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Inhibition of *Listeria monocytogenes* by a bacteriocinogenic *Lactobacillus sake* strain in modified atmosphere-packaged Brazilian sausage

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Abstract

Lactobacillus sake 2a is a bacteriocinogenic strain isolated from "lingüiça frescal", a Brazilian sausage. The combined effect of modified-atmosphere (MA) packaging (100% CO_2 and 50% CO_2/50% N_2) and addition of *L. sake* 2a on inhibition of growth of *Listeria monocytogenes* was evaluated in "lingüiça" stored at 6 °C. By the end of the first week, the inhibition of *L. monocytogenes* due to MA was significant ($P \leqslant 0.05$) while the presence of *L. sake* 2a did not influence significantly the growth of the pathogen. After 14 days, a reduction of 1.3–1.4 log in counts of *L. monocytogenes* was observed in samples containing *L. sake* 2a only or MA packaged only, while a reduction of 3.5 log was detected in those submitted to both treatments. Results indicate that inhibition of *L. monocytogenes* in "lingüiça frescal" by the bacteriocinogenic *L. sake* 2a is enhanced by the packaging of the product in MA.

Keywords: *Listeria monocytogenes*; *Lactobacillus sake*; Bacteriocins; Modified atmosphere; Meat products

1. Introduction

Listeria monocytogenes is a pathogenic microorganism that has been isolated from many types of foods. This microorganism is ubiquitous in the environment and the International Commission on Microbiological Specifications for Foods (1996) considers that when the contamination level that is lower than 100 CFU/g at the point of food consumption, the food is acceptable for individuals who are not at risk. Thus, proper control of multiplication of *L. monocytogenes* within the food may minimize the risk of foodborne listeriosis (International Commission on Microbiological Specifications for Foods, 1996; Jay, 1996).

In meat products, *L. monocytogenes* may represent a serious health hazard due to survival and active proliferation at refrigeration temperatures. Additional hurdles should be used in combination with cold storage to enhance the safety of refrigerated meat products. Among the possible hurdles, the packaging under modified atmosphere (MA) seems to be the most effective (García de Fernando, Nychas, Peck, & Ordóñez, 1995). Sheridan, Doherty, McDowell, Blair, and Harrington (1995) demonstrated that proliferation of *L. monocytogenes* was completely inhibited in lamb meat stored at 5 °C packaged under 100% CO_2, while atmospheres containing only 20 or 50% of CO_2 were less inhibitory. Mano et al. (1995) also reported complete growth inhibition in refrigerated pork meat packaged under 100% N_2, 20% CO_2/80% O_2 and 40% CO_2/60% O_2. Farber, Cai, and Ross (1996) observed that the inhibition was temperature and pH dependent. Many additional studies confirm that modified atmospheres in packaging of many types of meat products interfere with the survival and growth of *L. monocytogenes* (García de Fernando et al., 1995; Hart, Mead, & Norris, 1991; Hudson & Franco, 1994; Hugas, Pagés, Garriga, & Monfort, 1998).

Bacteriocinogenic lactic acid bacteria can also exert an inhibitory activity against *L. monocytogenes* in meats and meat products (Ahn & Stiles, 1990; De Martinis & Franco, 1998; Lewus, Kaiser, & Montville, 1991; Schil-

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linger & Lucke, 1989). Recently, Nilsson, Chen, Chikindas, Huss, Gram, and Montville (2000) reported that nisin and CO_2 atmosphere acted synergistically on the cytoplasmic membrane of *L. monocytogenes* by enhancing membrane permeabilization.

''Lingüiça'', a popular Brazilian meat product, is a mixture made of minced pork, curing salts and spices filled in natural gut casings. The percentage of fat varies according to the meat used in the manufacturing. The product has a pH around 6.0 and is frequently consumed undercooked, representing a risk to human health. De Martinis and Franco (1998) reported that a *Lactobacillus sake* strain (*L. sake* 2a) isolated from lingüiça was capable of inhibiting growth of *L. monocytogenes* in the product. The inhibition was due to a bacteriocin also active against other Gram positive pathogens (De Martinis & Franco, 1997).

In this study, the combined effect of MA packaging and use of the bacteriocinogenic *L. sake* 2a strain on the inhibition of growth of *L. monocytogenes* in ''lingüiça'' was evaluated. A possible synergistic effect of these two hurdles could be an interesting technological tool to increase the safety and extend the shelf life of this product.

2. Materials and methods

2.1. Microbial strains

L. sake 2a, isolated from lingüiça (De Martinis & Franco, 1997) and *L. monocytogenes* F5069, resistant to chloramphenicol and erythromycin, kindly provided by Dr. Thomas J. Montville, Rutgers—The State University of New Jersey, NJ, USA.

2.2. Preparation of inocula

L. monocytogenes F5069 was grown in 5 ml BHI containing 5 μg/ml of chloramphenicol (Sigma Chemical CO., St. Louis, MO) and 0.5 μg/ml erythromycin (Sigma Chemical CO., St. Louis, MO), at 35 °C for 18–24 h. *L. sake* 2a was grown in 5 ml MRS broth containing 0.5% glucose, at 30 °C for 18–24 h. Both cultures were centrifuged at 1600 ***g*** for 20 min and washed three times with equal volume of 0.85% saline. The resuspended pellets were used for inoculation of lingüiça and the supernatant of *L. sake* 2a culture was used to test for bacteriocin activity. The approximate number of CFUs in the cultures of *L. monocytogenes* and *L. sake* was determined by plating on Tryptic Soy Agar supplemented with 6% yeast extract (TSAYE) and deMan Rogosa Sharpe agar (MRS), respectively. TSAYE and MRS agar plates were incubated for 48 h at 37 and 30 °C, respectively. All culture media were from Oxoid Ltd. (Basingstoke, UK).

2.3. Testing for bacteriocin activity

The well-diffusion test according to Harris, Daeschel, Stiles, and Klaenhammer (1989) was used. Melted BHI (20 ml) containing 1% agar was inoculated with a fresh culture of *L. monocytogenes* F5069 to achieve a concentration of 10^5–10^6 CFU/ml and transferred to a empty Petri dish. After solidification, wells (3–4 mm diameter) were cut in the medium and filled with 40 μl of the neutralized supernatant of the *L. sake* culture, prepared as described earlier. Plates were incubated at 30 °C for 24 h and observed for the presence of growth inhibition halos around the wells.

2.4. Preparation and inoculation of lingüiça

For 1 kg of product, the following ingredients were used: 967g of pork shank, 20 g salt, 1 g sugar, 7 g spices (a ready-to-use commercial product), 3 g emulsifier, 2.5 g Exacor (ascorbic acid plus silicon dioxide, Lab. Exato Ind. e Com. Ltda., São Paulo, Brazil) and 150 ppm sodium nitrite. The pork meat was minced in a sterile mincer and mixed with the ingredients. The mixture was divided into portions of 800 g, transferred to plastic bags and treated with gamma irradiation (10 kGy) for elimination of indigenous microorganisms. Irradiation was done at Empresa Brasileira de Irradiação (EMBRARAD, Cotia, SP, Brazil). Remaining contaminants were enumerated by plating on Plate Count Agar (PCA), MRS agar, PALCAM and TSAYE with 5 μg/ml of chloramphenicol and 0.5 μg/ml erythromycin. MRS agar plates were incubated at 30 °C for 48 h under anaerobic conditions using Anaerogen paper sachets (Oxoid Ltd., Basingstoke, UK). PCA, PALCAM and TSAYE plates were incubated at 37 °C for 48 h. Microbial cultures were serially diluted in 0.85% saline. Inocula of microorganisms were added to each bag containing the irradiated mixture of meat and ingredients, using the appropriate dilution to obtain 10^5 CFU of *Lactobacillus sake* 2a and 10^4 CFU of *L. monocytogenes* per gram of product. Non-inoculated negative controls were also prepared. Inoculated samples were homogenized by external hand massaging of bags. The inoculated and non-inoculated mixtures were aseptically introduced into pork gut casings, previously sterilized by irradiation (10 kGy). The filled casings were tightly tied at every 10–12 cm with sterile string, forming segments of approximately 50 g. The segments were separated using sterile scissors, transferred to disposable plastic trays and wrapped with plastic film. For those submitted to packaging under modified atmosphere, Cryovac barrier bags (O_2 transmission rate 30 cm^3/m^2/24 h at 23 °C, 1 atm and 0%RH, Cryovac Brasil Ltda., São Paulo) were used. For the remaining samples, an oxygen-permeable polyethylene film was used.

2.5. Packaging in modified atmosphere

After removal of air, the desired combination of gases (50% CO_2/50% N_2 or 100% CO_2, White Martins Praxair Inc., São Paulo, Brazil) was introduced in the wrapped trays containing the lingüiça segments using a sealer with gas injection system (Engevac, São Paulo, Brazil). All lingüiça segments were maintained under refrigeration (6 °C). The chemical composition of the atmosphere during storage was monitored by gas chromatography, using a Varian Star 3400 CX (Walnut Greek, USA) chromatograph, equipped with a thermal conductivity detector and a CG-300 electronic integrator (Souza, Saad, & Oliveira, 2001).

2.6. Microbiological counts in the stored lingüiça segments

Counts of *L. monocytogenes* and *L. sake* 2a in lingüiça segments were determined weekly over a period of 28 days, in duplicates. For sampling, 25 g of lingüiça segments were homogenized with 225 ml of 0.1% peptone water, using a stomacher. Further decimal dilutions were prepared, using 0.1% peptone water as diluent. Counts of *L. monocytogenes* were done by spread plating on PALCAM and TSAYE containing 5 μg/ml of chloramphenicol (Sigma Chemical CO., St. Louis, MO) and 0.5 μg/ml erythromycin (Sigma Chemical CO., St. Louis, MO) and incubation at 37 °C for 48 h. Counts of *L. sake* were done by spread plating on MRS agar and incubation at 30 °C for 48 h under anaerobic conditions using Anaerogen paper sachets (Oxoid Ltd., Basingstoke, UK).

2.7. pH measurement

At each sampling, the pH of the lingüiça samples was monitored using pH indicator strips (Merck, Darmstadt, Germany) in the homogenates.

2.8. Sensory evaluation

Triangle tests, performed according to Meilgaard, Civille, and Carr (1999), were done to detect taste differences between lingüiça prepared with and without *L. sake*, packaged in air and in MA. The comparisons done were: (1) lingüiça with and without *L. sake*, packaged in air; (2) lingüiça with and without *L. sake*, packaged in 50%CO_2/50%N_2; (3) lingüiça with and without *L. sake*, packaged in 100%CO_2; (4) lingüiça without *L. sake* packaged in air and in 50%CO_2/50%N_2; (5) lingüiça without *L. sake* packaged in air and in 100%CO_2; (6) lingüiça without *L. sake* packaged in air and with *L. sake* in 50%CO_2/50%N_2; (7) lingüiça without *L. sake* packaged in air and with *L. sake* in 100%CO_2. Before testing, the samples were boiled in water during 10 min, deep-fried in hot oil for 6–8 min, let drain on absorbing paper and cut into 2–3 mm wide slices.

Each comparison was performed with three coded samples (two identical and one odd). Sixteen panelists, selected among the laboratory staff, were asked to taste the samples and select the odd. The number of correct answers was counted and compared to the reference Table T8 (Critical Number of Correct Responses in a Triangle Test) in Meilgaard et al. (1999), which contains the minimum number of correct responses required for significance at a stated α level for the corresponding number of panelists. In this study, the level of significance (α) was 0.05. The assumption of "no difference" was rejected when the number of correct responses was greater than or equal to 9 (tabled value).

2.9. Statistical tests

Four replications of each experiment were performed. The best combination of gas composition and bacteriocin activity for each time interval was determined by multiple comparisons using Tukey's test. The SAS 6.12 software (SAS Institute Inc., Cary, NC, USA Copyright @ 1989–1996, Release 6.12 TS level 0020) was used for these determinations.

3. Results and discussion

Variations in concentration of gases in the packages along the storage period (29 days) of the lingüiça samples were insignificant (data not shown), indicating that the mechanical barriers for gas exchange were effective during the study.

The initial pH of lingüiça was 6.1. The pH of samples containing the *L. sake* 2a strain dropped to 5.7 after 14 days under refrigeration and to 5.4 by the end of the sampling time. This drop in pH was considered insufficient to explain the inhibitory effect on the growth of *L. monocytogenes* detected in the products containing the *L. sake* strain. According to Jay (1999) *Listeria monocytogenes* is able to grow well at pH as low as 4.1. Previous papers have shown that the inhibition of *Listeria* spp. in meat products containing bacteriocin-producing lactic acid bacteria was greater than in products containing non bacteriocinogenic strains, for the same range of pH, indicating that the inhibition is more likely to be due to the bacteriocin than to the presence of lactic acid (Campos, Mazzota, & Montville, 1997; Hugas et al., 1998)

No microbial growth was observed in the negative controls.

As shown in Fig. 1, the MA packaging did not influence the growth of *L. sake* 2a in refrigerated lingüiça. From a 10^5 CFU/g initial level of contamination, the number of viable cells increased to 10^8 CFU/g after 15

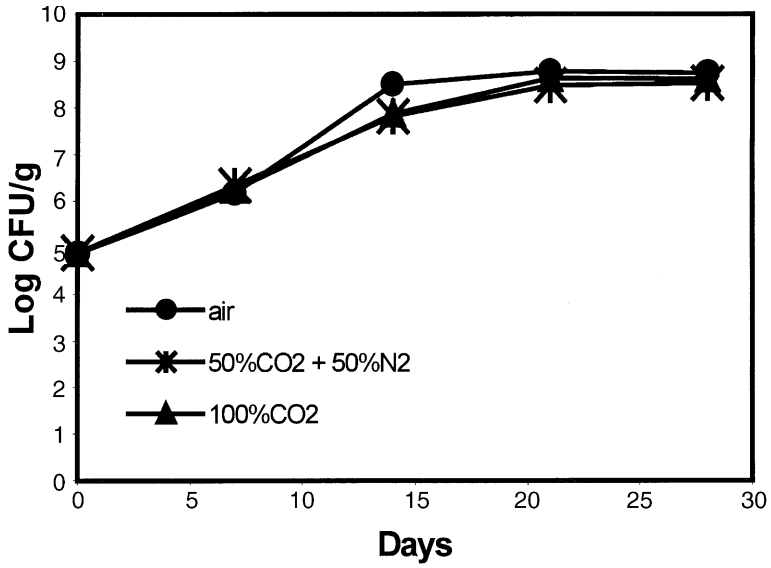

Fig. 1. Growth of *Lactobacillus sake* 2a in lingüiça packaged with air, 50% CO_2/50% N_2 or 100% CO_2.

days and did not change significantly afterwards, regardless the type of MA packaging.

In contrast, the type of MA packaging affected the growth of *L. monocytogenes* in lingüiça (Fig. 2). As indicated in Table 1, in samples containing only *L. monocytogenes* and packaged in air, the counts increased 1.9 log in 7 days, 3.5 log in 14 days, 4.7 log in 21 days and 5.8 log in 28 days. In similar samples but packaged in a mixture of 50% CO_2/50% N_2, the increases were lower: 0.5 log in 7 days, 0.9 log in 14 days, 1.5 log in 21 days and 2.0 log in 28 days. The effect of 100% CO_2 was even more intense. The growth of *L. monocytogenes* was completely inhibited by this atmosphere: the number of viable cells remained practically constant up to the end of the sampling time.

The effect of *L. sake* 2a in the inhibition of growth of *L. monocytogenes* in refrigerated lingüiça according to the type of packaging is shown in Table 1 and Fig. 3. In samples containing *L. sake* 2a, without MA packaging, the counts of *L. monocytogenes* increased 1.4 log after 7 days, 2.1 log after 14 days, and then remained constant up to 28 days. In these samples, the counts of *L. monocytogenes* after 7 days were 0.4 log lower than in samples without *L. sake* 2a. The differences were 1.3 log for 14 days, 2.6 log for 21 days and 3.7 log for 28 days. In samples packaged with the 50% CO_2/50% N_2 mixture, these differences were 0.1, 0.9, 1.9 and 2.6 log for 7, 14, 21 and 28 days, respectively. In samples packaged with the 100% CO_2, the differences up to 14 days remained constant, but after 21 and 28 days, the viable counts were lower than the initial counts (reduction of 0.7 log), suggesting a bactericidal effect.

The statistical evaluation of these results indicated that counts of viable *L. monocytogenes* in samples containing bacteriocinogenic *L. sake* 2a and packaged under 50% CO_2/50% N_2 and 100% CO_2 differed significantly from those in samples containing *L. sake* 2a but packaged with oxygen-permeable film (Table 1).

Table 1
Counts of *Listeria monocytogenes* (log CFU/g) in lingüiça with or without *Lactobacillus sake* 2a packaged in air, 50%CO_2/50%N_2 or 100% CO_2, during storage at 6 °C

Type of packaging	Microorganism	Days[a]				
		0	7	14	21	28
Air	*L. monocytogenes* only	3.5 a A	5.4 a B	7.0 a C	8.2 a D	9.3 a E
	L. monocytogenes + *L. sake* 2a	3.6 a A	5.0 a B	5.7 b B	5.6 b B	5.6 b B
50% CO_2 + 50% N_2	*L. monocytogenes* only	3.5 a A	4.0 b AB	4.4 c BC	5.0 b CD	5.5 b D
	L. monocytogenes + *L. sake* 2a	3.6 a AB	3.9 b A	3.5 d AB	3.1 c B	2.9 c B
100% CO_2	*L. monocytogenes* only	3.7 a A	3.9 b A	3.7 d A	3.7 d A	3.6 d A
	L. monocytogenes + *L. sake* 2a	3.7 a A	3.8 b A	3.5 d AB	3.0 c B	2.9 c B

[a] For each column, means followed by the same lowercase letter are not significantly different ($P \leqslant 0.05$). For each line, means followed by the same uppercase letter are not significantly different ($P \leqslant 0.05$).

Table 2
Sensory evaluation of lingüiças with or without *Lactobacillus sake* 2a, packaged in air, 50% CO_2/50% N_2 or 100% CO_2

Treatments			Number of panelists that correctly identified the odd sample[a]	Conclusion[b] ($\alpha \leqslant 0.05$)
Sample in air	X	Sample in air with *L. sake* 2a	6	Non different
Sample in 50% CO_2 + 50% N_2	X	Sample with *L. sake* 2a in 50% CO_2 + 50% N_2	6	Non different
Sample in 100% CO_2	X	Sample with *L. sake* 2a in 100% CO_2	5	Non different
Sample in air	X	Sample in 50% CO_2 + 50% N_2	9	Different
Sample in air	X	Sample in 100% CO_2	11	Different
Sample in air	X	Sample with *L. sake* 2a in 50% CO_2 + 50% N_2	9	Different
Sample in air	X	Sample with *L. sake* 2a in 100% CO_2	9	Different

[a] Total of panelists: 16.
[b] According to table T8 in Meilgaard et al. (1999).

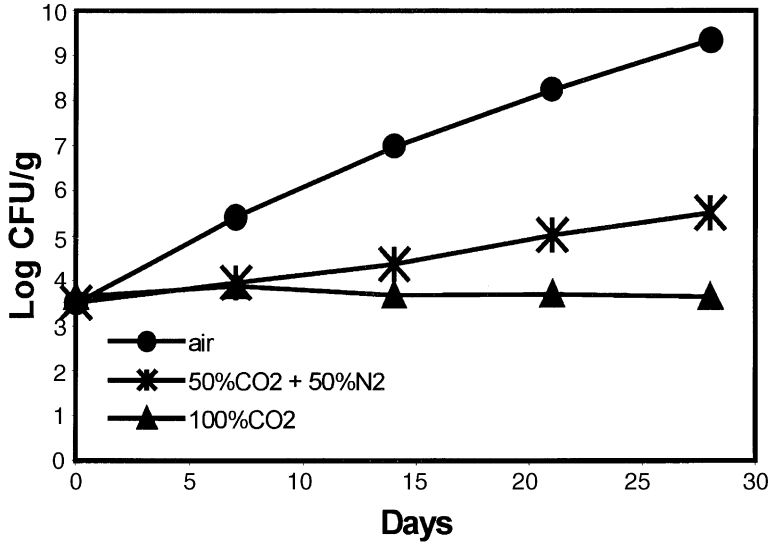

Fig. 2. Growth of *Listeria monocytogenes* F5069 in lingüiça packaged with air, 50% CO_2/50% N_2 or 100% CO_2.

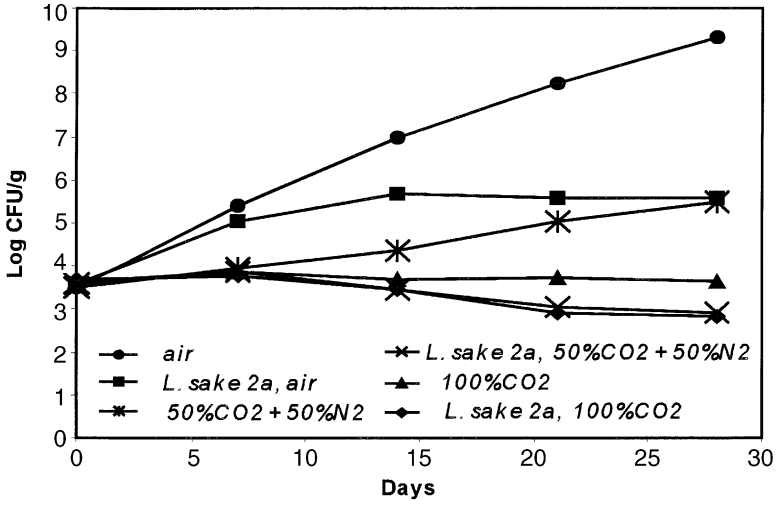

Fig. 3. Combined effect of *Lactobacillus sake* 2a and packaging in air, 50% CO_2/50% N_2 or 100% CO_2 on the growth of *Listeria monocytogenes* F5069 in lingüiça.

Results are similar to those reported previously by De Martinis and Franco (1998), for the same product packaged with oxygen-permeable film and for the same microorganisms. Hugas et al. (1998), working with bacteriocins and MA packaging, demonstrated that inhibition of *Listeria* spp. in fresh and cooked meat products could not be achieved by the sole application of vacuum or a mixture of 20% CO_2/80% O_2. However, the addition of sakacin resulted in immediate destruction of *Listeria* spp. in the products packaged in MA. Similar data were generated by Schöbitz, Zaror, Leon, and Costa (1999) who concluded that the inhibitory substance produced by *Carnobacterium piscicola* and vacuum packaging were able to completely inhibit the growth of *L. monocytogenes* in meat.

Szabo and Cahill (1998) studied the combined effect of modified atmosphere (100%N_2, 40% CO_2:60% N_2 and 100% CO_2), temperature and nisin on the growth of *L. monocytogenes* in buffered tryptone soya broth at 4 and 12 °C. Of the treatments evaluated, 100% CO_2 exerted the greatest inhibition. Nilsson et al. (2000) reported a 4 log reduction of *L. monocytogenes* counts in broth with nisin maintained in 100% CO_2 atmosphere. These authors concluded that CO_2 modifies the fatty acid composition of the cellular membrane of *L. monocytogenes*, improving the fluidity and playing an important role in the efficiency of nisin action. In both studies, the efficiency of the method in food products was not investigated, but the synergistic effect was clearly demonstrated.

The results of sensory evaluation of lingüiças prepared with *L. sake* 2a and packaged under air, 50% CO_2 + 50% N_2 and 100% CO_2 are shown in Table 2. After 11 days under refrigeration, the panelists did not detect the presence of *L. sake* in the product, regardless of the packaging atmosphere. However, the taste of samples packaged in 50% CO_2/50% N_2 differed significantly from those packaged in air ($P \leqslant 0.05$). The same occurred with samples packaged under 100% CO_2. These differences in taste could be detected as early as 5 days under refrigeration.

The results of our study indicate that modified atmosphere and the bacteriocin produced by *L. sake* 2a have a synergistic effect on the inhibition of growth of *L. monocytogenes* in lingüiça. This synergism, attributable to the increased sensitivity of the pathogen to the bacteriocin in the presence of CO_2, may also be caused by a possible enhanced bacteriocin production by *L. sake* 2a under modified atmosphere. However, further studies are needed to elucidate this hypothesis. In addition, the practical applications of the conclusions drawn from the study, such us the adequacy and cost of use of 100% CO_2 for packaging and interference of the indigenous flora on the shelf life of the product, still need to be evaluated.

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