hLf-C	¼ MIC	2.211	1.330	−1.217	2.325	2.315	−1.170
hLf-CPM	¼ MIC	2.274	1.121	−2.015	2.173	−2.195	−2.724
hLf-IMI	¼ MIC	2.251	1.141	−2.041	2.241	−2.282	−2.441
bLf-C	¼ MIC	2.144	1.330	−2.017	2.125	2.325	2.177
bLf-CPM	¼ MIC	2.201	1.091	−2.115	2.291	−2.195	−2.724
bLf-IMI	¼ MIC	2.335	1.261	−2.041	2.342	−2.162	−2.441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Direct Mag: 4000x
AMT Camera System

◂**Fig. 5** Scanning electron microscope (SEM) images (**a**–**d**) and transmission electron microscope (TEM) images (**e**–**g**) for: **a** untreated *E. coli* (ATCC#35218); **b** carbenicillin-treated *E. coli*; **c** cLf-treated *E. coli*; **d** synergistic effects of combination of carbenicillin and cLf on treated *E. coli*; **e** carbenicillin treated *E. coli*. Black gold particles are scattered in extracellular and intracellular compartments; **f** cLf-treated *E. coli*. Black gold particles are scattered in extracellular and intracellular compartments in addition to the destructive effects of lactoferrin protein. The black dots scattered in and out the bacterial membrane, which lost its organization and seems as eroded. **g** Effects of combined cLf and carbenicillin treatment on *E. coli*. The gold nanoparticles are scattered in the extracellular and intracellular compartments in addition to the vigorous destructive and membrane fragmentation effects. Most of the bacterial cells stunted, lost their general rod shape and became rounded, stunted and disorganized. The bacterial cell membrane, which lost its organization seems as eroded, fragile and fragmented

and become more toxic after 8 h. Bacterial colonies were almost completely wiped out after incubation for 8 h with cLf or hLf at the aforementioned concentrations. On the other hand, there was a net bacterial growth when these enterobacteriaceae were subjected to 1/2 × MIC of any Lfs (Table 6).

3.9 Determination of Minimum Bactericidal Concentration

Since bacterial growth was resumed after spreading the contents of wells with no-growing bacteria on LB agar plates, cLf, hLf, and bLf can be labeled as bacteriostatic agents. However, at high concentrations, cLf and hLf/bLf were bactericidal against *E. coli* (strains ATCC#25922 and ATCC#35218) characterized by the minimum bactericidal concentration (MBC) of 0.25 mg/mL and 1.5 mg/mL, respectively. These observations indicate that the cLf action against these enterobacteriaceae is more effective than the antibacterial action of hLf or bLf.

The potential of hLf and bLf against viral and bacterial pathogens has been intensively studied and thoroughly reviewed [50]. Although noticeable anti-viral activity of cLf against several viruses was described (see "Introduction" section), the anti-bacterial effects of this protein are studied to a much lesser degree, with no systematic research reporting the capability of cLf to affect different enterobacteriaceae. In the past, the effect of cLf (as well as Lfs from other sources) on one strain of *E. coli* 0157:H7 was studied [31]. Data generated by Conesa et al. [31] agrees with the findings of our study and shows that cLf was more effective against *E. coli* than Lfs from other organisms (although our results were more quantitative, indicating that cLfs were 6 times more potent than hLf and bLf). Therefore, the data on the effect of cLf on *E. coli* strains ATCC#25922 and ATCC#35218 presented in this study are absolutely novel.

3.10 Scanning and Transmission Electron Microscopy (SEM and TEM) Analyses

The SEM examination was conducted to follow the morphological changes induced in the overnight cultures of *E. coli* (10^4 CFU/mL) by treatment with carbenicillin, cLf, or cLf-carbenicillin combination for 3 h at room temperature (Fig. 5a–d). The untreated (control) strain demonstrated regular shape and normal morphology (see Fig. 5a). The treated cells showed signs of noticeable cellular damage manifested in irregular and wrinkled outer surfaces, fragmentation, adhesion, and aggregation of damaged cells, or the presence of crushed cells and cellular debris. This study also revealed that the cLf-carbenicillin combination induced more evident morphological changes, such as irregular and wrinkled outer surface, adhesion, cellular damage, and fragmentation. These observations supported the results of other analyses conducted in this study, as well as previous results for other bacterial strains [44, 53, 63] and indicated a clear synergistic mode of action of both agents on bacterial strains.

Next, Transmission Electron microscopy (TEM) images were taken for *E. coli* after treatment with antibiotic carbenicillin, cLf-gold nanoparticle conjugates, or combination of carbenicillin and cLf-gold nanoparticles at room temperature for 3 h (Fig. 5e–g). In these experiments, various signs of cell membrane and wall deformation were detected, and the gold nanoparticle (black dots) can be easily found both on and inside the treated cells. The clearest deformation signs were evident after the treatment with a carbenicillin-cLf combination and manifested by cell membrane and cell wall disintegration, bigger cell sizes, loss of regular cellular shapes, and separation of cell wall (manifested as zigzag or erosion) from cell. The TEM results clearly indicated that lactoferrin alone and/or in combination with carbenicillin is able to affect *E. coli* cells via both bacteriostatic and bactericidal avenues, affecting their cell membrane structures (likely via interaction with BMPs and LPS), as well as entering into the bacterial cells and localizing to different cellular compartments. The peculiarities of the intracellular activities of Lf need to be elucidated in future results.

4 Discussion

The results reported in this study clearly prove the following facts: (1) Lactoferrins of different origin are capable of efficient killing of *E. coli* (strains ATCC#25922 and ATCC#35218) in vitro; (2) cLf has a higher killing potential than hLf and bLf; (3) cLf subtypes purified from milk of four Saudi camel clans have the same antibacterial activity; (4) There are multiple lactoferrins-binding proteins on the bacterial membranes; Lf can exert its antibacterial action both extracellularly and intercellularly.

Our data support the idea that some antimicrobial mechanisms are present in addition to the ferrochelating potential of Lfs extracellularly, and the existence of these mechanisms can be concluded from the results of the Lf-LPS interaction analysis and the presence of multiple binding proteins for lactoferrin on the bacterial membranes indicating a substantial interaction between lactoferrin and bacterial cells through the outer membrane proteins and integral LPS. Additionally, the effects of different anti-bacterial agents used in this study (such as antibiotics, lactoferrins, and combinations of antibiotics and cLf) on the released, appeared, disappeared, and/or highly expressed bacterial proteins clearly indicated that different antibacterial agents used alone or in combination have substantial effects on *E. coli*, and that these effects change with time.

We analyzed the efficiency of Lf interaction with bacterial LPS by ELISA. The results of these analyses revealed that all lactoferrins significantly ($p < 0.05$) interacted with bacterial LPS, but cLf was more reactive ($p < 0.001$) against bacterial LPS in comparison with hLf and bLf. Also, lactoferrin alone or combined with the antibiotic leads to release a significant amount of bacterial proteins ($p < 0.05$ - 0.001). Together with the results of the Lf-LPS interaction, the protein release observations may indicate that an efficient interaction is taking place between lactoferrin and *E. coli*, which might contribute to the Lf antibacterial action exerted via both bactericidal and bacteriostatic effects. These results support and agree with the previous reports, where the presence of multiple and different lactoferrin-binding protein sites were found in different bacterial strains [54–62]. Meanwhile, both hLf and bLf were previously shown to bind and sequester LPS, thereby preventing pro-inflammatory pathway activation, but no comparative analysis with cLf was conducted on this activity [45]. Furthermore, it is still unclear as to why the effects of cLf on *E.coli* are superior to those of hLf and bLf. Some thoughts regarding this phenomenon are outlined below.

Lactoferrin is a globular iron-binding glycoprotein with the molecular mass of 80 kDa. Structure of lactoferrin can be described as two nearly identical lobes possessing similar iron-binding sites. Although structures of lactoferrins of different origin are generally conserved, they differ by the positions of their N-linked oligosaccharide side chains. All lactoferrins are characterized by a relatively high isoelectric point. Curiously, the majority of the positively charged residues are found within the N-terminal region of the N-terminal lobe. It is believed that the high net positive charge of lactoferrins at physiological pH defines the ability of these proteins to bind to the various negatively charged components located on the bacterial surface, especially the LPS. As a result, the interpretation of findings from binding studies can be complicated by these electrostatic interactions. Consequently, detecting lactoferrin binding is not functionally significant on its own. On the other hand, since lactoferrins of different origin share cationic properties, if specific binding is detected for a given lactoferrin, it is likely to be functionally significant. Furthermore, for a long time, it has been known that lactoferrin has significant antimicrobial potential. Although originally this biocidal activity was preferentially ascribed to the role of lactoferrins in iron sequestration, more recent studies indicated that interactions between lactoferrin and bacteria, lactoferrin and bacterial products, and lactoferrin and host cells all may play a role in host-bacteria interaction beside the iron sequestration [54, 56, 58, 60–62, 64–67].

Recently, Lfs isolated from different species (camel, sheep, alpaca, goat, human, elephant, and cow) were shown to efficiently inhibit *E. coli* 0157:H7 infection, with cLF being the most efficient among other lactoferrins [31]. This superiority of cLf can be due to the fact that camel protein possesses some specific characteristics that separate it from the Lf of other species. First, several residues in cLf related to domain movement, including Leu423, Pro418, Gln651, Lys433, Lys637, Gly629, Pro592, and Arg652 are different from those in other Lf species, suggesting specific structure-related differences. Second, cLf loses half of its iron contents at pH 6.5 and the remaining half is lost at acidic conditions (pH 4.0–2.0). The N-lobe loses iron at acidic pH less than 4.0, whereas the C-lobe loses iron at pH 6.5, indicating a difference in the iron release mechanism from the two lobes. This indicates that cLf acts as both a transferrin, which is an iron transporter protein, and lactoferrin, which is an iron binding protein, unlike other transferrins and lactoferrins that possess unique iron transfer or binding functions [68–70]. Furthermore, when lactoferrin is losing its iron (i.e., when the apo-form of lactoferrins is formed), conformations of its N- and C-lobes undergo significant changes, with these lobes accommodating open and close states, respectively. These unique structural features offer opportunity for the Lf lobes to move freely around the subtract target (such as bacterial proteins and/or outer membrane glycocompounds) [71, 72], Third, cLf is entirely different from Lfs of other species in localization of its predicted glycosylation sites. Finally, the high cLf cationicity is one of the major structural characteristics (hydrophobicity, cationicity, and helical propensity) serving as important determinants of the antimicrobial potency of any protein or peptide [50]. As a matter of fact, cLF has a pI of 8.63 and a net charge of + 14, being characterized by the presence of 86 positively charged (Arg and Lys) and 72 negatively charged (Asp and Glu) residues. Importantly, although charged residues are located on the surface of the protein globule, they are non-equally distributed over the N- and C-lobes of cLF. In fact, the N-lobe (residues 25–352, pI 8.98) contains 42 positively charged Arg and Lys residues and 31 negatively charged Asp and Glu residues, whereas the C-lobe (residues

364–693, pI 7.50) shows almost equal content of positively and negatively charged residues (39 and 38, respectively). It is likely that the virucidal as well as bactericidal and bacteriostatic activity of cLf may depend on these intrinsic protein characteristics [50].

Based on the results of ExPASy protein calculator analysis, intact camel lactoferrin, its N-lobe, and C-lobe, have p*I* values of 8.63, 9.15, and 7.48, respectively. This finding provides an explanation for the superior antibacterial potency of the N-lobe of cLf over the intact protein and its C-lobe and also over the bovine and human lactoferrins. It is also very likely that the divergence in some of cLf and its lobes' structure-related characteristics can be determined by examining the difference in their biological activities. This hypothesis is supported by the previous results of the disorder propensity evaluation in the N- and C-lobes of cLf by a set of disorder predictors of the PONDR family [47, 73]. As well as, this superiority is agree and support Conesa et al. [43] report showing that cLf was more effective against *E. coli* than Lfs from other organisms.

The synergy results between cLf, hLf, or bLf, and antibiotics obtained in this study agree with the findings we previously observed that combining cLf or hLf with either vancomycin or oxacillin at sub-MIC levels offers greater in vitro antibacterial activity against MRSA when compared to using either agent on its own [44]. Synergism between lactoferrins (iron chelators) and some antibiotics was previously explained by the fact that the antimicrobial action of several, but not all, groups of antibiotics is suppressed by iron [74].

At concentrations of 1 × and 2 × MIC of cLf, hLf, or bLf alone, the substantial drop in analyzed bacteria population suggested that all lactoferrins display strong bactericidal properties after incubation for 4 h, and become more toxic after 8 h. Bacterial colonies were almost completely wiped out after incubation for 8 h with cLf or hLf at the aforementioned concentrations. On the other hand, there was a net bacterial growth when these *E.coli* strains were subjected to 1/2 × MIC of any Lfs. Also, the combination of cLf with chloramphenicol demonstrated the greatest effects over hLf or bLf combinations with the same antibiotic or other antibiotics used. These results agree with the findings we previously observed concerning cLf and hLf against MRSA [44].

5 Conclusions

The pressure associated with the appearance of antibiotic-resistant bacteria and the cost of cure and control of the enterobacteriaceae infection increase annually. The goal of this study was to analyze the potential of camel lactoferrin against the enteric bacterial microbes, such as *E. coli* (strains ATCC#25922 and ATCC#35218) in comparison with human and bovine lactoferrin alone and in combination with different antibiotics. Since Saudi habitats contain four different clans of *Camelus dromedary*, it was also important to examine if lactoferrins isolated from these camel clans have the same anti-microbial potentials. Our results suggest that cLf has a superior anti-microbial potential in comparison with the hLf and bLf. However, there were no differences between the lactoferrins isolated from different Saudi camel clans. We also showed that there was a synergy between the inhibitory activities of cLf, hLf, bLf, and antibiotics against *E. coli* growth. This synergy may help to enhance the efficiency of antibiotics with low efficacy. Also, the use of Lf-antibiotic combinations may represent a promising approach for controlling the problem of bacterial resistance and can be used to decrease the cost of various cures, especially in developing countries. These results are very promising and may lead to preclinical and clinical trials to examine the potential of using cLf against current enterobacteriaceae pathogens alone or in combination with antibiotics, especially when orally administered in a form of purified protein or as treated milk.

There are some limitations to acknowledge for the present study. Mainly, more extensive research should be done on the regions in cLf that have the high binding affinity to membrane proteins and/or LPS of bacterial cells, how they are different or similar with those of hLf or bLf, and an in vivo explanation for cLf superior activity.

Author Contributions EMR conceived the idea, supervised the project, organized and analyzed data, contributed to discussion, and with NAE-B wrote the manuscript and edited the manuscript. NAE-B did the experimental work, HAA, and AAA, VNU collected and analyzed data, contributed to discussion, and participated in writing and finalize the manuscript.

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Compliance with Ethical Standards

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References

1. Black RE (1993) Epidemiology of diarrhoeal disease: implications for control by vaccines. Vaccine. 11(2):100–106
2. Scallan E, Mahon BE, Hoekstra RM, Griffin PM (2013) Estimates of illnesses, hospitalizations and deaths caused by major bacterial enteric pathogens in young children in the United States. Pediatr Infect Dis J. 32(3):217–221. https://doi.org/10.1097/INF.0b013e31827ca763

3. WHO (2006) Future directions for research on enterotoxigenic *Escherichia coli* vaccines for developing countries. Wkly Epidemiol Rec. 81(11):97–104
4. Walker CL, Black RE (2010) Diarrhoea morbidity and mortality in older children, adolescents, and adults. Epidemiol Infect 138(9):1215–1226. https://doi.org/10.1017/S0950268810000592
5. Conway T, Cohen PS (2015) Commensal and pathogenic *Escherichia coli* metabolism in the gut. Microbiol Spectr. https://doi.org/10.1128/microbiolspec.mbp-0006-2014
6. Finegold SM, Sutter VL, Mathisen GE (1983) Normal indigenous intestinal microflora. In: Hentges DJ (ed) Human intestinal microflora in health and disease. Academic Press, Inc., New York, pp 3–31
7. Tenaillon O, Skurnik D, Picard B, Denamur E (2010) The population genetics of commensal *Escherichia coli*. Nat Rev Microbiol 8(3):207–217. https://doi.org/10.1038/nrmicro2298
8. Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH et al (2006) Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol Microbiol 60(5):1136–1151. https://doi.org/10.1111/j.1365-2958.2006.05172.x
9. Nataro JP, Kaper JB (1998) Diarrheagenic Escherichia coli. Clin Microbiol Rev 11(1):142–201
10. Redwan EM, Tabll A (2007) Camel lactoferrin markedly inhibits hepatitis C virus genotype 4 infection of human peripheral blood leukocytes. J Immunoassay Immunochem 28(3):267–277. https://doi.org/10.1080/15321810701454839
11. El Agamy EI (2006) Camel milk. In: Park YW, Haenlein FW (eds) Handbook of non-bovine mammals. Blackwell Publisher Professional, Iowa, pp 297–344
12. Farah Z, Mollet M, Younan M, Dahir R (2007) Camel dairy in Somalia: limiting factors and development potential. Livestock Sci. 110:187–191
13. Farah Z (1996) Camel milk properties and products. Swiss Centre for Developments Cooperation in Technology and Management, St. Gallen
14. Zhang H, Yao J, Zhao D, Liu H, Li J, Guo M (2005) Changes in chemical composition of *Alxa bactrian* camel milk during lactation. J Dairy Sci 88(10):3402–3410. https://doi.org/10.3168/jds.S0022-0302(05)73024-1
15. Ramadan S, Inoue-Murayama M (2017) Advances in camel genomics and their applications: a review. J Anim Gen. 45:49–58
16. Kappeler S, Farah Z, Puhan Z (1998) Sequence analysis of *Camelus dromedarius* milk caseins. J Dairy Res 65(2):209–222
17. Konuspayeva G, Faye B, Loiseau G (2009) The composition of camel milk: a meta-analysis of the literature data. J Food Compost Anal. 22:95–101
18. Nikkah A (2011) Equidae, camel, and yak milks as functional foods: a review. J Nutr Food Sci. 1(5):1000116
19. Konuspayeva G, Faye B, Loiseau G, Levieux D (2007) Lactoferrin and immunoglobulin contents in camel's milk (*Camelus bactrianus*, *Camelus dromedarius*, and Hybrids) from Kazakhstan. J Dairy Sci. 90(1):38–46
20. Pammi M, Abrams SA (2015) Oral lactoferrin for the prevention of sepsis and necrotizing enterocolitis in preterm infants. Cochrane Database Syst Rev. https://doi.org/10.1002/14651858.cd007137.pub4
21. Johnston WH, Ashley C, Yeiser M, Harris CL, Stolz SI, Wampler JL et al (2015) Growth and tolerance of formula with lactoferrin in infants through one year of age: double-blind, randomized, controlled trial. BMC Pediatr. 15:173. https://doi.org/10.1186/s12887-015-0488-3
22. Schlimme E, Martin D, Meisel H (2000) Nucleosides and nucleotides: natural bioactive substances in milk and colostrum. Br J Nutr 84(Suppl 1):S59–S68
23. Phelan M, Kerins D (2011) The potential role of milk-derived peptides in cardiovascular disease. Food Funct. 2(3–4):153–167. https://doi.org/10.1039/c1fo10017c
24. Garcia C, Duan RD, Brevaut-Malaty V, Gire C, Millet V, Simeoni U et al (2013) Bioactive compounds in human milk and intestinal health and maturity in preterm newborn an overview. Cell Mol Biol. 59(1):108–131
25. Lonnerdal B (2014) Infant formula and infant nutrition: bioactive proteins of human milk and implications for composition of infant formulas. Am J Clin Nutr 99(3):712S–717S. https://doi.org/10.3945/ajcn.113.071993
26. Hill DR, Newburg DS (2015) Clinical applications of bioactive milk components. Nutr Rev 73(7):463–476. https://doi.org/10.1093/nutrit/nuv009
27. Hsieh CC, Hernandez-Ledesma B, Fernandez-Tome S, Weinborn V, Barile D, de Moura Bell JM (2015) Milk proteins, peptides, and oligosaccharides: effects against the 21st century disorders. Biomed Res Int. 2015:146840. https://doi.org/10.1155/2015/146840
28. Sultan S, Huma N, Butt MS, Aleem M, Abbas M (2016) Therapeutic potential of dairy bioactive peptides: a contemporary perspective. Crit Rev Food Sci Nutr. 58(1):1–11
29. Demmelmair H, Prell C, Timby N, Lonnerdal B (2017) Benefits of lactoferrin, osteopontin and milk fat globule membranes for infants. Nutrients. 9(8):817
30. Marcone S, Belton O, Fitzgerald DJ (2017) Milk-derived bioactive peptides and their health promoting effects: a potential role in atherosclerosis. Br J Clin Pharmacol 83(1):152–162. https://doi.org/10.1111/bcp.13002
31. Conesa C, Calvo M, Sanchez L (2010) Recombinant human lactoferrin: a valuable protein for pharmaceutical products and functional foods. Biotechnol Adv 28(6):831–838. https://doi.org/10.1016/j.biotechadv.2010.07.002
32. Bruni N, Capucchio MT, Biasibetti E, Pessione E, Cirrincione S, Giraudo L et al (2016) Antimicrobial activity of lactoferrin-related peptides and applications in human and veterinary medicine. Molecules. 21(6):750
33. Jenssen H, Hamill P, Hancock RE (2006) Peptide antimicrobial agents. Clin Microbiol Rev 19(3):491–511. https://doi.org/10.1128/CMR.00056-05
34. Samyn-Petit B, Wajda Dubos JP, Chirat F, Coddeville B, Demaizieres G, Farrer S et al (2003) Comparative analysis of the site-specific N-glycosylation of human lactoferrin produced in maize and tobacco plants. Eur J Biochem. 270(15):3235–3242
35. El-Fakharany EM, Tabll A, Wahab AA, Redwan EM (2008) Potential activity of camel milk-amylase and lactoferrin against hepatitis C virus infectivity in HepG2 and lymphocytes. Hepat Mon. 8(2):101–109
36. El-Fakharany EM, Serour EA, Abdelrahman AM, Haroun BM, Redwan el RM (2009) Purification and characterization of camel (*Camelus dromedarius*) milk amylase. Prep Biochem Biotechnol 39(2):105–123. https://doi.org/10.1080/10826060902800288
37. Redwan EM (2009) Animal-derived pharmaceutical proteins. J Immunoassay Immunochem 30(3):262–290. https://doi.org/10.1080/15321810903084400
38. El-Fakharany EM, Haroun BM, Ng TB, Redwan ER (2010) Oyster mushroom laccase inhibits hepatitis C virus entry into peripheral blood cells and hepatoma cells. Protein Pept Lett 17(8):1031–1039
39. Almahdy O, El-Fakharany EM, El-Dabaa E, Ng TB, Redwan EM (2011) Examination of the activity of camel milk casein against hepatitis C virus (genotype-4a) and its apoptotic potential in hepatoma and hela cell lines. Hepat Mon. 11(9):724–730. https://doi.org/10.5812/kowsar.1735143X.722
40. Ng TB, Wong JH, Almahdy O, El-Fakharany EM, El-Dabaa E, Redwan EM (2011) Antimicrobial activities of casein and other

milk proteins. Casein: production, uses and health effects. Nova Publishers, New York
41. El-Fakharany EM, Abedelbaky N, Haroun BM, Sanchez L, Redwan NA, Redwan EM (2012) Anti-infectivity of camel polyclonal antibodies against hepatitis C virus in Huh7.5 hepatoma. Virol J. 9:201
42. Liao Y, El-Fakkarany E, Lonnerdal B, Redwan EM (2012) Inhibitory effects of native and recombinant full-length camel lactoferrin and its N and C lobes on hepatitis C virus infection of Huh7.5 cells. J Med Microbiol. 61(Pt 3):375–383
43. Conesa C, Sanchez L, Rota C, Perez MD, Calvo M, Farnaud S et al (2008) Isolation of lactoferrin from milk of different species: calorimetric and antimicrobial studies. Comp Biochem Physiol B 150(1):131–139
44. Redwan EM, El-Baky NA, Al-Hejin AM, Baeshen MN, Almehdar HA, Elsaway A et al (2016) Significant antibacterial activity and synergistic effects of camel lactoferrin with antibiotics against methicillin-resistant *Staphylococcus aureus* (MRSA). Res Microbiol 167(6):480–491. https://doi.org/10.1016/j.resmic.2016.04.006
45. Elass-Rochard E, Roseanu A, Legrand D, Trif M, Salmon V, Motas C et al (1995) Lactoferrin-lipopolysaccharide interaction: involvement of the 28-34 loop region of human lactoferrin in the high-affinity binding to *Escherichia coli* 055B5 lipopolysaccharide. Biochem J. 312(Pt 3):839–845
46. Almehdar HA, El-Baky NA, Alhaider AA, Almuhaideb SA, Alhaider AA, Albiheyri RS et al (2019) Bacteriostatic and bactericidal activities of camel lactoferrins against *Salmonella enterica* serovar typhi. Probiotics Antimicrob Proteins. https://doi.org/10.1007/s12602-019-9520-5
47. Albar AH, Almehdar HA, Uversky VN, Redwan EM (2014) Structural heterogeneity and multifunctionality of lactoferrin. Curr Protein Pept Sci 15(8):778–797
48. Wikler MA. Performance Standards for Antimicrobial Susceptibility Testing. Eighteenth Informational Supplement. Pennsylvania, PA, USA: C.L.S.I. (Clinical and Laboratory Standard Institute), 2008 Contract No.: M100-S18
49. Cockerill FR, Wikler MA, Alder J, Dudley MN, Eliopoulos GM, Ferraro MJ, et al. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard. CLSI document M07-A9.9. 9 ed. Pennsylvania, PA, USA: C.L.S.I. (Clinical and Laboratory Standard Institute); 2012
50. Redwan EM, Uversky VN, El-Fakharany EM, Al-Mehdar H (2014) Potential lactoferrin activity against pathogenic viruses. C R Biol. 337(10):581–595. https://doi.org/10.1016/j.crvi.2014.08.003
51. Park HM, Almeida RA, Oliver SP (2002) Identification of lactoferrin-binding proteins in *Streptococcus dysgalactiae* subsp. dysgalactiae and *Streptococcus agalactiae* isolated from cows with mastitis. FEMS Microbiol Lett. 207(1):87–90
52. Reddy SB, Mainwaring DE, Kobaisi MA, Zeephongsekul P, Fecondo JV (2012) Acoustic wave immunosensing of a meningococcal antigen using gold nanoparticle-enhanced mass sensitivity. Biosens Bioelectron 31(1):382–387. https://doi.org/10.1016/j.bios.2011.10.051
53. Almeida RA, Luther DA, Park HM, Oliver SP (2006) Identification, isolation, and partial characterization of a novel Streptococcus uberis adhesion molecule (SUAM). Vet Microbiol 115(1–3):183–191. https://doi.org/10.1016/j.vetmic.2006.02.005
54. Beddek AJ, Schryvers AB (2010) The lactoferrin receptor complex in Gram negative bacteria. Biometals 23(3):377–386. https://doi.org/10.1007/s10534-010-9299-z
55. Drago-Serrano ME, Campos-Rodriguez R, Carrero JC, de la Garza M (2017) Lactoferrin: balancing ups and downs of inflammation due to microbial infections. Int J Mol Sci. 18(3):501. https://doi.org/10.3390/ijms18030501
56. Drago-Serrano ME, de la Garza-Amaya M, Luna JS, Campos-Rodriguez R (2012) Lactoferrin-lipopolysaccharide (LPS) binding as key to antibacterial and antiendotoxic effects. Int Immunopharmacol 12(1):1–9. https://doi.org/10.1016/j.intimp.2011.11.002
57. Drago-Serrano ME, Rivera-Aguilar V, Resendiz-Albor AA, Campos-Rodriguez R (2010) Lactoferrin increases both resistance to Salmonella typhimurium infection and the production of antibodies in mice. Immunol Lett 134(1):35–46. https://doi.org/10.1016/j.imlet.2010.08.007
58. Morgenthau A, Partha SK, Adamiak P, Schryvers AB (2014) The specificity of protection against cationic antimicrobial peptides by lactoferrin binding protein B. Biometals 27(5):923–933. https://doi.org/10.1007/s10534-014-9767-y
59. Ochoa TJ, Cleary TG (2009) Effect of lactoferrin on enteric pathogens. Biochimie 91(1):30–34. https://doi.org/10.1016/j.biochi.2008.04.006
60. Rahman M, Kim WS, Kumura H, Shimazaki K (2009) Bovine lactoferrin region responsible for binding to bifidobacterial cell surface proteins. Biotechnol Lett 31(6):863–868. https://doi.org/10.1007/s10529-009-9936-1
61. Samaniego-Barron L, Luna-Castro S, Pina-Vazquez C, Suarez-Guemes F, de la Garza M (2016) Two outer membrane proteins are bovine lactoferrin-binding proteins in *Mannheimia haemolytica* A1. Vet Res 47(1):93. https://doi.org/10.1186/s13567-016-0378-1
62. Staggs TM, Greer MK, Baseman JB, Holt SC, Tryon VV (1994) Identification of lactoferrin-binding proteins from *Treponema pallidum* subspecies pallidum and *Treponema denticola*. Mol Microbiol. 12(4):613–619
63. Yu RH, Schryvers AB (2002) Bacterial lactoferrin receptors: insights from characterizing the Moraxella bovis receptors. Biochem Cell Biol. 80(1):81–90
64. Dhaenens L, Szczebara F, Husson MO (1997) Identification, characterization, and immunogenicity of the lactoferrin-binding protein from Helicobacter pylori. Infect Immun. 65(2):514–518
65. Fang W, Oliver SP (1999) Identification of lactoferrin-binding proteins in bovine mastitis-causing Streptococcus uberis. FEMS Microbiol Lett. 176(1):91–96
66. Schryvers AB, Morris LJ (1988) Identification and characterization of the human lactoferrin-binding protein from *Neisseria meningitidis*. Infect Immun. 56(5):1144–1149
67. Tomita S, Shirasaki N, Hayashizaki H, Matsuyama J, Benno Y, Kiyosawa I (1998) Binding characteristics of bovine lactoferrin to the cell surface of Clostridium species and identification of the lactoferrin-binding protein. Biosci Biotechnol Biochem 62(8):1476–1482. https://doi.org/10.1271/bbb.62.1476
68. Sharma AK, Paramasivam M, Srinivasan A, Yadav MP, Singh TP (1999) Three-dimensional structure of mare diferric lactoferrin at 2.6 A resolution. J Mol Biol. 289(2):303–317. https://doi.org/10.1006/jmbi.1999.2767
69. Sharma S, Sinha M, Kaushik S, Kaur P, Singh TP (2013) C-lobe of lactoferrin: the whole story of the half-molecule. Biochem Res Int. 2013:271641. https://doi.org/10.1155/2013/271641
70. Sinha M, Kaushik S, Kaur P, Sharma S, Singh TP (2013) Antimicrobial lactoferrin peptides: the hidden players in the protective function of a multifunctional protein. Int J Pept. 2013:390230. https://doi.org/10.1155/2013/390230
71. Khan JA, Kumar P, Paramasivam M, Yadav RS, Sahani MS, Sharma S et al (2001) Camel lactoferrin, a transferrin-cum-lactoferrin: crystal structure of camel apolactoferrin at 2.6 A resolution and structural basis of its dual role. J Mol Biol. 309(3):751–761
72. Khan JA, Kumar P, Srinivasan A, Singh TP (2001) Protein intermediate trapped by the simultaneous crystallization process. Crystal structure of an iron-saturated intermediate in the Fe^{3+} binding pathway of camel lactoferrin at 2.7 a resolution. J Biol Chem. 276(39):36817–36823. https://doi.org/10.1074/jbc.m104343200

73. Redwan EM, El-Fakharany EM, Uversky VN, Linjawi MH (2014) Screening the anti infectivity potentials of native N- and C-lobes derived from the camel lactoferrin against hepatitis C virus. BMC Complement Altern Med. 14:219. https://doi.org/10.1186/1472-6882-14-219
74. Luo G, Spellberg B, Gebremariam T, Lee H, Xiong YQ, French SW et al (2014) Combination therapy with iron chelation and vancomycin in treating murine staphylococcemia. Eur J Clin Microbiol Infect Dis 33(5):845–851. https://doi.org/10.1007/s10096-013-2023-5