Selection of commercial protective cultures to be added in Sardinian fermented sausage to control *Listeria monocytogenes*

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Abstract

Sardinian fermented sausage "Salsiccia Sarda" is a Mediterranean-style, semi-dry, fermented, RTE product, representing the main pork meat product in Sardinia (Italy). The high variability that characterizes the technological processes applied in different production plants results in sausages with different chemico-physical features sometimes permissive for the growth of *Listeria monocytogenes*. In order to guarantee the hygienic-sanitary quality of the final product and to innovate the manufacturing process, the main objective of this study was to evaluate the use of different commercial protective cultures to control *L. monocytogenes* growth in the Sardinian fermented sausage. In the first step, *in vitro* tests were carried out to evaluate the effectiveness of five freeze-dried bioprotective cultures availabe on the market in limiting the growth of *L. monocytogenes*. The two protective cultures that showed the best *in vitro* results were selected for a challenge test on artificially contaminated Sardinian fermented sausages. Moreover, the protective culture that showed the best results in inhibiting the growth of *L. monocytogenes* according to *in vitro* and challenge test experiments, was included into real production settings and validated in three producing plants. As a result, it was observed that protective cultures represent an important technological innovation for the Sardinian fermented sausage processing plants as they allow to control *L. monocytogenes* growth without altering the composition, the microflora and the chemical-physical characteristics of the product, thus ensuring safety and quality. Protective cultures also showed to reduce *Enterobacteriaceae* mean levels at the end of ripening and not to affect the natural concentration of lactic acid bacteria and coagulase-negative staphylococci.

Introduction

Listeriosis caused by *Listeria monocytogenes* (*L. monocytogenes*) is one of the most severe food-borne diseases under EU surveillance. According to the report published in 2021 by the European Food Safety Authority and the European Center for Disease Prevention and Control, referring to 2020 data, *L. monocytogenes* was ranked the fifth most commonly reported zoonosis agent, with 1,876 confirmed invasive human cases of listeriosis and 16 outbreaks. A decrease in cases and outbreaks was observed (2,621 confirmed cases and 21 outbreaks in 2019), but the overall growing trend for listeriosis in 2016–2020 did not show any statistically significant modification (EFSA & ECDC, 2021). The fatality rate of *L. monocytogenes* infections in the EU was 13.0% in 2020, showing the highest number of fatal cases among foodborne infections. The pathogen is therefore a significant burden for public health, causing hospitalization, high morbidity and mortality, notably among the elderly (EFSA & ECDC, 2021).

The most significant infection source of *L. monocytogenes* for humans is represented by foods, especially ready-to-eat (RTE) that can be contaminated during or after processing and do not undergo any treatment that ensures their safety before consumption (Neri *et al.*, 2019). In the EU during 2020, *L. monocytogenes* was detected in 3.0% of RTE pig meat products, with a 0.9% increase respect to 2019 (EFSA & ECDC, 2021). *L. monocytogenes* has been identified at every point of the pig-meat supply chain (Kanuganti *et al.*, 2002; Thèveneot *et al.*, 2006; Meloni *et al.*, 2013). Contamination is often due to the presence of *L. monocytogenes* in raw materials (Thevenot *et al.*, 2006): pigs can be carriers of *L. monocytogenes* in their intestines most often as asymptomatic shedders (Esteban *et al.*, 2009, Boscher *et al.*, 2012) and not being identified neither in the farm nor in the slaughterhouse during ante and post mortem inspections, can potentially contaminate the carcasses (Kanuganti *et al.*, 2002; Fosse *et al.*, 2009). The incidence of the microorganism increases when going further in the pork processing industry from the slaughterhouse to the following steps (*e.g.* cutting, mincing), also due to cross-contamination which occurs by the environment and equipment of the processing plants, in consideration of *L. monocytogenes* capability of contaminating surfaces with bacterial attachment and biofilm formation (Autio *et al.*, 2000; Peccio *et al.*, 2003).

Salsiccia Sarda or Sardinian fermented sausage (SFS) is the main pork meat product in Sardinia (Italy): it is a Mediterranean-style, semi-dry, fermented, RTE product included in the National List of traditional food products (Twentieth revision of the list of traditional agri-food products, Italian Republic, 2020). The SFS production sector includes small businesses, with distinctly artisanal productions, and larger plants with standardized industrial processing. For this reason, the manufacturing process is characterized by a great variability, most of all in artisanal plants that are strongly influenced by customs and family recipes (Meloni, 2015). Fermented sausages are usually made using lactic acid bacteria (LAB) and nitrate-reducing coagulase-negative staphylococci (CNS), which are often naturally present in the meat or added by inoculation of starter cultures during the mixing step (Greco *et al.*, 2005). The safety of the SFS depends on the application of

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several sequential "hurdles" at different stages of the fermentation and ripening process (Mangia *et al.,* 2007; Meloni, 2015; Piras *et al.,* 2019). Safety of the final product is therefore ensured by the presence of multiple factors and specific physico-chemical conditions, such as pH, water activity (a_w), sodium chloride, nitrates and nitrites, which interact in limiting microbial growth (Piras *et al.,* 2019). In SFSs, pH values comprised between 5.3-5.5 and a_w values ≤ 0.920 indicate correct acidification drying processes (Greco *et al.,* 2005): products reporting these values at the end of ripening can be included in the category of RTE products unable to support the growth of *L. monocytogenes* (Regulation EC No. 2073/2005). However, the aforementioned high variability that characterizes the technological processes, especially in small artisanal production plants, results in products having different chemical and physical features, which can sometimes be permissive for *L. monocytogenes* growth. In fact, either strongly contaminated raw materials or inadequately applied production process steps (*e.g.,* insufficient fermentation or inappropriate dry-curing) can determine a deficiency in the development of the hurdles and the creation of favorable conditions for *L. monocytogenes* growth (Mureddu *et al.,* 2014).

Therefore, it is necessary to implement technological innovations into the SFS production process in order to guarantee the hygienic-sanitary quality of the final product and to ensure that the level of contamination by *L. monocytogenes* does not exceed 100 CFU/g up to the end of the shelf life as established by the EU legislation (Regulation EC No. 2073/2005) for the whole commercial life.

The use of bioprotective cultures has been gaining interest recently, especially in the dairy and meat industries, because they are safe for consumption and naturally dominate the microbiota of many foods. Protective cultures consist of bacteria that are specifically selected for their ability to inhibit the growth of pathogens or microbiological spoilage agents (Young and O'Sullivan, 2011). Protective cultures can exert a bioprotective or inhibitory effect against other microorganisms, due to their competition for nutrients and the production of bacteriocins and other antagonistic compounds such as organic acids, hydrogen peroxide and enzymes (Davidson *et al*., 2015). Furthermore, protective cultures can delay the development of spoilage microorganisms, extending the shelf-life. The faster metabolism of protective cultures outcompetes pathogens for the available nutrients and thus offers protection against *L. monocytogenes*, as a further barrier in the hurdles technique of protection (Youg and O'Sullivan, 2011). To the best of our knowledge, no available studies investigated the use of bioprotective cultures against *L. monocytogenes* in SFS. In this framework, the main objective of this study was to evaluate the effectiveness of different commercial protective cultures in inhibiting the development and the growth of *L. monocytogenes* in the SFS.

Materials and methods

Study set-up

The project was divided into different steps: 1) in the first phase, the *in vitro* efficacy of 5 freeze-dried protective cultures against *L. monocytogenes* was assessed; 2) in the second phase, the protective cultures that showed the best *in vitro* results against the pathogen were used during a challenge test for the production of the SFS; 3) in the third step, the protective culture that showed the best results during the challenge test was used during the manufacturing process of three SFS processing plants in Sardinia, representative of the entire regional sector.

Bioprotective cultures

Five lyophilized bioprotective cultures (A, B, C, D, E) available on the market, consisting of a mix of lactic acid bacteria (LAB) and micrococci, were selected. The selection criteria were represented by their proven activity against *L. monocytogenes* and their adaptability to both meat substrate and temperatures typically used in the production process of SFS. In particular, the species included in the composition of bioprotective culture A were *Lactobacillus sakei, Pediococcus acidilactici, Staphylococcus carnosus* and *Staphylococcus carnosus* subsp. *utilis*. Bioprotective culture B consisted of a mixture of *Pediococcus acidilactici.* The composition of bioprotective culture C included *Lactobacillus plantarum*. Bioprotective culture D included bacteriocin-producing strains of *Carnobacterium* ssp. Species included in bioprotective culture E were a mixture of different *Carnobacterium* spp. strains. According to the manufacturer's instruction, the cultures were individually rehydrated by dilution in 0.85% sterile NaCl solution immediately before their use.

Samples

SFSs were manufactured according to the technological process applied by producing plants representative of the sector. Briefly, the production process involved selection, chopping and mincing of pork meat and fat, followed by mixing with curing ingredients, spices and authorized additives, including nitrates and nitrites at maximum concentrations of 150 mg/kg each (Reg. EC 1333/2008). Starter cultures consisting of lactic acid bacteria (LAB) and nitrate-reducing coagulase-negative staphylococci (CNS) were added during the mixing step. After overnight refrigerated storage, the mixture was stuffed in natural bowel (mutton or beef). The fermentation stage continued during the next steps of initial dipping (20-22°C for 24h, 70-80% humidity) and drying (2-3 days with progressive decrease of temperature and humidity). Ripening was carried out for about 20 days in storerooms at 15°C and 70–75% humidity. The production process is summarized in Figure 1. The finished products were cylindrical in shape, with a length of 40-45 cm and a diameter of 3-4 cm, folded with the characteristic horseshoe shape; the weight was between 300 and 600 grams. Each SFS was regarded as a sample.

Step one: *in vitro* assessment

Two *L. monocytogenes* reference strains (American Type Culture Collection, ATCC 19111 and National Collection of Type Cultures, NCTC 10887) and three *L. monocytogenes* wild type strains, were used for the challenge tests. *L. monocytogenes* wild type strains were isolated from naturally contaminated SFSs samples and producing plants environment from previous investigations and identified by PCR according to the protocol by Lyu *et al.* (2013). The wild-type strains were selected in order to evaluate the effectiveness of the bioprotective cultures on isolates that were already adapted to plants' environments. *L. monocytogenes* strains were stored at -80°C and revitalized after incubation at 37°C for 18-24 h.

The well diffusion assay method was used according to the protocol defined by Cosentino *et al.* (2012), with modifications. In a first phase the selected protective cultures was tested as it is in order to test its anti-*Listeria* activity. Afterwards the selected protective cultures was tested as a "cell free" supernatant. The antimicrobial activity was expressed as the diameter of the inhibition zones around the wells. The bioprotective cultures were considered effective against *L. monocytogenes* if an inhibition zone greater than 15 mm was measured (Maragkoudakis *et al*., 2009).

Step two: challenge test

The definition of the protocol for the challenge test was conducted according to the "EURL Lm Technical guidance document on challenge tests and durability studies for assessing shelf-life of ready-to-eat foods related to *L. monocytogenes*" (version

4 July 2021). An experimental inoculum was set up consisting of a mixture of n. 5 strains of *L. monocytogenes* (n. 2 reference strains + n. 3 wild type strains) to challenge SFSs. The experimental inoculum was added in the mixing machine, together with the other ingredients. In order to account for natural contamination levels and, at the same time, to obtain a level of inactivation of *L. monocytogenes* estimated around 1-2 log at the end of the ripening, the inoculum level was set between 10 and 100 CFU/gr.

The bioprotective cultures that had showed the best *in vitro* results were selected for the challenge test of SFSs. According to the manufacturer's instruction, the freeze-dried cultures were resuspended in sterile 0.85% NaCl to obtain a final concentration of ca. 10^7 CFU/mL.

In a pilot producing plant located at the Department of Veterinary Medicine of Sassari University, n. 3 batches of SFSs were made with ingredients obtained by 3 representative production plans were prepared (total of 9 batches). Four types of samples for each batch were produced: 1) negative control samples (C, n. 36 samples), 2) positive control samples added with *L. monocytogenes* broth culture (CL, n. 36 samples), 3) samples added with protective culture A and *L. monocytogenes* (CLA, n. 36 samples) and 4) samples added with protective culture B and *L. monocytogenes* (CLB, n.36 samples). All the samples were produced according to the process shown in figure n. 1. *L. monocytogenes* broth cultures and protective culture were added during the mixing step. Triplicate samples of each of the nine batches of SFS were analyzed at four analysis times: after stuffing (T0), 24h after stuffing (T1), 6 days after stuffing (end of drying phase, T6) and 20 days after stuffing (end of ripening, T20). A summary of the experimental design with the test units, sampling point and analysis conducted during the challenge study is reported in Table 1.

Microbiological profile

At each time-point, *L. monocytogenes* quantitative and qualitative detection (UNI EN ISO 11290-1/2:2017) was carried out on both control and treated samples. Mesophilic lactic acid bacteria (LAB) were investigated according to ISO 15214:1998, using De Man, Rogosa and Sharpe agar (MRS agar, Biolife, Milan, Italy). For the enumeration of micrococci, Coagulase-Positive Staphylococci (CPS) and Coagulase-Negative Staphylococci (CNS), Mannitol Salt Agar (MSA, Biolife, Milan, Italy) was used. Finally, for *Enterobacteriaceae* enumeration the EN ISO 21528-2:2017 was applied.

Physico-chemical and composition analysis

On each sample, pH and a_w were determined using pH meter GLP 22 (Crison Instruments SA, Barcelona, Spain) and Aqualab CX3 (Decagon, Pullman, Washington, USA). Moisture, fat and protein (expressed as %) were determined by the FoodScanLab (FOSS, Analytic, Hillerød, Denmark) using the Near-Infrared Transmittance (NIT) technology and a previously set calibration curve. Analyzes were performed in triplicate on a homogenized sample representative of the product.

Step three: application of the protective culture during the manufacturing processes of three SFS producing plants

In the third step, the protective culture that showed the best results in inhibiting the growth of *L. monocytogenes* according to *in vitro* and challenge test experiments, was included into the normal production process of three SFS producing plants (P1, P2, P3). The aim of this experimental application was to identify any changes in physico-chemical and composition characteristics, as well as microbiological profile, of the SFSs added with the protective culture.

Three batches of SFSs were produced at each plant. Two types of samples were manufactured: 1) negative control samples without protective culture (n. 27 samples) and 2) samples inoculated with protective culture during the mixing step of the production process (n. 27 samples). Triplicate samples of each batch of SFS were analyzed at the end of ripening for a total of n. 54 samples.

Microbiological profile

On each sample, *L. monocytogenes* quantitative and qualitative detection was carried out according to UNI EN ISO 11290-1/2:2017. Moreover, from an initial suspension and decimal dilution, mesophilic lactic acid bacteria (LAB), micrococci, Coagulase-Positive Staphylococci (CPS), Coagulase-Negative Staphylococci (CNS) and *Enterobacteriaceae* enumeration was conducted as described before. *Salmonella spp*. presence was determined according to ISO 6579-1:2020.

Physico-chemical and composition analysis

On each sample at the end of ripening, physico-chemical and composition analyses were conducted, as described before.

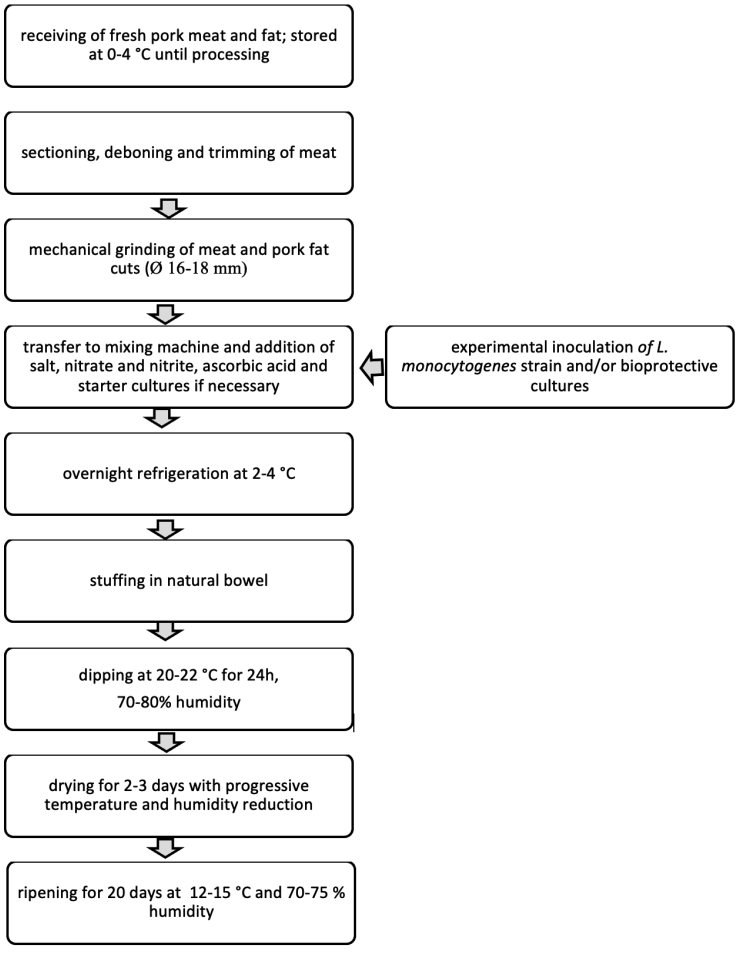

Figure 1. Process flow diagram of SFS.

Statistical analysis

Differences among average microbiological group counts (log_{10} cfu/g) and pH, over time (T0, T1, T6 and T20) and among treatments were compared using Fisher's least significant difference test. Statistical analyses were performed with Statgraphics Centurion XIX software (Stat Point Technologies, Warrenton, VA, USA).

Results

In vitro assessment

The results showed that the protective cultures with the most efficient anti-*Listeria* effect were cultures A and B. Both cultures showed a clear inhibition zone around the well greater than 15 mm on all tested *Listeria* strains. On the other hand, bioprotective cultures C, D and E showed an inhibition zone less clear and smaller than 15 mm. Based on *in vitro* tests results the cultures A and B were used for the challenge test.

Challenge test

Both protective cultures A and B, selected during the *in vitro* test, showed *in vivo* efficacy against *L. monocytogenes*. However, protective culture A showed the strongest anti-*Listeria* effect: in samples added with protective culture A (CLA samples), a reduction in the number of *L. monocytogenes* was detected in the product starting from T1 analysis time, with a significant decrease ($P<0.01$) between T0 and T1 when a reduction > 1 log was observed. Afterwards, mean *L. monocytogenes* levels remained stable until T20, without significant differences. The anti-*Listeria* efficacy detected for protective culture A was consistent in all three experimental production batches. *L. monocytogenes* was not detected with the qualitative method. Regarding samples added with protective culture B (CLB samples), even though an anti-*Listeria* activity was detected, it was lower in all batches representative of the three producing plants compared to that observed in CLA samples (<1 log if compared to the experimental inoculation). In these types of samples, the pathogen was also detected at T20 with the qualitative method.

In positive control samples (CL samples) *L. monocytogenes* was detected using both the quantitative method (T0, T1 and T6 analysis times) and the qualitative method (T0, T1, T6 and T20 analysis time).

As expected, LAB mean levels were significantly higher ($P<0.01$) in samples added with the protective cultures A and B respect to control samples at T0, T1 and T6, but comparable at T20. Also micrococci and CNS showed higher mean levels in samples added with the protective culture at T0 and T1, while at the following analysis time, the trend was more irregular. These differences in LAB, micrococci and CNS levels are probably due to the use of the protective cultures that, as said, consisted of microorganisms belonging to the genera *Lactobacillus* and *Staphylococcus*. *Enterobacteriaceae* mean counts in control samples were consistent, with values between 3 and 4 log_{10} CFU/g in all analysis times; on the other hand, counts were lower in samples added with the protective culture A respect to control samples with significant differences ($P<0.05$) starting from T1,

Table 1. Type of analysis, testing time and relative minimum number of test units performed per batch during the challenge test.

Analysis	Samples	Analysis time				Total
		T0	T1	T6	T20	
Detection and enumeration of *L. monocytogenes*						
Intrinsic properties: pH and aW; composition (%): moisture; fat; protein	C	9	9	9	9	36
	CL	9	9	9	9	36
	CLA	9	9	9	9	36
	CLB	9	9	9	9	36
Total						144

C: negative control samples; CL: positive control samples; CLA: samples added with protective culture A and *L. monocytogenes* broth culture of; CLB: samples added with protective culture B and *L. monocytogenes* broth culture; T0: after stuffing; T1: 24h after stuffing; T6: 6 days after stuffing; T20: 20 days after stuffing (end of ripening).

Table 2. *L. monocytogenes*, LAB, micrococci, Coagulase Negative Staphylococci and *Enterobacteriaceae* mean values (log_{10} CFU/g; $\bar{x}$ ± S.D.) in SFS samples during the challenge test.

Parameters	Samples	Analysis time			
		T0	T1	T6	T20
Listeria monocytogenes	C	0 3	0 4	0 3	0 3
	CL	1.04 ± 0.62 ^{b2}	1.81 ± 0.78 ^{a1}	1.50 ± 0.92 ^{ab1}	1.01 ± 1.22 ^{b1}
	CLA	1.36 ± 0.48 ^{a1}	0.33 ± 0.78 ^{b3}	0.34 ± 0.70 ^{b3}	0.16 ± 0.43 ^{b23}
	CLB	1.13 ± 0.64 ^{a12}	1.31 ± 0.63 ^{a2}	0.75 ± 0.74 ^{b2}	0.46 ± 0.63 ^{b2}
LAB	C	3.80 ± 1.14 ^{c4}	7.17 ± 0.59 ^{b2}	7.83 ± 0.42 ^{a3}	7.75± 0.46 ^{a1}
	CL	4.48 ± 0.93 ^{c3}	7.23 ± 0.47 ^{b2}	7.84 ± 0.50 ^{a3}	7.78 ± 0.36 ^{a1}
	CLA	6.56 ± 0.35 ^{b1}	8.16 ± 1.88 ^{a1}	8.24 ± 0.26 ^{a2}	7.70 ± 0.57 ^{a1}
	CLB	6.17 ± 0.47 ^{b2}	8.21 ± 0.34 ^{b1}	8.46 ± 0.34 ^{a1}	7.83 ± 0.32 ^{c1}
Micrococci and CNS	C	3.04 ± 0.74 ^{c3}	5.53 ± 0.50 ^{bc12}	5.99 ± 0.65 ^{ab1}	6.03 ± 0.73 ^{a1}
	CL	3.22 ± 0.70 ^{c3}	5.06 ± 1.30 ^{b3}	5.69 ± 0.46 ^{a1}	5.37 ± 0.83 ^{ab2}
	CLA	4.75 ± 0.77 ^{b1}	5.72 ± 1.30 ^{a1}	5.84 ± 0.66 ^{a1}	5.86 ± 0.64 ^{a1}
	CLB	4.07 ± 0.65 ^{b2}	5.49 ± 0.52 ^{a12}	5.73 ± 0.85^{a1}	5.80 ± 0.73 ^{a12}
Enterobacteriaceae	C	2.44 ± 0.83 ^{b1}	3.55 ± 0.50 ^{a12}	2.96 ± 1.50 ^{ab12}	3.79 ± 1.80 ^{a1}
	CL	2.58 ± 0.96 ^{c1}	3.47 ± 1.21 ^{ab12}	4.15 ± 0.96 ^{a1}	2.99 ± 1.94 ^{c12}
	CLA	2.78 ± 1.02 ^{a1}	3.01 ± 1.31 ^{a2}	2.78 ± 1.69 ^{a23}	1.87± 1.93 ^{b3}
	CLB	2.83 ± 0.57 ^{b1}	3.81 ± 0.75 ^{a1}	2.21 ± 1.77 ^{b3}	2.56 ± 1.70 ^{b23}

C: negative control samples; CL: positive control samples; CLA: samples added with protective culture A and L. monocytogenes broth culture of; CLB: samples added with protective culture B and L. monocytogenes broth culture; T0: after stuffing; T1: 24h after stuffing; T6: 6 days after stuffing; T20: 20 days after stuffing (end of ripening). Means in the same row with different superscript letter were significantly different (P< .05); means in the same column among biopreservative treatments with different superscript number were significantly different (P<0.05).

at T6 and T20. Results regarding *L. monocytogenes*, LAB, micrococci, CNS and *Enterobacteriaceae* counts in challenge test are reported in Table 2.

pH values (± SD) were 5.48±0.09 for the final products without protective culture (C and CL) and 5.30 ± 0.01 for those added with the culture (CLA and CLB). The a_w (± SD) was 0.83±0.01 for C and CL samples and 0.84±0.01 for CLA and CLB samples. The composition analysis of control samples (C and CL) showed, in the final products, average percentage (%) values of 33.06±2.25 for fat, 23.53±1.16 for protein, 38.51±4.24 for moisture. Samples added with protective cultures had mean percentage values (% ± SD) of 31.03±0.65 for fat, 37.74±1.05 for protein and 27.10±1.65 for moisture. pH, a_w and composition results (± SD) in SFS samples during the challenge test are reported in Table 3.

Use of protective culture into the production process

L. monocytogenes was never detected neither in samples with the addition of protective culture A nor in control samples analyzed at the end of ripening (qualitative and quantitative method). Analyses showed the presence of *Listeria spp.* (qualitative method) in control samples of n. 2 producing plants, in all production batches; however, in samples added with the protective culture A, *Listeria spp.* was never detected. *Enterobacteriaceae* mean levels were 2.31±1.64 $\log_{10}$ CFU/g in control samples and 2.14±1.62 in samples added with the protective culture. LAB, micrococci and CNS, showed higher mean levels in samples added with the protective culture, with values between 7-8 $\log_{10}$ CFU/g for LAB and 4-5 $\log_{10}$ CFU/g for micrococci and staphylococci. However, no significant differences (P>0.05) for LAB, micrococci and CNS mean levels were found between control samples and samples added with protective cultures. Results regarding *L. monocytogenes*, LAB, micrococci, CNS and *Enterobacteriaceae* counts are reported in Table 4. pH showed mean values (± SD) of 5.67±0.33 for samples without protective culture and of 5.46±0.19 for samples added with the culture. The a_w (± SD) was 0.891±0.03 for samples without protective culture and 0.897±0.02 for samples added with the protective culture. The composition analysis showed average percentage values (% ± SD) of 35.62±3.55 for moisture, 25.68±7.09 for fat, 32.01±6.04 for protein in samples with the protective culture. Similar results were found as regards the composition of samples without the protective culture: mean values of 35.95±4.50 for moisture, 26.63±7.03 for fat, and 31.89±6.66 for protein.

Table 3. pH, a_w and physico-chemical mean values ($\bar{x}$ ± S.D.) in SFS samples during the challenge test.

Parameters	Samples	Analysis time			
		T0	T1	T6	T20
pH	C	5.72 ± 0.15	5.63 ± 0.15	5.41± 0.35	5.54 ± 0.30
	CL	5.70 ± 0.14	5.62 ± 0.13	5.30 ± 0.32	5.43 ± 0.34
	CLA	5.69 ±0.13	5.41 ± 0.23	5.19 ± 0.31	5.31 ± 0.27
	CLB	5.69 ± 0.14	5.41 ± 0.12	5.19 ± 0.27	5.31 ± 0.27
a_w	C	0.975 ± 0.005	0.977 ± 0.004	0.946 ± 0.012	0.830 ± 0.040
	CL	0.979 ± 0.006	0.979 ± 0.004	0.948 ± 0.010	0.846 ± 0.047
	CLA	0.979 ± 0.003	0.979 ± 0.005	0.945 ± 0.009	0.846 ± 0.047
	CLB	0.978 ± 0.004	0.980 ± 0.004	0.945 ± 0.009	0.838 ± 0.047
Fats (%)	C	13.95 ± 2.21	14.22 ± 2.78	20.87 ± 2.59	34.65 ± 3.95
	CL	14.42 ± 2.65	13.94 ± 2.27	20.85 ± 1.30	31.47 ± 1.95
	CLA	14.49 ± 2.60	13.58 ± 1.76	20.66 ± 1.20	30.58 ± 1.42
	CLB	14.07 ± 2.06	13.31 ± 1.94	20.68 ± 1.81	31.49 ± 1.14
Moisture (%)	C	65.30 ± 1.62	64.76 ± 2.21	46.10 ± 0.82	20.53 ± 1.00
	CL	65.00 ± 1.84	65.05 ± 1.89	46.52 ± 0.15	26.54 ± 4.91
	CLA	65.01 ± 1.80	65.60 ± 1.57	46.71 ± 0.88	28.27 ± 4.63
	CLB	65.35 ± 35	65.70 ±1.69	46.45 ± 0.11	25.93 ± 5.23
Proteins (%)	C	19.44 ± 2.74	17.93 ± 1.40	27.30 ± 1.69	39.34 ± 6.43
	CL	18.17 ± 1.08	17.91 ± 1.14	26.82 ± 2.38	37.69 ± 7.10
	CLA	18.11 ± 1.08	18.15 ± 1.01	27.49 ± 1.62	37.00 ± 6.70
	CLB	18.22 ± 1.08	18.15 ± 1.01	27.46 ± 2.01	38.49 ± 7.89

C: negative control samples; CL: positive control samples; CLA: samples added with protective culture A and *L. monocytogenes* broth culture of; CLB: samples added with protective culture B and *L. monocytogenes* broth culture; T0: after stuffing; T1: 24h after stuffing; T6: 6 days after stuffing; T20: 20 days after stuffing (end of ripening).

Table 4. *L. monocytogenes*, LAB, micrococci, Coagulase Negative Staphylococci and *Enterobacteriaceae* mean values $\log_{10}$ CFU/g; $\bar{x}$ ± S.D. (positive samples/total) in SFS samples produced without and with the addition of protective culture A.

Producing plants	Samples	Parameters			
		Listeria monocytogenes	LAB	Micrococci and CNS	*Enterobacteriaceae*
P1	C	0	7.69 ± 0.17 (9/9)[1]	5.39 ± 0.37 (9/9)2	2.58 ± 1.14 (7/9)[1]
	A	0	7.53 ± 0.24 (9/9)[1]	5.93 ± 0.41 (9/9)[1]	2.35 ± 1.37 (7/9)[1]
P2	C	0	7.89 ± 0.09 (9/9)[2]	5.22 ± 0.28 (9/9)2	3.91 ± 0.43 (9/9)[1]
	A	0	8.29 ± 0.29 (9/9)[1]	5.55 ± 0.17 (9/9)[1]	3.06 ±0.25 (9/9)[1]
P3	C	0	7.89 ± 0.21 (9/9)[1]	5.77 ± 0.53 (9/9)[1]	0.67 ± 1 (3/9)[1]
	A	0	7.72 ± 0.42 (9/9)[1]	5.56 ± 0.35 (9/9)[1]	0.23 ± 0.67 (1/9)[1]

C: control samples; A: samples added with protective culture A. Means in the same column among biopreservative treatments with different superscript number were significantly different (P<0.05).

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pH, a_w and composition results (± SD) in SFS samples are reported in Table 5.

Discussion

There are several sources of *L. monocytogenes* contamination during the manufacturing process of fermented sausages. Raw meat may be contaminated at the slaughterhouse and during the production process of sausage in particular when good manufacturing and hygienic practices (GMP and GHP) are not correctly applied. Afterwards, the pathogen may survive during the shelf-life of the product due to its high tolerance to low pH conditions and high salt concentrations, especially if the standard hurdle technologies are not correctly applied, in particular when short ripening times are used (Meloni *et al*., 2014; Mureddu *et al.*, 2014; Mataragas *et al.*, 2015). Therefore, it is necessary to provide the SFS with protection against the growth of pathogens. A possible strategy is the use of bioprotective cultures aimed to control *L. monocytogenes* multiplication.

In our study, the *in vitro* analysis allowed to test the efficacy of different protective cultures and identify the ones with a stronger anti-*Listeria* activity.

The challenge test results showed that the use of protective culture A allowed to control *L. monocytogenes* growth in SFS samples: *L. monocytogenes* showed a reduction of 1 log approximately in the first 24 h after production. At the end of ripening, *L. monocytogenes* was not detectable using the qualitative method. The anti-*Listeria* efficacy was uniformly detected in all three production batches.

The species included in the composition of the bioprotective culture A were *Lactobacillus sakei*, *Pediococcus acidilactici*, *Staphylococcus carnosus* and *Staphylococcus carnosus* subsp. *utilis*. These microorganisms have previously shown to be able of inhibiting *L. monocytogenes* growth in meat products; in particular, *Lactobacillus sakei* demonstrated an antimicrobial effect due to its capacity to produce organic acids, hydrogen peroxide and bacteriocins (Zagorec *et al.*, 2017; Pedonese *et al.*, 2020). Hugas *et al.* (1995) demonstrated the ability of *L. sakei* to inhibit the growth of *L. monocytogenes* in a model sausage system and in dry fermented sausages. Similar results have been observed by other Authors with regard to the bacteriocins produced by *Pediococcus acidilactici* (Nielsen *et al.*, 1990; Nieto-Lozano *et al.*, 2006). *S. carnosus* is not usually considered able to produce specific growth inhibitors against pathogens and its addition to meat products is usually aimed at improving the flavor and color (Tjener *et al.*, 2004; Janssens *et al.*, 2013). However, co-cultures of *S. carnosus* and *L. sakei* can assure the quality of raw-cured meat products based on metabolic functions of both species. Tjener *et al.* (2004) and Leroy *et al.* (2005) reported that *L. sakei* co-cultured with *S. carnosus* in meat products generated an effective fermentation process in sausage manufacturing in which *S. carnosus* plays a fundamental role as a competitor, especially in the first 48 h of fermentation, while *L. sakei* maintains its effectiveness during the following stages of ripening. Therefore, in this study, the observed reduction in cell concentration of *L. monocytogenes* is most likely due to the mutualistic relationship between the microbial components of the protective culture used, the production of organic compounds and bacteriocins. This finding is concordant with what was reported by Blanco-Lizarazo *et al.* (2016), who performed an *in vitro* test to evaluate the microbial interactions between *L. monocytogenes*, *L. sakei* and *S. carnosus* in conditions that mimic fermentation in meat; the Authors concluded that *L. sakei* and *S. carnosus* can successfully be used in protective cultures thanks to their ability to dominate the competition for nutrients and control *L. monocytogenes* growth.

As regards pH and a_w evaluated during the challenge test, values reported were consistent with what was found by other Authors and typical of the product (Meloni *et al*., 2013; Piras *et al.*, 2019). Significant differences were found in samples added with the protective cultures compared to control samples (negative control) starting from T1 ($P<0.01$), at T6 ($P<0.05$) and also at T20 ($P<0.05$). Moreover, in samples added with protective cultures A and B, a slight decrease of pH mean values was observed at analysis times T6 and T20, which is compatible with an increase in lactic acid production by the cultures' microorganisms (LAB and Coagulase Negative Staphylococci). This decrease was statistically significant ($P<0.01$) in CLA samples between T0 and T1, and in CLB samples between T0 and T1 and T1 and T6 ($P<0.01$). In this regard, Leroy and De Vuyst (2005) indicated that *L. sakei* had a higher production rate of lactic acid, compared with other LABs with a bioprotection potential, such as *L. curvatus* and *L. amylovorus*. At the end of ripening time (T20) a slight increase in pH mean values,, was detected in all kind of samples, and was significant in CLB samples ($P<0.01$). Such increase is most likely due to the proteolytic effect of yeasts and molds, which play an important role in proteolysis and lipolysis to develop aroma during the manufacturing process and use lactic acid as substrate at the end of the drying step (Thévenot *et al.*, 2005). However, the composition analyses have shown that the use of protective cultures in the production process of SFS does not affect the typical composition characteristics of the product.

Following the results obtained in the challenge test, the protective culture identified as the most effective against the growth and persistence of *L. monocytogenes* was supplied to three producing plants in order to include the selected culture into their standard production process and evaluate its effect on the chemical, physical and microbiological characteristics of the SFS at the end of ripening. The use of protective cultures has proved to be easy and quick

Table 5. pH, aw and physico-chemical mean values ($\bar{x}$ ± S.D.) in SFS samples produced without and with the addition of protective culture A.

Producing plants	Samples	Parameters						
		pH ± SD	Aw ± SD	Ashes (%)	Fats (%)	Proteins (%)	NaCl (%)	Moisture (%)
P1	C	5.76 ± 0.20	0.867 ± 0.03	4.74 ± 0.62	20.50 ± 4.60	39.43 ± 5.62	5.26 ± 0.38	34.63 ± 6.01
	A	5.67 ± 0.22	0.877 ± 0.03	4.63 ± 0.51	18.40 ± 1.64	38.69 ± 4.48	5.06 ± 0.36	37.70 ± 3.45
P2	C	5.70 ± 0.18	0.921 ± 0.006	5.30 ± 0.31	25.09 ± 0.91	26.85 ± 0.84	4.05 ± 0.10	40.53 ± 1.57
	A	5.43 ± 0.15	0.926 ± 0.004	5.28 ± 0.29	26.08 ± 0.87	26.92 ± 0.65	4.04 ± 0.10	40.07 ± 1.45
P3	C	5.32 ± 0.08	0.885 ± 0.032	4.27 ± 0.70	34.31 ± 6.34	29.38 ± 1.33	4.57 ± 0.48	31.69 ± 6.72
	A	5.30 ± 0.07	0.889 ± 0.040	4.30 ± 0.85	32.56 ± 6.18	30.44 ± 2.53	4.50 ± 0.42	33.08 ± 7.42

C: control samples; A: samples added with protective culture A.

(freeze-dried mixture of selected microorganisms) to use; furthermore, it does not require modifications of the regular production process steps, as the culture can simply be added into the mixture before the stuffing phase. The economic commitment for the producing plants is therefore limited and adequate to what is required by the Food Sector Operators. The microbiological analyses at the end of ripening (ca. 20 days) showed that, in the samples added with the protective culture A, neither *L. monocytogenes* nor *Listeria spp*. were detected (quantitative and qualitative method). This data confirms the results obtained by the challenge test, demonstrating that the use of protective cultures represents a technological innovation capable of eliminating *L. monocytogenes* or reducing its levels within the limits set by European Regulations (Reg. CE n.2073/2005) in meat products. Microbiological analyses aimed at determining the prevalence of non-pathogenic microorganisms showed that the addition of the protective culture A in the production process of the three processing plants, influenced *Enterobacteriaceae* counts levels that were always lower in samples added with the protective cultures, although this difference was never significant. Moreover, *Enterobacteriaceae* levels were reduced during ripening until non-detectable levels in some samples at the end of processing after the addiction of the protective culture. These results are consistent with those reported by Martìn *et al.* (2021), who demonstrated the decrease in *Enterobacteriaceae* counts until non-detectable levels at the end of ripening in a dry-cured fermented sausage inoculated with *L. sakei*.

As regard physicochemical- parameters evaluated after application in the production process, it was shown that while the presence of the protective culture does not affect final a_w values, it does influence, although not significantly (P>0.05), the final pH values with an acidifying effect, compatible with an increase in the production of lactic acid by the protective cultures' microorganisms. The technological innovation has therefore allowed stronger acidification of the product, which represents a further obstacle for the growth of pathogens, improving the product from a safety point of view. The composition analysis, carried out using NIT technology, showed similar values in samples with and without protective culture, highlighting how the inclusion of this technological innovation in the SFS production process does not affect the typical composition characteristics of the finished product.

The results of this project validated the efficacy, both *in vitro* and in the product, of some protective cultures to reduce or inhibit the growth of *L. monocytogenes*. Furthermore, the data obtained will allow SFS producing plants to define, with scientific evidence, the healthiness and hygiene of the product added with specific protective cultures.

Conclusions

The use of protective cultures within the standard production process of the SFS represents a significant technological innovation for the producing plants that allows controlling, for the whole commercial life, the possible growth of *L. monocytogenes* up to values that are not compatible with the current legislation (Reg. 2073/2005), ensuring a high hygienic and sanitary quality of the product. It has been demonstrated that protective cultures control the growth of *L. monocytogenes* without altering the typical composition, microflora and physico-chemical characteristics of the SFS. Finally, this techological innovation is simply and ready to use, it is not expensive for the Food Business Operators and does not require modification and/or implementation of the production process, since protective cultures can be directly added during the normal mixing step of fat, meat, spices and other ingredients.

References

ANSES, 2021. EURL Lm Technical guidance document on challenge tests and durability studies for assessing shelf-life of ready-to-eat foods related to L. monocytogenes, version 4 of 1 July 2021.

Autio T, Sateri T, Fredriksson-Ahomaa M, Rahkio M, Lunden J, Korkeala H, 2000. Listeria monocytogenes contamination pattern in pig slaughterhouses. J Food Prot 63:1438-42.

Blanco-Lizarazao CM, Sotelo-Dìaz I, Llorente-Bosquetes A, 2016. In vitro modelling of simultaneous interactions of Listeria monocytogenes, Lactobacillus sakei, and Staphylococcus carnosus. Food Sci Biotechnol 25:341-8.

Boscher E, Houard E, Denis M, 2012. Prevalence and Distribution of Listeria monocytogenes Serotypes and Pulsotypes in Sows and Fattening Pigs in Farrow-to-Finish Farms. J Food Prot 75:889–95.

Chasseignaux E, Gerault P, Toquin M, Salvat G, Colin P, Ermel G, 2002. Ecology of Listeria monocytogenes in the environment of raw poultry meat and raw pork meat processing plants. FEMS Microbiol Lett 210:271-5.

Cosentino S, Fadda ME, Deplano M, Melis R, Pomata R, Pisano MB, 2012. Antilisterial activity of nisin-like bacteriocin-producing Lactococcus lactis subsp. lactis isolated from fermented Sardinian dairy products. J Biomed Biotechnol 2012, ID 376428 doi:10.1155/2012/376428

Davidson PM, Techathuvanan C, 2015. The use of natural antimicrobials in food, chapter in: Taylor TM, Handbook of natural antimicrobials for food safety and quality, Woodhead Publishing, 2015.

European Food Safety Authority and European Centre for Disease Prevention and Control, 2021. The European Union One Health 2020 Zoonoses Report. EFSA J 19:6971.

European Food Safety Authority, 2018. Listeria monocytogenes contamination of ready-to-eat foods and the risk for human health in the EU. EFSA J 16:5134

Esteban JI, Oporto B, Aduriz G, Juste RA, Hurtado A, 2009. Fecal shedding and strain diversity of Listeria monocytogenes in healthy ruminants and swine in northern Spain. BMC Vet Res 2009;5:2.

European Commission (EC). Commission regulation (EC) no 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. In: Off. J. Eur. Union 2005, L 338/1, 22/12/2005.

European Commission, 2008. Regulation (EC) no 1333/2008 of the European Parliament and of the council of 16 December 2008 on food additives. In: Official Journal, L 354/16, 31/12/2008.

Fosse J, Seegers H, Magras C, 2009. Prevalence and Risk Factors for Bacterial Food□Borne Zoonotic Hazards in Slaughter Pigs: A Review. Zoon Publ Health 56:429–54.

Greco M, Mazzette R, De Santis EPL, Corona A, Cosseddu AM, 2005. Evolution and identification of lactic acid bacteria isolated during the ripening of Sardinian sausages. Meat Sci 69:733–9.

Hugas M, Garriga M, Aymerich MT, Monfort JM, 1995, Inibition of Listeria in dry fermented sausages by the bacteriocinogenic Lactobacillus sakei CTC494. J Appl Microbiol 75:322-30.

ISO, 1992. Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of mesophilic lactic acid bacteria — Colony-count technique at 30 degrees C. ISO Norm 15214:1998. International Standardization

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Organization ed., Geneva, Switzerland.

ISO, 2017. Microbiology of the food chain - Horizontal method for the detection and enumeration of Listeria monocytogenes and of Listeria spp. - Part 1: Detection method. ISO Norm 11290-1:2017. International Standardization Organization ed., Geneva, Switzerland.

ISO, 2017. Microbiology of the food chain - Horizontal method for the detection and enumeration of Listeria monocytogenes and of Listeria spp. - Enumeration method. ISO Norm 11290-2:2017. International Standardization Organization ed., Geneva, Switzerland.

ISO, 2017. Microbiology of the food chain - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination - Part 1: General rules for the preparation of the initial suspension and decimal dilutions. ISO Norm 6887:2017. International Standardization Organization ed., Geneva, Switzerland.

ISO, 2017. Microbiology of the food chain - Horizontal method for the detection and enumeration of Enterobacteriaceae - Part 2: Colony-count technique. ISO Norm 21528:2017. International Standardization Organization ed., Geneva, Switzerland.

ISO, 2020. Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of Salmonella - Part 1: Detection of Salmonella spp. ISO Norm 6579-1:2020. International Standardization Organization ed., Geneva, Switzerland.

Italian Republic, 2020. Aggiornamento dell'Elenco nazionale dei prodotti agroalimentari tradizionali ai sensi del Decreto Ministeriale 8 settembre 1999, n.350. Regolamento recante norme per l'individuazione dei prodotti tradizionali di cui all'articolo 8, comma 1, del decreto legislativo 30 aprile 1998, n. 173. Prot. Uscita N.0001375 del 10/02/2020

Janssens M, Myter N, De Vuyst L, Leroy F, 2013. Community dynamics of coagulase-negative staphylococci during spontaneous artisan-type meat fermentations differ between smoking and moulding treatments. Int J Food Microbiol 166:168-75.

Kanuganti SR, Wesley IV, Reddy PG, McKean J, Hurd HS, 2002. Detection of Listeria monocytogenes in pigs and pork. J Food Prot 65:1470–4.

Leroy F, De Vuyst L, 2005. Simulation of the effect of sausage ingredients and technology on the functionality of the bacteriocin-producing Lactobacillus sakei CTC 494 strain. Int J Food Microbiol 100:141-52.

Ryu J, Park SH, Yeom YS, Shrivastav A, Lee SH, Kim YR, Kim HY, 2013. Simultaneous detection of Listeria species isolates from meat processed foods using multiplex PCR. Food Control 32:659-64.

Mangia NP, Murgia MA, Garau G, Deiana P, 2007. Microbiologia e valutazione igienico-sanitaria della salsiccia sarda. Ind Aliment Italy 46:533-6.

Maragkoudakis PA, Mountzouris KC, Psyrras D, Cremonese S, Fischer J, Cantor MD, Tsakalidou E, 2009. Functional properties of novel protective lactic acid bacteria and application in raw chicken meat against Listeria monocytogenes and Salmonella enteritidis. Int J Food Microbiol 130:219-26.

Martìn I, Rodrìgez A, Sànchez-Montero L, Padilla P, Còrdoba JJ, 2021. Effect of the Dry-Cured Fermented Sausage "Salchichón" Processing with a Selected Lactobacillus sakei in Listeria monocytogenes and Microbial Population. Foods 10:856.

Mataragas M, Rantsioua K, Alessandria V, Cocolin L, 2015. Estimating the non-thermal inactivation of Listeria monocytogenes in fermented sausages relative to temperature, pH and water activity. Meat Sci 100:171-8.

Meloni D, 2015. Presence of Listeria monocytogenes in Mediterranean-Style Dry Fermented Sausages. Foods 4:34-50.

Meloni D, Consolati SG, Mazza R, Mureddu A, Fois F, Piras F, Mazzette R, 2014. Presence and molecular charachterization of the major serovars of Listeria monocytogenes in ten Sardinian fermented sausage processing plants. Meat Sci 97:443-50.

Meloni D, Piras F, Mureddu A, Fois F, Consolati SG, Lamon S, Mazzette R, 2013. Listeria monocytogenes in five Sardinian swine slaughterhouses: Prevalence, Serotype and Genotype Characterization. J Food Prot 76:1863–7.

Mureddu A, Mazza R, Fois F, Meloni D, Bacciu R, Piras F, Mazzette R, 2014. Listeria monocytogenes persistence in ready-to-eat sausages and in processing plants. Ital J Food Saf 3:12–5.

Neri D, Antoci S, Iannetti L, Ciorba AB, D'Aurelio R, Del Matto I, Di Leonardo M, Giovannini A, Prencipe VA, Pomilio F, Santarellia GA, Miglioratia G, 2019. EU and US control measures on Listeria monocytogenes and Salmonella spp. in certain ready-to-eat meat products: An equivalence study. Food Control 96:98-103.

Nielsen JW, Dickson JS, Crouse JD, 1990. Use of a bacteriocin produced by Pediococcus acidilactici to inhibit Listeria monocytogenes associated with fresh meat. Appl Environ Microbiol 56:2142-5.

Nieto-Lozano JC, Reguera JI, Peláez-Martínezb MC, Hardisson de la Torre A, 2006. Effect of a bacteriocin produced by Pediococcus acidilactici against Listeria monocytogenes and Clostridium perfringens on Spanish raw meat. Meat Sci 72:57-61.

Peccio A, Auto T, Korkeala R, Rosmini R, Trevisani M, 2003. Listeria monocytogenes occurrence and characterization in meat-producing plants. Lett Appl Microbiol 37:234-8.

Pedonese F, Torracca B, Mancini S, Pisano S, Turchi B, Cerri D, Nuvoloni R, 2020. Effect of a Lactobacillus sakei and Staphylococcus xylosus protective culture on Listeria monocytogenes growth and quality traits of Italian fresh sausage (salsiccia) stored at abusive temperature. Ital J Anim Sci 19:1363–74.

Piras F, Spanu C, Mocci AM, Demontis M, De Santis EPL, Scarano C, 2019. Occurrence and traceability of Salmonella spp. in five Sardinian fermented sausage facilities. Ital J Food Saf 8:8011.

Thévenot D, Delignette-Muller ML, Christieans S, Vernozy-Rozand C, 2005. Fate of Listeria monocytogenes in experimentally contaminated French sausages. Int J Food Microbiol 101:189–200.

Thévenot D, Dernburg A, Vernozy-Rozand C, 2006. An updated review of Listeria monocytogenes in the pork meat industry and its products. J Appl Microbiol 101:7-17.

Tjener K, Stahnke LH, Andersen L, Martinussen J, 2004. The pH-unrelated influence of salt, temperature and manganese on aroma formation by Staphylococcus xylosus and Staphylococcus carnosus in a fermented meat model system. Int J Food Microbiol 97:31-42.

Young NWG, O'Sullivan GR, 2011. The influence of ingredients on products stability and shelf life. In: Kilcast D, Subramaniam P. Food and Beverage Stability and Shelf Life, Woodhead Publishing, 2011.

Zagorec M, Champomier-Vergès MC, 2017. Lactobacillus sakei: A Starter for sausage fermentation, a protective culture for meat products. Microorganisms 5:56.