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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.

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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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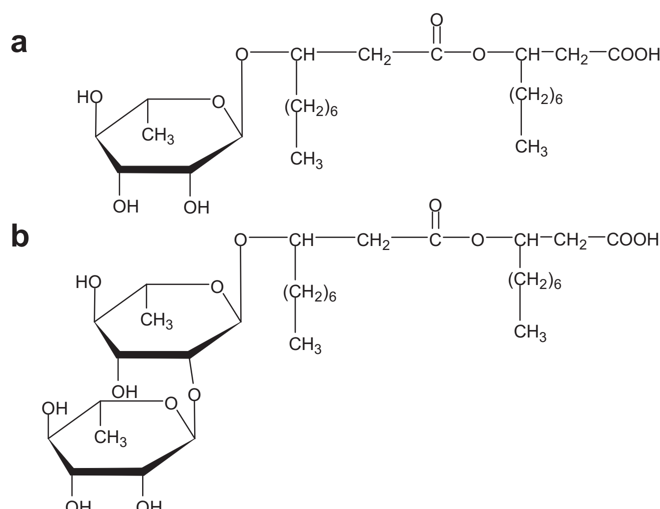

Fig. 1. Chemical structure of mono-rhamnolipid (a) and di-rhamnolipid (b).

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%

tetrazolium bromide (MTT-Sigma Aldrich) solution was added to the wells to confirm the presence or absence of growth. The minimal inhibitory concentration (MIC) was determined as the lowest concentration of rhamnolipid that visually inhibits the microbial growth in comparison with the positive control (no rhamnolipid). The MIC value of nisin was determined by this methodology using concentrations ranging from 5 IU/mL to 2560 IU/mL.

Minimal bactericidal concentration (MBC) was determined by spreading a sample from wells, where no bacterial growth was observed, on TSYEA plates that were incubated at 37 °C for 24 h. MBC was determined as the lowest MIC concentration where no viable growth was obtained.

2.4. Determination of combined effect of rhamnolipid and nisin

The fractional inhibitory concentration (FIC) of nisin and rhamnolipid against the wild-type isolates L12 and L17 was determinate using the checkerboard test (Verma, 2007). The FIC index was calculated employing the minimal inhibitory concentrations (MIC) of the antimicrobial compounds alone and the respective MIC when the compounds were combined. The classification of the antimicrobial interaction was made using the following parameters: when the FIC index is ≤ 0.5 the interaction is synergistic, when the FIC index is >0.5 and ≤ 4 the interaction is indifferent and the FIC index >4 defines the antagonistic interaction.

The checkerboard test was elaborated using a 96-well microtiter plate with serial dilutions of the nisin and rhamnolipid (Verma, 2007). The dilutions of rhamnolipid were prepared in the horizontal rows and the nisin in the vertical columns. The antimicrobial concentrations ranged from the MIC value to seven serial twofold dilutions. The micro-broth dilution technique was performed as described above (MIC determination). The FIC index ($\sum$FIC) was determined using the following equation:

$$\sum \mathrm{FIC} = \mathrm{FIC}_{\mathrm{RL}} + \mathrm{FIC}_{\mathrm{nisin}}$$

$$\sum \mathrm{FIC} = \frac{(\text{MIC of RL in combination})}{(\text{MIC of RL})} + \frac{(\text{MIC of nisin in combination})}{(\text{MIC of nisin})}$$

2.5. Survival curve

The effect of nisin and rhamnolipids separately and in combination on the growth of two *L. monocytogenes* wild-type isolates (L12 and L17) was evaluated through the construction of a survival curve. The minimal inhibitory concentration (MIC) of each compound was utilized when they were tested alone. In the case of combination, it was the lowest concentration of nisin and rhamnolipid, found in the checkerboard test, that showed bactericidal activity against the isolates. The bactericidal concentration in combination of the antimicrobials for the isolate L12 was 78.1 μg/mL to RL and 160 IU/mL to nisin, and for the isolate L17 was 156.2 μg/mL to RL and 320 IU/mL to nisin.

The assay was conducted in glass tube, filled with 4.5 mL of TSYE broth and 0.5 mL of 10-fold-concentrated of antimicrobial solution, the tube was agitated using a vortex and 0.5 mL of bacterial inoculum standardized at 10^8 CFU/mL was added. The tube was incubated at 37 °C and the number of viable cells was determined using the drop method (Miles, Misra, & Irwin, 1938) at different time intervals.

3. Results

3.1. The rhamnolipids antimicrobial activity

The MIC values of rhamnolipids against the cultures of *L. monocytogenes* are showed in Table 1. The values varied from 78.1 μg/mL to 2500 μg/mL and the 2500 μg/mL concentration was the predominant value (Fig. 2). Among the 32 cultures tested 90.6% were sensitive to rhamnolipids and the antimicrobial activity was primarily bacteriostatic since only four cultures presented MBC values at the concentrations tested (data not shown).

3.2. Combined effect of rhamnolipids and nisin against *L. monocytogenes*

The combined effect of commercial nisin and rhamnolipid was evaluated against two wild-type isolates of *L. monocytogenes* with different susceptibility to rhamnolipid, being the L12 more sensitive (MIC 156.2 μg/mL) and L17 less sensitive (MIC 2500 μg/mL).

Before evaluating the antimicrobial interaction, the antimicrobial activity of commercial nisin was determined against the L12 and L17 isolates and the results are shown in Table 2. The interaction between nisin and rhamnolipid performed by the checkerboard test, resulted in FIC index 0.18 to isolate L12 and 0.078 to isolate L17. For both isolates the FIC index was lower than 0.5 which represents a synergistic interaction between nisin and rhamnolipid.

3.3. Survival curve

The survival curve shows the effect of nisin, rhamnolipids and their combination against the growth of two *L. monocytogenes* isolates. The survival curve of isolate L12 (Fig. 3) shows that the RL in the concentration of 156.2 μg/mL presented a bactericidal effect (confirming MBC test). The nisin in the concentration of 320 IU/mL inhibited completely the growth of L12 in 30 min of incubation. The combination with 1/2 MIC of rhamnolipid and 1/2 MIC of nisin was bactericidal in 30 min of incubation.

For the isolate L17, the RL in the concentration of 2500 μg/mL reduced the cell population by 4 log units after 8 h (suggesting a bactericidal effect) however, after 24 h the population reached the initial number (Fig. 4) corroborating with results of MBC test where

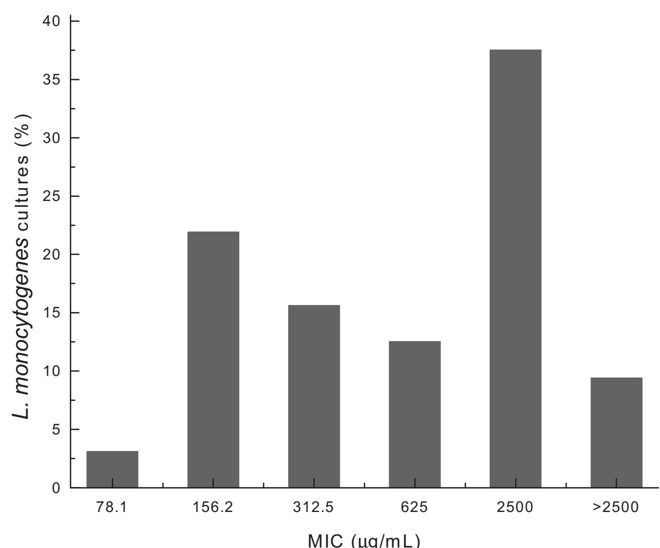

Fig. 2. Sensitivity profile of *Listeria monocytogenes* cultures to rhamnolipid.

Table 2
Minimal inhibitory concentration and fractional inhibitory concentration of nisin and rhamnolipid against two wild-type isolates of *L. monocytogenes*.

Listeria monocytogenes	Rhamnolipid (μg/mL)			Nisin (IU/mL)			FIC index	Interaction
	MIC	MIC_c	FIC	MIC	MIC_c	FIC		
L12	156.2	19.1	0.12	320	20	0.062	0.18	Synergistic
L17	2500	39.1	0.016	640	40	0.062	0.078	Synergistic

MIC_c – minimal inhibitory concentration in combination.

a bacteriostatic effect was observed for this isolate. The nisin (640 IU/mL) was bactericidal in 4 h of treatment and the combination of 156.2 μg/mL of RL and 320 IU/mL of nisin inhibited completely the bacterial population in 2 h of treatment.

4. Discussion

This work evaluates the antimicrobial activity of rhamnolipid biosurfactants against *L. monocytogenes* as well the effect of their combination with nisin.

The results presented shown that the rhamnolipids have antimicrobial potential against *L. monocytogenes* since the 90.6% of tested cultures were sensitive. The antimicrobial activity of rhamnolipids has been described for many bacteria (Benincasa et al., 2004; Haba et al., 2003) however, *L. monocytogenes* was poorly investigated. In fact, the only report we have found in literature, describes that rhamnolipids can control the growth of *L. monocytogenes* 432651 with an MIC of 6 μg/mL (Weimer, 2008).

The mechanism of rhamnolipid action against microorganisms is not yet elucidated but, it is supposed that the cell membrane is the target, because these molecules can increase the permeability of microbial cells (Sotirova et al., 2008). In preliminary studies, we have observed an increase in cell permeabilization promoted by rhamnolipids however, no correlation with the antimicrobial activity was demonstrated (data not shown).

Since rhamnolipids act on the cell membrane, the variations in the susceptibility of *L. monocytogenes* cultures, with MIC ranging from 78.1 to 2500 μg/mL, would be most certainly related to differences in the lipid membrane composition of the cells (Sotirova et al., 2008). Verheul, Russell, Van't Hof, Rombouts, and Abee (1997) evaluated the membrane composition of two *L. monocytogenes* Scott A strains, one sensitive and the other resistant to nisin, and showed the resistant cells possessed a modified phospholipid composition.

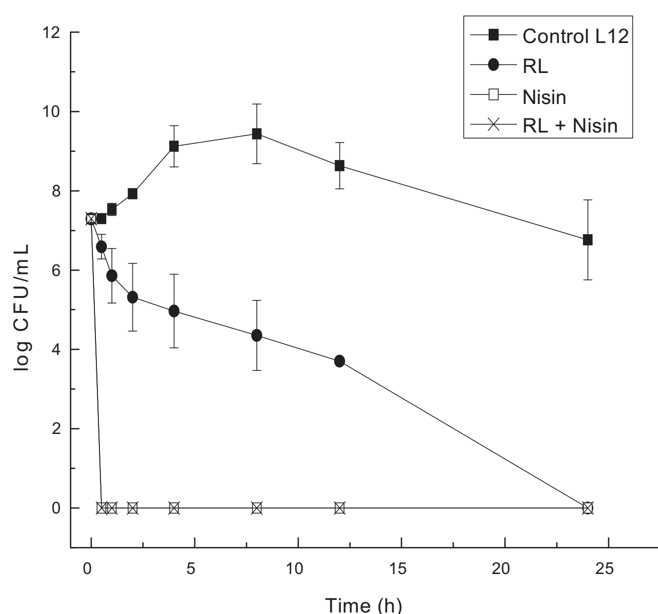

Fig. 3. Growth curve of *L. monocytogenes* L12 with RL (156.2 μg/mL), nisin (320 IU/mL), combination of RL (78.1 μg/mL) + nisin (160 IU/mL) and the control.

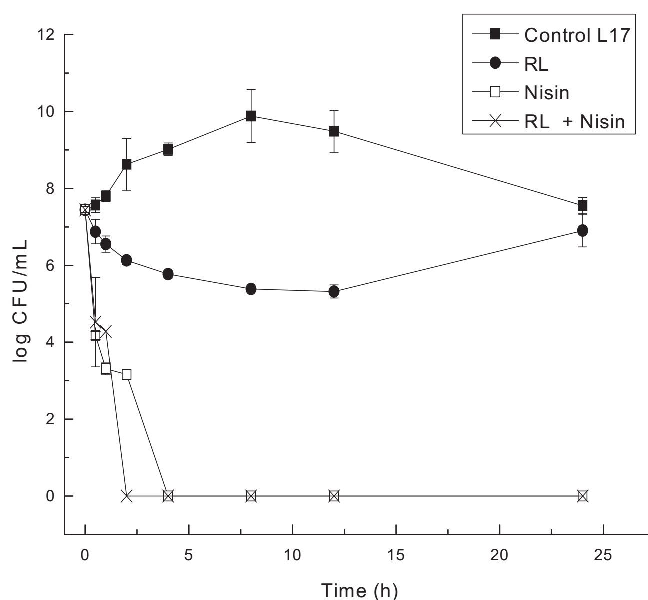

Fig. 4. Growth curve of *L. monocytogenes* L17 with RL (2500 μg/mL), nisin (640 IU/mL), combination of RL (156.2 μg/mL) + nisin (320 IU/mL) and the control.

The interaction between antimicrobials is synergistic when the combined activity is greater than the additive effect of the antimicrobials; the synergistic interaction allows the use of lower dosages and in some cases can extend the range of actuation (Berenbaum, 1989). With the checkerboard test it was possible to evaluate the interaction between nisin and the rhamnolipids. This combination showed a synergistic effect against two wild-type *L. monocytogenes* isolates (L12 and L17) with different susceptibility to rhamnolipids (Table 2).

The mechanism of interaction between nisin and RL should be elucidated, however since, both antimicrobials act on the same target it is most likely that the interaction occurs on the plasma membrane.

The lipid membrane composition of cell is important to nisin activity, since this antimicrobial is a cationic bacteriocin and the presence of lipids with negative charges can increase its binding to the cell membrane and consequently enhances the cell sensitivity (Cleveland, Montville, Nes, & Chikindas, 2001). Li, Chikindas, Ludescher, and Montville (2002) verified that in the presence of the anionic surfactant Tween 20, the *L. monocytogenes* cells altered the composition of lipid bilayer by increasing the number of anionic lipids. The cells adapted with the surfactant showed higher sensitivity to nisin due to the increase in the negative charges on the cell surface and in the electrostatic interaction between the cationic peptide and the anionic membrane.

The rhamnolipids have an anionic character mainly because of their carboxylate group (Fig. 1); and in solution, they interact with lipid bilayer and the negative groups tend to organize on the cell surface increasing the negative charges (Nguyen, Youssef, McInerney, & Sabatini, 2008) hence, promoting the binding of nisin, which can lead to the synergistic effect observed in this work. As rhamnolipids increase the cell membrane permeability, this effect could also contribute to increase cell susceptibility to nisin. A previous study described that the combination of rhamnolipids and

the antimicrobial syringopeptin improve membrane permeabilization when compared with their use alone (Weimer, 2008).

The survival curves show the effect of the rhamnolipid, nisin and their combination on the growth of *L. monocytogenes*. The survival curve of isolate L12 (Fig. 3) shows that the interaction of nisin and rhamnolipid, in concentrations lower than their individual MIC values, eradicated the population after 30 min of incubation. This result is similar to the treatment using nisin alone. However, when in combination, it was possible to use a lower concentration of this antimicrobial agent to reach the same inhibitory effect. The survival curve of L17 isolate (Fig. 4) demonstrated that the combination allowed a reduction not only in the concentration of the antimicrobials but also in the response times of the agents. The isolate L17 has MIC of 2500 μg/mL to rhamnolipid and in the presence of nisin this value decreases to 156.2 μg/mL moreover, the rhamnolipid individually did not eradicated the population but, when combined with nisin, a bactericidal effect could be clearly observed.

The emergence of tolerance in food pathogenic bacteria, combined with the increasing demand for "natural" and safety additives stimulates the search for new bio-preservatives to control food pathogens. The antimicrobial activity here shown, associated with the characteristics of surface activity, biodegradability and low toxicity of rhamnolipids, present the opportunity to be an important option for the control of *L. monocytogenes* with application in food industry.

5. Conclusions

Our findings have demonstrated the potential antimicrobial activity of rhamnolipids against *L. monocytogenes*. The activity of rhamnolipids was primarily bacteriostatic and their combination with nisin resulted in a synergistic action improving the efficacy of both antimicrobials.

Further work is required to verify the effect of pH, temperature and salt concentration in the antimicrobial activity of rhamnolipids even individually and in combination. The activity of rhamnolipids against other food pathogens especially Gram-negative bacteria should also be investigated.

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