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Effects of salts of organic acids on the survival of *Listeria monocytogenes* in soft cheeses

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Summary The purpose of this study was to determine the effect of sodium lactate and sodium propionate, both in combination with sodium acetate, on strains of *Listeria monocytogenes* in artificially inoculated soft cheeses. Minas Frescal and Coalho cheeses, inoculated with a mix of *L. monocytogenes* 1/2a and Scott A, underwent two treatments: 2% (w/v) sodium lactate in combination with 0.25% (w/v) sodium acetate and 2% (w/v) sodium propionate in combination with 0.25% (w/v) sodium acetate. The samples were analysed immediately and after 7 days at 10 °C. The growth of the pathogen was inhibited in cheeses containing the salts of organic acids, and the effects of treatment were statistically significant ($P < 0.05$). However, there was no difference between the types of treatment applied. Our data demonstrate that the effectiveness of the salts of organic acids depended on the initial concentration of *L. monocytogenes* and that a higher concentration of the salts is necessary to ensure sustained inactivation of target pathogens because they are weakly antilisterial when the soft cheeses are stored at 10 °C.

Keywords Cheese, food safety, *Listeria*, organic acids.

Introduction

Since the 1980s, the increase in outbreaks of human listeriosis and the possible relationship with contaminated food have been a concern of health authorities. In Brazil, there is no description of outbreaks of foodborne listeriosis, but the presence of *Listeria monocytogenes* in various products, including cheeses, is reported in several studies. Reports have indicated that these food products are susceptible to recontamination with *L. monocytogenes* after heat processing. Because of its psychrotropic character, the bacterium is a pathogen of concern in refrigerated food products, such as meat (cooked and fresh), cheese and milk (Cox, 1989; Rocourt & Cossart, 1997). Because *L. monocytogenes* is capable of growing at refrigerated temperatures, antimicrobial strategies to overcome the tolerance of *L. monocytogenes* for low temperatures are essential, and the industry has sought more effective methods to combat the pathogen.

Cheeses are frequently considered to be possible vehicles of contamination for *L. monocytogenes*. These foods are exposed to a series of conditions that are favourable to the development of microorganisms. These conditions are handicraft production, which includes intense manipulation during the processing; the storage and distribution of the cheese; the high activity of water, which is mainly in soft cheeses with middle range and high humidity; the mixture of different ingredients included in the cheese; and finally, the fact that cheeses are kept under refrigeration, which favours the multiplication of *L. monocytogenes*.

Minas Frescal and Coalho are traditional cheeses that are very popular in Brazil. The cheeses are produced by enzymatic coagulation of cow's milk with rennet and/or other appropriate enzymes and are either complemented or not by the action of specific lactic bacteria. These cheeses are classified as soft. However, Minas Frescal has very high humidity, while Coalho cheese has middle range humidity at half-cooking or cooking mass.

The antimicrobial effect of salts of organic acids, alone or in combination with other food additives, has been examined and reported (Anderson & Marshall, 1990; Mbandi & Shelef, 2002; Ukuku *et al.*, 2005).

Organic acids with short chains, and/or their salts, are frequently used as chemical decontaminants and are

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generally recognised as safe compounds (Bolder, 1997; Alakomi *et al*., 2000). They are extensively used in meat and poultry industries to enhance antimicrobial benefits (Shelef & Yang, 1991; Weaver & Shelef, 1992; Harmayani *et al*., 1993; Serdengecti *et al*., 2006).

Traditionally, lactic acid, a weak organic acid, has been widely used to control the growth of pathogenic bacteria in foods for several decades. The antimicrobial activity occurs through the diffusion of lactic molecules into microbial cells until equilibrium is reached, in accordance with the pH gradient, causing membrane disruption, inhibition of essential metabolic reactions, stress on intracellular pH, homeostasis, the accumulation of toxic anions and the ultimate death of microbial cells (Ibrahim *et al*., 2008).

Some studies have demonstrated that *L. monocytogenes* is more acid tolerant that the majority of food pathogens. Even so, the sensitivity of the organism to organic acids varies in accordance with the nature of the acid added (Sorrells *et al*., 1989). Furthermore, its tolerance to acid can be strengthened through the exposure of the organism to moderately acidic conditions (Kroll & Patchett, 1992).

The present study was undertaken to evaluate the inhibitory effect of two salts of organic acids, sodium lactate and sodium propionate, both in combination with sodium acetate, on strains of *L. monocytogenes* in artificially inoculated Minas Frescal and Coalho cheeses immediately and after 7 days under refrigerated temperature.

Materials and methods

Cheeses

Minas Frescal is classified as a soft cheese with very high humidity, raw mass and a soft or lightly acidic flavour. Coalho is a rennet cheese, classified as having middle range or high humidity, half-cooking or cooking mass. The distinctive characteristics of the elaboration process of the cheeses include approximately 40 min of coagulation, the cut and mixture of the mass, partial removal of the serum, heating of the mass with hot water or indirect steam (Coalho cheese) until attainment of half-cooking (45 °C) or cooking mass (between 45 and 55 °C), the addition of salt (sodium chloride) to the mass, pressing, drying, packing and storage.

Bacterial strains, media and culture conditions

The bacteria used in this study were a strain of *Listeria monocytogenes*, serotype 1/2a, isolated from Brazilian fresh sausage and *Listeria monocytogenes* Scott A - ATCC 15313 (serotype 4b).

The cultures of *L. monocytogenes* were stored in Hogness medium (1.3 mM K_2HPO_4. $3H_2O$; 1.3 mM KH_2PO_4; 2.0 mM citrate-Na.2 H_2O; 1.0 mM $MgSO_4$.7 H_2O; 4.4% (v/v) glycerol) and frozen at −80 °C. Before use, the *L. monocytogenes* cultures were activated in tryptic soy broth supplemented with 0.5% (w/v) yeast extract (TSB-YE) and kept at 35 °C overnight in a shaker (Cientec Equipamentos para laboratœrio, Cientec Incubadora Shaker, model CT-712, Araraquara, SP, Brazil) at 150 rpm.

Bacterial survival, following treatment with the organic acids or no treatment, was determined by colony-forming units (CFU g^{-1}) on lithium chloride-phenylethanol-moxalactam (LPM, DIFCO, Detroit, MI, USA) agar with added 0.1% esculin and 0.05% ferric citrate ammoniac.

Preparation of bacteria inoculums

Listeria monocytogenes 1/2a and Scott A (serotype 4b) were cultured by loop inoculation of 10 mL volumes of tryptic soy broth containing 0.5% (w/v) yeast extract (TSB-YE), which was then incubated at 35 °C for 18–20 h in a shaker at 150 rpm. The cell suspensions were transferred to sterile Eppendorf tubes, and inoculum levels were confirmed by surface plating duplicate samples onto LPM agar. The plates were incubated at 35 °C for 24 h before colony counts were obtained. Overnight cultures of the strains were diluted appropriately to obtain the required bacterial load (approximately 10^5 CFU mL^{-1}) before inoculation in the samples.

Sample inoculation and treatment application

Minas Frescal and Coalho cheeses were purchased at a local supermarket in Salvador, Bahia, Brazil. The samples were transported in insulated, iced containers to the laboratory for analysis. After aseptically removing the package under a class II biosafety cabinet (Labconco model 36210 class BII; Labconco, Kansas City, MO, USA), six 30-g samples of Minas Frescal cheese and six 30-g samples of the Coalho cheese were transferred to a bag mixer. Three samples of each cheese were identified as time zero, and three were identified as 7 days. All samples were inoculated separately with a 1-mL volume of the mixture of *L. monocytogenes* 1/2a (approximately 6.2×10^5 CFU mL^{-1}) and Scott A (approximately 3.5×10^5 CFU mL^{-1}). Four samples of each cheese were submitted to treatments with organic acids (two for time zero and two for 7 days), and two of them were identified as control, one for time zero and one for 7 days. Inoculated samples were homogenised gently by hand to ensure an even distribution of organisms and air-dried under a class II biosafety cabinet for 30 min at 21 °C to allow for the attachment of bacteria to the cheese before undergoing treatment with organic acids (Singh *et al*., 2002). Two samples of each cheese were dipped separately into a solution

containing a mixture of 2% sodium lactate and 0.25% sodium acetate. After 20 min of incubation at room temperature, the excess organic acids were drained off. One sample of each cheese was immediately analysed, and the other was stored at 10 °C for seven days. The same procedure was conducted for the treatment carried out with sodium 2% propionate and 0.25% sodium acetate. The control samples were dipped in sterile water. These experiments were independently performed three times.

Enumeration of microorganism and pH measurement

For enumerating *L. monocytogenes*, each 30-g portion was added to 270 mL of 0.1% (w/v) peptone water with 0.01% (w/v) tween-80 and homogenised in a stomacher (ITR instrumentos para laboratórios Ltda., Esteio, RS, Brazil, 240 bpm) for 2 min. Serial dilutions were made in the same solution without tween-80 and spread onto LPM plates, in duplicate, and incubated at 35 °C for 48 h. Bacterial survival following treatment with organic acids or without organic acids (control) was determined by measuring colony-forming units (CFU).

The cheeses pH was measured initially and after 7 days by directly inserting the pH electrode (Sure Flow* pH electrode 9172BNWP) into the cheese homogenates (1:10 dilution) (3-Star Benchtop pH Meters; Thermo Scientific Orion, Cole Parmer, IL, USA).

Investigation of *Listeria* spp. in Minas Frescal and Coalho cheeses

The cheese samples were analysed for the presence or absence of *Listeria* spp., before inoculation with *L. monocytogenes* strains, by streaking Half Fraser enrichment broth on LPM agar (DIFCO) plates to check for the presence of typical *Listeria* spp. colonies. One *L. monocytogenes* positive control (*L. monocytogenes* Scott A, ATCC 15313) and one uninoculated media negative control were used for each set of concurrently analysed samples.

Statistical analysis

To investigate the effectiveness of organic acids on the elimination of *L. monocytogenes* 1/2a and Scott A, bacterial counts were determined by duplicate plating, and all the experiments described here were independently performed three times. All counts of bacteria (CFU g^{-1}) recovered from Minas Frescal and Coalho cheeses were transformed to logarithms before computing means and standard deviations. The decimal reduction (DR) in the population of the bacteria was calculated by the difference between the counts obtained in the control and each treatment. Log values of zero were assumed for samples in which *L. monocytogenes* was not detected (< 2 log CFU g^{-1}). Data were subjected to the Statistical Analysis System of variance and Tukey's multiple range (Software ASSISTAT, version 7.6 beta; 2011) to determine whether significant differences ($P < 0.05$) in the populations of *L. monocytogenes* existed among the mean log values.

Results

The microbiological evaluation of Minas Frescal and Coalho cheeses showed an absence of *Listeria* spp. in the samples investigated.

Minas Frescal cheese

The effects of 2% sodium lactate in combination with 0.25% sodium acetate and 2% sodium propionate in combination with sodium acetate 0.25% in Minas Frescal cheese are shown in Tables 1 and 2, respectively. These treatments reduced the growth rates of *L. monocytogenes* immediately and at 7 days at 10 °C when

Table 1 Reduction in viable counts of *Listeria monocytogenes,* a mix of serotype 1/2a and Scott A, in Minas Frescal cheese by 2% sodium lactate in combination with 0.25% sodium diacetate at time zero and after 7 days at 10 °C

	Listeria monocytogenes **(Log$_{10}$ CFU g^{-1})**					
	Time zero			**7 days**		
Experiment	**Control**	**Treatment***	**DR**	**Control**	**Treatment***	**DR**
1	5.9	3.4	2.5	6.6	5.6	1.0
2	6.0	3.8	2.2	6.5	5.0	1.5
3	5.1	3.5	1.6	6.5	5.6	0.9
Mean†	5.8 ± 0.5	3.6 ± 0.21	2.2	6.5 ± 0.05	5.4 ± 0.35	1.1

*2% (w/v) sodium lactate and 0.25% (w/v) sodium acetate.
†Mean ± standard deviation.
DR, Decimal reduction.

Table 2 Reduction in viable counts of *Listeria monocytogenes,* a mix of serotype 1/2a and Scott A, in Minas Frescal cheese by 2% sodium propionate in combination with 0.25% sodium diacetate at time zero and after 7 days at 10 °C

	Listeria monocytogenes **(Log$_{10}$ CFU g^{-1})**					
	Time zero			**7 days**		
Experiment	**Control**	**Treatment***	**DR**	**Control**	**Treatment***	**DR**
1	5.9	3.8	2.1	6.6	5.8	0.8
2	6.0	3.7	2.3	6.5	5.4	1.1
3	5.1	3.4	1.7	6.5	5.7	0.8
Mean†	5.8 ± 0.5	3.6 ± 0.21	2.2	6.5 ± 0.05	5.6 ± 0.21	0.9

*2% (w/v) sodium propionate and 0.25% (w/v) sodium acetate.
†Mean ± standard deviation.
DR, Decimal reduction.

compared with cheeses containing no salts. The results indicate that sodium lactate and sodium propionate, in combination with sodium acetate, may prevent the growth of this pathogen in soft cheese. The log reductions or DR in viable cells was calculated (Tables 1 and 2). A mean reduction in more than two log cycles in viable counts was observed when we used the sodium lactate (6.6×10^5 to 3.9×10^3 CFU mL^{-1}) or sodium propionate in combination with sodium acetate (6.6×10^5 to 4.3×10^3 CFU mL^{-1}), and the samples were analysed immediately (time zero). However, after 7 days at 10 °C, the numbers of the *L. monocytogenes* in untreated samples exceeded 6 log CFU g^{-1}, and in treated samples, the DR was the lowest, about 1.1 for sodium lactate and 0.9 unit for sodium propionate.

Coalho cheese

The effects of 2% sodium lactate in combination with 0.25% sodium acetate and 2% sodium propionate in combination with 0.25% sodium acetate in Coalho cheese are shown in Tables 3 and 4, respectively.

The addition of sodium lactate plus sodium acetate in Coalho cheese reduced the growth rates of *L. monocytogenes* when compared with cheese containing no salts. The results indicate that sodium lactate in combination with sodium acetate may prevent the growth of these pathogens in Coalho cheese. In samples stored under refrigeration, the organic acids were only weakly antilisterial.

Similar results to Minas Frescal cheese were found, but the DR achieved was the lowest value in the samples analysed immediately (time zero), 1.8 for samples treated with sodium acetate and 1.9 units for sodium propionate.

During storage in the refrigerator, the bacteria grew approximately 1 log cycle, and the DR was lower, 1.1 for samples treated with sodium acetate and 0.8 units for sodium propionate.

The results obtained from the pH analysis indicate that the values were smaller after storage of the cheeses under refrigeration (Table 5).

For both cheeses, the statistical analyses showed no difference between the treatments used ($P < 0.05$)

Table 3 Reduction in viable counts of *Listeria monocytogenes,* a mix of serotype 1/2a and Scott A, in Coalho cheese by 2% sodium lactate in combination with 0.25% sodium diacetate at time zero and after 7 days at 10 °C

	Listeria monocytogenes (Log_{10} CFU g^{-1})					
	Time zero			7 days		
Experiment	Control	Treatment*	DR	Control	Treatment*	DR
1	5.1	3.7	1.4	6.5	5.3	1.2
2	5.6	3.5	2.1	6.6	5.6	1.0
3	5.6	3.8	1.8	6.4	5.5	0.9
Mean†	5.4 ± 0.29	3.7 ± 0.15	1.8	6.5 ± 0.1	5.5 ± 0.15	1.0

*2% (w/v) sodium lactate and 0.25% (w/v) sodium acetate.
†Mean ± standard deviation.
DR, Decimal reduction.

Table 4 Reduction in viable counts of *Listeria monocytogenes,* a mix of serotype 1/2a and Scott A, in Coalho cheese by 2% sodium propionate in combination with sodium 0.25% diacetate at time zero and after 7 days at 10 °C

	Listeria monocytogenes (Log_{10} CFU g^{-1})					
	Time zero			7 days		
Experiment	Control	Treatment*	DR	Control	Treatment*	DR
1	5.1	3.3	1.8	6.5	5.7	0.8
2	5.6	3.7	1.9	6.6	5.8	0.8
3	5.6	3.7	1.9	6.4	5.7	0.7
Mean†	5.4 ± 0.29	3.6 ± 0.23	1.9	6.5 ± 0.1	5.7 ± 0.06	0.8

*2% (w/v) sodium propionate and 0.25% (w/v) sodium acetate.
†Mean ± standard deviation.
DR, Decimal reduction.

Table 5 Mean values of pH of Minas Frescal and Coalho cheeses at time zero and after 7 days at 10 °C

Cheese	Control	Treatment A*	Treatment B†
Minas Frescal			
Time zero	6.3	6.7	6.8
Seven days	5.6	6.2	5.9
Coalho			
Time zero	5.0	5.5	5.8
Seven days	5.3	5.4	5.6

*2% (w/v) sodium lactate plus 0.25% (w/v) sodium acetate.
†2% (w/v) sodium propionate plus 0.25% (w/v) sodium acetate.

Table 6 Mean counts of the *Listeria monocytogenes,* a mix of serotype 1/2a and Scott A, in samples of Minas Frescal cheese as a function of the use of the antimicrobial treatments at time zero and after 7 days at 10 °C

	Listeria monocytogenes (Log_{10} CFU g^{-1})	
	Time zero	7 days
Control	5.7*†	6.5*
Treatment A*	3.6‡	5.4‡
Treatment B‡	3.6‡	5.6‡

*2% (w/v) sodium lactate plus 0.25% (w/v) sodium acetate.
†Means followed by different letters are significantly different by Tukey's test ($P < 0.05$).
‡2% (w/v) sodium propionate plus 0.25% (w/v) sodium acetate.

Table 7 Mean counts of the *Listeria monocytogenes,* a mix of serotype 1/2a and Scott A, in samples of Coalho cheese as a function of the use of the antimicrobial treatments at time zero and after 7 days at 10 °C

	Listeria monocytogenes **(Log_{10} CFU g^{-1})**	
	Time zero	**7 days**
Control	5.4*†	6.5*
Treatment A*	3.7‡	5.5‡
Treatment B‡	3.6‡	5.7‡

*2% (w/v) sodium lactate plus 0.25% (w/v) sodium acetate.
†Means followed by different letters are significantly different by Tukey's test ($P < 0.05$).
‡2% (w/v) sodium propionate plus 0.25% (w/v) sodium acetate.

(Tables 6 and 7). A significant difference was seen by Tukey's test ($P < 0.05$) when compared with the control (Tables 6 and 7).

Discussion

Food safety issues linked to food-borne bacterial pathogens continue to be a major concern for the food industry. An important strategy for food safety is to develop new approaches for food preservation while satisfying the increased consumer demand for natural products with health benefits. Combinations of salts of organic acids are attractive in providing functional benefits for both food safety and human health.

Organic acids and their salts have also been suggested to have antimicrobial effects by causing hyper-acidification via proton donation at the plasma membrane interface of the microorganism and intracellular cytosolic acidification, an excess of which can disrupt the H^+-ATPase enzyme, which is required for ATP synthesis (Shetty & Wahlqvist, 2004; Lin *et al*., 2005; Kwon *et al*., 2007).

In the present study, it was demonstrated that the growth of *L. monocytogenes* could be prevented or inhibited in two different soft cheeses by sodium lactate and sodium propionate, both in combination with sodium acetate, immediately after the treatment (time zero). However, under refrigeration, the organic acids were only weakly antilisterial. These findings suggest that the exposure of the organism to moderately acidic conditions can be strengthened by its tolerance to acid and demonstrate the psychrotropic characteristics of the bacteria.

The initial pH in control samples was 6.3 and 5.0 for Minas Frescal and Coalho cheeses, respectively. Immediately, after the addition of lactate and propionate plus acetate, we observed a slow increase in pH in all samples. After 7 days of storage, the pH was reduced in control and in treated samples. The changes observed were less than one pH unit. Different results in studies with meat products were reported by Mbandi & Shelef (2002) showing a slow increase in pH in all samples during storage.

It is known that at lower pH values, the presence of undissociated lactate results in a more effective antimicrobial effect (McMahon *et al*., 1999).

On the other hand, the DRs in the counts of the bacteria at time zero demonstrate that organic acids efficiently inhibited *L. monocytogenes*. One possibility to explain these results is that a subpopulation of cells of *L. monocytogenes* tolerant/resistant to acid action is selected and increased up to seven days after incubation. The tolerance/resistance of *L. monocytogenes* to organic acids has been previously reported by Sorrells *et al*. (1989).

Studies have demonstrated the inhibition of *L. monocytogenes* in meat products that are packed with 2% sodium lactate and stored in temperatures of 1–7 °C (Unda *et al*., 1991; Qvist *et al*., 1994). This has also been demonstrated in pork liver sausage (Weaver & Shelef, 1992) using 3% sodium lactate. Other studies have demonstrated that 2.5% sodium lactate alone did not hinder the growth of *L. monocytogenes* in stored turkey meat at 4 °C, but when used in combination with 0.1% diacetate, the inhibition of the growth of the bacterium was verified by at least 6 weeks (Schlyter *et al*., 1993). The authors noted that the use of only 0.1% diacetate was inefficacious for the control of bacterial growth in the food.

According to Apostolidis *et al*. (2008), the addition of 2% sodium lactate in broth at pH 6 and 4 °C resulted in significant *L. monocytogenes* inhibition when compared to an untreated control after 20 days of storage. With sodium lactate alone, the authors demonstrated a 1.8-log reduction in bacteria counts. These results were similar to the present study when the samples were analysed immediately.

In the current study, the level of the initial inoculum was in the range of 5–6 log CFU mL^{-1}. In the study by Apostolidis *et al*. (2008), when the inoculum level was in the range of 4–5 log CFU mL^{-1}, the results showed that the degree of inhibition was lower when compared with initial inoculum levels of 3–4 log CFU mL^{-1}.

The kinetics of the bactericidal effect of lactic acid decontamination on meat-borne pathogens in an *in vitro* model indicated that 2% lactic acid decontamination at 37 °C for 30–90 s is suitable for elimination of *Salmonella* on meat but not for *L. monocytogenes* (Nettern *et al*., 1994). In the study of Sudershan *et al*. (2011), the antimicrobial effect of lactic acid, both *in vitro* and *in vivo,* indicated that 3% lactic acid was found to be more effective in reducing the count of selective food-borne pathogens.

According to Hwang *et al*. (2011), *L. monocytogenes* did not grow in ham containing 3% lactate at all storage

temperatures. The addition of lactate to ham at 1% or 2% concentrations reduced the growth rates of *L. monocytogenes* when compared with ham containing no lactate. The results indicate that lactate at lower levels slows the growth of these pathogens, which is what was observed in the current study.

The addition of a combination of 0.05% sodium benzoate and 0.05% sodium propionate and a combination of 0.05% sodium benzoate and 0.05% potassium sorbate in the cured bologna that was inoculated on the surface with *L. monocytogenes* (4 log CFU per package) was assessed by Glass *et al*. (2007). The results of the study showed that the agents prevented the growth of *L. monocytogenes* during the 13-week storage period at 4 °C, compared with a more than 3.5 log increase in listerial populations in the control bologna, to which no antimicrobial agents had been added. The authors suggest that low concentrations of antimycotic agents can prevent *L. monocytogenes* growth in certain ready-to-eat meats.

In conclusion, the data from the present study suggest that sodium lactate (2%) and sodium propionate (2%), in combination with sodium acetate (0.25%), can be used as an antimicrobial agent to reduce the growth of *L. monocytogenes*.

However, it is important to mention that the initial concentration of *L. monocytogenes* inoculated in the cheeses was higher than the one that occurs in food natural contamination. In fact, the industry needs efficient technologies to guarantee food safety, and higher concentrations of the salts are necessary because they are weakly antilisterial when the soft cheeses are stored at 10 °C.

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