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Comparison of Antilisterial Effects of Two Strains of Lactic Acid Bacteria during Processing and Storage of a Portuguese Salami-like Product "Alheira"

Manuela Vaz-Velho*[a], Samuel Jácome[a], Lúcia Noronha[b], Svetoslav Todorov[c], Susana Fonseca[a], Rita Pinheiro[a], Alcina Morais[b], Joana Silva[b] ,Paula Teixeira[b]

[a] Escola Superior de Tecnologia e Gestão – Instituto Politécnico de Viana do Castelo, Avenida do Atlântico s/n, 4900-348 Viana do Castelo, Portugal
[b] CBQF – Centro de Biotecnologia e Química Fina, Escola Superior de Biotecnologia, Centro Regional do Porto da Universidade Católica Portuguesa, Rua Dr. António Bernardino Almeida, 4200-072 Porto, Portugal
[c] Departamento de Alimentos da Faculdade de Ciências Farmacêuticas – Universidade de São Paulo, Av. Prof. Lineu Prestes, 580, Bloco 14, 05508-900 São Paulo, Brasil
mvazvelho@estg.ipvc.pt

Traditional smoked and/or cured salami-like products are much appreciated by Southwest European consumers. However modern consumers require products that not only have high appealing sensory attributes but are also safe. In this work the effect of the application of two bacteriocinogenic lactic acid bacteria (LAB) *Lb. plantarum* ST202 and *Lb. sakei* ST153 for controlling *Listeria monocytogenes* growth in "Alheira", a traditional salami-like product, was investigated. The paste of ''Alheira',' produced by an industrial meat company, was sterilized by autoclaving before inoculation and *L. monocytogenes* growth was evaluated during 15 days of storage.
Both LAB strains showed antilisterial activity in the inoculated paste, but as in preliminary experiments with sterilized minced pork meat only strain ST153 presented activity against *L. monocytogenes*. it was decided, for further industrial tests, to use only this strain. Therefore, the effect of *Lb. sakei* ST153 against *L. monocytogenes* in industrially processed "Alheira", packed under vacuum or under modified atmosphere (MA) (20 % CO_2 and 80 % N_2), was evaluated during 7 days storage at 4 ºC. The effect of the inoculation of the bacteriocinogenic culture in the organoleptic properties of "Alheira" was also investigated. A quantitative descriptive sensory test was performed by a semi-trained panel after 5 days storage.
No significant differences were observed in the microbiological and sensory quality of "Alheira" packed under vacuum or MA. A significant reduction in the level of *L. monocytogenes* was observed in the presence of *Lb. sakei* ST153 (2 log cycles reduction during the first 7 days). Results showed that panellists did not find significant differences between "Alheira" with *Lb. sakei* ST153 addition and commercial control, except for mass connection, acid taste and atypical taste. Despite that, both samples were scored above conformity limit. Thus, the application of LAB is a very promising mean of preventing *L. monocytogenes* growth in "Alheira".

1. Introduction

"Alheira" is a tradicional fermented smoked sausage. Its origin dates back to the late fifteenth century and is associated with the presence of Jewish communities in the region of Tras-os-Montes in northern Portugal. It is a product consisting of a mixture of beef, chicken, pork, bread and condiments. It shows a light brown color and a cylindrical shape recalling a horseshoe with about 20 to 25 cm length.
The actual consumers interest on traditional food products as well as on natural ones, with no synthetic chemicals addition, are recognized as key market trends that play an important role in new product development.

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The lactic acid bacteria (LAB) are part of the typical microbial flora of cured and smoked products, either by natural presence or by addition as starter cultures. This approach offers an indirect way to add lactic acid bacteria and bacteriocins, to a meat product and seems more acceptable for producers and consumers in foods like fermented sausages and vacuum-packed, meat with a relatively short shelf life, where high initial inoculum numbers (10^6–10^7 CFU/g) (Aymeric *et al*., 2006). Furthermore, in fresh and fermented meat, the applied bacteria must be able to compete with the endogenous microflora. Lactic acid bacteria can inhibit the growth of *L. monocytogenes* by a variety of antimicrobial agents such as organic acids, bacteriocins, and the competition for nutrients in the product (Talon and Leroy, 2006).

Besides their role as a technological agent in fermented products, the LAB also impart a number of nutritional and sensory characteristics assessed by the consumer such as color, flavor, texture, digestability and nutritional quality. Added LAB (i) should be salt and nititrite tolerant (grow at 6 % NaCl and 100 ppm nitrite) (ii) must grow at a temperature range of 27 ºC to 43 ºC (iii) must not produce off-odors and (iv) must be innocuous to human health (Smith and Palumbo, 1983). Preservation by the addition of LAB results from a decreased pH resulting from the formation of lactic acid which itself may be sufficient to antagonize many organisms, including *L. monocytogenes*. Acetic and propionic acids act in a similar manner to lactic acid. These organic acids play an important role in certain fermented foods, and it is known that acetic acid has a synergistic antimicrobial effect in the presence of lactic acid (Papagianni, 2012).

In last decade, a large variety of bacteriocins (LAB peptides) have already been identified and characterized, which allowed a considerable research progress in biocontrol. Several studies focused on antimicrobial ability of different bacteriocins isolated or in mixtures (Todorov and Vaz-Velho, 2008; Todorov *et al*., 2010).

Nisin is the only antimicrobial agent allowed to be introduced directly in food products. United States Food and Drug Administration recognized nisin in 1988 with a GRAS label and European Union in 1995 authorized the use of nisin (E234) in food products by directive 95/2/EC.

New strategies that take in consideration the quality and sensory characteristics of Portuguese traditional smoked and cured pork products, maintaining their safety and enlarging their shelf life are under investigation (Albano *et al*., 2009). Biopreservation or biocontrol, through introduction of natural substances, is an interesting technology that creates adverse conditions *in situ* to pathogenic development and consequently assures microbiological safety, and simultaneously allows chemical additives reduction, maintaining nutritional and sensorial product characteristics. In order to evaluate the influence of antimicrobial agents on the microbiological safety and stability of traditional smoked and cured meat products, it was investigated the effect of the application of two bacteriocinogenic LAB *Lb. plantarum* ST202 and *Lb. sakei* ST153, for controlling *L. monocytogenes* growth in “Alheira”.

Lb. sakei ST153, isolated from Salpicão, a traditional salami-like portuguese product, produces a bacteriocin ST153 that has a narrow spectrum of activity, is heat resistant and stable between pH 2.0 and 10.0, not adsorbing to the surface of the producer cell and are produced at higher levels during the stationary phase of fermentation in a presence of 2/% (w/v) D-Glucose (Todorov et al., 2013). *Lb. plantarum* ST202, isolated from Beloura, a traditional sausage-like Portuguese product, produces a bacteriocin ST202 with a broad spectrum of activity, heat resistant and stable between pH 2.0 and 10.0, adsorbing to the surface of the producer cell and are produced at higher levels during the stationary phase of fermentation in a presence of 2/% (w/v) D-Glucose (Todorov et al., 2010). These behaviours suggest that these bacteriocins may be produced at high levels during all phases of fermented meat processing, therefore their producers can be used as starter cultures in the production of fermented meat products like ”Alheiras” and also acting as ‘natural’ inhibitors of undesirable microorganisms improving the safety of these products.

2. Materials and Methods

2.1 Assays performed in Paste of ‘‘Alheira’’

2.1.1 Microbiological analysis

Paste of ‘‘Alheira’’, before stuffing, was used in these experiments. This paste was produced by an industrial meat company and, on the day of its production, transferred to the laboratory at 4 ºC and sterilized by autoclaving before being inoculated. The antagonistic effect of each of two strains of LAB (*Lb. plantarum* ST202 and *Lb. sakei* ST153) on *L. monocytogenes* was studied. The organisms were sub-cultured twice (24 h at 30 ºC) in 10 mL MRS broth from Pronadisa (*Lb. plantarum* ST202 and *Lb. sakei* ST153) and in TSB broth from Lab M (*L. monocytogenes*), using a 1% v/v inoculum. An aliquot (250 mL) of each bacterial suspension (10^7 CFU/mL for LAB strains and 10^4 CFU/mL for the *L. monocytogenes*) was

added to 25 g of sterilized paste of ‘‘Alheira’’ contained in stomacher bags. After assuring good mixing of the inoculum with the paste (manually massaging of the exterior of the bags), the samples were stored at 4 ºC for 15 d. At days 0, 1, 3, 7, 10 and 15, inoculated paste samples were analysed for growth of the inoculated strains. The experimental conditions were: (1) uninoculated paste as control, (2) paste inoculated with *L. monocytogenes*, (3) paste inoculated with ST202, (4) paste inoculated with ST153. Each trial was performed in triplicate.

2.1.2 Microbiological sampling and analysis

A 1 g sample was weighed aseptically into a sterile tube with 9 mL of 1/4 -strength Ringer's solution and homogenized (by vortexing). Serial decimal dilutions in sterile ¼ -strength Ringer's solution were prepared and 20 µL samples of the appropriate dilutions were spotted, in duplicate,on selective agar plates. Counts were performed on MRS from Pronadisa incubated at 30 ºC for 72 h under microaerophilic conditions (LAB) and on ALOA Agar (Biorad) incubated at 30 ºC for 72 h (*L. monocytogenes*).

2.2 Assays performed in the Industrial Plant

2.2.1 Microbiological analysis

The inoculum of *Lb. sakei* ST153 was performed as described in section 2.2.1. Thereafter, the LAB was transported under refrigerated conditions to a industrial plant and subsequently inoculated in paste of "Alheira" before stuffing (500 mL of *Lb. sakei* ST153 in a concentration of 10^9 CFU/mL was added to 10 kg of paste of "Alheira"); mixed, stuffed and smoked accordingly in the industrial plant. "Alheiras" were then packed under vacuum or under MA and further transported to the laboratory under refrigerated conditions and divided in 3 different conditions: i) control samples (commercial "Alheiras" from the same batch); ii) alheiras with *Lb. sakei* ST153 and with the addition of *L. monocytogenes* (added in the laboratory; 1 mL of a 10^6 UFC/g suspension of *L. monocytogenes*) and iii) "Alheiras" with *Lb. sakei* ST153. "Alheiras" were stored at 4 ºC until further analysis. The microorganisms were enumerated as described in 2.2.2 immediately after the inoculation (t=0 days) and after 7 days of storage.

2.2.2 Sensory Analysis

In order to evaluate the effect of LAB addition on the sensory profile of "Alheira" packed under vacuum or under MA, a quantitative descriptive sensory test (QDA) was performed, involving previous sessions for main descriptors definition, their scale limits as well as verbal anchors by panel consensus. A final sheet with 16 descriptors, each one with a 13-point scale, and an overall quality index with a 5-point scale (1 and 2 - under conformity (with defect), 3 - limit of conformity, 4 and 5 - above conformity) was developed. Then, "Alheira" with *L. sakei* ST153 addition and a control (commercial sample with no LAB addition), packed under vacuum or under MA, were evaluated by a semi-trained panel after 5 days storage at 4 °C.

2.2.3 Statistical analysis

Panel data were converted in a percentage intensity scale. An analysis of variance (two-way ANOVA) was carried out to assess the effects of packaging conditions and LAB addition on panel results using STATISTICA 7 and Microsoft® Office Excel 2007 tools.

3. Results and Discussion

The enumeration of LAB and *L. monocytogenes* in mass of "Alheira" is illustrated in figures 1 and 2. It was observed in both figures that, in the presence of *Lb. sakei* ST153 and *Lb. plantarum* ST202 *L. monocytogenes* remained constant until the end of storage (Figure 1 and 2, respectively). In the absence of the bacteriocinogenic strains, *L. monocytogenes* was able to growth in the "Alheira" paste. No influence in LAB populations was observed when inoculated with or without *L. monocytogenes.*

Our results are in agreement with previous studies (Albano *et al.*, 2007) where the growth of *L. innocua* 2030c population was significantly suppressed in the paste of ‘‘Alheira’’ when the samples were co inoculated with LAB strains. In this study the studied LAB strain was *Pediococcus pentosaceus*. This *L. innocua* strain showed similar physiological properties to *L. monocytogenes and it* was considered to be a suitable marker to replace the pathogen in similar experiments using LAB strains (*Carnobacterium* sp.) and their bacteriocins. (Vaz-Velho *et al*., 2001). Previously, Alves *et al*. (2003) demonstrated that when *Lb. sakei* and *L. monocytogenes* cultures were inoculated in a model meat gravy system, *in situ* bacteriocin production played an important role in preventing growth of *L. monocytogenes*.

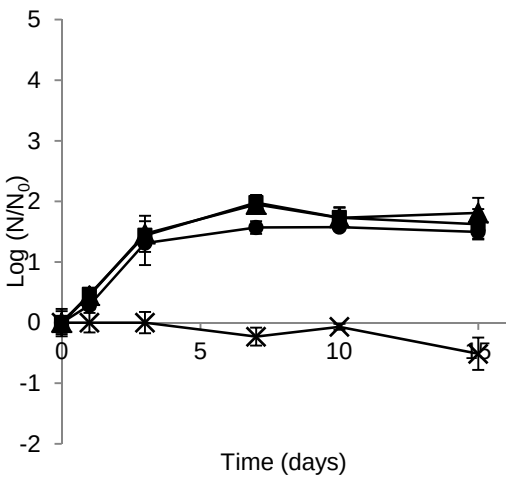


Figure 1: Enumeration of LAB: ■, paste of "Alheira" with Lb. sakei ST153; ▲, paste of "Alheira" with Lb. sakei ST153 and L. monocytogenes; Enumeration of pathogen: ●, paste of "Alheira" with L. monocytogenes; x past of "Alheira" with Lb. sakei ST153 and L. monocytogenes.

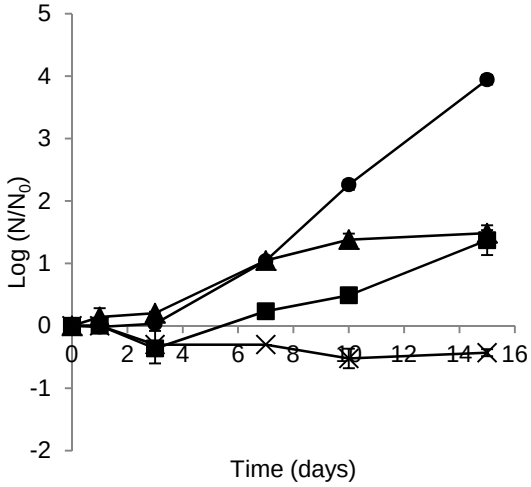


Figure 2: Enumeration of LAB: ■, paste of "Alheira" with Lb. plantarum *ST202; ▲, paste of "Alheira" with Lb. plantarum ST202 and L. monocytogenes; Enumeration of pathogen: ●, past of "Alheira" with L. monocytogenes; x paste of "Alheira" with Lb. plantarum ST202 and L.monocytogenes.*

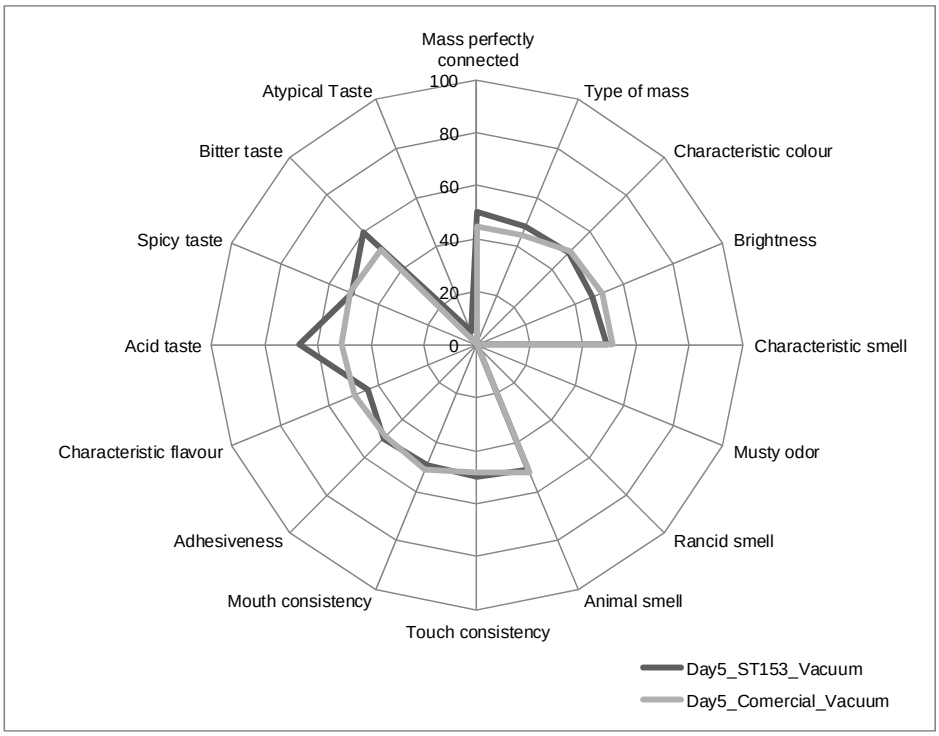
Both LAB strains showed antilisterial activity in the inoculated paste, but as in preliminary experiments with sterilized minced pork meat only strain ST153 presented activity against *L. monocytogenes*. It was decided, for further industrial tests, to use only this strain. Concerning the assay performed in the industrial plant the microbiological results are listed in Table 1. A decrease of 2 log in the number of *L. monocytogenes* was observed when *Lb. sakei* ST153 was present. As it was demonstrated in paste of "Alheira", the LAB strain was not affected by the presence of *L. monocytogenes*. No differences between samples packed under vacuum or MAP were observed (data not shown).

Table 1: Enumeration of Lb. sakei ST153 and L. monocytogenes immediately after the production (t=0 days) and after 7 days of storage at 4 ºC.

Sample	Bacteria Enumeration	Day 0 (log CFU/g)	Day 7 (log CFU/g)
Control	*L. monocytogenes*	6.5 ± 0.0	6.6 ± 0.1
Inoculated with *Lb. sakei* ST153 and *L. monocytogenes*	*Lb. sakei* ST153	9.5 ± 0.0	8.9 ± 0.1
Inoculated with *Lb. sakei* ST153 and *L. monocytogenes*	*L. monocytogenes*	6.1 ± 0.0	4.2 ± 0.1
Control	*Lb. sakei* ST153	9.0 ± 0.0	9.0 ± 0.05

The sensory profile of "Alheira" with and without *Lb. sakei* ST153 and packed under vacuum or MA are presented in Figure 3. No significant differences between vacuum and MA were found for all sensory descriptors (p-value > 0.05). Significant differences between "Alheira" with *Lb. sakei* ST153 and control were obtained only for "mass connection", "acid taste" and "atypical taste" descriptors. Higher intensity of mass connection, acid taste and atypical taste were observed for "Alheira" with *Lb. sakei* ST153 than control one. In fact these differences were perceived in the overall quality (1-5 scale) score of panelists, but were not sufficient to be considered under the limit score of conformity (3.8 and 4.6 for "Alheira" with *Lb. sakei* ST153 and control, respectively).

a)



b)

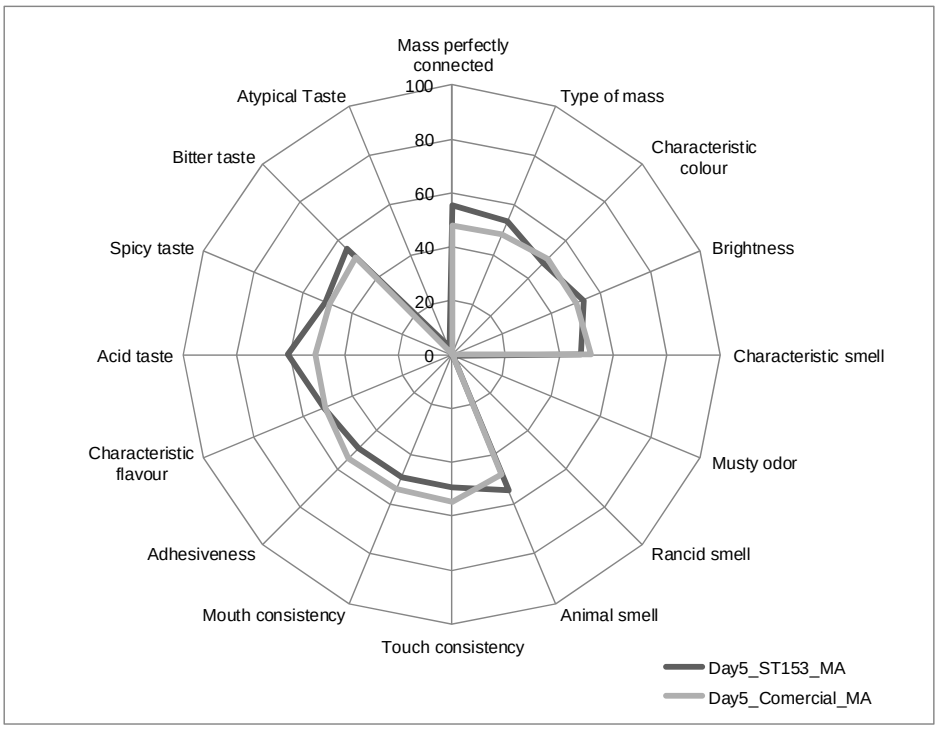


Figure 3: Sensory profile of "Alheira" after 5 days of storage at 4 °C in an intensity scale (%) of the main descriptors considered in a QDA for a) "Alheira" with Lb. sakei *ST153 and commercial "Alheira" both under vacuum packaging; b) "Alheira" with Lb.* sakei *ST153 and commercial "Alheira" both under MA packaging.*

4. Conclusions

No significant differences were observed in the microbiological and sensory quality of “Alheira” packed under vacuum or under MA. A significant reduction in the level of *L. monocytogenes* was observed in the presence of *Lb. sakei* ST153 (2 log cycles reduction during the first 7 days). Significant differences between “Alheira” with *Lb. sakei* ST153 and commercial control were obtained only for mass connection, acid taste and atypical taste but despite of that both samples were scored above conformity limit.
The results of this study showed the possibility of using *Lb. sakei* ST153 as a protective culture in the production of ”Alheira” being a promising methodology to improve the safety of these products with respect to *L. monocytogenes*.

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