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Antimicrobial activity of rhamnolipids against *Listeria monocytogenes* and their synergistic interaction with nisin

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ABSTRACT

The rhamnolipids (RL) are biodegradable biosurfactants which have low toxicity and surface active properties that can be useful for food processing industries. The objective of this study was to evaluate the antimicrobial potential of rhamnolipids against *Listeria monocytogenes*. Susceptibility tests were performed by the minimal inhibitory concentration (MIC) using the micro-broth dilution technique. The MIC values varied from 78.1 μg/mL to 2500 μg/mL with the 2500 μg/mL being the predominant value. Among the 32 tested cultures, 90.6% were susceptible to RL. Results showed that the rhamnolipid activity was primarily bacteriostatic. The interaction of rhamnolipid with nisin was also investigated. The combined effect of nisin and RL was evaluated against two wild-type isolates of *L. monocytogenes*, L12 sensitive to RL (MIC 156.2 μg/mL) and L17 less sensitive to RL (2500 μg/mL). The FIC indexes for the isolates were 0.18 and 0.078 for L12 and L17 respectively, indicating a strong synergistic effect. The survival curve of isolates L12 and L17 showed that the combination between nisin and RL was bactericidal at lower concentration than for the individual antimicrobials. For the L12 isolate 78.1 μg/mL of RL and 160 IU/mL of nisin eliminated the population after 30 min of incubation. The combination of 156.2 μg/mL of RL and 320 IU/mL of nisin reduced completely L17 population after 2 h of incubation. Rhamnolipids showed antimicrobial activity against *L. monocytogenes* and presented a synergistic effect when combined with nisin.

1. Introduction

Foodborne contaminations can cause more than 200 diseases in humans, and many factors contribute to the occurrence of diseases related with food consumption. Examples are the increase in world population with the consequent increase in the demand for food, increased popularity for eating outside the home, and the microbial genomic diversification and selection pressures which results in the emergence of new pathogens (Nyachuba, 2010).

Listeria monocytogenes is an important foodborne pathogen which can cause the serious illness, listeriosis (McLauchlin, 1996). This bacterium has been found in a wide variety of food products as raw meat, raw vegetables, dairy products and read-to-eat food (White, Zhao, Simjee, Wagner, & McDermott, 2002). *L. monocytogenes* is often linked to ready-to-eat food because it is able to grow at refrigeration temperatures and many outbreaks are associated with the consumption of these products (Gandhi & Chikindas, 2007; Liu, 2008). Furthermore, listeriosis can cause severe symptoms in susceptible human hosts like meningitis and abortion (Gandhi & Chikindas, 2007).

The increased number of listeriosis cases in the last years can be related to changes in food habits of the consumer, such as increased consumption of ready-to-eat food, and the rise in elderly age classes (CDC, 2010; Carpentier & Cerf, 2011). Therefore it is important to find alternatives for the control of *L. monocytogenes* in the food industry.

The rhamnolipids produced by *Pseudomonas aeruginosa* are glycolipids composed of one or two rhamnose molecules linked to one or two fatty acids alkyl chains (Fig. 1). They are synthesized as a mixture of homologs mainly composed of di-rhamnolipids and mono-rhamnolipids (Maier & Soberón-Chávez, 2000). Rhamnolipids biosurfactants show several useful properties for the processing industries such as surface-activity, emulsification, low toxicity and biodegradability (Nitschke & Costa, 2007). Furthermore, this biosurfactant has demonstrated antimicrobial activity against several microorganisms such as the Gram-positive bacteria, *Staphylococcus aureus*, *Bacillus subtilis*, *Clostridium perfringens*, the Gram-negative bacteria *Salmonella* Typhimurium, *Escherichia coli*, *Enterobacter aerogenes* and the fungi *Phytophthora infestans*, *Phytophthora capsici*, *Botrytis cinerea*, *Fusarium graminearum* and *Mucor*

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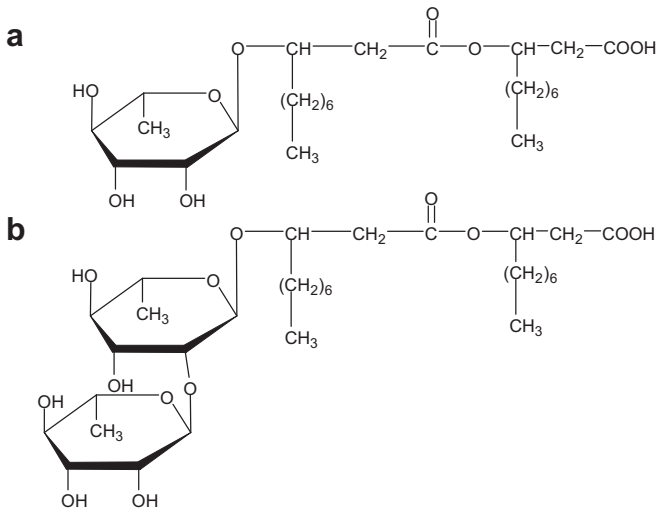

Fig. 1. Chemical structure of mono-rhamnolipid (a) and di-rhamnolipid (b).

spp. (Benincasa, Abalos, Oliveira, & Manresa, 2004; Haba et al., 2003; Sha, Jiang, Meng, Zhang, & Song, 2011).

The mechanism of rhamnolipid antimicrobial activity is not completely understood but their hypothetical site of action is the cell membrane, as they possess an amphipathic nature that allows its interaction with phospholipids (Ortiz et al., 2006). Some authors have suggested that rhamnolipids increase the membrane permeability with consequent alteration of this barrier causing cell damage (Sánchez et al., 2006; Sotirova, Spasova, Galabova, Karpenko, & Shulga, 2008).

In a recent study, we have demonstrated that the rhamnolipids, produced by *P. aeruginosa* PA1, inhibit the growth of *L. monocytogenes* ATCC 19112 and ATCC 7644 (Araujo et al., 2011). However, to verify the potential of rhamnolipids to control *L. monocytogenes*, it is necessary to evaluate minimal inhibitory concentration against a wide range of strains from different sources.

Nisin is an antimicrobial peptide produced by *Lactococcus lactis* which has a bactericidal effect against a broad range of Gram-positive bacteria including *L. monocytogenes*, and it is regularly utilized for the control of this pathogen in food products (McAuliffe, Ross, & Hill, 2001). Nisin forms pores in the membrane of sensitive cells, leading to the efflux of cellular constituents and the collapse of proton-motive force (Cotter, Hill, & Ross, 2005). Since both, nisin and rhamnolipids, have the cytoplasmic membrane as target we have hypothesize that their combination could be synergistic.

The aim of this study was to evaluate the antimicrobial potential of rhamnolipids against *L. monocytogenes* by the determination of minimal inhibitory concentration (MIC). The effect of the combination of rhamnolipids and nisin on the growth of *L. monocytogenes* was also investigated.

2. Materials and methods

2.1. Bacteria and culture medium

Thirty two *L. monocytogenes* cultures were used in the study, being five strains: ATCC 7644, ATCC 15313, ATCC 19112, ATCC 19117, SCOTT A; and twenty seven wild-type isolates: L01, L02, L03, L04, L06, 07, L08, L09, L10, L11, L12, L13, L14, L15, L16, L17, L18, L19, L20, L21, L22, L23, L24, L25, L26, L27 and L28. The origin of each isolate is described in Table 1. The *L. monocytogenes* cultures were stored in a freezer at −20 °C in tryptone soy broth (TSB, Acumedia) with 6 g/L of yeast extract (TSYE broth) and 10% (v/v) of glycerol added.

Table 1
Origin of the *Listeria monocytogenes* isolates and their respective MIC values for rhamnolipids.

Listeria monocytogenes	Source	MIC (μg/mL)
L01	PIE[a] (chilling chamber floor)	312.5
L02	PIE (cutting room wall)	78.1
L03	PIE (cutting room car)	312.5
L04	PIE (carcass after chilling room)	156.2
L06	PIE (scissors from cutting room)	>2500
L07	PIE (viscera inspection box)	2500
L08	PIE (poultry breast)	156.2
L09	PIE (Staff hand)	156.2
L10	Human clinical isolate	2500
L11	Dairy industry floor	>2500
L12	Artisan cheese	156.2
L13	Minas fresh cheese	156.2
L14	Minas fresh cheese	312.5
L15	Minas fresh cheese	625
L16	Minas fresh cheese	2500
L17	PIE (hook)	2500
L18	PIE (scissors)	625
L19	PIE (floor)	2500
L20	PIE (internal area of channel)	625
L21	Frozen bread cheese	>2500
L22	Gouda cheese	312.5
L23	Toscana sausage	2500
L24	Grated parmesan cheese	2500
L25	Minas cheese	2500
L26	Minced meat	2500
L27	Ham	156.2
L28	Sausage	312.5
ATCC 7644		2500
ATCC 15313		2500
ATCC 19122		2500
ATCC 19117		156.2
SCOTT A		625

[a] Poultry industrial environment.

2.2. Chemicals

Commercial rhamnolipid JBR599 (Jeneil Biosurfactant Co.) with 99.0% purity, was dissolved in distilled water and stored at 4 °C. Commercial nisin (Silver Elephant 2.5% purity, 10^6 International Units/g), was dissolved with 0.02 mol/L HCl solution and stored at 4 °C. The solutions were sterilized by membrane filtration (0.22 μm).

2.3. Determination of minimal inhibitory concentration

Antimicrobial activity of commercial rhamnolipid was tested against the 32 *L. monocytogenes* cultures by the micro-broth dilution method using 96 U-shaped wells microdilution plates (Woods & Washington, 1995). Briefly, 100 μL of sterile TSYE broth were dispensed into all the wells and 100 μL of rhamnolipid solution (5000 μg/mL) were added on the first column, serially dilutions were made to obtain final concentrations ranging from 4.9 μg/mL to 2500 μg/mL. The bacterial inoculum was prepared on tryptone soy yeast extract agar (TSYEA, Acumedia) incubated for 24 h at 37 °C. After the incubation some colonies were suspended in saline solution (NaCl 0.86%) and adjusted to approximately 10^8 CFU/mL using 0.5 McFarland standard. All the wells, except negative control column, were inoculated with 10 μL of *L. monocytogenes* standardized inoculum. The microplates were incubated for 24 h at 37 °C. The tests were conducted in quadruplicate and at least three independent replicates. After visual inspection, 20 μL of 0.1%