

Increase of Glutathione by *Listeria monocytogenes* Scott A Cm^rEm^r When It Is Challenged by Gallic Acid and Nisin

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Abstract

This work investigates the activities of nisin and gallic acid, used either alone or in combination, against *Listeria monocytogenes* Scott A Cm^rEm^r. The antimicrobial activity was determined using agar diffusion tests. The growth profile was evaluated by measurements of optical density and the numbers of viable cells. Lipid peroxidation was measured by the thiobarbituric acid-reactive substances (TBARS) technique, and the levels of reduced glutathione (GSH) were determined by the 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) method. Growth of the pathogen was inhibited by the combined use of nisin and gallic acid, with an inhibition zone of approximately 11 mm at concentrations of 8 mg/ml and 100 IU/ml, respectively. No significant reduction in the number of viable pathogen cells was observed after 18 hours of exposure to nisin and gallic acid. There was a significant increase in the GSH concentration, and a decrease in lipid peroxidation, when the pathogen was exposed to increasing concentrations of nisin and gallic acid, used either alone or in combination, for a period of 18 hours. It was concluded that the *Listeria monocytogenes* cell line studied was able to resist the action of oxidizing agents by increasing the synthesis of GSH.

Keywords: *Listeria monocytogenes*, nisin, gallic acid, glutathione

1. Introduction

Listeria monocytogenes is an important food-borne pathogen. It causes approximately 2500 new cases of listeriosis every year in the US. The main targets of the bacteria are the new-born, pregnant women, the elderly, and those with immune system deficiencies, with a mortality rate of 37%. Control of the organism is difficult, due to its widespread distribution in nature, intrinsic physiological resistance, capacity to adapt, and ability to grow at low temperatures (McEntere, Carman, & Montiville, 2004).

The combined use of techniques such as low temperatures, acidification, and natural or artificial preservatives can complicate the control of *L. monocytogenes* in foods, since these procedures alter the physicochemical characteristics of the materials (Rohani et al., 2011; Valero et al., 2006).

As part of the continuing drive to improve food safety, natural substances have been investigated for use as antimicrobial and antioxidant agents that could improve the organoleptic quality, acceptability, and shelf-life of foods (Rohani et al., 2011). These substances include nisin and gallic acid.

Nisin, a well-known bacteriocin with Generally Recognized as Safe (GRAS) status, is used in many countries worldwide. It shows inhibitory action against Gram-positive bacteria including *L. monocytogenes*. The anti-listerial properties of nisin have been studied in a variety of foodstuffs, while some microorganisms can develop resistance to the compound (Bozariar & Nychas, 2006; Murdock et al., 2007).

Many different approaches have been proposed with the aim of increasing the antibacterial activity of nisin and extending its range of applications, including the combination of the drug with other substances possessing antibacterial activity (He & Chen, 2006). One possibility is the combination of nisin with gallic acid, which exhibits recognized antioxidant, antimicrobial, antimutagenic, and anticarcinogenic properties (Savi et al., 2005). The present work investigates the effects of nisin and gallic acid, used either alone or in combination, against the bacterium *Listeria monocytogenes* Scott A Cm^rEm^r.

2. Materials and Methods

2.1 Microorganism and Conditions of Growth

The *Listeria monocytogenes* Scott A Cm^rEm^r (Foegeding et al., 1992) strain was employed, which is resistant to the antibiotics chloramphenicol and erythromycin. Before use, the culture was reactivated for 24 hours at 37°C in tryptic soy broth containing 0.6% yeast extract (TSB-YE), with addition of 5 µg/ml of chloramphenicol and 0.5 µg/ml of erythromycin (both obtained from Sigma-Aldrich Chemical Co., Germany), and 20% glycerol (Synth, São Paulo, Brazil). A culture of the pathogen was kept at -20°C in TSB-YE broth containing 20% glycerol. For the biochemical tests, the culture was propagated in TSA-YE agar medium for 18 hours at 30°C, under constant agitation at 100 rpm.

2.2 Preparation of the Antimicrobial Agents

The stock solution of gallic acid (GA) (Sigma-Aldrich, St. Louis, USA) was prepared at a concentration of 0.2 g/ml by dissolution in ethanol. The stock solution of nisin (N) (Nisaplin, Applin & Barrett, UK) was prepared at a concentration of 0.1 g/ml (100,000 IU) by dissolution in 0.02 M HCl. The stock solutions were diluted in sterile water according to the concentrations required.

2.3 Antibacterial Activity

2.3.1 Agar Diffusion Assay

The agar diffusion test was used to investigate the antibacterial effects of nisin and gallic acid (Harris, Daeschel, & Klaenhammer, 1989). Agar plates were inoculated with the bacterial culture grown overnight in liquid medium and containing approximately 10⁹ UFC/ml. Holes were made in the agar, into which were placed 40 µL of each test substance (N at concentrations of 12.5, 25, 50, and 100 IU/ml, GA at concentrations of 2, 4, and 8 mg/ml, and GA+N at concentrations of 0.5 mg/ml + 6.25 IU/ml, 1 mg/ml + 25 IU/ml, and 2 mg/ml + 50 IU/ml), together with the corresponding vehicles. In agar diffusion test was not possible to use concentrations of gallic acid above 8 mg/ml due low solubility of this compound. After incubation at 30°C for 24 hours, the zone of inhibition was measured, to an accuracy of 0.5 mm. The test was performed in triplicate, and the results expressed as means ± standard deviations (SD).

2.3.2 Growth Curve of *Listeria monocytogenes* Scott A Cm^rEm^r in the Presence of Nisin and Gallic Acid

TBS-YE was used as the liquid growth medium in this experiment. Nisin and gallic acid were added to the medium in order to obtain final concentrations of 200 mg/100 ml and 400 mg/100 ml (GA), 250 IU/100 ml and 500 IU/100 ml (N), and 200 mg/100 ml + 250 IU/100 ml (GA+N). Ethanol was added to the medium to obtain a final concentration of 5% (v/v), the maximum concentration of ethanol present in the gallic acid solution. The medium was inoculated with 7% of a bacterial culture grown overnight at 30°C under constant agitation at 100 rpm. Bacterial growth was evaluated using the optical density at 600 nm, and by determination of the number of viable cells grown on TSA-YE agar.

2.4 Determination of Lipid Peroxidation Induced by Nisin and Gallic Acid in *Listeria monocytogenes* Scott A Cm^rEm^r

After incubation of *L. monocytogenes* for 18 hours at 30°C, under constant agitation at 100 rpm, a 10⁹ CFU/ml density of bacteria was incubated with nisin and gallic acid for a further 18 hours under the same conditions. The concentration of GA in the medium was varied between 4 and 40 mg/ml, and that of N between 100 and 500 IU/ml. The same concentrations were used for the mixture (GA+N). After incubation, the samples were centrifuged at 2,500 x g for 10 minutes, and the pellet of cellular material was washed twice with sterile pH 7.4 PBS buffer. The pellet was resuspended using sterile 0.1 M phosphate buffer (pH 7.4), with addition of 60 mg/ml of lysozyme to aid disruption of the bacterial cell walls, and sonicated for 30 minutes. Lipid peroxidation was determined according to the method proposed by Bird and Draper (1984), adding a suspension of 12% trichloroacetic acid (TCA) and 0.73% thiobarbituric acid (TBA), with heating at 100 °C for one hour, cooling to ambient temperature, centrifuging at 10,000 x g, and measurement of the absorbance of the supernatant at 535

nm. The extent of lipid oxidation was indicated by the formation of malondialdehyde (MDA) and other substances that react with TBA, and the values were expressed as percentages relative to the control.

2.5 Determination of the Concentration of Reduced Glutathione (GSH) after Exposure of *Listeria monocytogenes* Scott A *Cm^rEm^r* to Nisin and Gallic Acid

Reduced glutathione was determined according to the method proposed by Tietz (1969). In this technique, the sulfhydryl groups of GSH interact with 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), producing GSTNB (an oxidized form of GSH), which leads to the release of 5-thio-2-nitrobenzoic acid (TNB), a yellow-colored compound. For determination of the GSH content, *L. Monocytogenes* was first incubated in TSB-YE medium for 18 hours at 30°C, under constant agitation at 100 rpm. A 10^9 CFU/ml density of bacteria was then incubated with nisin and gallic acid for a further 18 hours under the same conditions. The concentration of GA added to the medium was varied between 4 and 40 mg/ml, and that of N between 100 and 500 IU/ml, whether used alone or in combination. After incubation, the samples were centrifuged at $2,500 \times g$ for 10 minutes, and the cellular pellet was washed twice with sterile pH 7.4 PBS buffer. The pellet was resuspended in 12% TCA, and submitted to ultrasonication for 30 minutes to aid disruption of the cell walls. The sample was then centrifuged at $5,000 \times g$ for 10 minutes, and the concentration of GSH in the supernatant was determined. The procedure consisted of mixing the cellular supernatant with the reaction medium (75 μ M DTNB and 0.1 M phosphate buffer, at pH 8.0). The color change was measured at 412 nm after 15 minutes incubation at ambient temperature. At the same time, a GSH standard curve was generated using concentrations of between 1 nM and 1 μ M. The GSH values were calculated after interpolation by linear regression, and expressed as percentages relative to the control.

2.6 Statistical Analysis

The experiments were repeated three times. The average values were submitted to analysis of variance (ANOVA), followed by Tukey's post-hoc test. A value of $p < 0.05$ was considered to indicate a significant difference.

3. Results

3.1 Antibacterial Activity

The agar diffusion test was used to evaluate the anti-*Listeria* activities of different concentrations of nisin and gallic acid, used either alone or in combination. The greatest effect was obtained using GA, with a zone of inhibition varying between 7.25 ± 0.479 and 14.75 ± 0.85 mm, for concentrations of 2 and 8 mg/ml, respectively. The greatest effect for nisin occurred at a concentration of 100 IU/ml, with a zone of inhibition of 10.25 ± 0.479 mm. When GA and N were used in combination, the maximum inhibition zone was 11.5 ± 0.289 mm. There was no evidence of any appreciable synergistic effect, since GA at 4 mg/ml and N at 100 IU/ml produced inhibition zones of 8.5 ± 0.645 mm and 10.25 ± 0.479 mm, respectively (Figure 1).

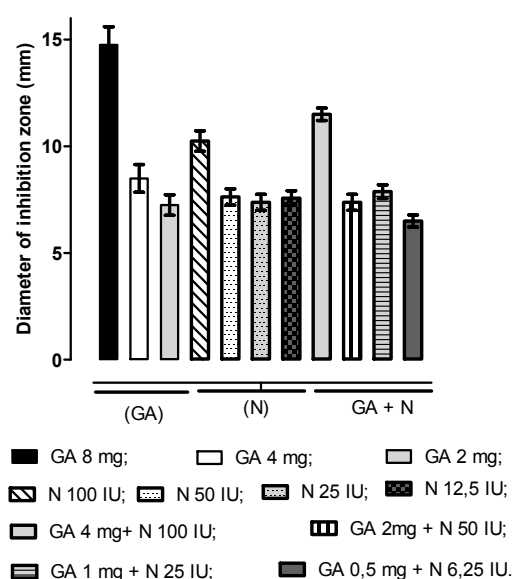


Figure 1. Antimicrobial activity of nisin (N) and gallic acid (GA) against *Listeria monocytogenes* Scott A *Cm^rEm^r*

3.2 Growth Curve of *Listeria monocytogenes* Scott A Cm^rEm^r in the Presence of Nisin and Gallic Acid

The effects of the different concentrations of nisin and gallic acid (used either alone or in combination) on the growth of *Listeria monocytogenes* are illustrated in Figure 2. At a concentration of 400 mg/100 ml, gallic acid caused 34% inhibition of the growth of the microorganism after 10 hours of exposure (Figure 2A), with the effect continuing up to 18 hours of exposure. At a concentration of 250 IU/100 ml, nisin did not promote any significant inhibition of growth up to 18 hours of exposure (Figure 2B); however, at 500 IU/100 ml there was a significant inhibition of bacterial growth, of 23%. There was a significant reduction in growth, of 45%, after 8 hours of exposure using a combination of nisin at 250 IU/100 ml and gallic acid at 200 mg/100 ml (Figure 2C), which was maintained up to around 12 hours. After this period of exposure, the reduction in growth was only 12%.

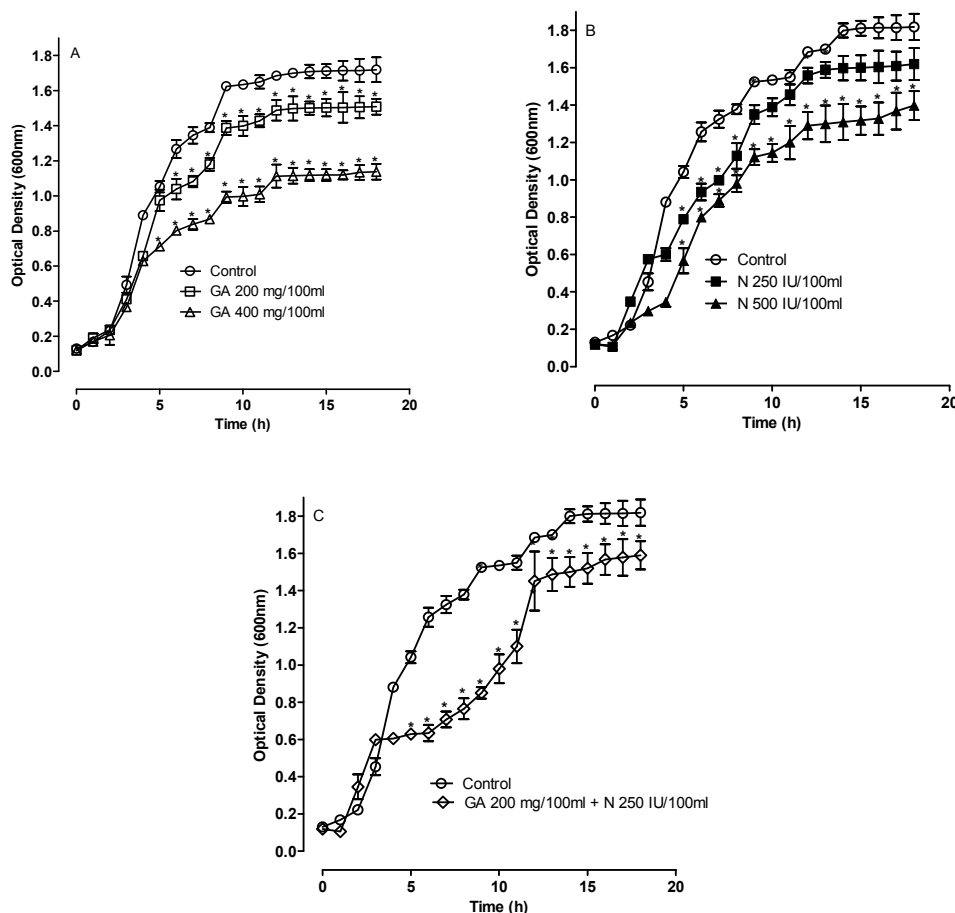


Figure 2. Growth curves for *Listeria monocytogenes* Scott A Cm^rEm^r in TSB-YE supplemented with different concentrations of gallic acid (GA) and nisin (N), either alone or in combination

A: GA at 200 and 400 mg/100 ml, and control (5% ethanol). **B:** N at 250 and 500 IU/100 ml, and control (distilled water). **C:** GA plus N at concentrations of 200 mg/100 ml and 250 IU/100 ml, and control (5% ethanol plus distilled water). Each point represents the mean $\pm$ standard deviation of three determinations.

3.3 Number of Viable Cells after Exposure of *Listeria monocytogenes* Scott A Cm^rEm^r to Nisin and Gallic Acid

After 18 hours of exposure of *L. monocytogenes* to nisin and gallic acid, either alone or in combination, a significant reduction in the number of viable cells was only obtained for nisin used alone (Figure 3). Hence, even though gallic acid reduced the cell density by 34% after 18 hours of exposure to a concentration of 400 mg/100 ml, it was not able to significantly reduce the number of viable cells.

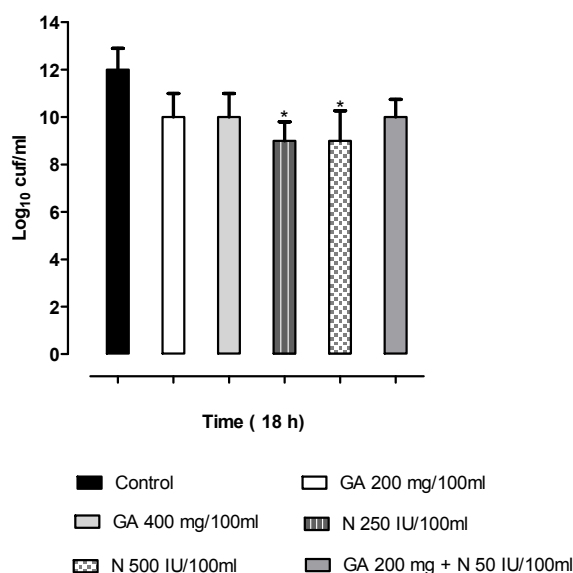


Figure 3. Viable cells of *Listeria monocytogenes* Scott A Cm^rEm^r after 18 hours/ of culture in TSB-YE medium supplemented with different concentrations of gallic acid (GA) and nisin (N), either alone or in combination, after Each point represents the mean $\pm$ standard deviation of three determinations. * $p < 0.05$ in comparison with the respective controls

3.4 Determination of Lipid Peroxidation and the Concentration of Glutathione in *Listeria monocytogenes* Scott A Cm^rEm^r after Exposure to Nisin and Gallic Acid

After 18 hours of exposure of *L. Monocytogenes* to nisin and gallic acid, either alone or in combination, greatest lipid peroxidation was induced using 4 mg/ml of gallic acid, since the concentration of GSH in the cells was maintained equal to that of the control. The use of higher concentrations resulted in less lipid peroxidation, with the microorganism showing resistance by increased concentrations of GSH (Figure 4, A-B). Nisin was only able to induce lipid peroxidation at a concentration of 0.1 mg/ml, with higher concentrations resulting in resistance of the bacteria due to increased GSH concentrations (Figure 4, C-D). When nisin and gallic acid were used in combination, the effects were similar to those observed for the individual compounds, with reduced lipid peroxidation associated with increased GSH concentrations (Figure 4, E-H).

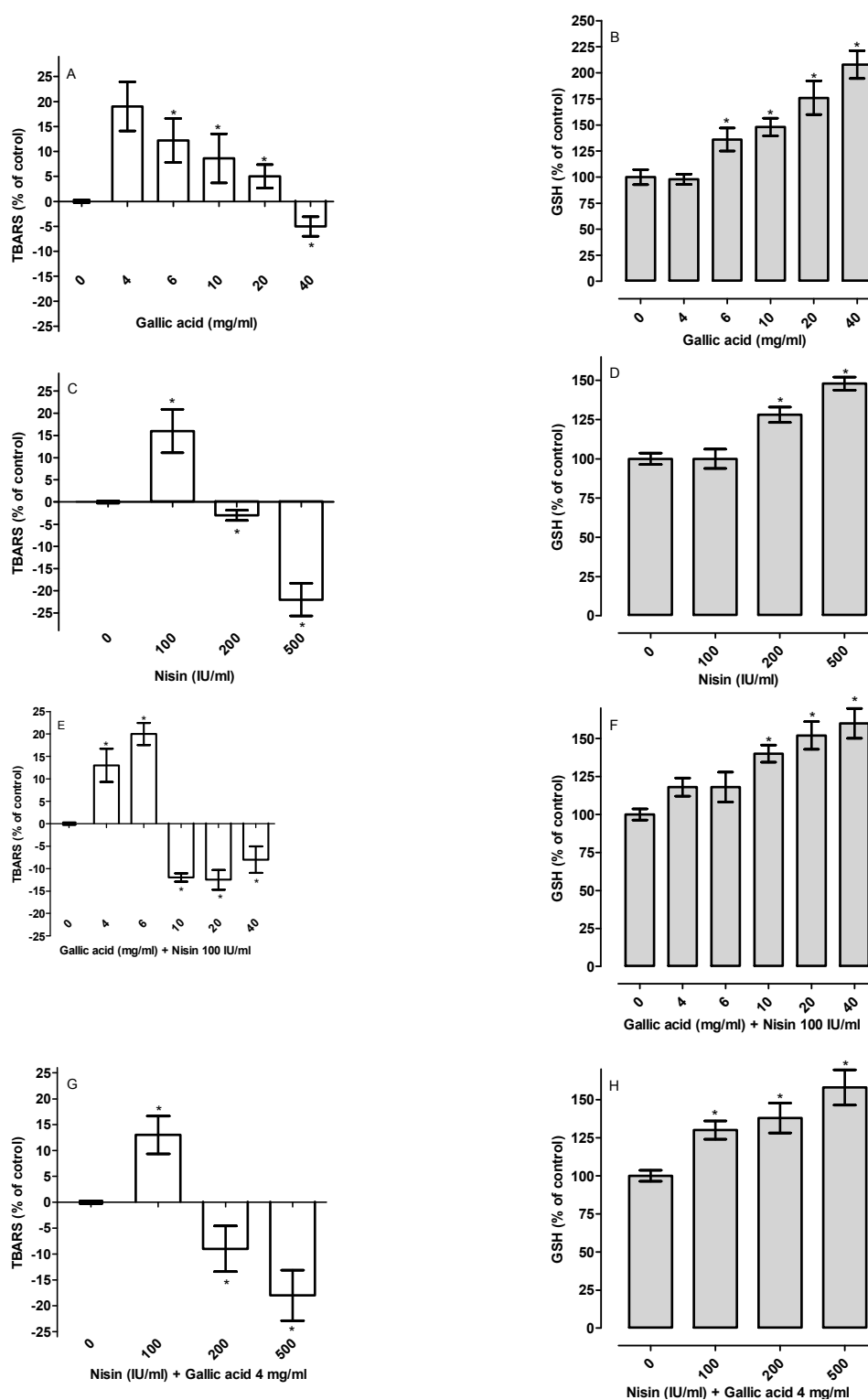


Figure 4. Effect of different substances on lipid peroxidation (%) and the concentration (%) of reduced glutathione (GSH) in cultures of *L. monocytogenes* Scott A Cm^rEm^r, after 18 hours exposure to different concentrations of the compounds

A: lipid peroxidation versus gallic acid. **B:** concentration of GSH versus gallic acid. **C:** lipid peroxidation versus nisin. **D:** concentration of GSH versus nisin. **E:** lipid peroxidation versus gallic acid combined with 0.1 mg/ml of nisin. **F:** GSH versus gallic acid combined with 0.1 mg/ml of nisin. **G:** lipid peroxidation versus nisin combined with 4 mg/ml of gallic acid. **H:** concentration of GSH, versus nisin combined with 4 mg/ml of gallic acid. Each point represents the mean $\pm$ standard deviation of three determinations. * $p < 0.05$ in comparison with the respective controls.

4. Discussion

The results showed that gallic acid at a concentration of 8 mg/ml was able to promote inhibition of bacterial growth, as assessed using the diameter of the zone of inhibition (Figure 1). Meanwhile, when a 7% concentration of bacteria was exposed to different concentrations of nisin and gallic acid, alone or in combination, the compounds were unable to reduce the number of viable cells after 18 hours (Figure 3). These findings indicate that the bacterial strain employed was resistant to the action of both nisin and gallic acid. Other authors have suggested that gallic acid can act synergistically with antibiotics used against *Pseudomonas aeruginosa*, and that it is effective against the herpes simplex virus, suggesting that it could be a likely candidate for use in topical medicines for treatment of the virus (Jayaraman et al., 2010; Kratz et al., 2008).

Studies have shown that several different strains of *L. monocytogenes* are resistant to the action of nisin (Abee et al., 1994; Crandall & Montville, 1998; Azizoglu & Kathniou, 2010). However, some strains are sensitive, indicating that nisin might be able to be used as a food additive to inhibit the proliferation of *L. monocytogenes* and other pathogenic bacteria present in contaminated food (Samelis et al., 2005; Hara et al., 2009). In United States of American nisin is used at maximum concentration of 10000 IU/g. Here we used a lower concentration (500 IU/ml) in according to Brazilian legislation (Cleveland et al., 2001).

The *L. monocytogenes* Scott A Cm^rEm^r strain was shown to be resistant to the action of nisin and gallic acid, used either alone or in combination, which could be related to the capacity of the bacteria to increase concentrations of GSH, and therefore become resistant to oxidative stress. Work by Azizoglu and Kathariou (Azizoglu & Kathniou, 2010) indicated that growth of *L. monocytogenes* under aerobic conditions is associated with higher activity of the catalase enzyme, promoting greater resistance to oxidative stress. The present results support this possibility, and suggest that the resistance of the strain employed could be related to increased bacterial antioxidant activity, reflected in an increase in GSH concentrations and a consequent reduction in lipid peroxidation under aerobic conditions. There are few published reports that have related the resistance of the pathogen to antioxidant activity. Some of the earlier work has suggested that the resistance of *L. monocytogenes* to nisin is associated with alterations in membrane lipids that increase the rigidity of the bacterial membrane (Ming & Daeschel, 1993; McEntire, Montville, & Chikindas, 2003; McEntere, Carman, & Montville, 2004). Other work has proposed that resistance to nisin may be associated with greater sensitivity to the action of acids, which could be mediated by cellular depletion of ATP (McEntire, Montville, & Chikindas, 2003; McEntere, Carman, & Montville, 2004).

The combination of nisin and gallic acid was shown to be ineffective, since the diameter of the inhibition zone was smaller in the presence of both compounds than when the compounds were used individually (Figure 1). Probably this antagonistic effect was due potential antioxidant of gallic acid. When the combination was used, there was a reduction in cell density after 18 hours of exposure; however, there was no significant reduction in the number of viable cells (Figures 2C and 3). This effect may be related to recovery of injured but not death cells by treatment (18 hours) after culture in agar plate. Similar findings were reported by Vaquero, Rodrigues and Nadra (2007), using gallic acid alone, where optical density measurements showed that there was inhibition of cell growth at concentrations in excess of 200 mg/L, while only concentrations above 500 mg/L were effective in reducing the numbers of viable cells.

Resistance of the *L. monocytogenes* strain was associated with a concentration-dependent increase in GSH levels in the presence of stressor agents. Some studies have found that GSH can react with electrophilic compounds, promoting cellular detoxification and consequently bacterial resistance to the antimicrobial action of the compounds, especially in strains of *E. coli*, where GSH appears to play the key role in bacterial resistance (Liu et al., 2009; Pittman, Robinson, & Poole, 2005). It can therefore be concluded that the strain of *L. Monocytogenes* studied in the present work was able to resist the action of oxidizing agents by increasing the synthesis of GSH. In light of this, further studies using this bacterial strain should be conducted to investigate the activities of important antioxidant enzymes and their involvement in microbial resistance to the action of antibacterial agents. Maybe an alternative model to evaluate the antagonistic action of different compounds against pathogens is using complex food systems. Milk and derivatives is an option.

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