Differential Antimicrobial Effectiveness of Camel Lactoferrin-Oleic Acid and Bovine Lactoferrin-Oleic Acid Complexes against Several Pathogens

Nawal Abd El-Baky*

Abstract
Considering the superior biological activities of Camel Lactoferrin (cLf) over lactoferrin from other animal species; which we previously confirmed and continuing the analysis of antimicrobial effectiveness of cLf; we started in previous studies, the current study aimed to formulate a protein-fatty acid complex of cLf and Oleic Acid (OA) and to compare it's in vitro antimicrobial activities against different pathogens with those of a similar Bovine Lactoferrin (bLf)-OA complex. Antimicrobial activity of these complexes was evaluated by agar disc diffusion method, broth microdilution assay, and ELISA-estimating Lf and its complexes binding to bacterial outer membrane proteins. Agar disc diffusion assay results revealed that inhibitory activity of both free cLf and cLf-OA against 13 test pathogens (Methicillin-Resistant Staphylococcus Aureus (MRSA), Staphylococcus aureus, Bacillus cereus, Escherichia coli, Salmonella typhi, Shigella sonnei, Klebsiella pneumonia, Pseudomonas aeruginosa, Proteus vulgaris, Serratia marcescens, Candida albicans, Aspergillus niger, and Aspergillus flavus) noticeably exceeded that of corresponding bLf and bLf-OA. Additionally, free OA exhibited antimicrobial activity against MRSA, S. aureus, B. cereus, and C. albicans and to a lesser extent against E. coli, K. pneumoniae as well as A. niger and A. flavus. Consequently, synergy was evident between cLf/bLf and OA (mostly higher in case of cLf) in prepared complexes against MRSA, S. aureus, B. cereus, and C. albicans. cLf-OA demonstrated 4 times lower Minimum Inhibitory Concentration (MIC) values against MRSA, B. cereus, and C. albicans than bLf-OA; indicating more superiority in case of cLf-OA than free cLf that showed only twice the activity of bLf. ELISA signals confirmed binding of biotinylated cLf/bLf and cLf/bLf-OA to bacterial membrane proteins. This study proves that cLf obtains enhanced antimicrobial activities after complex formation with fatty acids such as OA even than its free form which has already superior activity than otherLf species; thus this complex may be used as a cure of various microbial infections.

Keywords: Antimicrobial; bovine lactoferrin; camel lactoferrin; oleic acid; protein-fatty acid complexes

Introduction
Lactoferrin is an 80 kDa iron-binding glycoprotein, which is found in numerous secretory fluids, for example milk [1]. Lf displays antimicrobial activity against a variety of pathogenic microorganisms besides modulating the immune system. Most published studies aimed to reveal therapeutic utilization of bLf and Human Lactoferrin (hLf) for treatment of various infectious and inflammatory diseases, but fewer studies have been reported on cLf. Evidences point to the differences in the biological (antimicrobial) activities between cLf and other lactoferrins. It was found that cLf was the most active lactoferrin against many pathogens [2, 3].

Lactoferrin has a variety of biological functions, many of which not related to its iron-binding capability [4]. Lf is an important part of the innate immune system. Besides its major biological function, which is binding and transport of iron ions, lactoferrin has various other functions such as antibacterial, anti-fungal, antiparasitic, antiviral, antiallergic, catalytic, and anticancer functions. Lf exhibits diverse inhibitory effects against microorganisms, including stasis, cidal, synergistic, bacterial adhesion blocking, opsonic, and cationic mechanisms. Due to broad-spectrum activities of Lf against various bacteria, fungi, parasites, and viruses, along with its immunomodulatory and anti-inflammatory functions, lactoferrin seems to have great impact on practical medicine [5].

OA; the key monounsaturated fatty acid of olive oil has the ability of in vitro killing of different bacterial and fungal pathogens. Long-chain unsaturated fatty acids such as OA and linoleic acid are bactericidal to various pathogens including MRSA and Helicobacter pylori [6, 7]. Also, they have in vitro killing activity against C. albicans [8].

Both LF and α-Lactalbumin (α-LA) have similar iron-binding region structure [9]. It was confirmed that these proteins release the bound ions at acid pH to produce a more open structure; a property that favors OA binding [10-12].
Therefore, in this study, complexes of cLf and bLf with OA were obtained. Then for the first time, their differential antimicrobial activity against several pathogens was evaluated by agar disc diffusion method and broth micro dilution assay, meanwhile, estimating LF binding to bacterial outer membrane proteins by ELISA.

**Materials and Methods**

**Antimicrobial Agents**

cLf and bLf were purified from camel and bovine milk after processing at our lab according to the protocol described by Redwan & Tabil (2007) [13]. Both purified cLf and bLf preparations were sterilized by filtration through 0.22 µm syringe filter (TFF, St. Louis, Mo., USA) and stored at −20°C until use. The iron saturation of both lactoferrins, checked by spectrophotometry, was of approximately 10% in case of bLf and 35% in case of cLf (partially iron-saturated) [14]. The protein content was tested by the Folin phenol reagent [15]. OA (C18:1 9 cis, ≥ 99.0% purity, Sigma-Aldrich) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Discs of 5 antibacterial agents, including carbenicillin (10 µg), vancomycin (30 µg), fusidic acid (10 µg), gentamicin (10 µg), and chloramphenicol (30 µg) were purchased from Mast Diagnostics (Mersseyside, UK). Nystatin and amphotericin-B antifungal standards at concentration of 100 µg/ml were obtained from Sigma-Aldrich.

**Determination of cLf and bLf Activity**

Activity of both lactoferrins was assayed according to the procedure described by Ye et al. with slight modifications [16]. Samples of lactoferrin (50 µl) were added to the mixture containing 15 µl of 50 mM Tris-HCl buffer (pH 8.0), and 75 µl of 300 mM dihydro-nicotinamide-adenine-dinucleotide-phosphate, 300 mM Nitroblue Tetrazolium (NBT), and 30 µM of Phenazine Methosulfate (PMS). The absorbance was checked at 0 and 5 min of reaction at 580 nm. L-ascorbic acid was used as a control. The calculations were based on standard curve prepared with different concentrations of NBT. LF activity was expressed in IU per milligram of protein.

**Preparation of cLf-OA and bLf-OA Complexes**

Both lactoferrins were dissolved in 10 mM phosphate buffered saline (PBS, pH 8.0) to different concentrations. OA was added directly to each protein solution at 50 molar equivalents (OA: LF). After vortexing for 30 s, the mixtures were incubated for 20 min at 45°C in a water bath. Finally, excess fatty acid in the complexes was removed by centrifugation at 4°C followed by ultra filtration using a 3000 kDa cut-off membrane.

**Oleic Acid Determination in the Prepared Complexes**

OA concentration in the prepared complexes was determined according to the colorimetric method of Duncombe [17]. In brief, protein complexes were shaken with chloroform and copper solution. Copper amount in chloroform is corresponding to the amount of OA in the test samples, which is examined by adding sodium diethylthiocarbamate as a color developer. Copper reagent (2.5 ml) consisting of 9 volumes of 1 M triethanolamine, 1 volume of 1 N acetic acid, and 10 volumes of 10% (w/v) copper sulphate was added to either 500 µl standard samples or 500 µl test samples. After shaking vigorously with a vortex mixer, 5 ml chloroform were added to the solution and shaken vigorously for 1 min. Then, 3 ml of the lower layer were carefully transferred to another test tube containing 500 µl of 0.1% sodium diethylthiocarbamate in butanol and absorbance values were recorded at 440 nm. Experiment was done three times, each in triplicate and the results were presented as mean ± SEM.

**Test Microorganisms and Growth Conditions**

MRSA clinical isolate was obtained from blood of a patient at Almery University Hospital (Alexandria, Egypt) and subjected to the confirming BD GeneOhm™ MRSA assay. The *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231 strains were purchased from Becton Dickinson (France). *Escherichia coli* ATCC 25922, *Salmonella typhi* ATCC 19430, and *Shigella sonnei* ATCC 25931 were obtained from American type culture collection (ATCC, USA). Bacillus cereus, Klebsiella pneumonia, Pseudomonas aeruginosa, Proteus vulgaris, Serratia marcescens, Aspergillus niger, and Aspergillus flavus were collected from Al-Azhar University Mycology Center (Cairo, Egypt), and Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Assiut Branch (Egypt).

A 100 µl aliquot culture of each bacterial strain was added to 2.5 ml of Luria Bertani (LB) broth, incubated at 37°C for 24 h, and then stored at −80°C after addition of 20% glycerol to be used as seeds stock. Yeasts such as *C. albicans* and fungi such as *A. niger* and *A. flavus* were maintained on Sabouraud’s dextrose agar at 4°C. To determine in vitro antibacterial activity, Cation-Adjusted Mueller-Hinton (CAMH) broth and Mueller-Hinton agar were used. While, in vitro antifungal activity was determined using Sabouraud’s dextrose broth and Sabouraud’s dextrose agar.

**Agar Disc Diffusion Assay**

Susceptibility screening of test microorganisms to cLf, bLf, OA, cLf-OA, bLf-OA, and different antibacterial and antifungal standards was carried out using the agar disc diffusion technique on Mueller-Hinton agar for bacteria and Sabouraud’s dextrose agar for fungi. Plates were overlaid with 100 µl of standardized inoculum suspension using McFarland standard, then wells were bored into the agar media by using a sterile 6 mm cork borer, and about 100 µl of solutions containing different concentrations of cLf, bLf, OA, cLf-OA, and bLf-OA were added into each well. After incubated for 24 h at 37°C in case of *C. albicans* and bacterial cultures, respectively, and for 5 days at 25°C in case of fungal cultures. Then plates were examined for the presence of the inhibition zones. The zone of inhibition was measured and interpreted using the Clinical and Laboratory Standards Institute (CLSI) zone...
were incubated at 37 °C and 25 °C, respectively for 24 h, whereas plates inoculated with fungi were incubated at 25 °C for 5 days. The MICs were determined by measuring the absorbance at 600 nm for test bacterial strains and *C. albicans* and calculating fungal sporulation using hemocytometer. The MIC was defined as the lowest concentration at which growth was completely inhibited. All MIC determinations were performed in duplicate. Bacteria in CAMH broth and fungi in Sabouraud’s dextrose broth were used as control of growth.

Detection of Lf and its Complexes Binding to Bacterial Membrane Proteins by ELISA

Bacterial membrane fractions were prepared from Gram-positive and Gram-negative bacteria under investigation. Test bacterial cells were harvested via centrifugation of their cultures and then washed with PBS at pH 7.4. Cells were suspended in 0.5 mg/ml herbinycin A, 0.1 mM sodium vanadate, 25 mg/ml leupeptin, 50 mg/ml aprotinin, 750 mg/ml benzamidine and 1 mM phenylmethylsulfonyl fluoride in carbonate/bicarbonate pH 9.6 buffer as a blank or 50 µl of biotinylated cLf/bLf at a concentration of 2 mg/ml as positive and Gram-negative bacteria under investigation. Test organisms wells. After 2 h of incubation at 37°C, the plate was washed 5 times with PBS, and 50 µl of alkaline phosphatase conjugated streptavidin (BIO-RAD, Alfred Nobel, Hercules, USA) diluted 1:1000 was added, followed by an incubation of 1 h at 37°C. After washing five times, p-Nitrophenyphosphate (p-NPP) was added for color development and optical density was calculated at 405 nm using an ELISA micro titer plate reader (Micro Plate Reader, BIO-RAD, USA). Results were represented as mean ± SD of three replicates.

Results and Discussion

Purification of cLf and bLf

Both lactoferrins were purified from skimmed milk by one-step affinity chromatography using heparin-Sepharose column and eluted by 0.0-1.0 M NaCl gradient. Purified fractions were analyzed by SDS-PAGE and then by ELISA (data not shown).

Single discrete band was obtained on 12% SDS polyacrylamide gel of the two proteins and estimated to be 80 kDa as shown in Figure 1.

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Agar Disc Diffusion Assay

cLf and bLf possessed antibacterial besides antifungal activities against a total of 13 test microorganisms (10 bacteria, 1 yeast, and 2 fungi; Tables 1, 2, and 3) and produced concentration dependent inhibition zones Figure 2. The mean diameter of inhibition zone of cLf, evaluated against the test organisms ranged between 7.3 mm against A. flavus at a concentration of 0.5 mg/ml and 39.7 mm against P. aeruginosa at a concentration of 1 mg/ml. On the other hand, the mean diameter of inhibition zone of bLf, evaluated against the test organisms ranged between 10.0 mm against A. niger at a concentration of 1 mg/ml and 42.0 mm against P. aeruginosa at a concentration of 1 mg/ml.

Figure 2: Differential antimicrobial effectiveness of cLf-OA and bLf-OA complexes against several pathogens. 1: represents different antibacterial or antifungal standards; 2: represents cLf/bLf-OA at concentration of 0.0625 mg/ml in case of Gram-positive bacteria and 0.125 mg/ml in case of Gram-negative bacteria and fungi; 3: represents cLf/bLf-OA at concentration of 0.125 mg/ml in case of Gram-positive bacteria and 0.25 mg/ml in case of Gram-negative bacteria and fungi; 4: represents cLf/bLf-OA at concentration of 0.25 mg/ml in case of Gram-positive bacteria and 0.5 mg/ml in case of Gram-negative bacteria and fungi; 5: represents cLf/bLf-OA at concentration of 0.5 mg/ml in case of Gram-positive bacteria and 1 mg/ml in case of Gram-negative bacteria and fungi; 6: represents sterile water used as negative control.
It was observed that neither cLf nor bLf showed any inhibition zones at concentration of 0.125 mg/ml against any of tested bacterial and fungal pathogens except against S. aureus in case of cLf which gave an inhibition zone of 19.3 mm mean diameter.

Table 1 showed that growth of MRSA, S. aureus, and B. cereus was inhibited by cLf at concentrations of 0.25-1 mg/ml, bLf at concentrations of 0.5-1 mg/ml (except for S. aureus that was inhibited by bLf at 0.25-1 mg/ml), cLf-OA at 0.0625-0.5 mg/ml, and bLf-OA at 0.25-0.5 mg/ml (except for S. aureus that was inhibited by bLf-OA at 0.125-0.5 mg/ml). This indicates a noticeable increase (this increase was mostly higher in case of cLf) in antibacterial activity against test Gram-positive bacteria of both cLf and bLf by binding to OA in the complexes prepared; a suggestion correlates with the previously published studies, which confirmed that oleic acid was active against many Gram-positive bacteria thus synergy was evident between cLf/bLf and OA [20, 21]. OA exhibited antibacterial activity against the Gram-positive bacteria MRSA, S. aureus, and B. cereus (zones of inhibition ranging from 10.0-15.7 mm were observed after overnight incubation Table 1).

Table 1: Antibacterial activity of cLf-OA and bLf-OA complexes against tested Gram-positive bacteria

<table>
<thead>
<tr>
<th>Strains</th>
<th>cLf (mg/ml)</th>
<th>bLf (mg/ml)</th>
<th>OA</th>
<th>cLf-OA (mg/ml)</th>
<th>bLf-OA (mg/ml)</th>
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<tr>
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<td>0.0625</td>
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<td>MRSA</td>
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<td>21</td>
<td>30.7 R</td>
<td>16.0</td>
<td>19.7</td>
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<tr>
<td>S. aureus</td>
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<td>30.7</td>
<td>36</td>
<td>20.3</td>
<td>27.0</td>
</tr>
<tr>
<td>B. cereus</td>
<td>20</td>
<td>23.7</td>
<td>32.7 R</td>
<td>19.0</td>
<td>20.3</td>
</tr>
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</table>

As presented in Table 2, growth of E. coli, S. sonnei, and S. typhi was inhibited by cLf and cLf-OA at concentrations of 0.25-1 mg/ml in addition to bLf and bLf-OA at concentration of 1 mg/ml. Klebsiella pneumonia was sensitive to cLf and cLf-OA at concentration of 1 mg/ml but was not sensitive to bLf at 0.25-1 mg/ml or bLf-OA at 0.125-1 mg/ml and only showed sensitivity towards 2 mg/ml bLf (12.0 mm zone of inhibition) and 2 mg/ml bLf-OA complex (15.3 mm zone of inhibition). cLf/cLf-OA and bLf/bLf-OA inhibited growth of P. aeruginosa, P. vulgaris, and S. marcescens at concentrations of 0.5-1 mg/ml and 1 mg/ml, respectively. The inhibitory activity of OA was lower against Gram-negative E. coli and K. pneumoniae compared to test Gram-positive bacteria (zones of inhibition of 3.7 mm and 3.3 mm, respectively, were observed after overnight incubation) while it has no effect on growth of other test Gram-negative bacteria Table 2. Thus, no change occurred in antibacterial activity against Gram-negative bacteria of both cLf and bLf by binding to OA in the complexes prepared. This agrees with results obtained by Dillica et al. who reported that OA was inactive against the Gram-negative species they tested [20].

Table 2: Antibacterial activity of cLf-OA and bLf-OA complexes against tested Gram-negative bacteria

<table>
<thead>
<tr>
<th>Strains</th>
<th>cLf (mg/ml)</th>
<th>bLf (mg/ml)</th>
<th>OA</th>
<th>cLf-OA (mg/ml)</th>
<th>bLf-OA (mg/ml)</th>
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<tr>
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<tr>
<td>E. coli</td>
<td>22.3</td>
<td>26.3</td>
<td>35.7 R</td>
<td>23.7</td>
<td>26.7</td>
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<tr>
<td>S. sonnei</td>
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<td>29.3</td>
<td>31.0 R</td>
<td>23.7</td>
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<td>S. typhi</td>
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<td>P. aeruginosa</td>
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<td>36.3</td>
<td>39.7 R</td>
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<tr>
<td>S. marcescens</td>
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<td>35.7</td>
<td>37.3 R</td>
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</tr>
<tr>
<td>P. vulgaris</td>
<td>R</td>
<td>22.7</td>
<td>25.0 R</td>
<td>30.7</td>
<td>R</td>
</tr>
</tbody>
</table>

a - Mean of three assays; OA - oleic acid at concentration of 10 mM; CB – Carbenicillin; FC - Fucidic acid; VA - Vancomycin antibacterial standard discs at concentrations of 10, 10, and 30 µg/ml, respectively; R - Resistant (no inhibition zone); NT – Not tested.

b - Mean of three assays; OA - oleic acid at concentration of 10 mM; GM - Gentamicin antibacterial standard disc at concentration of 10 µg/ml; R-Resistant (no inhibition zone); b-P. aeruginosa and P. vulgaris were not sensitive to GM but were sensitive to chloramphenicol disc (30 µg) and showed inhibition zones of 19.0 mm and 8.3 mm mean diameter, respectively.
Table 3 showed that cLf/cLf-OA and bLf/bLf-OA inhibited growth of *A. niger* and *A. flavus* at 0.5-1 mg/ml and 1 mg/ml, respectively. Sensitivity of *C. albicans* to cLf, cLf-OA, bLf, and bLf-OA was observed at concentrations of 0.5-1, 0.125-1, 1, and 0.5-1 mg/ml, respectively. Additionally, oleic acid was inhibitory to *C. albicans* after 18 h of incubation and to a lesser extent against *A. niger* and *A. flavus* Table 3; causing a noticeable elevation (this elevation was higher in case of cLf) in antifungal activity against *C. albicans* both cLf and bLf by binding to OA in the complexes prepared. These synergy results are in agreement with those of Kabara et al., who found OA to be inhibitory to *C. albicans*[22].

Overall results revealed that inhibitory activity of cLf and cLf-OA against test microorganisms noticeably exceeded that of bLf and bLf-OA as previously confirmed [2, 3].

### Table 3: Antifungal activity of cLf-OA and bLf-OA complexes

<table>
<thead>
<tr>
<th>Strains</th>
<th>Mean diameter of inhibition zone (±1 mm)</th>
<th>cLf-OA (mg/ml)</th>
<th>bLf-OA (mg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>AMP</td>
<td>cLf (mg/ml)</td>
<td>bLf (mg/ml)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td></td>
<td>R</td>
<td>20.7</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td></td>
<td>R</td>
<td>8.7</td>
</tr>
<tr>
<td><em>A. flavus</em></td>
<td></td>
<td>R</td>
<td>7.3</td>
</tr>
</tbody>
</table>

- Mean of three assays; OA - oleic acid at concentration of 10 mM; AMP – Amphotericin-B antifungal standard at concentration of 100 µg/ml; R - Resistant (no inhibition zone); b - *A. niger* and *A. flavus* were not sensitive to amphotericin-B but were sensitive to nystatin at concentration of 100 µg/ml and showed inhibition zones of 9.0 mm and 8.0 mm mean diameter, respectively.

### The MIC Values of Antimicrobial Agents

cLf showed MIC values of 0.25, 0.125, 0.25, 1, 0.5, 0.5, 0.5, 0.5, 0.5, and 0.5 mg/ml for MRSA, *S. aureus*, *B. cereus*, *K. pneumonia*, *P. aeruginosa*, *S. marcescens*, *P. vulgaris*, *A. niger*, and *A. flavus*, respectively, while 0.25 mg/ml for *E. coli*, *S. sonnei*, and *S. typhi* indicating that it achieved twice and 4 times, respectively higher inhibitory activity than that of bLf against these pathogens Table 4. These data provided further support to the notion that cLf exerted higher antimicrobial activity than bLf.

In view of the MIC results, synergy between cLf or bLf and OA in the prepared complexes was observed against MRSA, *S. aureus*, *B. cereus*, and *C. albicans* causing a 4, 2, 4, and 4 times, respectively increase in cLf antimicrobial activity and a 2 times increase in bLf antimicrobial activity against all of these pathogens. Whereas, the combinations of cLf or bLf and OA in the complexes displayed no synergistic effect against *E. coli*, *K. pneumoniae*, *A. niger* and *A. flavus*.

Interestingly, OA concentrations in the prepared complexes were significantly (P<0.05) lower than its MICs against sensitive test pathogens, thus the higher antimicrobial activity of cLf/bLf-OA than free forms was not due to a higher OA concentration in the prepared complexes but confirmed the differential participation of lactoferrin proteins in this elevated complex antimicrobial activity.

### Table 4: MIC values of cLf-OA and bLf-OA complexes against various pathogens

<table>
<thead>
<tr>
<th>Test organism</th>
<th>MIC values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cLf (mg/ml)</td>
</tr>
<tr>
<td>MRSA</td>
<td>0.25</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>0.125</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>0.25</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0.25</td>
</tr>
<tr>
<td><em>S. sonnei</em></td>
<td>0.25</td>
</tr>
<tr>
<td><em>S. typhi</em></td>
<td>0.25</td>
</tr>
<tr>
<td><em>K. pneumonia</em></td>
<td>1</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>0.5</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td>0.5</td>
</tr>
<tr>
<td><em>P. vulgaris</em></td>
<td>0.5</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>0.5</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>0.5</td>
</tr>
<tr>
<td><em>A. flavus</em></td>
<td>0.5</td>
</tr>
</tbody>
</table>

R - Resistant (no antimicrobial activity).
Detection of Lf and its Complexes Binding to Bacterial Membrane Proteins by ELISA

Biotinylated cLf, bLf, cLf-OA, or bLf-OA reacted significantly (P<0.05) with bacterial membrane fractions preparations of all test Gram-positive and Gram-negative bacteria compared to blank; carbonate/bicarbonate pH 9.6 buffer Table 5. Wells of ELISA microtiter plate coated with biotinylated cLf or bLf as positive control gave mean ±SD of 0.829±0.012 and 0.75±0.03, respectively.

We previously confirmed that biotinylated cLf was recognized by two membrane proteins of MRSA [3]. Additionally, bacterial outer membrane protein OmpC of E. coli and S. typhi was found to complex with the antibacterial eukaryotic protein camel lactoferrin [23].

![Table 5: Detection of Lf and its complexes binding to bacterial membrane proteins by ELISA](image)

<table>
<thead>
<tr>
<th>Test sample/ organism</th>
<th>OD at 405 nm (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cLf a</td>
</tr>
<tr>
<td>Blank</td>
<td>0.05±0.007</td>
</tr>
<tr>
<td>MRSA</td>
<td>0.31±0.012</td>
</tr>
<tr>
<td>S. aureus</td>
<td>0.26±0.02</td>
</tr>
<tr>
<td>B. cereus</td>
<td>0.30±0.01</td>
</tr>
<tr>
<td>E. coli</td>
<td>0.37±0.07</td>
</tr>
<tr>
<td>S. sonnei</td>
<td>0.34±0.12</td>
</tr>
<tr>
<td>S. typhi</td>
<td>0.32±0.05</td>
</tr>
<tr>
<td>K. pneumonia</td>
<td>0.35±0.073</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>0.31±0.12</td>
</tr>
<tr>
<td>S. marcescens</td>
<td>0.27±0.02</td>
</tr>
<tr>
<td>P. vulgaris</td>
<td>0.29±0.12</td>
</tr>
</tbody>
</table>

*Biotinylated cLf, bLf, cLf-OA, or bLf-OA reacted significantly (P<0.05) with bacterial membrane fractions preparations compared to carbonate/bicarbonate pH 9.6 buffer used as a blank.

The observed differences in biological activities between cLf and other species of Lf are likely due to the variance in some of its structure-related characteristics. cLf was found to comprise 689 amino acids and contain 17 disulfide bridges and 4 predicted glycosylation sites, one of them in the N-lobe and three found in the C-lobe. The pattern of disulfide bonds in cLf is the same as that found in hLf, but the positions of predicted glycosylation sites are totally different in cLf. Besides, the amino acid sequence of cLf is 70% identical to the sequences of other lactoferrins, but the first 50 residues or so of cLf N-termini show an identity of less than 40%. Some residues associated with movement of domains in the protein are different in cLf from those found in other lactoferrins, revealing the likelihood of specific structural differences [29, 30]. We also previously proved that elevated levels of intrinsic disorder in the N-terminal region of cLf can influence the functionality of this region [3].

All the tested microorganisms in this study are of significance as human pathogens, mostly showing resistance to many antibiotics, and chosen to be both Gram-negative and Gram-positive bacteria as well as fungi to indicate broad spectrum activity of the formulated cLf/bLf-OA complexes. S. aureus can cause infection in tissues and sites with lowered host resistance as in case of damaged skin or mucous membranes. It is a very common cause of infection in hospitals, mostly capable of infecting newborn babies and surgical patients. Strains of S. aureus differ in their degree of susceptibility to particular antibiotics [31]. Moreover, methicillin-resistant strains; MRSA have emerged which complicate the treatment of staphylococci infections because methicillin is considered as the first option in treatment of S. aureus infection and also because resistance to methicillin means resistance to all β-lactam antibiotics. The epidemic of MRSA infections occurs mostly in hospitals. MRSA has become one of the leading causes of death in hospitalized patients around the world [32].

B. cereus causes a minority of food borne illnesses (2–5%), resulting in severe nausea, diarrhea, and vomiting. Bacillus food borne infections arise because of survival of the bacterial endospores when food is improperly cooked. It produces beta-lactamases, thus it is resistant to beta-lactam antibiotics [33].
E. coli is a pathogen associated with acute gastroenteritis in infants up to 2 years old and rarely in adults with lowered resistance besides infections of urinary tract. Outbreaks of gastroenteritis can cause high fatality rates in maternity nurseries and institutions caring for young children. Antibiotics have insignificant role in treatment of acute stage in severe cases of gastroenteritis as they are not fast enough to stop further body fluid loss [34]. *Shigella sonnei* causes shigellosis (bacillary dysentery) and produces Shiga toxins that target the vascular endothelium, inhibiting protein synthesis within target cells by a mechanism similar to that of ricin [35]. On the other hand, *S. typhi* spreads by food or water contaminated with feces resulting in typhoid fever, with a risk of death of about 20% without treatment [36]. While antibiotics are capable of shortening the span of a diarrheal infection, particularly if administered early, pathogenic *Shigella* and *Salmonella* species are often resisting the effects of common antibiotics, including ampicillin, trimethoprim-sulfamethoxazole, and third generation cephalosporins.

*K. pneumonia* is a rare cause of bacterial pneumonia but its significance lies in high case mortality in such cases. It is resistant to multiple antibiotics and can produce extended-spectrum beta-lactamases against all beta-lactam antibiotics, except carbapenems [37]. *P. aeruginosa* is a multidrug resistant pathogen associated with serious diseases-hospital-acquired infections such as ventilator-associated pneumonia and sepsis syndromes [38]. *S. marcescens* causes an opportunistic infection in respiratory tract, urinary tract, the eye (keratitis, conjunctivitis, endophthalmitis, and tear duct infections), and wounds. Most *S. marcescens* strains are resistant to numerous antibiotics because of the presence of R-factors; intrinsically resistant to macrolides, ampicillin, and first-generation cephalosporins (such as cephalaxin) [39]. *P. vulgaris* is found in individuals in long-term care facilities and hospitals and those with compromised immune systems [40].

*Candida* infects immunocompromised patients diagnosed with serious diseases such as HIV and cancer. *Candida* commonly causes nosocomial infections. It affects high risk patients who recently undergone surgery, a transplant or are in the Intensive Care Units, leading to malnutrition and interference with the absorption of medication. *A. niger* causes fungal ear infections while *A. flavus* is a common cause of fungal sinusitis and cutaneous infections and noninvasive fungal pneumonia.

**Conclusion**

This study revealed that cLf and bLf could bind OA and exhibited a much stronger antimicrobial activity than their free forms, especially in case of cLf. Additionally, inhibitory activity of cLf and cLf-OA against test microorganisms noticeably exceeded that of corresponding bLf and bLf-OA. Our study undoubtedly confirmed the presence of no effect by OA binding to lactoferrins on Lf binding to bacterial membrane proteins.

**Competing Interests**

The author declares having no competing interests.

References


38. Balch T, Smith R. Pseudomonas aeruginosa: Infections and Treatment. Infectious Disease and Therapy Series. 1994(9); 615.
