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INFLUENCE OF SALT LEVEL, STARTER CULTURE, FERMENTABLE CARBOHYDRATES, AND TEMPERATURE ON THE BEHAVIOUR OF *L. MONOCYTOGENES* IN SLICED CHOURIÇO DURING STORAGE

J. García-Díez* and L. Patarata

CECAV, Centro de Ciência Animal e Veterinária, Universidade de Trás-os-Montes e Alto Douro, Quinta de Prados, 5000-801, Vila Real. Portugal

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Sliced ready-to-eat traditional meat products presented in individual packaging with more convenience to the consumer is the way that food industry tries to adapt to the new consumer tendencies.

The current work assessed the survival of *Listeria monocytogenes* in a contaminated sliced vacuum packed chouriço with different sugar concentrations, two salt levels, and presence/absence of *Lactobacillus sakei* as starter culture along the storage period at two temperatures (8 ºC and 22 ºC). Neither the inoculation with *L. sakei*, nor the addition of carbohydrates influenced the survival of the pathogen. Higher amount of salt resulted in a significant reduction of *L. monocytogenes,* and storage at the higher temperature displayed a safer product. After 7 days, *L. monocytogenes* was not detected in any samples. The study of the association of the factors contributing for *L. monocytogenes* survival by logistic regression showed that increasing the level of salt addition from 1.5% to 3% reduces the odds of survival of *L. monocytogenes* to about one fifth (0.174). Worthy of attention is the significant reduction in the odds (OR=0.028) of finding viable *L. monocytogenes* when the samples are stored at room temperature (22 ºC), when compared to low temperature (8 ºC).

The general and indiscriminate use of refrigeration for meat products might increase the risk of presence of *L. monocytogenes*. The competitive advantage that *L. monocytogenes* has at low temperatures, as the potential inhibition of LAB activity, is probably the reason of the observed behaviour. The amount of salt was an important hurdle to control *L. monocytogenes* growth, so, manufacturing meat products with lower salt contents to meet the demands of healthy products might represent a risk for safety, since high levels of salt together with low a_w are the keys for their conservation.

Keywords: chouriço, sliced, vacuum-packaging, *L. monocytogenes, L. sakei*, storage

Traditional Portuguese meat products are characterized by a wide number of products of considerable gastronomic, social, and economical importance, the chouriço being the most representative of these meat products (Melo et al., 1991). These kinds of products have a well-founded reputation as safe products. The changes in their composition, mainly in their pH and water activity (a_w), and the presence of salt, nitrites, spices, and other ingredients, are the hurdles that hinder the growth of food-borne pathogens (Ordóñez & de la Hoz, 2007).

Nowadays, consumers demand more ready-to-eat products without added chemical preservatives, low salt levels, or attractive packaging presentations. In consequence, these social changes have conditioned the strategies of meat industry. Thus, the efforts to adapt them, the development of sliced ready-to-eat traditional meat products presented in individual packaging with more convenience to the consumer is a way to adapt to the new consume tendencies. These kinds of food commodities are often presented in vacuum or modified atmosphere packaging and stored at refrigeration temperature, and represent a positive

* To whom correspondence should be addressed.
Phone: +351259350000; fax: +351259350480; e-mail: juangarciadiez@gmail.com

economic impact to the meat industry. However, their food safety risks are higher than the traditional products due to the slicing process.

Listeria monocytogenes is a well-known food-borne pathogen of meat industry due to its ubiquity and survival capacity under adverse conditions (Gómez et al., 2014), and responsible for listeriosis disease. In one hand, individuals with a good health status are usually not affected, however, even they can develop listeriosis or listerial gastroenteritis if a large number of *L. monocytogenes* cells are consumed. On the other hand, listeriosis in risk population (pregnant women, new-born infants, children or inmunocompromised individuals) has a severe impact, and it is responsible for abortion, meningitis, or septicemic syndrome (Tham & Danielsson-Tham, 2013).

L. monocytogenes can grow at low temperatures, survives at low a_w and pH values, and persists for long periods of time in food processing environments due to their biofilm forming ability and resistance to sanitizers (Gandhi & Chikindas, 2007). Since *L monocytogenes* is ubiquitous in nature and ready-to-eat products are exposed to the environment during slicing process, *L. monocytogenes* can easily adhere to the products (Moore, 2004; Pérez-Rodríguez et al., 2010). In consequence, the effect of the several hurdles naturally present in this kind of products (i.e. pH, a_w), the correct implementation of an HACCP system, and pre-requisite programs are the most valuable tools available to ensure the safety of these products (Wallace & Williams, 2001).

Safety issues regarding *L. monocytogenes* in traditional dry-cured meat products during manufacturing have been previously reported (Encinas et al., 1999; Díez & Patarata, 2013), although research on its control in sliced dry-cured meat products is scarce. Thus, the objective of this work was the study of the effects of salt level, *L. sakei* starter culture, fermentable carbohydrates, and storage temperature in sliced vacuum-packaged chouriço on the behaviour of *L. monocytogenes.*

1. Materials and methods

1.1. Microorganisms and growth conditions

Four indigenous strains of *L. monocytogenes* (Table 1) were previously isolated from chouriço or its production environment, and identified by a species-specific PCR technique (Simon et al., 1996; Talon et al., 2007).

Table 1. Strains of *L. monocytogenes* used in the experiment

Strain	Source
NCTC 7973	National Collection of Type Cultures, Salisbury, UK).
EDS-B-LM02	Chouriço batter
JI-Me-LM03	Meat used in meat products preparation
EDS-E-LM02	Environment of meat products preparation
MPI-E-LM01	Environment of meat products preparation

Strains isolated from meat products or environment of its production are from UTAD/LP laboratory collection

The experiments were conducted with a cocktail composed of the four indigenous strains and one reference strain. The pathogen was grown on brain heart infusion agar (BHI – Biokar, Beauvais, France) and incubated for 24 h at 30 ºC.

To screen the inhibition activity against *L. monocytogenes,* a total of 13 strains of lactic acid bacteria, belonging to the species *L. sakei,* were used in this study. These strains were maintained at –18 °C. Strains were subcultured twice in Man-Rogosa-Sharpe medium (MRS, Biokar) before use, and were incubated for 18 to 24 hours at 30 ºC. Cultures for inoculation were grown individually overnight, harvested by centrifugation, washed twice, and resuspended in 0.85% NaCl to obtain an optical density (OD) of 600 nm of ca. 0.5.

1.2. Antagonistic activity against foodborne pathogens

The agar spot test method (SCHILLINGER & LUCKE, 1989) was used to determine the antagonism of the LAB activity against the pathogens. Thirteen strains of *L. sakei* (Table 2) were tested individually against a cocktail of five strains of *L. monocytogenes*.

Table 2. Antagonistic effect of different strains of indigenous *Lactobacillus sakei* against *Listeria monocytogenes**

L. sakei strain	*L. monocytogenes*
Ls 1320	++
Ls 1322	–
Ls 1336	–
Ls 1343	–
Ls 1345	+
Ls 1358	+
Ls 1376	++
Ls 1377	(+)
Ls 1378	–
Ls 1382	–
Ls 1394	–
Ls 1305	(+)
ATCC 15521	–

–: no inhibition zone; (+): inhibition zone under 5 mm; +: inhibition zone over 5 mm; ++: inhibition zone over 10 mm; *: mixture of *L. monocytogenes* strains (NCTC 7973, EDS–B–LM02, JI–Me–LM03, EDS–E–LM02, MPI–E–LM01)

A cell suspension in 0.85% NaCl of each *L. sakei* strain was dotted (10 µl) onto the surface of MRS agar with only 0.2% glucose (MRS 0.2) and was anaerobically incubated for 24 h at 30 ºC. For *L. monocytogenes*, 100 µl of the cell suspension cocktail to be tested for sensitivity (approximately 8 log CFU ml^{-1}) was inoculated in 7 ml of melted brain heart infusion broth supplemented with 0.7% agar (brain heart infusion soft agar) and poured over

the plate on which the producer was grown. After anaerobic incubation for 24 h at 30 ºC, the plates were checked for inhibition zones. The results were recorded as follows: (–), no inhibition zone; (+), inhibition zone under 5 mm; +, inhibition zone over 5 mm; and ++, inhibition zone over 10 mm. The pH of the supernatant of the 24-h growth of *L. sakei* strains in MRS broth with only 0.2% glucose was recorded. As the pathogens were used in a cocktail of five strains (four indigenous plus one reference strain), the potential cross-inhibition between the strains was previously screened using the agar spot test method described above by replacing MRS 0.2 with brain heart infusion agar for all the possible combinations of the strains of pathogens tested.

1.3. Manufacturing of chouriço

To test the influence of the (i) presence or absence of the indigenous *L. sakei* starter culture, (ii) presence or absence of 0.75% dextrose plus 0.75% lactose, and (iii) the level of salt (1.5 or 3%), four replicates were conducted for each condition. To screen the effects more efficiently, the chouriço de vinho (hereafter referred as chouriço) was prepared using meat with very low initial contamination, which was obtained by immersing the pork belly in ethanol, burning the surface, and aseptically handling the inner part. All eight batches (3 kg each) were manufactured with ground pork belly (15-mm grinder plate; Mainca, Barcelona, Spain), 5% regional red wine (11% ethanol, pH 3.8), 125 mg kg^{-1} $NaNO_2$, 125 mg kg^{-1} KNO_3, and the variable ingredients of the batch. The *L. sakei* starter culture, selected from the 13 strains tested for antagonistic activity, was suspended in 10 ml of 0.85% NaCl to achieve an initial concentration of 6 log CFU g^{-1}. The same amount of 0.85% NaCl was added to the samples without the starter culture. After mixing (10 min; Mixer RM-35, Mainca), the batter was rested for 24 h at 4 ºC before stuffing. The chouriços de vinho were then stuffed into natural thin pork gut, tied in a horseshoe shape, and suspended to drain the excess marinade for 4 h in a drying chamber at 4 ºC with 85% relative humidity. They were then smoked for 3 h in a smoking chamber (Thermaxs 100EC, Begarat, Berlin, Germany) with smoke generated from beechwood scraps. The initial temperature of the smoke chamber was 18 ºC. From the start of the process, the temperature inside the chouriços de vinho was monitored at 10-min intervals and never exceeded 35 ºC. The chouriços de vinho were then dried at 15 ºC in an 85% relative humidity environment (Aralab Fitoclima, Rio de Mouro, Portugal) for 30 days.

1.4. Chouriço contamination

Samples of each finished chouriço batch were aseptically sliced and contaminated with a suspension of *L. monocytogenes* (mixture of ATCC 7973 and 4 strains) in 0.9% NaCl to obtain 10 to 100 CFU g^{-1} with 0.1 ml dropped onto the surface. Then, slices of chouriço were vacuum packaged and stored at 4 ºC and 22 ºC. Microbial analyses were made after 24 h, 7, 15, and 30 days. The detection was carried out as described in ISO 11290-1 (1996) and confirmed by a species-specific PCR technique.

1.5. Chemical analysis

The chemical composition was determined according to ISO recommended procedures: moisture (ISO, 1997), total fat (ISO, 1973), protein (Kjeldahl N×6.25) (ISO, 1978), and NaCl (ISO, 2011). Water activity was measured in a Hygroscope DT apparatus with a WA40 cell maintained at 20±2 ºC. The pH was measured directly in the chouriço itself using a pH meter (model MicropH 2002, Crison).

1.6. Statistical analysis

The influence of temperature, salt content, presence/absence of *L. sakei,* and presence/absence of carbohydrates in the presence/absence of *L. monocytogenes* was assessed by chi-square test and by binary logistic regression.

2. Results and discussion

The dry-cured Portuguese chouriço prepared in the present study presented an average crude chemical composition of 34.18±1.51% moisture, 24.50±1.72% protein, 37.36±1.72% fat, and 2.46±0.08% salt.

The results of presence/absence of *L. monocytogenes* in chouriço influenced by temperature, salt content, presence/absence of *L. sakei,* and presence/absence of carbohydrates are presented in Table 3. Neither the inoculation with *L. sakei*, nor the addition of carbohydrates influenced the survival of *L. monocytogenes*. On the contrary, higher amount of salt resulted in a significant reduction of the survival of the pathogen and the higher storage temperature resulted in a safer product. After 7 days, *L. monocytogenes* was not detected in any of the samples.

Table 3. Presence of *Listeria monocytogenes** under the effect of study (results expressed %)

	*L. monocytogenes**		Chi-square test (P)
	Absence	Presence	
Temperature (ºC)			
8 ºC	69	31	11.852; 0.001
22 ºC	100	0	
Salt content (%)			
1.5%	50	50	5.236; 0.022
3%	88	12	
L. sakei			
Presence	63	37	0.582; ns
Absence	75	25	
Carbohydrates			
Presence	81	19	2.327; 0.127
Absence	56	44	

Ns: not significant; *: mixture of *L. monocytogenes* strains (NCTC 7973, EDS-B-LM02, JI-Me-LM03, EDS-E-LM02, MPI-E-LM01)

To study the association of the factors contributing to *L. monocytogenes* survival, a binary logistic regression was performed, using the occurrence of the pathogen as the predicted variable and the storage temperature and salt level as predictors. It was observed that increasing the level of salt addition in the formulation of chouriço from 1.5% to 3% reduces the odds (Table 4) of survival of *L. monocytogenes* to about the one fifth (0.174). Worthy of attention is the significant reduction in the odds (OR=0.028) of finding viable

L. monocytogenes when the samples are stored at room temperature (22 ºC), when compared to low temperature (8 ºC, reference category). Similar resistance behaviour was reported by Byelashov and co-workers (2009) in vacuum packaged pepperoni.

Table 4. Results of the logistic regression model for the presence of *L. monocytogenes* in sliced chouriço considering the scores PC 1 (temperature 8 ºC) and PC3 (salt content 1.5%) as predictors

Variable	Beta	SE	*p*	Odds ratio	95% CI
Temperature (ºC)	–3.59	1.500	0.017	0.028	0.001–0.520
Salt content (%)	–1.74	0.845	0.039	0.174	0.033–0.912
Constant	–0.005	0.496	0.015		

SE: standard error; CI: confidence interval

Presence of *Listeria monocytogenes* in ready-to-eat meat products had been described as a potential risk for consumers (Gormley et al., 2010; Kramarenko et al., 2016).

Residual activity presented in the product against *L. monocytogenes* was most efficient during the first 24 hours in chouriço with high salt contents (3%). However, for the first 7 days of storage, the temperature played an important role in its conservation, since reductions of *L. monocytogenes* counts were higher in chouriço stored at 22 ºC. An important reduction of *L. monocytogenes* in the 7 first days of storage was also reported by Menéndez and co-workers (2015) in Spanish chorizo vacuum packaged and stored at 20 ºC.

The absence of *L. monocytogenes* counts in samples analysed at 15 and 30 days of storage at both temperatures (8 ºC and 22 ºC) indicates that the resistance mechanisms of *L. monocytogenes* are not enough to survive in unfavourable environment (Gandhi & Chikindas, 2007). Several author reported reductions of *L. monocytogenes* counts in dry-cured sausages during the storage period, being greater at lower levels of pH and a_w at the same temperature (Lindqvist & Lindblad, 2009; Menéndez et al., 2015).

Moreover, Porto-Fett and co-workers (2008) verified that reduction of *L. monocytogenes* counts were greater in sliced, contaminated meat sausages than batter contamination during the manufacturing process. In contrast, Mena and co-workers (2004) and Vitas and co-workers (2004) reported the presence of *L. monocytogenes* in sliced dry-cured meat products (including chouriço).

Survival of *L. monocytogenes* during manufacturing of dry-cured chouriço has also been published in the literature. Díez and Patarata (2013) reported its absence in the finished product. However, the isolation of *Listeria* spp. by Encinas and co-workers (1999) in similar chouriço indicates that resistance of *L. monocytogenes* during the drying process (and presence in the finished product) or contamination during the slicing process could compromise the safety of this product if storage conditions are not adequate. This seems to be affirmed by the isolation of *L. monocytogenes* of sliced chouriço at retail level (Mena et al., 2004; Vitas et al., 2004).

Lactobacillus sakei has been reported as a protective culture with antilisterial activity (Barbosa et al., 2014). In our study, *L. sake* played no significant role in the reduction of *L. monocytogenes* counts, however, its presence in combination with the hurdles of vacuum-packaging and time-temperature could cause competition for micronutrients utilization that might affect *L. monocytogenes* growth (Kröckel, 2013).

Commission Regulation (EC) 1441/2007 regarding microbiological criteria for foodstuffs determines that ready-to-eat foods able to support the growth of *L. monocytogenes* cannot present counts over 100 CFU g^{-1} after placed on the market during their shelf-life. Our results may be interesting to meat processors, since the application of appropriate salt levels and/or temperature storage may accomplish the legal requirements and guarantee food safety.

3. Conclusions

The general and indiscriminating usage of refrigeration for meat products, even when it is not necessary, might increase the risk of presence of *L. monocytogenes*. The competitive advantage that *L. monocytogenes* has at low temperatures, while LAB activity is suppressed, is probably the reason to the observed behaviour. The amount of salt in the product was revealed to be an important hurdle to control *L. monocytogenes* growth, however, consumers demand healthy product with low salt content leading producers to use lower amounts of it. However, we are still unsure of the risk that this reduction might represent for the safety of these products, as high levels of salt and consequently low a_w are the main hurdles for their conservation.

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