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Research article

Evaluation of a bacteriocinogenic *Lactobacillus plantarum* strain on the microbiological characteristics of "Alheira de Vitela"

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Abstract: Lactic Acid Bacteria (LAB) and their bacteriocins can be successfully used as natural preservatives in meat products. This work aimed to investigate the effect of fresh and lyophilized starter cultures of an autochthonous bacteriocinogenic LAB strain (*Lactobacillus plantarum* ST153Ch: bac + culture) on the microbiological characteristics of "Alheira", a traditional Portuguese smoked product. "Alheira" with the addition of fresh or lyophilized culture (ca. 10^8 cfu/g) and "Alheira" control (no bacteriocinogenic culture added) were produced by an industrial meat company. The antilisterial activity of this culture in this food matrix was investigated, with some samples being inoculated with *Listeria monocytogenes* (ca. 10^5 cfu/g). Detection of *L. monocytogenes*, *Salmonella* spp., sulphite reducing clostridia, *Yersinia enterocolitica* and enumeration of *L. monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Enterobacteriaceae*, lactic acid bacteria, yeasts and moulds were performed immediately after production and at 3, 7, 15, 21, 28, 60 and 90 days of storage at 4 ℃, according to ISO methodologies. Also, a 16S rRNA Gene Analysis was performed of the microbial communities of "Alheira" with and without the lyophilized bacteriocinogenic culture. Pathogenic and indicator organisms were not detected or were below acceptable levels in all samples. LAB counts increased during storage and reached similar values after 15 days (ca. 10^{10} cfu/g) in all samples. There was a clear trend for a higher reduction of *L. monocytogenes* in the presence of the bioprotective culture, more pronounced during the initial 15 days of storage. From the analysis of the microbial communities of samples of "Alheiras" at different stages of fermentation, *Leuconostocaceae* and *Lactobacillaceae* predominated

in all the samples and *Lactobacillus* was the genus more prevalent in "Alheiras" after 60 days of storage with the addition of bacteriocinogenic culture.

Keywords: food safety; bioprotection; fermented meat sausages; microbiota; *L. monocytogenes*

Abbreviations: LAB: lactic acid bacteria; bac +: bacteriocinogenic; PGI: Protected Geographical Indication; S2BLAF: *Lb. plantarum* ST153Ch fresh; S2BLA: *Lb. plantarum* ST153Ch lyophilized; S2BC non-inoculated; S2BLAF + LM: *Lb. plantarum* ST153Ch fresh plus cocktail of *L. monocytogenes*; S2BLA + LM: *Lb. plantarum* ST153Ch lyophilized plus cocktail of *L. monocytogenes*; S2BC + LM: cocktail of *L. monocytogenes*

1. Introduction

"Alheira" is a traditional fermented smoked meat sausage, typical of the North of Portugal, which production dates back to the late fifteenth century. Nowadays, "Alheira" is a well-known Portuguese product with some varieties with Protected Geographical indication (PGI) status. "Alheiras" vary considerably in their final compositions and production processes. The relevant common elements in the production process are the boiling of various meats in lightly salted and spiced water; soaking the thinly sliced bread in some of the broth, formed during the boiling of the meats, until it is soft enough; adding meat in small pieces, spices and olive oil and/or fat drippings to the bread/broth mixture; there is no addition of starter cultures; stuffing the paste into pork intestinal or cellulose-based casings when everything is completely mixed and the salt and spices adjusted to the desirable taste (variable). Traditionally, "Alheiras" are smoked at low but uncontrolled temperature (generally < 37 °C) and uncontrolled humidity [1], but industrially smoking is performed at higher temperatures, in ovens, and cooled rapidly in cooling chambers. Several authors observed that LAB constitute the predominant microbiota of "Alheira", with particular incidence of *Lactobacillus* spp. and *Enterococcus* spp. [1,2] from the early stages of fermentation process (during the smoking). However, pathogenic organisms, such as *L. monocytogenes*, *Salmonella* spp. and *St. aureus* have already been detected [2,3] which could affect the safe consumption of this food. "Alheira" have a pH ~5.1, 1.1% NaCl, ± 0.95 of water activity, 52.3% of moisture, 13% of lipids, 14% total protein and 13% of carbohydrates. These physicochemical parameters and composition, indicated that, and according to the accepted limits for these parameters, pH, salt content and moisture *per se*, do not assure the microbiological safety of this product. Furthermore, this is a product that is consumed cooked, but some studies suggested that internal temperatures may often not be sufficient to kill all of the pathogens originally present [4], which could justify the use of bioprotective cultures or other natural antimicrobial components.

One of the main challenges in the production of traditional fermented meat products is to improve competitiveness by identifying innovations, which comply with safety policies and regulations, and guarantee food safety, while at the same time meeting consumer demands and expectations [5]. There is an increasing interest in the use of LAB as natural preservatives, due to the potential production of antimicrobial metabolites such as bacteriocins [6–8]. So, LAB cultures and/or their bacteriocins are very important for bioprotective strategies to control spoilage and pathogenic microorganisms such as *L. monocytogenes* [6–9]. It is recommended to use strains that are well adapted to specific environment

characteristics of the food matrices in which they are going to be used, preferably isolates recovered from these or from similar foods, for optimal performance and bacteriocin production [9–11].

Lactobacillus plantarum ST153Ch originally identified as *Lb. sakei* but subsequently re-identified as *Lb. plantarum*, is an autochthonous strain isolated from "Salpicão", a traditional Portuguese salami-like product [12]. Vaz-Velho et al. [13] showed the possibility of using this strain as a protective culture to improve the safety of fermented meat sausages with respect to *L. monocytogenes*. Todorov et al. [12] showed that this strain produces an anti-listerial bacteriocin (ST153Ch) that is heat resistant and stable at low temperatures, stable between pH 2.0 and 10.0 and produced at higher levels during the stationary phase of fermentation in the presence of 2% (w/v) D-Glucose [12].

Food safety challenges are constantly changing and require new approaches and tools. Omics methodologies are rapidly transforming our approaches to the detection, prevention, and removal of foodborne pathogens [14], but also to monitor changes in microbiota composition of these products, the interactions between new bio-control agents and microbiota, which is directly correlated to its taste, aspect, safety and functional characteristics [15,16]. This could be a very interesting approach for industrial application.

The aim of this work was to evaluate the effect of a fresh and a lyophilized bioprotective *Lb. plantarum* ST153Ch culture, at an industrial scale, on the microbiological characteristics of "Alheira de Vitela", during the 90 days of storage at 4 ℃. Simultaneously the antilisterial activity of *Lb. plantarum* ST153Ch was also evaluated. Changes in microbiota were also investigated by 16s rRNA gene analysis in "Alheira de Vitela" produced with and without the bacteriocinogenic culture.

2. Materials and methods

2.1. Microorganisms and growth conditions

Lactobacillus plantarum ST153Ch (formerly *Lc. sakei* [12] and reclassified following 16 sRNA sequencing) was grown in de Man, Rogosa Sharpe (MRS) broth (Lab M, Bury, UK) at 30 ℃ for 48 h.

Listeria monocytogenes Scott A [17] and *L. monocytogenes* 3701 (isolated from a fermented meat sausage (ESB-UCP) were grown in Tryptone Soy Broth (TSB; Biokar) supplemented with 0.6% (w/v) of yeast extract (LabM; TSBYE) at 37 ℃ for 18–22 h.

All bacterial strains were sub-cultured twice under appropriate conditions before use in experiments. All strains were stored at −20 ℃ in the presence of 30% (v/v) glycerol.

2.2. Preparation of fresh and lyophilized culture

One liter of MRS broth was inoculated (1% v/v) with *Lb. plantarum* ST153Ch and after 24 h at 30 ℃, cells were centrifuged at 17200x g, for 10 minutes, at 4 ℃. Cells were resuspended in 100 mL of sterilized deionized water, in order to have 10^9 CFU/mL. For fresh culture, cells were kept at 4 ℃ until the inoculation procedure (fresh culture was prepared on the same day of inoculation).

For preparation of lyophilized culture, the method used was adapted from Barbosa et al. [18]: cells were initially frozen at −80 ℃ overnight, and then desiccated under vacuum (2 ATM) for 4 days in a freeze-drier (SB4 Armfield, UK) at room temperature; and the condenser was cooled at −48 ℃. Before lyophilization process, *Lb. plantarum* ST153Ch reached ~10^{10} CFU/mL and after the concentration was ~10^9 CFU/mL.

2.3. Manufacture of "Alheira de Vitela" and sampling procedures

"Alheira" samples were manufactured in a meat plant according to traditional recipes and techniques. The ingredients used were as follows: Veal meat (35%), wheat bread (wheat flour, baking powder and salt), pork meat, rooster, cooking broth, spices, onion, olive oil and salt. The sausage mixture, before stuffing in natural casing, was divided into three similar portions, each one with 45 kg: one was inoculated with *Lb. plantarum* ST153Ch fresh (S2BLAF), another with *Lb. plantarum* ST153Ch lyophilized (S2BLA) and the other one was non-inoculated (S2BC), to act as a control. *Lactobacillus plantarum* ST153Ch, fresh or lyophilized (prepared as indicated in section 2.2) was added before stuffing, in order to reach ~10^8 CFU/g in the final product. Lyophilized culture was suspended in 3 L of water and sucrose (6%w/v) and then added to the fresh sausage mixture. Fresh culture was directly added to the sausage mixture. Sausages were smoked in ovens, with a slight smoke of holm-wood, in controlled humidity, for some hours. Then, ''Alheiras" were packed (in packages composed with materials of polyolefin and polyamide), under modified atmosphere (20% CO_2 and 80% N_2) and stored for 90 days at 4 ℃. Three independent batches were produced.

Microbiological analyses of all the samples were performed before smoking (−1 day), immediately after smoking (0 day) and during storage at times 3, 7, 15, 21, 28, 60 and 90 days.

2.4. Anti-listerial activity of the bacteriocinogenic Lb. plantarum ST153 culture in ''Alheira"

Listeria monocytogenes strains were sub-cultured twice (24 h at 37℃) in TSB broth using a 1% v/v inoculum. Each culture was centrifuged and mixed in Ringer's solution. An aliquot (300 µL) of the cocktail of *L. monocytogenes* suspension (10^7 CFU/mL for each strain of *L. monocytogenes*) was inoculated with a sterilized syringe in 300 g of each sample of "Alheira". This procedure was done after smoking, in the microbiology laboratory, in order to reach 10^5 CFU/g of sample of ''Alheira": one batch was inoculated with *Lb. plantarum* ST153Ch fresh plus cocktail of *L. monocytogenes* (S2BLAF + LM), another batch with *Lb. plantarum* ST153Ch lyophilized plus cocktail of *L. monocytogenes* (S2BLA + LM) and the other one only with cocktail of *L. monocytogenes* (S2BC + LM), to act as a control.

2.5. Microbiological analysis

Twenty-five gram samples were added to 225 mL of sterile buffered peptone water (Biokar Diagnostics, Beauvais, France), and homogenized in a stomacher for 2 min. Appropriate decimal dilutions were prepared in Ringer's solution for microbial enumeration according to ISO Standards: LAB on de Man, Rogosa and Sharpe Agar (MRS, Biokar Diagnostics; [19]) incubated at 30 ℃ for 72 h; *Enterobacteriaceae* on RAPID' *Enterobacteriaceae* medium (Bio-Rad, CA, USA; [20]) and *St. aureus* on Baird Parker Agar (BPA, Bio-Rad; [21]), with rabbit plasma fibrinogen confirmation [22], both incubated at 37 ℃ for 48 h; *Escherichia coli* on Tryptone Bile X-glucuronide Agar [23] incubated at 44 ℃ for 24 h; *Bacillus cereus* on Mannitol Egg York Polymyxin Agar Base (VWR International, Pennsylvania, USA; ISO 7932:2004/Amd 1 [24]), and yeasts and molds on Rose-Bengal Chloramphenicol Agar (Oxoid, Hampshire, UK; NP 3277-1:1987 [25]) incubated at 25 ℃ for 5 days. Also the detection of some agents was performed using the ISO methodologies: detection and

enumeration of *L. monocytogenes* [26,27], detection of *Salmonella* spp. [28], sulfite- reducing *Clostridium* spores [29] and detection of *Yersinia* [30]. Every analysis was performed in duplicate.

2.6. 16S rRNA gene analysis of microbial communities of "Alheira"

Ten grams of "Alheira" samples were homogenized individually in a stomacher for 2 min. From this, 1 g was used for DNA extraction. The DNA was extracted according to the instructions of "The GRS Genomic DNA Kit–Tissue–for Stool" (GRISP, Porto, Portugal).

The samples used were: "Alheira" inoculated with *Lb. plantarum* ST153Ch lyophilized (S2BLA): before smoking (S2BLA (−1 day)), after smoking (S2BLA (0 day)) and after 60 days of storage (S2BLA (60 days)); and the control non-inoculated (S2BC): before smoking (S2BC (−1 day)), after smoking (S2BC (0 day)) and after 2 months storage (S2BC (60 days)).

Samples were prepared for Illumina Sequencing by 16S rRNA gene amplification of the bacterial community. The DNA was amplified for the hypervariable V3-V4 region with specific primers and further reamplified in a limited-cycle PCR reaction to add sequencing adapters and dual indexes. First PCR reactions were performed for each sample using KAPA HiFi HotStart PCR Kit according to the manufacturer's suggestions, 0.3 μM of each PCR primer: forward primer Bakt_341F 5'-CCTACGGGNGGCWGCAG-3' and reverse primer Bakt_805R 5'-GACTACHVGGGTATCTAATCC-3' [31,32] and 12.5 ng of template DNA in a total volume of 25 μL. The PCR conditions involved a 3 min denaturation at 95 ℃, followed by 25 cycles of 98 ℃ for 20 s, 55 ℃ for 30 s and 72 ℃ for 30 s and a final extension at 72 ℃ for 5 min. Second PCR reactions added indexes and sequencing adapters to both ends of the amplified target region according to manufacturer's recommendations [33]. Negative PCR controls were included for all amplification procedures. PCR products were then one-step purified and normalized using SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, USA) [34], pooled and pair-end sequenced in the Illumina MiSeq® sequencer with the V3 chemistry, according to the manufacturer's instructions [33] at Genoinseq (Cantanhede, Portugal).

All 16S rRNA data were analyzed with Kraken v1 [35] using the pre-built MiniKraken 8Gb database (https://ccb.jhu.edu/software/kraken/) with default parameters. At each taxonomic rank, classification falling below a threshold of 0.1% of all data were excluded from the subsequent analysis (i.e. classification represented by less than ~100 reads).

2.7. Statistical analysis

The comparison of the growth of *L. monocytogenes* without and with the bacteriocinogenic culture (fresh or lyophilized) through the storage time was carried out by one-way Analysis of Variance (ANOVA). The LSD-Tukey test was used to determine the significant differences ($p < 0.05$) among group means. Statistical analysis was done using SPSS 24.0.0.0 software for Windows.

3. Results and discussion

Since food safety has become an increasingly important international concern, the application of antimicrobial peptides from LAB, that target foodborne pathogens without toxic or other adverse effects, has received great attention. LAB that produce bacteriocins with antilisterial activity

have been used in fermented meat sausages as bioprotective cultures since the beginning of the 90's [6,7,36–41]. Our work follows this trendline, and expects to validate the effect of this bioprotective culture at industrial scale and over long storage times.

The behaviour of *L. monocytogenes* and LAB in "Alheira" samples (control and inoculated with bacteriocinogenic culture) is presented in Figure 1 and Table 1, respectively. No differences were observed when *L. monocytogenes* strains were tested alone or combined in a cocktail (data not shown), showing that they don't interfere between them. In the first three days of storage, it was observed a slight increase in *L. monocytogenes* counts. In parallel, a more pronounced growth was registered for LAB achieving ca. 9–10 log CFU/g. Also, and as observed in Table 1, the decrease in pH occurs at the same time as the number of lactic acid bacteria increases. After this period and until day 15, counts of the pathogen remained at the same level in control samples and decreased ca. 0.5 and 1.0 log CFU/g in samples inoculated with lyophilized and fresh bacteriocinogenic cultures, respectively, while counts of LAB increased ca. 1 log cycle in all the samples. From day 15 until the end of storage, counts of LAB remained stable (varied less than < 1 log cycle) while counts of *L. monocytogenes* decreased in all the samples. Although there were no significant differences ($p > 0.05$) in the reduction of the pathogen between samples with and without bacteriocinogenic culture, there was a clear trend for a higher reduction of *L. monocytogenes* in the presence of the protective culture. It is important to highlight that results represent the mean of three independent batches produced at industrial level and consequently several factors, e.g. the composition of the indigenous microbiota of the raw materials and final products, were neither constant nor controlled. Most of the results published in previous articles were performed at laboratory or pilot scale [7, 36–41]. The work published by Vaz Velho et al. [13] was developed at smaller industrial scale, with only fresh culture and only at 7 days of storage. Also, these authors showed a reduction of *L. monocytogenes* less than 1 log. This current work was performed at industrial scale, with all the variability resulting therefrom. Moreover, this work used two different forms of the starter culture: lyophilized and fresh and analyses were performed during 90 days of storage. This well demonstrates the importance of validating studies at the industrial level and the importance of following the entire processes, until the end of storage time. For the industry it is crucial to follow product alterations until the end of storage, especially if we keep in mind that consumers are increasingly looking for safe products with long shelf-life.

The pathogenic organisms investigated, (*Salmonella*, *S. aureus*, *Yersinia*, sulphite reducing *Clostridium* spores and *E. coli*) were not detected in any sample. Also, counts of *Enterobacteriaceae* and *B. cereus* were below the detection limit of the enumeration technique for 0, 3, 7, 15, 21, 28, 60 and 90 days of storage (Table 2).

Overall, for 16S rRNA metagenomic analysis almost all reads generated (~99%) were classified into a taxonomic rank for all the "Alheira de Vitela" samples, for the different stages of fermentation and with and without the lyophilized bacteriocinogenic culture, with more than 95% of them being classified at the family and genus level (Table 3). Regarding family analysis, *Leuconostocaceae* and *Lactobacillaceae* predominated in all the samples (Figure 2A). *Lactobacillaceae* was the predominant family in samples with addition of bacteriocinogenic culture and *Leuconostocaceae* was prevalent in control samples. At the genus level, the microbiome distribution was similar to the family classification, with the main genus being *Lactobacillus, Weissella* and *Leuconostoc* (Figure 2B). At both taxonomic levels, higher microbial diversity could be observed before and after smoking, as compared to 60 days after storage where samples presented two main families or genus. Of note, "Alheiras" sampled after 60 days of storage (60 days) with the addition of bacteriocinogenic culture

were mostly populated by *Lactobacillus*, whereas without this addition, "Alheiras" were equally populated by *Leuconostoc* and a higher portion of *Weisella*.

At the species level, it was possible to observe lower microbial diversity in the samples before smoking (labeled as −1 day), with the sample without culture presenting higher diversity than the sample with culture (Figure 2C). After smoking, the microbial diversity of the inoculated sample increased, especially when compared to the control, with a more pronounced trend after 60 days of storage, when microbial diversity was greater. Through time, in both tested conditions, a decrease in *Leuconostoc* and *Weisella* species could be observed, although this was more accentuated in samples with bacteriocinogenic culture, as only traces of *Weisella* species were observed and *Leuconostoc* species were only present below 7%. After 60 days of storage, the most predominant specie identified was *Lb. sakei* that, as mentioned above, was the previous classification of inoculated *Lb. plantarum* strain, which is likely due to the characteristics of the database used. Also, it is interesting to observe the change in microbiome of samples before and after fermentation, showing the role of LAB in this process. Although there are differences in the microbiota, these are slight, which makes it impossible to draw final conclusions. So, we believed that further studies are still needed to explain the observed differences.

Studying the changes in microbial populations can provide useful information to follow natural fermentation dynamics, particularly when using bioprotective cultures. Many different researches have been carried out unravelling the structure of the microbial consortia in dairy [42,43], meat [44] and vegetable foods [45].

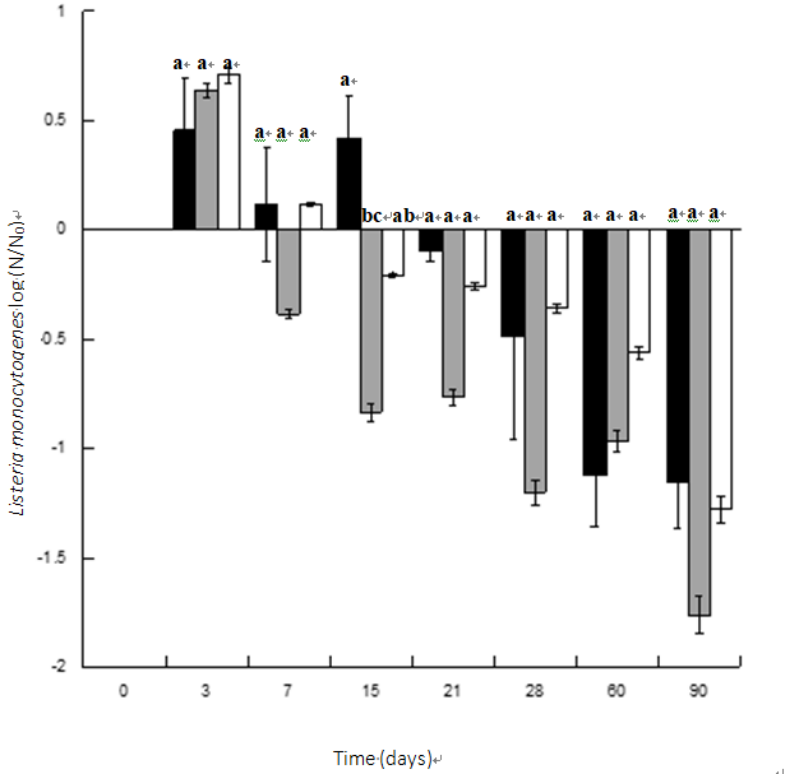

Figure 1. Behaviour of *L. monocytogenes* in "Alheira de Vitela", during storage at 4 °C in samples inoculated with *Lb. plantarum* ST153Ch and cocktail of *L. monocytogenes*. (■) S2BC + LM; (■) S2BLAF + LM; (□) S2BLA + LM. For each time, columns with the same letter are not significantly different ($p > 0.05$).

Table 1. Growth of lactic acid bacteria and pH values in "Alheira de Vitela", during storage at 4 °C in samples inoculated with *Lb. plantarum* ST153Ch and samples inoculated with *Lb. plantarum* ST153Ch plus cocktail of *L. monocytogenes*.

Samples	"Alheira" with *Lb. plantarum*						"Alheira" with *Lb. plantarum* plus cocktail of *L. monocytogenes*					
	S2BC (non-inoculated)		S2BLAF (fresh)		S2BLA (lyophilized)		S2BC + LM (only inoculated with *L. monocytogenes*)		S2BLAF + LM (Fresh)		S2BLA + LM (lyophilized)	
Time (days)	Log (CFU/g)	pH	Log (CFU/g)	pH	Log (CFU/g)	pH	Log (CFU/g)	pH	Log (CFU/g)	pH	Log (CFU/g)	pH
−1*	4.0 ±0.12	5.45 ±0.04	8.3 ±0.55	5.65 ±0.03	7.8 ±1.5	5.56 ±0.06		nd		nd		nd
0	7.6 ±1.10	4.72 ±0.06	9.1 ±1.46	4.75 ±0.05	7.8 ±1.9	4.65 ±0.06	7.3 ±0.95	4.70 ±0.02	8.0 ±1.66	4.73 ±0.03	8.53 ±1.48	4.80 ±0.04
3	9.0 ±0.06	4.62 ±0.03	9.3 ±0.59	4.59 ±0.03	9.8 ±0.6	4.60 ±0.02	9.3 ±0.81	4.62 ±0.01	9.8 ±0.40	4.65 ±0,03	9.87 ±0.06	4,65 ±0,04
7	9.7 ±0.55	4.54 ±0.09	9.5 ±0.96	4.53 ±0.04	9.2 ±0.7	4.5 ±0.07	10.3 ±0.40	4.55 ±0.05	9.4 ±1.30	4.64 ±0.05	10.07 ±0.71	4.60 ±0.03
15	10.0 ±0.41	4.39 ±0.05	10.2 ±0.46	4.42 ±0.07	10.5 ±0.5	4.40 ±0.03	10.4 ±0.31	4.44 ±0.08	10.7 ±0.42	4.57 ±0.02	10.53 ±0.55	4.50 ±0.07
21	11.0 ±0.00	4.25 ±0.06	10.6 ±0.29	4.30 ±0.08	11.1 ±0.0	4.31 ±0.10	10.8 ±0.12	4.43 ±0.06	10.4 ±0.46	4.45 ±0.06	11.07 ±0.06	4.50 ±0.04
28	10.7 ±0.67	4.11 ±0.08	10.7 ±0.69	4.14 ±0.04	10.9 ±0.2	4.14 ±0.06	10.6 ±0.87	4.13 ±0.02	11.0 ±0.20	4.25 ±0.04	11.03 ±0.06	4.20 ±0.06
60	10.9 ±0.06	3.60 ±0.04	10.8 ±0.85	3.67 ±0.02	10.6 ±0.6	3.70 ±0.08	10.7 ±0.29	3.70 ±0.02	10.7 ±0.44	3.90 ±0.07	10.97 ±0.12	4.00 ±0.07
90	10.5 ±0.15	3.58 ±0.09	10.4 ±0.44	3.52 ±0.06	10.4 ±0.4	3.54 ±0.04	10.4 ±0.17	3.57 ±0.04	10.4 ±0.40	3.65 ±0.05	10.60 ±0.44	3.70 ±0.05

* before smoking.

Table 2. Microbial characterization of "Alheira de Vitela" at 4 °C for 0, 3, 7, 15, 21, 28, 60 and 90 days of storage.

"Alheira"	Enumeration (log CFU/g)					Presence in 25g				
	Bacillus cereus	Yeasts	Moulds	*Enterobacteriaceae*	*E .coli*	Coagulase positive staphylococci	*Listeria* spp.	*Listeria monocytogenes*	*Salmonella* spp.	SRC spores[a]
S2BC	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	–	–	–	–
S2BLAF	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	–	–	–	–
S2BLA	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	–	–	–	–
S2BC + LM	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	–	–	–	–
S2BLAF + LM	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	–	–	–	–
S2BLA + LM	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	–	–	–	–

Continued on next page

"Alheira"	Enumeration (log CFU/g)						Presence in 25g			
	Bacillus cereus	Yeasts	Moulds	*Enterobacteriaceae*	*E .coli*	Coagulase positive staphylococci	*Listeria* spp.	*Listeria monocytogenes*	*Salmonella* spp.	SRC spores[a]
S2BC	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	–	–	–	–
S2BLAF	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	–	–	–	–
S2BLA	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	–	–	–	–
S2BC + LM	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	–	–	–	–
S2BLAF + LM	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	–	–	–	–
S2BLA + LM	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	–	–	–	–
S2BC	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	–	–	–	–
S2BLAF	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	–	–	–	–
S2BLA	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	–	–	–	–
S2BLAF + LM	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	–	–	–	–
S2BLA + LM	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	–	–	–	–

Legend: [a]SRC spores; sulphite reducing clostridial spores, presence (+) or absence (−) in 1, 0.1 or 0.01 g sample.

Table 3. Global results of the 16SrRNA metagenomic analysis using Kraken.

Sample	Total # of paired reads generated	Percentage of classified reads (%)			
		Total	Up to family	Up to genus	Up to species
S2BLA (−1 day)	88285	99.93	94.94 (0.15)	94.71 (0.35)	30.03 (1.08)
S2BLA (0 day)	77182	98.71	98.59 (0.67)	98.02 (0.86)	50.32 (1.71)
S2BLA (60 days)	83671	99.77	97.53 (1.07)	96.87 (1.19)	81.41 (1.74)
S2BC (−1 day)	91240	99.91	98.17 (0.23)	98.07 (0.21)	55.04 (0.8)
S2BC (0 day)	62382	99.08	98.72 (0.69)	98.52 (0.72)	84.29 (1.18)
S2BC (60 days)	76074	99.64	97.39 (0.14)	96.98 (0.34)	68.14 (0.73)

Percentages are relative to the total number of classified reads. Values in parenthesis refer to the percentage of reads excluded at each taxonomic rank. "Alheira de Vitela" non-inoculated as a control (S2BC) and inoculated with *Lb. plantarum* ST153Ch lyophilized (S2BLA): before smoking (−1 day), after smoking (0 day) and 60 days of storage (60 days).

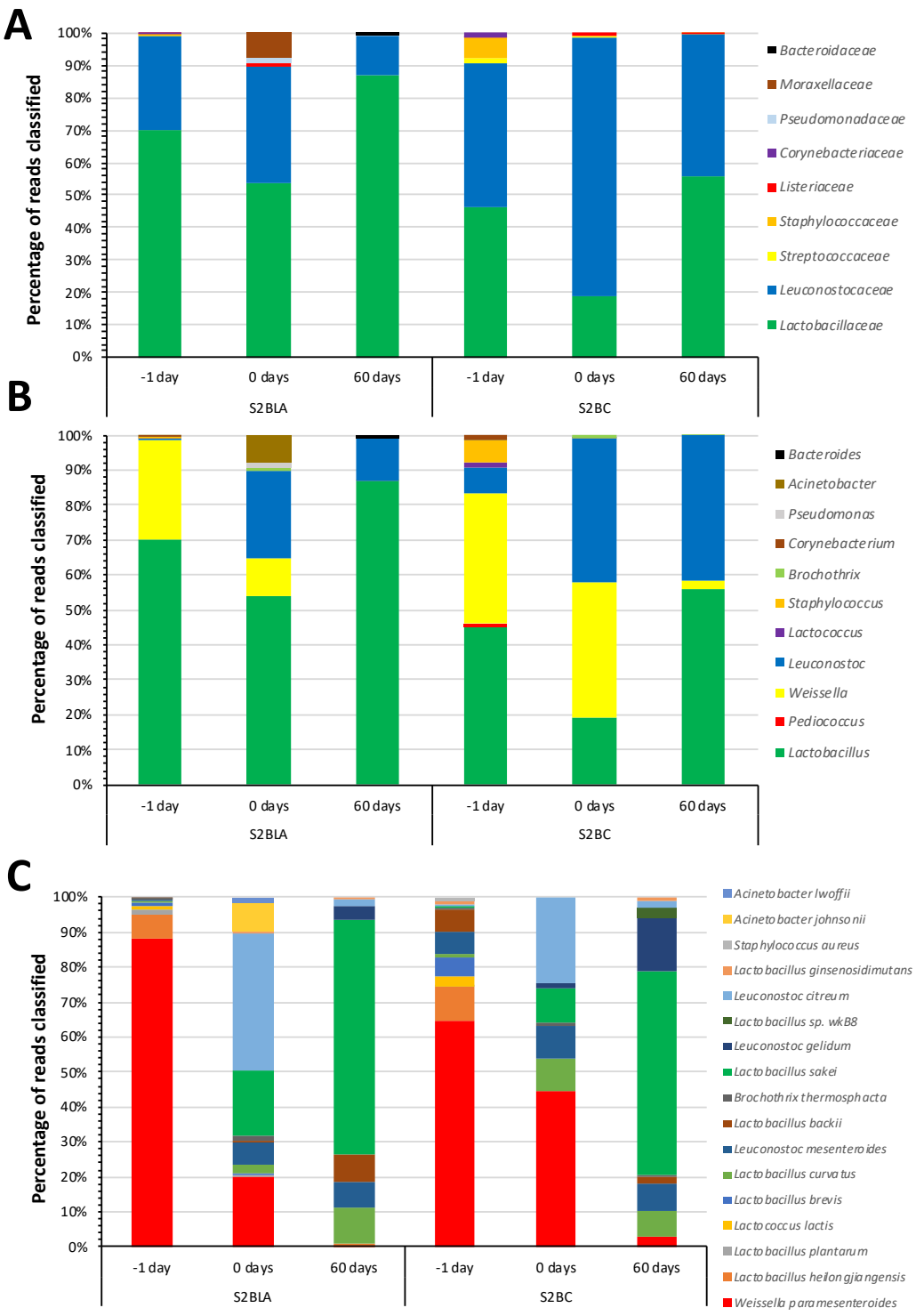

Figure 2. Results for 16s rRNA gene analysis of microbial communities of "Alheira de Vitela". Reads were classified using Kraken and (A) families, (B) genus and (C) species identified are presented. Percentages are relative to the total number of reads classified at each taxonomic level. Classifications falling below 1% in all samples are not presented and are detailed in Supplementary Table S1.

4. Conclusion

Lactobacillus plantarum ST153Ch showed a slight antilisterial activity *in situ*, with a reduction of ~0.5–1 log until 15 days of storage of "Alheira de Vitela". However, until the end of storage, counts of *L. monocytogenes* decreased in all the samples, while counts of LAB remained stable. Additionally, no significant differences were found between application methodologies (fresh or lyophilized LABs), therefore industry might be able to choose the most appropriate technique according to their manufacturing process. From the analysis of the microbial communities it was confirmed the absence of the pathogens investigated by culture dependent methods and it was demonstrated that the addition of the bioprotective culture altered the microbiota of "Alheira". At different stages of fermentation with and without the bac + culture, *Leuconostocaceae* and *Lactobacillaceae* predominated in all samples and, as expected, *Lactobacillus* was the genus more prevalent in "Alheiras" sampled after 60 days of storage with the addition of the bacteriocinogenic culture.

Nevertheless, the few differences observed, there was a reduction in the *L. monocytogenes* counts in the early storage days, which could justify the use of these cultures. For the industry it is very important to control the development of a pathogen at the beginning of storage time when products are already available to consumers.

Conflict of interest

All authors declare no conflicts of interest in this paper.

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