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Bactericidal effect of bovine lactoferrin, LFcin, LFampin and LFchimera on antibiotic-resistant *Staphylococcus aureus* and *Escherichia coli*

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Abstract Increased prevalence of antibiotic-resistant bacteria has become a major threat to the health sector worldwide due to their virulence, limited therapeutic options and distribution in both hospital and community settings. Discovery and development of new agents to combat antibiotic-resistant bacteria is thus needed. This study therefore aimed to evaluate the ability of bovine lactoferrin (LF), peptides from two antimicrobial domains lactoferricin B (LFcin17-30) and lactoferrampin (LFampin265-284) and a chimeric construct (LFchimera) containing both peptides, as potential bactericidal agents against clinical isolates of antibiotic-resistant *Staphylococcus aureus* and *Escherichia coli.* Results in kinetics of growth show that LF chimera and peptides inhibited the growth of both bacterial species. By confocal microscopy and flow cytometry it was observed that LF and FITC-labeled peptides are able to interact with these bacteria and cause membrane permeabilization, as monitored by propidium iodide staining, these effects were decreased by preincubation with lipopolysaccharide in *E. coli*. By electron microscopy, a clear cellular damage was observed in bacteria after treatments with LFchimera and peptides, suggesting that interaction and membrane disruption are probably involved as a mechanism of action. In conclusion, results show that LFchimera, LF and peptides have potential as bactericidal agents in the antibiotic-resistant strains of *S. aureus* and *E. coli* and also the work strongly suggest that LFcin17-30 and LFampin265-284 acts synergistically with antibiotics against multidrug resistant EPEC and MRSA in vitro.

Keywords Lactoferrin · Lactoferrin chimera · Peptides · Bactericidal · Resistance

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Introduction

The current worldwide increase in antibiotic-resistant bacteria and, simultaneously, the downward trend in the development of new antibiotics have serious implications to the public health sector. Resistant bacteria dramatically reduce the possibilities of treating infectious diseases effectively and multiply the risks of complications and a fatal outcome for patients with septicemia. Most vulnerable people are those with weakened immune defenses, such as cancer patients, malnourished children and HIV-positives, for whom adequate therapy to prevent and treat severe infections is often necessary for their survival (Hawkey and Jones 2009; Livermore 2009).

Large outbreaks of infant diarrhea due to enteropathogenic *Escherichia coli* (EPEC) have largely disappeared in industrialized countries; however, EPEC remains as an important cause of diarrhea in infants less than two years of age in developing countries. On the other hand, the O157:H7 enterohaemorrhagic *E. coli* (EHEC O157:H7) caused several large outbreaks of a food-borne haemorrhagic colitis in developed countries, for example in USA, from April to August of 2009, 95 persons were infected with this pathogen in two outbreaks associated with the consumption of beef and refrigerated prepackaged cookies (CDC 2009a, b).

Other important resistant bacterial strains are the Gram-positive antibiotic-resistant *Staphylococcus aureus* (MRSA). These strains are resistant to methicillin and other more common antibiotics such as oxacillin, penicillin and amoxicillin. MRSA occur most frequently in hospital and healthcare facilities patients (such as nursing homes and dialysis centers) who have weakened immune systems. These infections affect mainly skin, such as abscesses, boils, and other pus-filled lesions (Bartlett 2008). In hospitalized patients, MRSA have been a problem by their association with other affections becoming an important cause of death around the world (Nguyen et al. 2009). The proportion of hospital-onset *S. aureus* infections that were methicillin-resistant reached 64.4% in US intensive care units in 2003 (Klevens et al. 2007). In the hospital, MRSA infections are associated with greater lengths of staying, high mortality, and increased costs.

Lactoferrin (LF) is an iron-binding protein involved in a large spectrum of biological properties including antimicrobial action (Farnaud and Evans 2003). Digestion of LF with pepsin generates the fragment lactoferricin (LFcin17-30), which is more potent against bacteria compared with the native protein (Bellamy et al. 1992). A second antimicrobial LF synthetic peptide has been obtained and designated lactoferrampin (LFampin) (van der Kraan et al. 2004). Both, LF and these LF-peptides exert the bactericidal activity after cell membrane penetration and damage (Bolscher et al. 2009; Haney et al. 2007; Leon-Sicairos et al. 2009; van der Kraan et al. 2005a; van der Kraan et al. 2006; van der Kraan et al. 2005b; van der Kraan et al. 2005c). Therefore, LF and LF-peptides are a promising class of antimicrobial compounds to fight pathogenic microbes. Linking these two antimicrobial peptides into one molecule resulted in a strikingly stronger bactericidal activity than its constituent lactoferricin and lactoferrampin peptides (Bolscher et al. 2009; Leon-Sicairos et al. 2009). The bactericidal activity of this chimerical peptide called LFchimera was not hampered by high salt concentration in halophilic *Vibrio* bacterial multidrug-resistant strains (Leon-Sicairos et al. 2009). The aim of this study was to evaluate the bactericidal activity of bovine lactoferrin and LF peptides against antibiotic-resistant strains of *S. aureus* and *E. coli* from clinical origin.

Materials and methods

Materials

Bovine LF proximately 30% iron saturated was purchased to Sigma. Synthetic peptides LFcin17-30, LFampin265-284 and LFchimera, were obtained using F-moc chemistry as described before (Bolscher et al. 2009).

Bacterial strains and culture conditions

Enteropathogenic *E. coli* (EPEC E2348/69), multidrug resistant clinical isolate Enteropathogenic *E. coli* (MREPEC) and Enterohaemorragic *E. coli* (EHEC O157:H7) were grown in McConkey agar medium (Difco™) during 24 h at 37°C. *Staphylococcus aureus* (*S. aureus* ATCC 25923) and methicillin-resistant *S. aureus* (MRSA) were grown in LB agar under the same conditions. For viability assays, all the

strains were grown in LB broth at 37°C for 18 h with shaking (200 rpm). After that, bacteria were transferred to LB broth without iron for 2 h at 37°C, washed twice in PBS without iron by centrifugation at 4,500 × *g* for 5 min. Only bacteria in mid-log phase where used in the assays.

Growth inhibition in the presence of LF and LF-derived peptides

To test the bactericidal activity of bovine LF and synthetic LF peptides, approximately 2×10^8 CFU per ml of each strain were incubated in 96-well microplates (Corning) containing LB broth with peptide solutions at final concentration of 12.5 μM bLF, 1, 10, 20 and 40 μM LFcin17-30, LFampin 265-284, and LFchimera. Bacteria in LB were used as control of growth. 100 μg per ml of amikacin was used as control of growth inhibition. For synergistic assays, concentrations of 10 μM of LFcin17-30 and LFampin 265-284 combined with 50 μg per ml of ampicillin were used. Cultures were incubated at 37°C with constant agitation for 1 h and monitored by enumerating viable cells using the following methods: 1. Growth kinetics and 2. Obtaining the CFU from serial 10-fold dilutions prepared in LB agar (each strain after the treatment with LF and peptides) in an electronic counter (Count™, Heathrow Scientific).

Membrane permeabilization and damage to cells

Membrane permeabilization of each treatment was estimated by flow cytometry using the propidium iodide (PI) inclusion criterium. *E. coli* and *S. aureus* strains were cultured in LB as described before and then they were washed three times with LB without iron. A suspension of approximately 10^8 cells per ml was incubated with 40 μM of LFcin17, LFampin265-284 or LFchimera at 37°C during 1.5 h. After that, bacteria were washed again and incubated with PI (10 mg ml^{-1}) at 4°C, for 10 min. Then, bacterial suspension was washed five times with PBS (pH 7.4) and finally fixed and processed to being quantified under a FACScan (Becton–Dickinson). Control experiments were carried out in bacterial cells either without the addition of peptide (viability control), or with 0.1% SDS (which disrupts lipids in bacterial membrane), or 5 mM amikacin (which acts on the 30S subunit of ribosome and produces errors in genetic code reading during the process of translation). All of the experiments were repeated at least twice in duplicate for each assay.

Interaction between peptides and bacteria observed by confocal microscopy

Escherichia coli and *S. aureus* strains (10^8 cells per ml) were incubated for 30 min in LB without iron containing 1 mM FITC-labeled LFchimera, with the purpose to observe whether this peptide is internalized into the cell. Bacteria were centrifuged (5 min, 10,000 × *g*), resuspended and fixed (4% paraformaldehyde, pH 7.4 at 37°C during 30 min), washed twice and processed to be analyzed under confocal microscopy.

To search whether LF chimera is recognized by the bacterial membrane, cells were fixed, washed twice and incubated with 1 mM FITC-labeled LFchimera for 30 min. After that, bacteria were washed, processed and analyzed. All samples were visualized by using a confocal laser-scanning microscope (Leica, Heidelberg, Germany).

Electron microscopy

Escherichia coli and *S. aureus* strains (1.0×10^9 cells per ml) were incubated in LB without iron with 40 μM LFcin17-30, LFampin 285-284 and LFchimera, respectively, for 1.5 h at 37°C. Cells were harvested, resuspended in PBS (pH 7.4) and fixed with 4% paraformaldehyde plus 0.5% glutaraldehyde. Subsequently, bacteria were rinsed with distilled water and mounted on bare 200-mesh copper grids. Next, phosphotungstic acid (1%, pH 5.5, 30 s) was added, replicas were dehydrated and observed under a transmission electron microscope (IEM2000Ex), operated at 100 kV.

Competitive assay between LF-peptides and LPS in *E. coli*

To evaluate whether *E. coli* LPS was the target for the LF-peptides, we developed a competitive assay mixing 1 μM of LF chimera with 10 and 100 times the concentration of LPS and 20 μM LFcin17-30 and LFampin265-284 under the same proportion. The LF peptides–LPS mix and bacteria were incubated at 37°C with shaking for 30 min. After that, the

bactericidal activity of LF peptides–LPS mix was determined as described in the bactericidal activity assays.

Results

Bactericidal activity of LF and LF peptides on *E. coli* and *S. aureus* strains

The capacity of LFcin17-30, LFampin265-284 and LFchimera to inhibit the growth of *E. coli* and *S. aureus* strains was tested in LB medium without iron. The MREPEC, EPEC E2348/69 and EHEC O157:H7 showed different susceptibility to bovine LF (Fig. 1). EHEC O157:H7 was the most susceptible to LF, followed of EPEC E2348/69 and MREPEC with a growth inhibition of 81, 76.8, and 36.7%, respectively.

Since the three strains were sensible to LF, then we tested these strains with the LF peptides. All of the treatments used in the *E. coli* strains showed a concentration-dependent effect. LFcin17-30 and LFampin265-284 inhibited more than 85–90% of growth (Fig. 2A–C). However, LFchimera showed the best inhibition of the bacterial growth (1 μM), similar to the effect with amikacin, that was used as a positive control of inhibition.

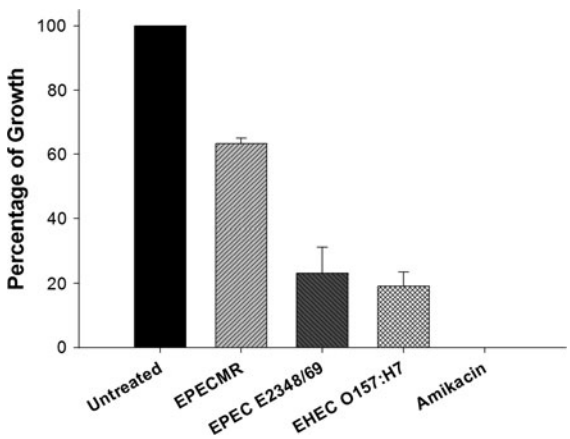

Fig. 1 Bactericidal activity of bovine Lactoferrin in *Escherichia coli* strains. EPEC E2348/69, MREPEC and EHEC O157:H7 were incubated in LB medium without iron, and with 12.5 μM of bovine LF. Culture optical density was determined every 30 min at 660 nm and results after 2 h incubation are shown. All experiments were made three times in triplicate. Percentage of viable cells was calculated relative to viable bacteria obtained in LB. The mean and deviation standard are indicated

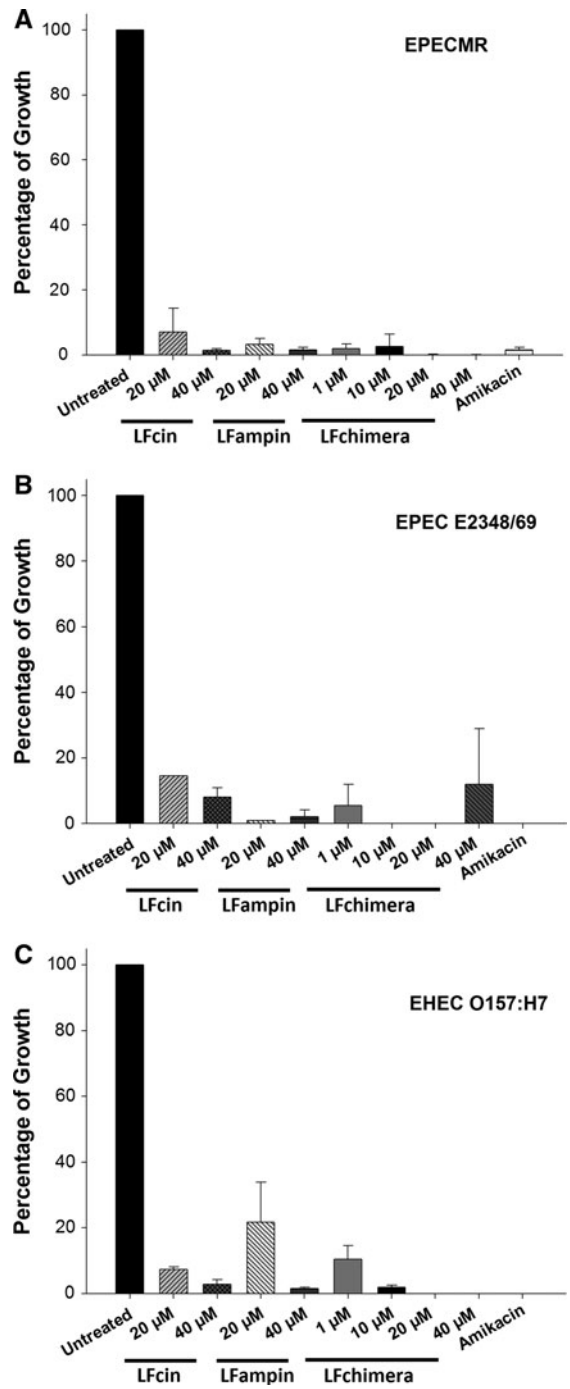

Fig. 2 Inhibition of the growth of *Escherichia coli* strains with synthetic peptides. EPEC E2348/69 (**A**), MREPEC (**B**) and EHEC O157:H7 (**C**) treated with bovine lactoferrin-synthetic peptides. The percentage of inhibition on the growth was determined by counting colony forming units (CFU) from serial 10-fold dilutions prepared in LB agar of each strain after the treatment with peptides. All experiments were made three times in triplicate. The mean and deviation standard are indicated

In *S. aureus* ATCC 25923 the peptides effectuated more than 85% of growth inhibition at all concentrations tested and again LFchimera was more efficient than the other peptides; a concentration of 10 μM inhibited more than 90% of bacterial growth (Fig. 3A). The same holds true for MRSA. In this bacterium LFcin17-30 and LFampin inhibit more than 85% of growth and LFchimera was the best bactericidal peptide. A concentration of 10 μM inhibited the growth in a similar way to amikacin, antibiotic used as a positive control of growth inhibition. It is important to clarify that amikacin was used at high concentration in order to eliminate this pathogen (100 μM) (Fig. 3B).

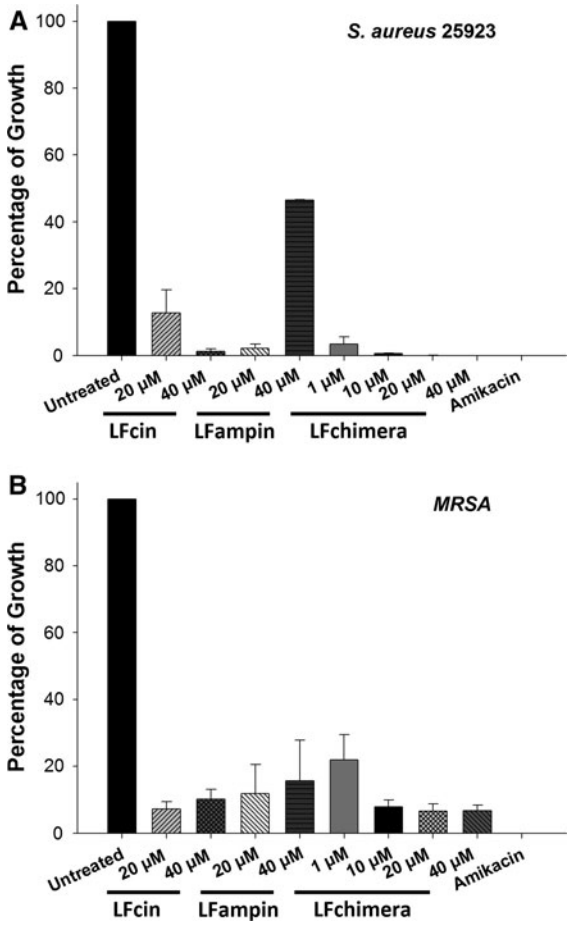

Fig. 3 Inhibition of the growth of *Staphylococcus aureus* with synthetic peptides. *Staphylococcus aureus* ATCC 25923 (**A**) and Methicillin-Resistant *Sthaphylococcus aureus* (**B**) were incubated in LB medium with the peptides and the percentage of growth was determined as before described for *E. coli* strains. All experiments were made three times in triplicate. The mean and deviation standard are indicated

Interaction of LFchimera with bacteria: membrane permeabilization and damages

The effect of LFchimera on the membrane of labelling the *E. coli* and *S. aureus* strains was analysed using FITC labelled LFchimera which showed similar bactericidal activity in all the strains tested (data not shown) using confocal microscopy (Fig. 4). We observed that FITC-LFchimera quickly bound to and internalized in EPECMR (panel A) EHEC O157:H7 (panel B) and *S. aureus* strains (panel C), suggesting that in the bactericidal mechanism a direct interaction is needed. Experiments with FITC-LFcin and FITC-LFampin were similar to results with FITC-LFchimera (Data not showed). Next, the effect of LFchimera on the bacterial membrane integrity was studied with the dye PI (that only penetrates dead cells), and quantified by flow cytometry. Figure 4D shows that LF chimera, LFcin and LFampin permeabilized the bacterial membrane of MREPEC, EHEC O157:H7 and MRSA; virtually all cells had taken up the fluorescent dye to the same level as after treatment with the detergent SDS and amikacin.

By using negative staining and electron microscopy, it was found that LFcin17-30, LFampin265-284 and LFchimera caused several damages in MREPEC (Fig. 5 panels B, C and D) and in EHEC (Fig. 5 panel E, F and G). Also damages in cell morphology were observed when *S. aureus* after incubation with LFcin17-30, LFampin and LFchimera (Fig. 5 panels J, K and L). In all cells treated with peptides, bacteria contained atypical vesicles, protrusions and filamentations, compared with untreated cells (Fig. 5, panels A, E and I).

These results suggest that LFchimera destabilize and permeabilize the bacterial membrane structure. MRSA incubated with LFchimera showed unusual cellular enlargement or cellular surrounding. All the data shown to us that LFchimera interact with bacteria; disrupt membrane integrity and could cytolysis occurs.

Bactericidal activity of peptides is inhibited by LPS

The treatments of LFcin17-30 and LFampin265-284 used in EPEC E2348/69 and EHEC O157:H7 effectuated 98% and 99% of growth inhibition, respectively, (Fig. 6A, B). However; when EPEC E2348/69

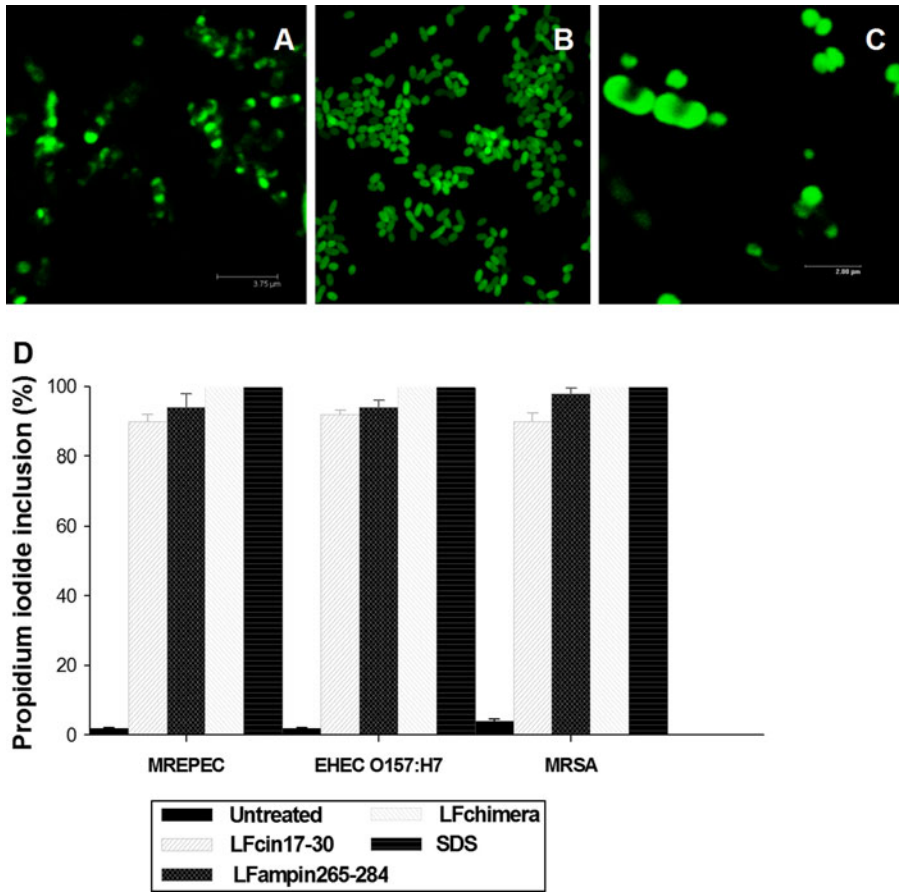

Fig. 4 Binding of FITC-LFchimera on multidrug resistant strains, and membrane permeabilization. Bacterial cultures of MREPEC (**A**), EHEC O157:H7 (**B**) and *S. aureus* (**C**) were incubated in medium with 1 μM of LFchimera, during 1 h at 37°C. Cells were washed and processed to be analyzed under confocal microscopy. **D** Percentage of cells stained with Propidium Iodide (PI). Bacteria were treated with 40 μM of LFcin17-30, LFampin265-284, LFquimera and SDS (0.12%) during 1 h at 37°C. Next, cells were washed and then incubated with10 μg/ml PI during 10 min at 4°C. Finally, bacteria were washed and processed to be analyzed under Flow Cytometry to measure the PI penetration on cells due to permeabilization after treatment with peptides

was incubated with a mixture of peptides and LPS the effect was reduced up to 94% for LFcin17-30 and 57% for LFampin265-284 (Fig. 6A). In EHEC O157:H7 incubated with the peptides–LPS mix the effect was similar to EPEC showing a growth inhibition of 72% for LFcin17-30 and 82.4% for LFampin265-284 (Fig. 6B). These data suggest that LPS in *E. coli* could be one of the targets for these peptides. On the other hand; LFchimera affected significantly the growth of *E. coli* at 1 μM. The percentage of growth was 13 and 28% in EPEC and EHEC, respectively, (Fig. 7A, B) and when the LFchimera was mixed with LPS the effect of the peptide was reduced drastically, showing percentage of growth from 38% for EHEC O157:H7 and 100% for EPEC E2348/69. LPS avoid the interaction of peptides and bacteria, this was confirmed by flow cytometry and confocal microscopy (data not shown) suggesting that LPS could interact with LF synthetic peptides.

Synergic effect between LFcin17-30, LFampin265-284 and ampicillin, in multidrug resistant strains LFchimera had a potent bactericidal activity on the multidrug-resistant EPEC and Methilcillin-resistant *S. aureus.* Both isolates exhibit a high level of resistant to ampicillin (Fig. 8A, B). In order to see if peptides help to ampicillin to kill bacteria several combinations of ampicillin and peptides were tested. In results, the use of 10 μM LFcin17-30 and LFampin265-284 alone did not affect the growth of MREPEC. Interestingly, a combination of ampicillin plus LFcin17-30 or ampicillin plus LFampin265-284 incremented drastically the growth inhibition (up to 94%) (Fig. 8A). The effect of ampicillin, LFcin17-30 and LFampin265-284 in MRSA were more pronounced than in MREPEC because the mixture of them with ampicillin resulted in a growth inhibition of 94.5, 84.4 and 88.6%, respectively (Fig. 8B). We did not make experiments of ampicillin with LFchimera, because LFchimera at a concentration of 1 μM

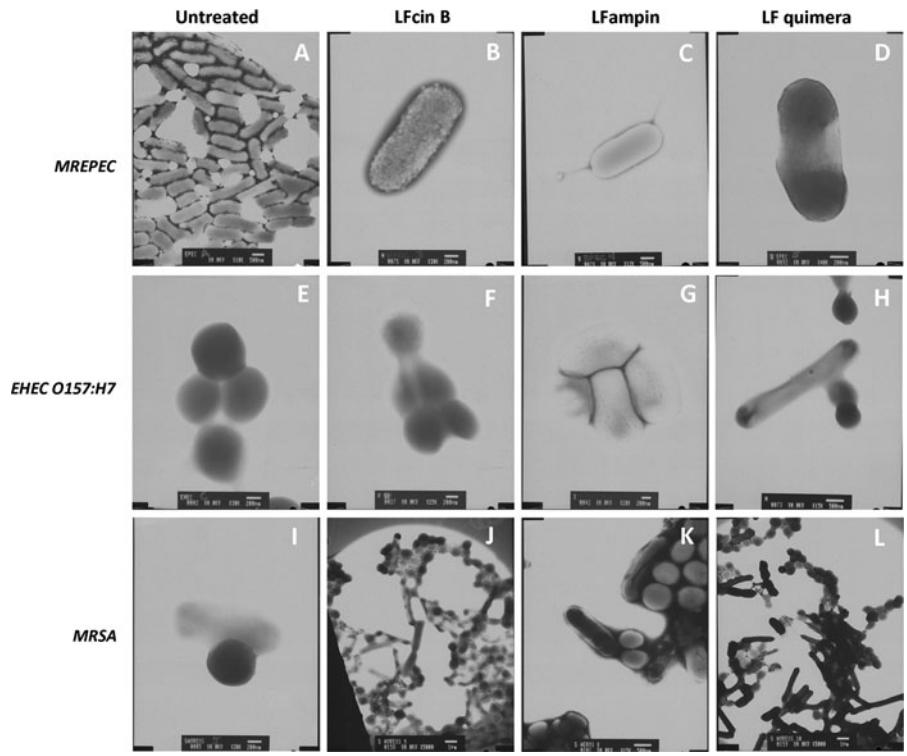

Fig. 5 Ultraestructural damages in bacteria treated with peptides. Bacteria were untreated (**A**, **E**, and **I**) or treated with LFcin17-30 (**B**, **F**, and **J**), LFampin265-284 (**C**, **G** and **K**) and LFchimera (**D**, **H**, and **L**) at a concentration of 40 μM of each peptide. Cells were processed to be analyzed under electron microscopy

is able to kill bacterial cultures. All these data showed that the combination of ampicillin with LFcin17-30 or LFampin265-284 incremented the inhibitory effect on growth (99.9%) for both peptides suggesting a synergistic effect occurs. These data strongly suggest that LFcin17-30 and LFampin265-284 act synergistically with antibiotics against MREPEC and MRSA in vitro.

Discussion

The recent increment of foodborne illness around the world is one of the major problems of health and *E. coli* is one of the three most frequent agents involved in this problem. Enteropathogenic *E. coli* (EPEC), is a leading cause of diarrhea in infants less than two years of age in developing countries such as México (Vidal et al. 2007). On the other hand, the recent outbreaks generated by enterohaemorrhagic *E. coli* O157:H7 in USA and the increment of Methicillin-Resistan *S. aureus* infections that was one of the most important causes of bacteremias in hospitals making these pathogens as important target to evaluate the bactericidal activity of these peptides.

In the present study we show that EPEC E2348/69, multidrug-resistant EPEC and EHEC O157:H7 were sensitive to bovine LF and LF synthetic peptides (Figs. 1, 2) being LFchimera the best of the peptides evaluated. The inhibition observed with LFchimera at 10 μM was similar to the bactericidal capacity of amikacin that was used as a positive control. Similar results were found for *S. aureus* strains.

These results are consistent with other authors that evaluate the microbicidal activity of porcine LFcinP20 in *E. coli* and *S. aureus* found minimum inhibitory concentration from 12 to 25 μM of this peptide (Chen et al. 2006). However, the effect of LFcin on EHEC O157:H7 was not enough to control this pathogen in ground beef (Venkitanarayanan et al. 1999).

LFcin17-30 and LFampin265-284 reduce drastically the sub-lethal doses of antibiotic in multidrug-resistant EPEC and MRSA (Fig. 8A, B), comparable to previous results with LFchimera and gentamicin to control *Vibrio parahaemoliticus* (Leon-Sicairos et al. 2009).

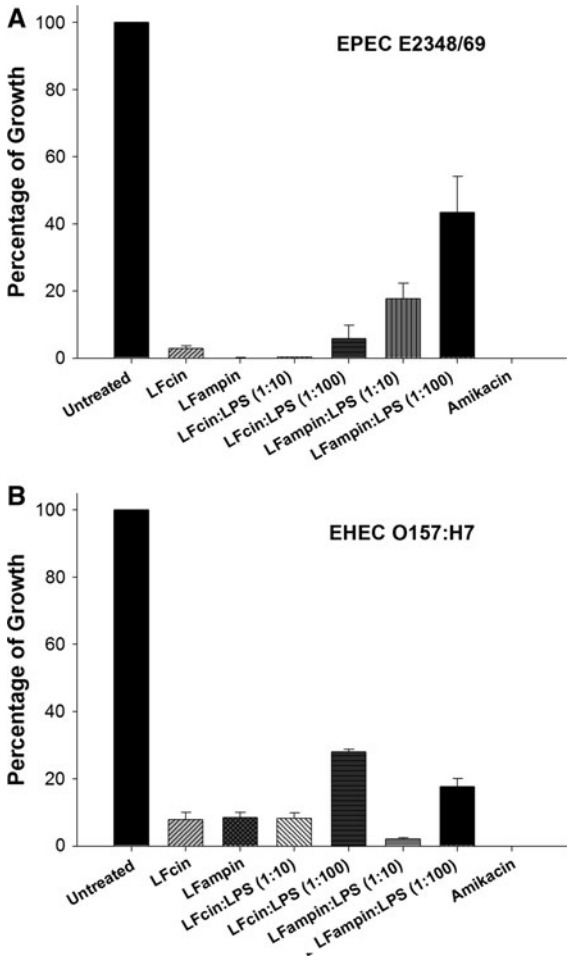

Fig. 6 Inhibition of the peptides bactericidal activity with LPS. 10 μM of LFcin or LFampin were incubated with 10 and 100 times the concentration of LPS and then were incubated with EPEC E2348/69 (**A**) and EHEC O157:H7 (**B**). The percentage of inhibition on the growth was determined by counting colony forming units (CFU) from serial 10-fold dilutions prepared in LB agar of each strain after the treatment with peptides. All experiments were made three times in triplicate. The mean and deviation standard are indicated

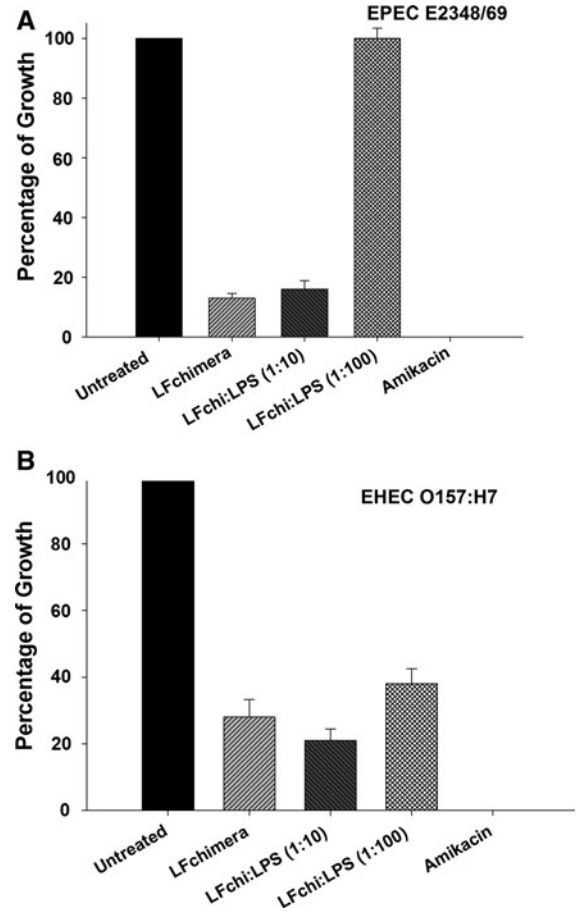

Fig. 7 Inhibition of the LFchimera bactericidal activity with LPS. 1 μM of LFchimera was incubated with 10 and 100 times the concentration of LPS and then were incubated with EPEC E2348/69 (**A**) and EHEC O157:H7 (**B**). The percentage of inhibition on the growth was determined by counting colony forming units (CFU) from serial 10-fold dilutions prepared in LB agar of each strain after the treatment with peptides. All experiments were made three times in triplicate. The mean and deviation standard are indicated

Besides, it has been reported that LF can directly interact with microbiological membranes (Leon-Sicairos et al. 2006; Orsi 2004), alter the membrane permeability through dispersion of membrane components such as lipopolysaccharides (LPS) in Gram-negative bacteria and lead to death of the organisms. Stable complexes between free LPS and both hLF and bLF are formed and then they are liberated leading membrane cell permeablization and cellular death (Weinberg 2007). Concerning the mechanism of action of LFchimera in *E. coli* and *S. aureus* strains, most probably the cell membrane is the target. The LFchimera caused indeed bacterial membrane permeabilization, determined by the internalization of the vital dye propidium iodide (Fig. 4D). Furthermore, formation and coalescence of multiple vesicles and blebs derived from the membrane were observed by electron microscopy, indicating fragmentation and disruption of the membrane (Fig. 5C, D), these damages are found in bacteria with programmed cell death Type II. LFchimera caused similar damage to all the strains tested.

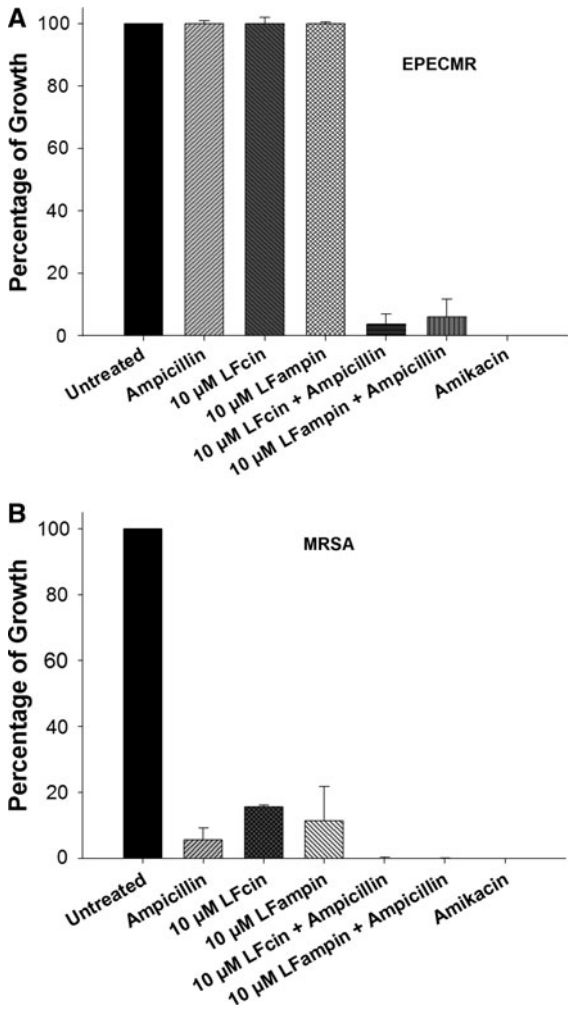

Fig. 8 Bactericidal synergism of LFcin17-30 and LFampin265-284 plus ampicillin, on Multidrug-Resistant strains. EPECMR (**A**) and MRSA (**B**) growth were evaluated in the presence of 10 μM of LFcin, LFampin, or 50 μg per ml of ampicillin. In other experiments each peptide was mixed with 50 μg per ml of ampicillin. The percentage of inhibition on the growth was determined by counting CFU from serial 10-fold dilutions prepared in LB agar of each strain after the treatment with each peptide, antibiotic and combination of ampicillin–LF synthetic peptides. Data are mean values of two experiments performed in triplicate. Standard deviations were less than 10% in each experiment

This finding suggests the possibility that LFchimera and peptides LFcin17-30 and LFampin265-284, may contribute to protection against infection by *E. coli* O157:H7, multidrug resistant EPEC and MRSA. Further studies are required to elucidate the in vivo protective effect of LFchimera and the peptides LFcin17-30 and LFampin265-284 against these infections in an animal model.

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