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# Melittin and its potential in the destruction and inhibition of the biofilm formation by *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* isolated from bovine milk

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ABSTRACT

*Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* stand out in veterinary and human medicine for their role in opportunistic infections and their pathogenic mechanisms, including the biofilms formation. It was investigated the antibacterial activity of melittin and antibiofilm of such bacteria. Twelve strains of these microorganisms isolated from bovine milk were used, as well as the strains *S. aureus* ATCC 12600, *E. coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 15442. The minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) were determined by broth microdilution technique. The biofilms were formed in 96-well plates and melittin on these colonies was added at different concentrations and times. Bacteria previously exposed to melittin were evaluated for inhibition of biofilm production. The MIC and MBC were respectively in μg/mL: *S. aureus* (6–7 and 32–64), *E. coli* (40–42.5 and 64–128) and *P. aeruginosa* (65–70 and 64–128). *S. aureus* biofilms were more sensitive to the action of melittin, since upon exposure to a concentration 10 times lower than the MIC for 4 h, was completely destroyed. In Gram negative bacteria, the pre-formed biofilm was destroyed only when exposed for 4 h under the MIC. With respect to inhibition of biofilm production, *S. aureus* was the most sensitive again because produced only 37.2% of the biofilm formed by the control (without previous exposure to melittin), when exposed to the MIC, and at a concentration hundred times smaller than MIC, this microorganism produced 75.2% of the biofilm. *E. coli* was the most resistant bacteria and produced 56.3% of the biofilm, even if previously exposed to melittin MIC. Melittin presents desirable effects in combating microorganisms studied both at your disposal, biofilm destruction and inhibition of the formation, and maybe used in future studies of new strategies to combat infections caused by these pathogens.



## 1. Introduction

The antimicrobial resistance to the active principles used in its combat are one of the main problems in medicine, constituting a great obstacle in the prevention and treatment of a series of bacterial infections [1]. The rate at which infectious pathogens become resistant to antimicrobials leads to the difficulty of treating the smallest and most common diseases, which may be causes of mortality [2]. According Chaudhary et al. [3] in a report by the World Health Organization, the resistance of common bacterial pathogens has reached alarming levels in several parts of the world and there are few treatment options available. The failure of traditional antibiotics to control infections leads to the search for alternative agents with new mechanisms of action, and the products of natural origin can offer a diversity of active principles and mechanisms of action, many still unknown, stimulating their research on the whole planet [4].

Natural antimicrobial peptides are essential to the life of several

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organisms (microscopically or not), and more than 750 compounds were identified in bacteria, fungi, viruses, plants, insects, mollusks, arachnids, amphibians, fish, birds and mammals [5], which is based on the results obtained in the literature. The studies, based on these peptides, have shown antibacterial, antiviral, antifungal and antiparasitic activities [4].

Melittin is a known water-soluble toxic peptide present in honey bees venom, comprising approximately 50% of its dry weight [6,7]. With only 26 amino acids in its conformation, it is the most active poison toxin. When stored in the venous vesicle, it is arranged tetramerically reducing its toxicity [8]. However, when the venom is released, it dissociates and, individually, its monomeric form becomes high toxicity [8,9]. It has a rapid cytolytic action, destabilizing membranes of different cell types [10], and this capacity is not restricted to animal cells, since it has known antibacterial and antifungal activities [11].

*Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* stand out in human and veterinary medicine due to their pathogenicity, virulence, distribution, diversity of clinical signs and problems related to public health. *S. aureus* is a Gram-positive spherical bacterium with a diversity of virulence factors [12]. It is a major cause of hospital admissions for causing a wide variety of symptoms such as cardiac, pulmonary, osteoarticular and skin and soft tissue infections, as well as the great potential to cause mainly foodborne toxinfections [13]. Lindsay and Holden [14] further state hospitalized patients are particularly exposed to new *S. aureus* infections due to the compromised immune system and, thus, this microorganism becomes a major cause of nosocomial infections, and the insertion of catheters colonized with *S. aureus* biofilms is one of the ways of new infections. *E. coli* and *P. aeruginosa* are Gram negative microorganisms also related to diseases in humans and animals, such as gastroenteritis, urinary infections, peritonitis and meningitis [15].

All these bacteria, however, have a virulence factor in common: the production of biofilms. These are defined as bacterial colonies adhered by protein filaments to a surface and surrounded by a matrix of organic polymers [16]. Biofilms contain particles of proteins, lipids, phospholipids, carbohydrates, minerals and vitamins, which form a kind of crust, under which microorganisms continue to multiply. Thus, microorganisms become more resistant to the action of disinfectants, physical agents and chemicals [17]. Hoiby et al. [18] reports that bacteria organized in biofilms are 10–1000 times more resistant to the action of antimicrobials in relation to planktonic bacteria. However, Costa et al. [19] state that strains of *E. coli* isolated from bovine milk and organized into biofilms were up to 2500 times more resistant to ampicillin and 2400 times more resistant to enrofloxacin when compared to free life bacteria.

Bacterial biofilms represent a danger in the food industry, since they can be a source of constant contamination of the product, since they form in places where food comes in contact, such as stainless steel pipes, utensils, tables, among others [20]. It may also be formed in the body during an infection, which gives the bacteria greater resistance to the available drugs and to the immune system of the host [21,22].

This study aimed to evaluate antibacterial activities and antibiofilms *in vitro* of the peptide melittin on *S. aureus*, *E. coli* and *P. aeruginosa* isolates.

## 2. Materials and methods

### 2.1. Microorganisms and solutions

Twelve strains of *S. aureus*, *E. coli* and *P. aeruginosa* isolated from bovine milk were tested from milk cooler tanks, as well as strains *of S. aureus* ATCC 12600, *E. coli* ATCC 8739 and *P. aeruginosa* ATCC 15442 that were used as control strains in all tests. The selection of the strains for use in the experiments was performed after biofilm production assay, as described in item 2.3. The strains that statistically differed ($p < 0.05$) and showed higher optical densities than the negative control densities plus three standart deviations were selected.

Bacterial strains were thawed and seeded in Columbia Agar, plus 5% defibrinated sheep blood. The plates were incubated in aerobiose at 37 °C for 24 h, then the colonies were removed and suspended in Müller-Hinton broth to a concentration of approximately $10^{5-6}$ colony forming units (CFU)/mL.

Melittin was commercially purchased from Sigma-Aldrich (St Louis, MO, USA), dissolved in sterile pH 7.2 phosphate buffer (PBS) and kept frozen at 20 °C until the moment of use.

### 2.2. Assays of antibacterial activity

For the determination of minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC), the microdilution technique was used according to the CLSI guidelines (CLSI M7-A6) [23]. Melittin was diluted and added together with the bacterial inoculums in 96-well polystyrene plates with flat bottom (KASVI®). The microplates were incubated at 37 °C for 24 h. MIC value was determined visually by comparison of bacterial growth with positive control. MIC was among the lowest concentration capable of promoting inhibition of bacterial growth and the lowest concentration which did not promote inhibition. To obtain the Minimum Bactericidal Concentration (MBC) values, 10 μL aliquots of all wells without bacterial growth were seeded into Petri dishes containing BHI Agar (Prolab®/São Paulo) and incubated at 37 °C for 48 h. MBC refers to the lowest concentration capable of causing bacterial elimination. All tests were performed in triplicate.

### 2.3. Biofilms productions

In 96-well plates with flat bottom,(KASVI®), 200 μL of bacterial inoculums containing $10^{5-6}$ CFU/mL were added in Müller-Hinton broth. The plates were kept in a bacteriological oven for 48 h at 37 °C for growth and biofilm formation. The quantification of biofilms was obtained through a methodology described by Tang et al. [24], when the supernatants were carefully aspirated and the wells subjected to two washes with 200 μL of PBS. Then, 200 μL of MTT solution (bromo-3- (4,5-dimethylthiazol-2yl) -2-5-diphenyl-2H-tetrazolate) was added to the wells and, after further incubation in microaerophylia for 4 h in the absence of light. The supernatant was again aspirated and 200 μL of dimethylsulfoxide (DMSO) was added to the wells for solubilization of the generated formazan salts. After slight stirring of the plates for 10 min for color homogenization, the absorbances were measured by spectrophotometry under wavelength of 496 nm. Biofilms formed by bacteria that did not undergo any treatment with melittin were used as controls for comparison with the means of the treatments. The tests were carried out in sixfold.

### 2.4. Biofilm destruction test

The assay was performed after the biofilm formation in the same manner as described previously. After 24 h of incubation the microplate wells were washed three times with sterile PBS for subsequent addition of 200 μL of melittin in a concentration equal to the previously established MIC (1 × MIC), ten times smaller (1/10 × MIC) and one hundred times smaller (1/100 × MIC). Plates were incubated at 37 °C for 1 h, 2 h and 4 h. Six wells were used for each treatment against each of the bacteria (six times). Positive controls were strains of the tested bacteria, grown on microplate