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Effect of a novel starter culture and specific ripening conditions on microbiological characteristics of nitrate-free dry-cured pork sausages

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ABSTRACT

This study evaluated the effect of a 'formulation of selected dairy starter cultures and commercial probiotics', associated with 'specific ripening conditions' in the production of nitrite and nitrate-free, low-acid, fermented pork sausage, produced in a small-scale plant in Umbria (Italy), and their effect on microbiological, physico-chemical and sensorial properties of the products. The following conditions were investigated throughout the experiment: (i) the use of the 'formulation of selected dairy starter cultures and commercial probiotics' *vs* no starter; (ii) the use of 'specific ripening conditions' *vs* 'classic ripening conditions'; (iii) the use of nitrate *vs* nitrite and nitrate-free sausages. Sensory properties were improved and pathogen control was greater in salami to which a 'formulation of selected dairy starter cultures and commercial probiotics' had been added, especially under 'specific ripening conditions'. The proposed formulation, together with the specific ripening conditions, greatly inhibited the growth of undesirable microbiota, reduced the isolation rate of pathogens and preserved the acceptability of the salami.

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Cold-adapted microorganisms; dry-cured sausage; *Enterococcus faecium*; *Lactococcus lactis* ssp. *lactis*; *Lactobacillus casei* ssp. *casei*; starter culture

Introduction

Salame nostrano is a typical, dry, fermented sausage from Umbria, Italy. The basic ingredients of *salame nostrano* are lean pork, pork fat, salt, pepper and garlic. The production of *salame nostrano* is characterised by the following steps: receipt and selection of raw meat, storage, dissection and cutting, cooling, weighing and pre-grinding, preparation and weighing of the ingredients, starter culture addition (when applicable), preparation of natural casing, grinding, blending and sacking (sausage making), fermentation, drying, ripening and final storage. A series of well-known hurdles (Leistner & Gorris 1994) have been associated with low drying, ageing and ripening conditions and even the fermentation phase did not exceed 12 °C. Over recent years, there has been growing consumer interest in salami produced with lower concentrations of additives, and *salame nostrano* is an example of how to integrate locally made products with safer technologies (Cenci Goga et al. 2008). If not carefully addressed, the removal of nitrites and nitrates from salami production can have two drawbacks: a lack of control over *Clostridium botulinum* toxin and inconsistent colour formation and stability (Zarringhalami et al. 2009). Several alternatives to nitrite have been tested (Pegg & Shahidi 2000) for their antimicrobial action and many different methods have been proposed to enhance colour intensity and uniformity, including lactic acid bacteria (LAB) and natural colourant (Zhang et al. 2007; Zarringhalami et al. 2009). The original recipe for *salame nostrano* does not include milk, milk powder, sugars or additives. Safety is ensured by the low fermenting and ripening temperature and colour is improved by the decrease in redox potential, which is a consequence of the multiplication of autochthonous, lactic acid bacteria, especially the naturally occurring cold-adapted microorganisms (Haga et al. 1994).

Nowadays, consumers prefer salami with a lower acidity, because the speed of fermentation can negatively affect the aroma in the product and the faster the acidification, the fewer the colour and aroma-enhancing enzymes produced (Marco et al. 2008; Barbut 2010). The particular ageing and ripening conditions for *salame nostrano* in Umbria, Italy, have been

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handed down for centuries. In our previous works, we discovered that the unusual ageing and ripening conditions are responsible for the selection and growth of naturally occurring, cold-adapted microorganisms, especially among the *Lactobacillus*, *Lactococcus* and *Enterococcus* genus (Cenci Goga et al. 2008; Cenci Goga et al. 2012). Since the 1980s, researchers have postulated that enterococci may contribute to the development of the typical sensory properties of certain fermented meat products, such as salami (Holley et al. 1988). In the food environment, *Enterococcus* spp. are mostly associated with dairy products and occasionally with meat. *Enterococcus* strains have been isolated from numerous, diverse non-dairy environments, including fermented sausages (Cintas et al. 1997). In fact, Enterococci are frequently detected at high levels in several kinds of fully ripened salami with no alteration in the sensorial properties (Dellapina et al. 1994). Enterococci fit within the general definition of lactic acid bacteria. Their association with the human environment and their beneficial interaction, both in food and in the human intestinal tract, combined with the long tradition of lactic fermented foods in many cultures, has led to their incorporation in several probiotic formulations, even though this genus is not 'generally recognized as safe' (GRAS).

In this work, an association of cold-adapted microorganisms (a commercial strain of *E. faecium* and three strains of lactic acid bacteria of dairy origin), together with low ripening conditions throughout fermentation, drying and ripening, was studied to investigate the effect on the microbiological, physico-chemical and sensory characteristics of *salame nostrano*, an Italian dry-cured sausage made of pork meat.

Materials and methods

Starter cultures

Bacterial strains used as starter cultures were taken from the collection of the Laboratorio di Ispezione degli Alimenti di Origine Animale: *Lactococcus lactis ssp. lactis*, strain 340; *L. lactis* ssp. *lactis*, strain 16; *Lactobacillus casei* ssp. *casei*, strain 208 and *Enterococcus faecium* UBEF-41. Lactococci and lactobacillus were isolated from traditional cheeses, manufactured in small-scale dairy plants in Umbria, Italy, whereas *Enterococcus faecium* UBEF-41 was isolated from the dietary supplement Enterelle formulation by Bromatech Srl. (Milan, Italy). The LAB of choice used in this study are the result of previous investigations and were chosen on the basis of an *in vitro* challenge test with selected pathogens and after acidifying activity tests, and growth-test at low temperatures (Cenci Goga et al. 2012; Cenci-Goga et al. 2015). Characterisation of *Enterococcus faecium* UBEF-41 is available at Unique Biotech Ltd. (Anonymous 2014), moreover the strain has been further studied before inclusion in the starter formulation by sequencing (see below) and testing for antibiotic susceptibility (see below). Before salami production, freeze-dried strains of the starter cultures were grown aerobically in Nutrient Broth (NB, Oxoid CM0001, Basingstoke, UK) at 37 °C for 24 h. Each strain was then sub-cultured in Skim Milk (BD Difco, Franklin Lakes, NJ, 232100) at 37 °C for 24 h. The total viable cells (TVC) count (on Nutrient Agar, NA, Oxoid CM0003, incubated at 37 °C on air for 24 h) at 24 h was approximately 1×10^9 cfu ml^{-1}.

Characterization of the commercial probiotic strains enterococcus faecium UBEF-41

Identification by 16S rRNA sequencing

The universal primers for eubacteria P27f (5′-GAG AGT TTG ATC CTG GCT CAG-3′) and P1495r (5′-CTA CGG CTA CCT TGT TAC GA-3′) were used to amplify a 16S rRNA gene segment. According to the amplification protocol reported by Osimani et al. (2015), the PCR conditions were 1 cycle at 95 °C for 5′, 35 cycles at 94 °C for 30″, 50 °C for 45″ and 72 °C for 2′, and final extension of 72 °C for 10′. The amplicons were sent to Microgem Lab (University of Naples, Italy) for purifying and sequencing. The 16S-rRNA sequencing around 1100 bp were analyzed through Sanger Sequencing and then compared with the closest relatives of the sequences obtained with those in the GenBank database using the BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/) from National Center of Biotechnology Information (NCBI). Isolates were allocated to a given species on percentages of sequence identity and on visual inspection of the concordance, using DIALIGN software (http://dialign.gobics.de/chaos-dialign-submission).

Antibiotic susceptibility profiles by disk diffusion susceptibility test

Antimicrobial resistance pattern was determined against a panel of 10 antibiotics (Ampicillin 10 μg/disk; Methicillin 5 μg/disk, Amoxycillin/Clavulanic Acid 20 + 10 μg/disk; Erythromycin 15 μg/disk; Vancomycin 30 μg/disk; Cyprofloxacin 5 μg/disk; Nalidixic acid 30 μg/disk; Tetracycline 30 μg/disk; Kanamycin 30 μg/disk; Gentamicin 10 μg/disk; Neomycin 30 μg/disk; Streptomycin 10 μg/disk), using the disk diffusion test with antimicrobial susceptibility disks

(Thermo Scientific, Oxoid, Basingstoke, UK). The antibiotics used for this study were cell wall synthesis, nucleic acid synthesis, folate synthesis and protein synthesis inhibitors. The agar disk diffusion method was performed on Mueller Hinton agar (Oxoid, CM0337), according to Clinical and Laboratory Standards Institute (CLSI 2011) guidelines and results were interpreted according to CLSI standard criteria (CLSI 2011).

Sausage manufacturing and ripening conditions

Nine repetitions of the trials were made on nine different days. Eight batches were produced at each repetition: with and without a 'novel formulation of starter cultures', with and without the 'addition of nitrates', under 'specific ripening conditions' and under 'classic ripening conditions'. All batches were prepared according to a typical, local recipe by an experienced craftsman at the small-scale processing plant of the Laboratorio di Ispezione degli Alimenti di Origine Animale.

The meat for each repetition came from the same farm and all animals were '*suino pesante italiano tipico*' [*typical Italian heavy pigs*] with a live weight of over 150 kg, and an age of over 9 months.

The meat formulation consisted of pork shoulder and boneless belly (80%) and back fat (20%). The meat was minced and mixed with NaCl (30 g kg^{-1}), pepper (5 g kg^{-1}), garlic (2 g kg^{-1}), white wine (2 ml kg^{-1}), sodium nitrate (E 251) (150 mg kg^{-1}), when applicable. The formulation of selected dairy starter cultures and commercial probiotics used for the inoculation of salami batter, when applicable, was prepared as described above. The formulation of selected dairy starter cultures and commercial probiotics cultures was added, where appropriate, to achieve initial levels of 10^7 cfu g^{-1}, with a cocci:bacilli:enterococci ratio of 2:1:1. All added cultures had reached a stationary phase of growth. For each of the nine repetitions, the meat was chopped in a cutter and once all the ingredients had been added, it was mixed in a blender and divided into different batters according to the experimental protocol. The batters were stuffed under vacuum into natural swine casing with a 30 mm diameter, commencing with the mixture without a starter culture and without nitrates. Individual sausages were formed by tying the casing at approximately 100 mm intervals to give sausages, which were 30 mm in diameter and 10 cm in length. After stuffing, the sausages were suspended in an ARC 180 cold storage chamber (Frigoimpianti, Bastia Umbra, Italy) for fermentation and the various ripening programmes were applied, as shown in Table 1. Figures 1 and 2 show the actual temperature and relative humidity recorded by the chamber data-logger).

Table 1. Fermentation and ripening conditions used for *salame nostrano* production.

	Classic ripening conditions (NO SPEC)		Specific ripening conditions (SPEC)	
Time	T, °C	RH, %	T, °C	RH, %
Hour 0–5	6	65	6	65
Hour 5–10	12	65	12	65
Hour 10–15	22	80	20	80
Hour 15–20	20	85	18	90
Hour 20–24	18	90	18	90
Day 1–6	16	90	12	80
Day 7–21	15	85	11	85

T: temperature; RH: relative humidity.

Sampling procedure

Samples for analysis were taken at time 0 (meat mixture immediately after stuffing), day 3 (middle fermentation), day 7 (end-fermentation), day 13 (middle ripening) and day 21 (end-ripening process). At each sampling time, three sausages per group were analysed and three subsamples were taken from each sausage. All analyses were conducted within 30 min after sampling.

Microbiological analysis

For the analysis of salami, approximately 25 g of sample were aseptically transferred to 225 ml of sterile, buffered, peptone water (Oxoid, CM1049), and homogenised in a stomacher (PBI International, Milan, Italy) for 1 min at low speed and 1 min at high speed at room temperature. Serial decimal dilutions in buffered peptone water were prepared and triplicate 1 ml or 0.1 ml samples of appropriate dilutions were poured or spread on the total count and on selective agar plates. The total aerobic mesophilic microbiota was determined on plate count agar (Oxoid), incubated at 30 °C for 72 h; lactic acid cocci spp. on M17 agar (Oxoid, CM0785) to which 10% v/v lactose was added, incubated at 37 °C for 48 h; Lactobacillus spp. on de Man, Rogosa, Sharpe (MRS) Agar (Oxoid) acidified to pH 5.5, incubated at 30 °C for 72 h under anaerobic conditions (Gas generating kit, Oxoid, BR0038); Enterococci on Slanetz and Bartley (SB) Agar (Oxoid, CM0377), incubated at 37 °C for 48 h; *Enterobacteriaceae* on Violet Red Bile Glucose (VRBG) Agar (Oxoid, CM0485), overlaid with 5 ml of the same medium and incubated at 37 °C for 24 h; coliform organisms on Violet Red Bile Lactose (VRBL) Agar (Oxoid, CM0107), overlaid with 5 ml of the same medium and incubated at 30 °C for

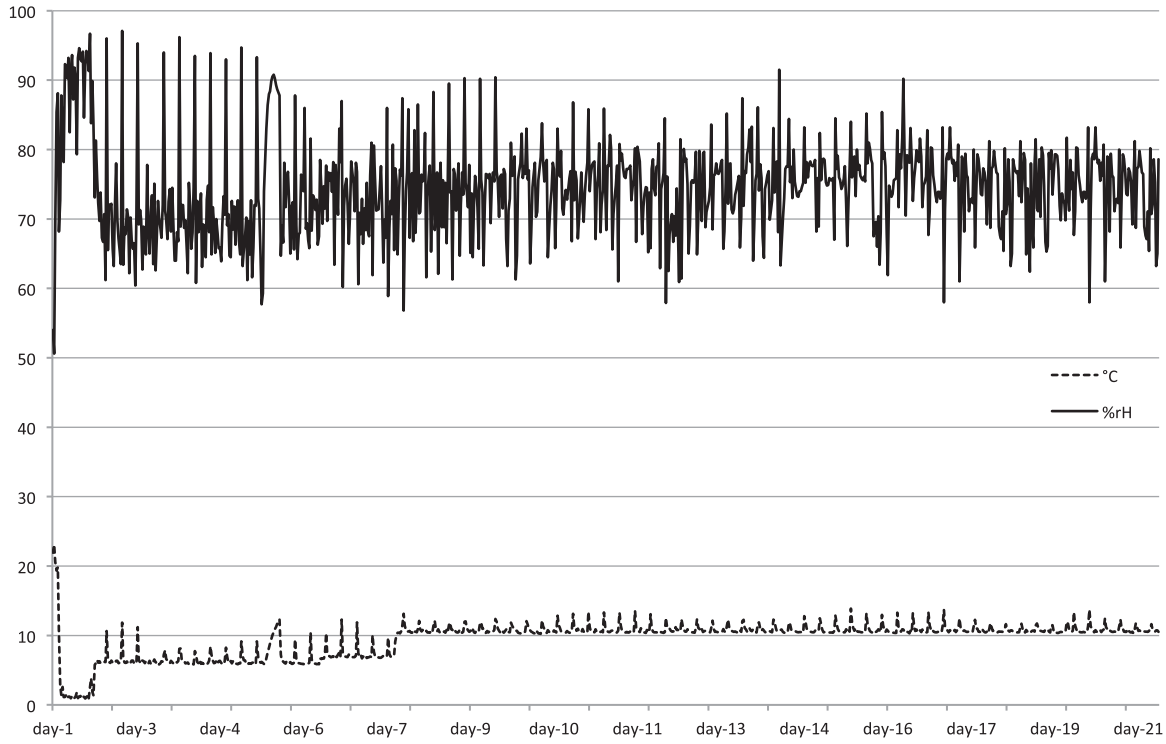

Figure 1. Temperature (---) and relative humidity (—) recorded throughout fermentation and ripening of *salame nostrano* under specific ripening conditions (SPEC).

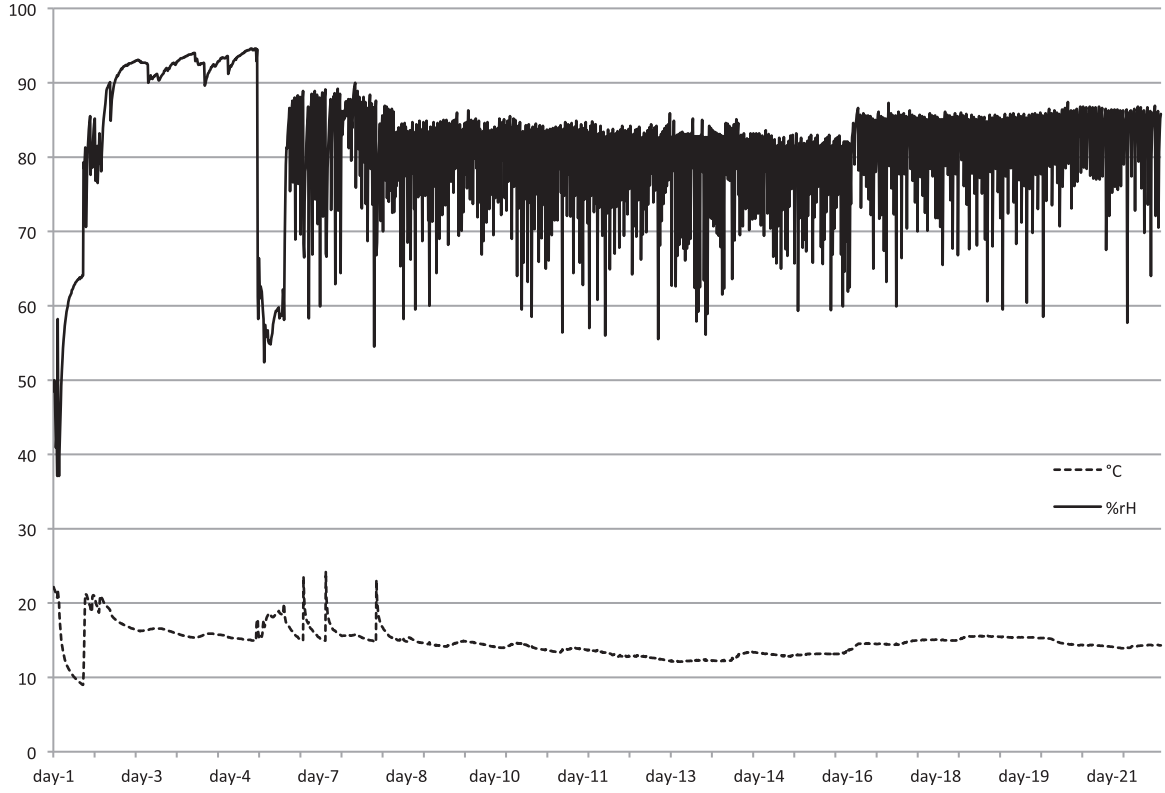

Figure 2. Temperature (---) and relative humidity (—) recorded throughout fermentation and ripening of *salame nostrano* under classic ripening conditions (NO SPEC).

24 h. For *Enterobacteriaceae* and coliform organisms, in case of counts below the detection limit as described in the analysis of results section, counts were repeated with the most probable number (MPN) method with brilliant green bile (2%) broth (Oxoid, CM0031). *Pseudomonas* spp. was determined on Pseudomonas agar base (Oxoid, CM0559) added with Pseudomonas CFC supplement (Oxoid, SR0103), incubated at 25 °C for 48 h. *Staphylococcus* spp. on Rabbit Plasma Fibrinogen (RFP) Agar (Oxoid, CM0961), with RFP Supplement (Oxoid, SR0122), incubated at 37 °C for 48 h, all black shining colonies were counted with and without halo. Presence/absence (P/A) analyses were conducted for sulphite reducing *Clostridium* spp., *C. botulinum*, *Listeria* spp, non-sorbitol-fermenting *Escherichia coli*, *Salmonella* spp. *Staphylococcus aureus*. Sulphite-reducing *Clostridium* spp. were determined on Perfringens Agar (Oxoid, CM0543) to which Perfringens selective supplement A (Oxoid, SR0076) and B (Oxoid, SR0077) were added. The ISO 6579 method was used (ISO 2007) to isolate *Salmonella* spp. The ISO 11290 method (ISO 1996) was used to isolate *Listeria* spp. and particularly *Listeria monocytogenes*. *C. botulinum* was determined on *C. botulinum* agar base with egg yolk emulsion and *C. botulinum* antimicrobic supplement (Biolife, Milan, Italy) incubated in anaerobic conditions for 48 h at 35 °C. Non-sorbitol-fermenting *E. coli* was determined on Sorbitol MacConkey agar with a cefixime-tellurite supplement (Oxoid) incubated on-air at 35 °C for 24 h. For *S. aureus*, after incubation in Rabbit Plasma Fibrinogen (RFP) Agar, all black, convex colonies with an opaque halo were examined under the microscope after Gram staining, and tested for thermonuclease production. Thermonuclease determination was performed according to the method described by Ibrahim (1981).

Physico-chemical and chemical analysis

With the sampling scheme used for microbiological analysis (eight salami productions, nine repetitions, three sausages per group at each sampling point, three subsamples per sausage), the salami were macerated in a chopper and appropriate portions of the homogenised sample were used for chemical analysis. A Double Pore F electrode (Hamilton Company, Reno, NV) hooked to an Eutech pH 2700 (Eutech Instrument Europe B.V.) was used to measure the pH by mixing 10 g of sausage with 90 ml of distilled water. Water activity (a_w), was measured with a HygroLab 3 dewpoint hygrometer (Rotronic, Huntington, NY) using the method described by the Association of Official Analytical Chemists (AOAC 1980). Calibration was performed using five saturated solutions of known a_w. Chemical composition and NaCl content were determined according to AOAC methods (AOAC 2000). a_w and pH were determined through ripening, whereas chemical composition and NaCl content were determined on the day of stuffing and at the end of the ripening process.

Sensory evaluation

Few days after the end of ripening, *Salame nostrano* was assessed for differences in odour and taste between the groups. Comparisons included all batches, except those positive for pathogens. Each comparison was evaluated using a triangle test (ISO 2004). Methods have already been described elsewhere (Cenci Goga et al. 2008; Cenci Goga et al. 2012). Briefly, samples (a 4 mm slice of salami) were placed on the day of testing into semi opaque plastic cups with plastic lids, assigned a random 3-digit code, and stored and maintained at room temperature until sensory testing. Samples were allowed to stand for at least 20 min before testing. All combinations of the two samples were presented within each sensory session for an equal number of times. Two sets of three samples were presented to each panelist, representing a balanced order of presentation. Panelists were instructed to identify the sample that smelled and tasted different in each group of three. There was additional space for comments, with instructions to describe any odour or taste associated with the unique sample. Thirty-six volunteers ($\geq$18 year) were recruited from the school of Veterinary Medicine of Perugia, to serve on each panel session. Each panelist contributed one observation per testing session, for a total of 36 observations per triangle test (each observation consisting of two sets of 3 samples). Testing was conducted in individual booths in the Laboratorio di Ispezione degli Alimenti di Origine Animale.

A descriptive sensory evaluation was also performed at the end of the ripening process. The panel consisted of six assessors selected among the staff at the Laboratorio di Ispezione degli Alimenti di Origine Animale, previously trained in descriptive analysis for cured meat products. The tasters were asked to test the dry-cured sausages for the following characteristics: colour uniformity, colour intensity, fat/lean connection, fat/lean distribution, acid flavour, rancid flavour, bitter flavour, salty flavour, mould flavour, spicy flavour, flavour intensity, elasticity, hardness, cohesiveness, chewiness, juiciness, fattiness. Each assessor was given sheets with a 7-point scale (non-numbered to avoid biased assessment) for each

characteristic: 7 = maximum intensity and 1 = minimum intensity. The evaluations were held in individual booths, built according to the criteria of the International Standards Organisation (ISO 2003). Samples were taken from the middle of the sausage by cutting off 2 cm from each edge. The sausage slices were 4 mm thick and were immediately served on a plastic dish covered with plastic film and coded with random, three-digit numbers. Water and unsalted bread were provided to cleanse the palate between samples. Assessments were carried out under natural light at a room temperature of 20 ± 2 °C. The individual scores for each assessor were then averaged to give a score for the taste panel as a whole. Three evaluations for each different sausage were made. Each evaluation was carried out in different test sessions at the same time of day, between 10 and 12 am. To reduce fatigue, assessors conducted no more than three tests per day, lasting a maximum of 1 h. The significance and the quantitative scale for each descriptor were discussed during the training sessions. A preparatory session was held prior to analysis, so that each assessor could thoroughly discuss and clarify each attribute to be evaluated.

Analysis of results

For bacterial counts the arithmetic means within each sampling was computed, and all data (geometric mean for microbiology) were subsequently elaborated with GraphPad InStat (La Jolla, CA), 3.0b and GraphPad Prism 6.0d for Mac OS X. The $\log_{10}$ of the arithmetic means for all microbiological analysis was calculated for each of the nine repetitions, following which all $\log_{10}$ data were analysed with GraphPad InStat, version 3.0b, for Mac OS X for the analysis of variance followed by the Tukey–Kramer multiple comparisons test.

For microbiological results statistical analysis compared the eight production methods by the analysis of variance (GLM) and canonical discriminant analysis (CDA) with the software SAS/STAT SAS 9.4. CDA is a dimension-reduction technique related to principal component analysis and canonical correlation. CDA finds linear combinations (canonical variables) of the quantitative variables (different microbiota groups) that have the highest possible multiple correlations with the groups and provide maximal separation between groups in much the same way that principal components summarize total variation. The canonical variable can show substantial differences between the groups, even though none of the original variables do. The GLM results are expressed as the estimated means (LSMean and MSE) and then compared by the Tukey–Kramer Test, adjusted for multiple comparisons.

The detectable colony limit was 10^2 cfu g^{-1} for spread plate and 10 cfu g^{-1} for pour plate and the confidence limit 95%, according to the classic formula $2s = 2\sqrt{x}$. Only values between 30 and 300 cfu were considered suitable for data analysis. When the count revealed lower values at the lowest dilution, the results were reported as <300 for pour plate and <3000 for spread plate and these data were not included for the calculation of general mean value. For MPN, when applied, the detection limit was 30 cfu g^{-1}. In case of quantitative analysis, samples showing at least one typical colony in the lowest dilution were defined as positive, otherwise the result was considered negative (Cenci Goga et al. 2005).

The data for each triangle test was analysed by the number of correct responses *vs* the total number of responses. Parameters were defined at $n = 36$, $\alpha = 0.01$, $\beta = 0.10$ and pd = 50%; the critical number of correct responses for significance was 20 out of 36 (Anonymous 2004). One test was administered per repetition, and tests were not replicated. One-way ANOVA and the Tukey–Kramer multiple comparisons test were used to analyse the data for descriptive sensory analysis. A p value of <0.05 was considered to be significant.

Results

Characterization of the commercial probiotic strains Enterococcus faecium UBEF-41

The isolate subjected to sequencing and BLAST analysis was closely related to known species, with ≥97% nucleotide sequence identity. It is commonly accepted that if two organisms share a 16S rRNA gene sequence identity higher than 97%, they have to be considered closely related. From BLAST analysis the strain was confirmed as *Enterococcus faecium*.

Antibiotic susceptibility was evaluated using zone diameter interpretive criteria after an average of two readings. Isolates were expressed as susceptible (S), intermediate (I) and resistant (R) according to CLSI published breakpoint interpretations based on pharmacokinetic and pharmacodynamic data (CLSI 2011). Susceptibility to inhibitors of cell wall synthesis: the strain showed resistance towards ampicillin and methicillin. Assays for β-lactamase inhibitors revealed partial resistance to amoxicillin–clavulanic acid. The strain was susceptible for vancomycin Susceptibility to inhibitors of nucleic acid synthesis: concerning quinolones, the isolate was resistant to nalidixic acid and cyprofloxacin.

Susceptibility to protein synthesis inhibitors: all strains were susceptible to tetracyclines, gentamicin and resistant to streptomycin and to erythromycin. Finally, intermediate resistance was observed to kanamycin and neomycin.

Microbial analysis

Total aerobic mesophilic microbiota, LAB and enterococci

Figure 3(a) shows the results. On the day of production, salami without starter had very low levels of LAB, while counts in salami made with starter were higher than 10^6 cfu g^{-1}. In fully ripened salami, LAB were above 10^6 cfu g^{-1} in all starter added groups ($>10^7$ cfu g^{-1} in salami made without added nitrates), whereas counts were below 10^7 cfu g^{-1} ($<10^7$ cfu g^{-1} in salami with added nitrates) in all batches without added starter. Enterococci were below 10^4 cfu g^{-1} in all sausages made without starter, and above 10^6 cfu g^{-1} for all salami with added starter on the day of stuffing. In fully ripened salami, Enterococci mean values were above 10^6 cfu g^{-1} for all starter groups and below 10^6 cfu g^{-1} for all groups made without starter.

Figure 3(b) shows the results for *Staphylococcus* spp., *Pseudomonas* spp., *Enterobacteriaceae* and coliform organisms. On the day of stuffing, mean *Staphylococcus* spp. counts were between 10^3 and 10^4 cfu g^{-1} for all groups. *Pseudomonas* spp. levels were between 10^5 cfu g^{-1}–10^6 cfu g^{-1} in all groups made without nitrates. *Enterobacteriaceae* were lower than 10^2 cfu g^{-1} for salami made with starter and without nitrates and below 10^3 cfu g^{-1} for other groups. Coliform organisms were above 10^2 cfu g^{-1} in all groups added with nitrates and between 10 cfu g^{-1} to nondetectable in other groups. At the end of ripening, *Staphylococcus* spp. were over 10^5 cfu g^{-1} for groups made without nitrates and without starter and below 10^5 cfu g^{-1} for all groups made with added starter and for the two groups without starter, but with added nitrates. *Pseudomonas* spp. counts were above 10^4 cfu g^{-1} in all salami made without starter and below 10^3 cfu g^{-1} in all groups made with added starter. *Enterobacteriaceae* were higher than 10^2 cfu g^{-1} in salami made without starter and lower than 10 cfu g^{-1} in all starter-added salami. Coliform organisms were never detected in salami made with starter and above 10 cfu g^{-1} in all salami without starter.

Table 2 shows the counts and the statistical evaluation data (analysis of variance followed by the Tukey–Kramer multiple comparisons test) for all groups at the end of ripening.

Pathogens

Table 3 shows sulphite-reducing clostridia, *C. botulinum*, *Listeria* spp., non-sorbitol-fermenting *E. coli*, *S. aureus* and *Salmonella* spp. Sulphite-reducing clostridia, *C. botulinum*, *Salmonella* spp. and non-sorbitol-fermenting *E. coli* were never found. *Listeria* spp. was isolated up to day 7 in all salami, whereas at day 13, isolation was negative only for salami made with nitrate, starter and ripened under classic ripening conditions. At the end of ripening, *Listeria* spp. was isolated only from those groups of salami made without nitrates and without starter. *L. monocytogenes* was never detected. *Staphylococcus aureus* was isolated from all groups at up to day 7, whereas at day 13, only salami aged under specific ripening conditions were negative (with and without starter in the case of salami made with added nitrate and only with starter in the case of salami produced without nitrate). At the end of ripening, all groups were negative for S. *aureus*, except the groups of salami made without nitrate and under classic ripening conditions.

Physico-chemical and chemical analysis

Figures 4 and 5 show a_w and pH. On day 13, a_w decreased to 0.84–0.86 in all groups ripened under specific conditions and was below 0.84 in all groups ripened under classic ripening conditions. Fully ripened salami was between 0.79 and 0.82. Mean pH values on the day of stuffing were between 5.96–5.99 and reached values between 5.61–5.80 on day 7 and values between 5.32–5.47 at the end of ripening for all batters. Table 4 shows the chemical composition: on the day of stuffing, proteins were 39.73, fat 48.64, ashes 8.92, (g 100 g^{-1} total solids), and similar data were obtained at the end of ripening. Moisture decreased from 60.68% (standard error: 0.40) on the day of stuffing to 26.68% (standard error: 0.72) in full ripened salami.

Sensory evaluation

Table 5 shows the results of the triangle test performed for all production with nitrates and for the only production without nitrates that was negative for pathogens (i.e. salami made with starter and ripened under specific conditions). For salami ripened under classic ripening conditions, the panelists detected differences ($\alpha = 0.01$) between salami made with starter and salami made without starter. For salami made with starter addition, differences were detected between salami ripened under classic *vs* specific ripening conditions ($\alpha = 0.10$). Differences were also detected

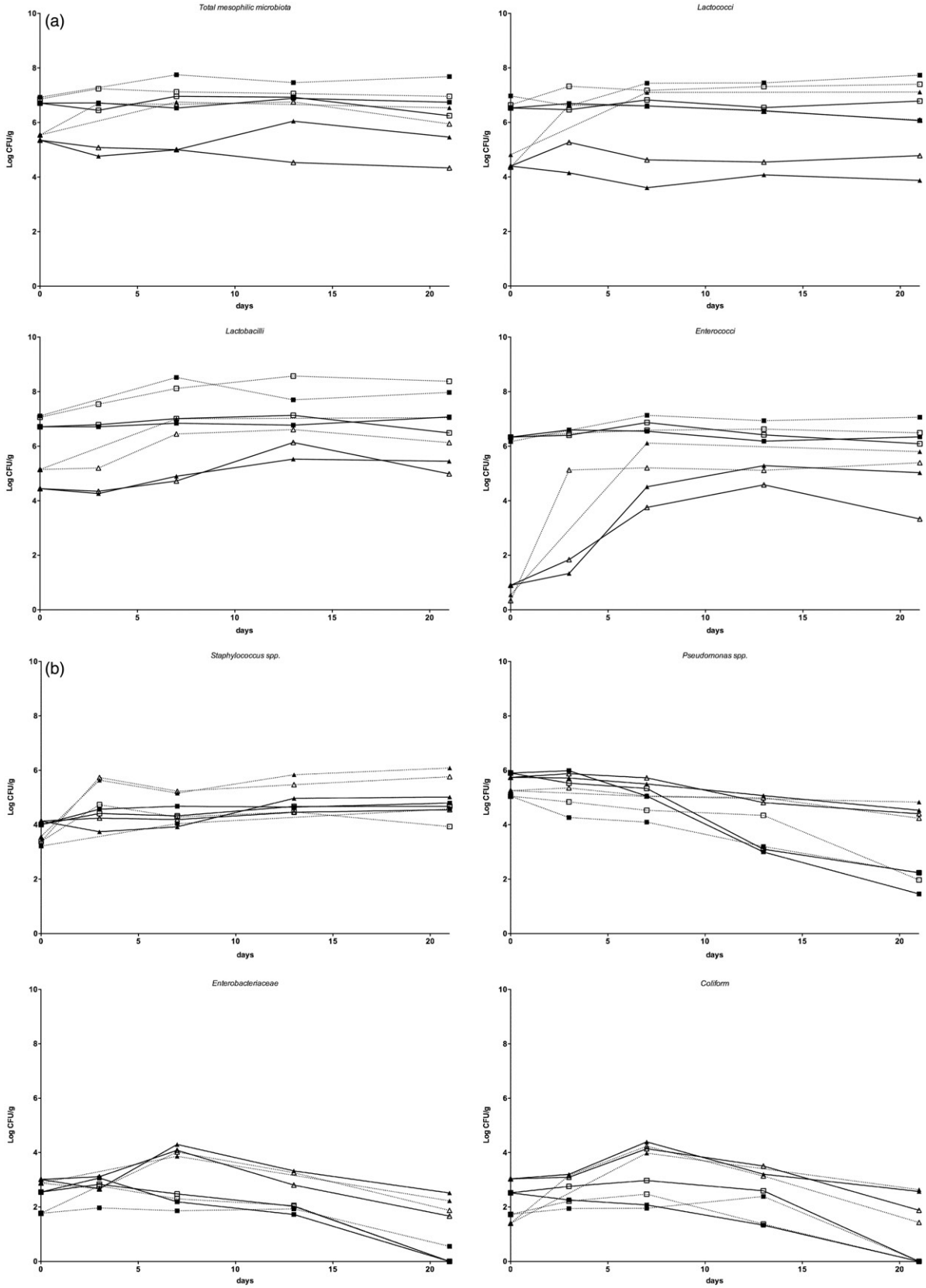

Figure 3. Evolution of microbiota during (a) fermentation and (b) ripening of *salame nostrano*. (—△—: nitrate, no starter, classic ripening; —▲—: nitrate, no starter, specific ripening; —□—: nitrate, starter, classic ripening; —■—: nitrate, starter, specific ripening; · · ·△· · ·: no nitrate, no starter, classic ripening; · · ·▲· · ·: no nitrate, no starter, specific ripening; · · ·□· · ·: no nitrate, starter, classic ripening, · · ·■· · ·: no nitrate, starter, specific ripening).

Table 2a. Counts at the end of ripening for total aerobic mesophilic microbiota, LAB and enterococci (Log CFU g^{-1}).

	NIT				NO NIT			
	NO STA		STA		NO STA		STA	
	NO SPEC	SPEC	NO SPEC	SPEC	NO SPEC	SPEC	NO SPEC	SPEC
Total aerobic microbiota								
Mean	4.33[a]	5.47[ab]	6.25[abc]	6.74[bc]	5.95[b]	6.54[bc]	6.96[bc]	7.68[c]
SD	1.96	0.53	0.30	0.54	0.28	0.24	0.11	0.50
n	7	9	9	9	9	8	9	9
SE	0.74	0.18	0.10	0.18	0.09	0.08	0.04	0.17
Lactococci								
Mean	4.78[a]	3.87[a]	6.78[bc]	6.07 [b]	6.09[b]	7.12[bc]	7.40[c]	7.73[c]
SD	0.46	0.59	1.05	0.62	0.27	0.22	0.27	0.61
n	9	9	9	9	9	9	9	9
SE	0.15	0.20	0.35	0.21	0.09	0.07	0.09	0.20
Lactobacilli								
Mean	4.98[a]	5.45[a]	6.49[bc]	7.07[c]	6.13[b]	7.05[c]	8.38[d]	7.97[d]
SD	0.81	0.32	0.30	0.32	0.47	0.14	0.14	0.16
n	9	9	9	9	9	9	9	9
SE	0.27	0.11	0.10	0.11	0.16	0.05	0.05	0.05
Enterococci								
Mean	3.33	5.03[a]	6.09 [bd]	6.34 [bd]	5.39[ac]	5.80[bc]	6.49[de]	7.06[e]
SD	0.54	0.33	0.12	0.31	0.39	0.17	0.17	0.36
n	9	7	9	9	9	9	9	9
SE	0.18	0.12	0.04	0.10	0.13	0.06	0.06	0.12

SD: standard deviation; SE: standard error; *n*: number of samples. Mean values with different superscripts in the same row are significantly different, $p < 0.001$).

Table 2b. Counts at the end of ripening for total *Staphylococcus* spp., *Pseudomonas* spp., *Enterobacteriaceae* and coliform organisms (Log CFU g^{-1}).

	NIT				NO NIT			
	NO STA		STA		NO STA		STA	
	NO SPEC	SPEC	NO SPEC	SPEC	NO SPEC	SPEC	NO SPEC	SPEC
Staphylococcus spp.								
Mean	4.55[b]	5.02[b]	4.68[b]	4.79[b]	5.76[a]	6.08[a]	3.93	4.58[b]
SD	0.33	0.22	0.27	0.10	0.19	0.13	0.30	0.14
n	9	9	9	9	9	9	9	9
SE	0.11	0.07	0.09	0.03	0.06	0.04	0.10	0.05
Pseudomonas spp.								
Mean	4.40[a]	4.53[a]	2.24[b]	1.46[b]	4.24[a]	4.83[a]	1.97[b]	2.22[b]
SD	0.19	0.45	0.21	0.59	0.26	0.29	1.15	0.98
N	9	9	9	9	9	9	9	9
SE	0.06	0.15	0.07	0.20	0.09	0.10	0.38	0.33
Enterobacteriaceae								
Mean	1.67[ab]	2.52[ab]	nd	nd	1.88[ab]	2.23[a]	nd	0.55[b]
SD	1.58	1.90	0.00	0.00	0.75	0.05	0.00	0.44
n	9	9	9	9	9	9	9	9
SE	0.53	0.63	0.00	0.00	0.38	0.02	0.00	0.15
Coliform organisms								
Mean	1.88[ab]	2.57[a]	nd	nd	1.43[ab]	2.64[a]	nd	nd
SD	1.79	1.61	0.00	0.00	1.50	0.06	0.00	0.00
n	9	8	9	9	9	9	9	9
SE	0.60	0.57	0.00	0.00	0.50	0.02	0.00	0.00

Means values with different superscripts in the same row are significantly different ($p < 0.001$). SD: standard deviation; SE: standard error; *n*: number of samples; nd: not determined.

between all groups made with starter *vs* all groups made without starter ($\alpha = 0.05$). No differences were detected for salami made without starter under classic *vs* specific ripening conditions and for salami ripened under specific ripening conditions in starter *vs* no starter groups. All the results of the sensory analysis for all groups are given in Figure 6 and Table 6: no statistically significant differences were detected among groups ($p < 0.05$).

Multivariate canonical discriminant analysis

A multivariate canonical discriminant analysis was performed to synthesise the effect of ripening and the use of starters and nitrites on the different microbiota groups to aid the visual interpretation of the differences in group. The classificatory variable was the combination of the three treatments at the end of the ripening and quantitative variables were the related

Table 3. Isolation of *S. aureus*, sulphite-reducing *Clostridium* spp., *C. botulinum*, *Salmonella* spp., *Listeria* spp. and non-sorbitol-fermenting *E. coli* from *salame nostrano* (positive samples/tested samples) during fermentation and ripening.

	NIT				NO NIT			
	NO STA		STA		NO STA		STA	
	NO SPEC	SPEC	NO SPEC	SPEC	NO SPEC	SPEC	NO SPEC	SPEC
Day-0								
Sulphite-reducing *Clostridium* spp.	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
C. botulinum	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
Salmonella spp.	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
Listeria spp.	5/9	5/9	5/9	5/9	6/9	5/9	5/9	6/9
Non-sorbitol-fermenting *E. coli*	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
S. aureus	2/9	3/9	3/9	2/9	2/9	2/9	2/9	2/9
Day-7								
Sulphite-reducing *Clostridium* spp.	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
C. botulinum	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
Salmonella spp.	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
Listeria spp.	4/9	3/9	2/9	4/9	6/9	5/9	5/9	6/9
Non-sorbitol-fermenting *E. coli*	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
S. aureus	2/9	3/9	3/9	2/9	2/9	2/9	2/9	2/9
Day-13								
Sulphite-reducing *Clostridium* spp.	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
C. botulinum	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
Salmonella spp.	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
Listeria spp.	2/9	3/9	0/9	1/9	5/9	5/9	2/9	2/9
Non-sorbitol-fermenting *E. coli*	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
S. aureus	2/9	0/9	1/9	0/9	2/9	2/9	2/9	0/9
Day-21								
Sulphite-reducing *Clostridium* spp.	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
C. botulinum	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
Salmonella spp.	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
Listeria spp.	0/9	0/9	0/9	0/9	4/9	3/9	0/9	0/9
Non-sorbitol-fermenting *E. coli*	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
S. aureus	0/9	0/9	0/9	0/9	2/9	0/9	1/9	0/9

counts of microbiota. Canonical Variable 1 (Can1) in Figure 7 shows how to relate the groups to the use of starters. More groups are far away from the centre and most are related: sausages without starters are on the right positive side; sausages with added starter and free of nitrites are on the far negative left side; sausages with added starter and nitrites are on the near negative left side. The raw canonical coefficients which best define the first canonical variable are positive for *Staphylococcus* spp. (1.7863), *Pseudomonas* spp. (0.9866) and coliforms (0.2730), and negative for lactobacilli (−1.9095) and enterococci (−1.5283). This means that the microbial groups mentioned above are more correlated with the related groups in the same sector as shown in Figure 8. Coliform and *Enterobacteriaceae* were found only in the salami without probiotics.

The Canonical Variable 2 (Can 2) axis best correlated to the effect of nitrite and ripening. On the right side, sausages made without added starter cultures need nitrites to control microbial groups, and free nitrites groups are in the upper right quadrant. Those with nitrite are in the lower side. The importance to use nitrites is shown by a greater dispersion along the second axis. On the left side, groups are concentrated along axis 1 to indicate the poor correlation with nitrites and ripening due to the use of starters. The raw canonical coefficients that most define the second-canonical variable are positive for the *Staphylococcus* spp. (3.7257) and *Lactobacill*i (2.0163) and negative for lactic acid cocci (-0.4695).

The univariate statistics for classificatory variable showed R^2 values ranging from 0.5608 for *Enterobacteriaceae* (EB) to 0.9524 for *Lactobacilli* (LB), and each variable is significant below 0.001. Obviously, the multivariate test is also highly significant. The R^2 canonical correlation between Can1 and the class variable, 0.9798, is not much larger than the corresponding R^2 for Can2, 0.9124. The Can1 discriminates 75% of the variability and the Can2 16%. Therefore, it is possible to correlate 91% of the total variability with two variables. This indicates a very strong contribution on the two axes of the discriminatory variables used.

Discussion

The growth of *Staphylococcus aureus, Pseudomonas* spp., *Enterobacteriaceae* and coliform organisms and *Listeria* spp. was inhibited in salami made without the addition of curing nitrate and with the proposed starter culture formulation along with specific ripening conditions. The pH value (5.96–5.99 on the day of

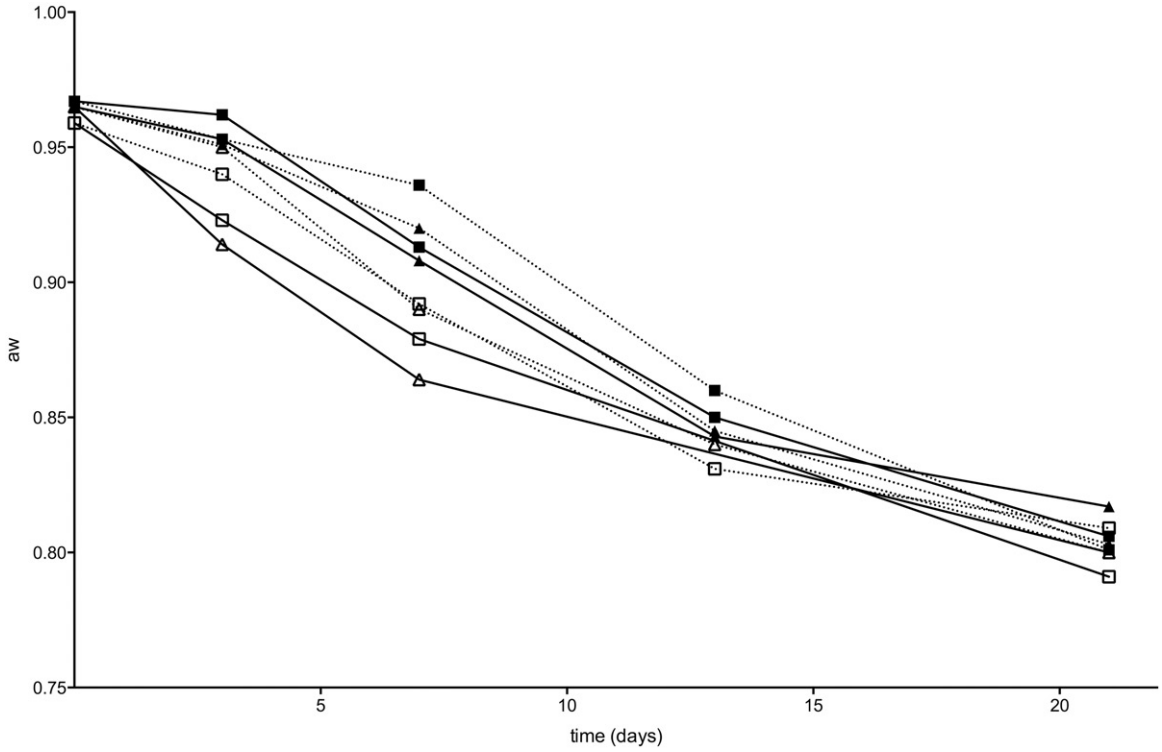

Figure 4. Changes of a_w in *salame nostrano* during fermentation and ripening (average values of five replicates, sampled in triplicate and standard error, SE). (—△—: nitrate, no starter, classic ripening; —▲—: nitrate, no starter, specific ripening; —□—: nitrate, starter, classic ripening; —■—: nitrate, starter, specific ripening; ···△···: no nitrate, no starter, classic ripening; · · ·▲· · ·: no nitrate, no starter, specific ripening; · · ·□· · ·: no nitrate, starter, classic ripening, · · ·■· · ·: no nitrate, starter, specific ripening).

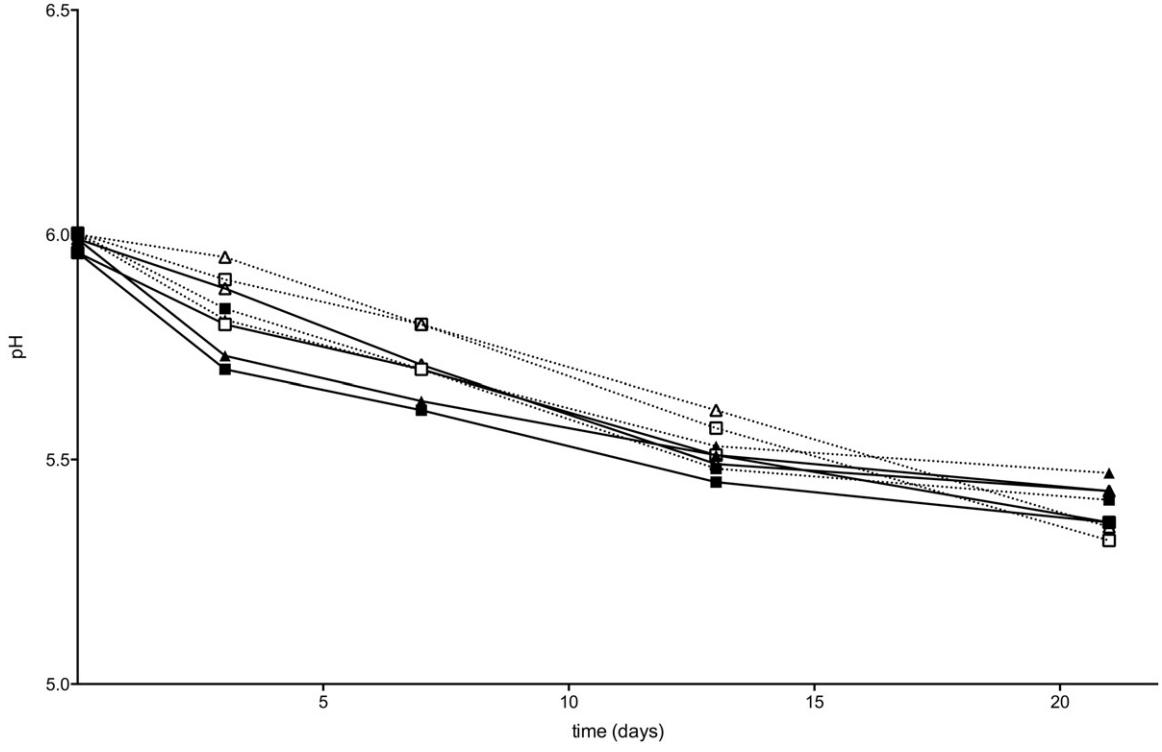

Figure 5. Changes of pH in *salame nostrano* during fermentation and ripening (average values of five replicates, sampled in triplicate and standard error, SE). (—△—: nitrate, no starter, classic ripening; —▲—: nitrate, no starter, specific ripening; —□—: nitrate, starter, classic ripening; —■—: nitrate, starter, specific ripening; · · ·△· · ·: no nitrate, no starter, classic ripening; · · ·▲· · ·: no nitrate, no starter, specific ripening; · · ·□· · ·: no nitrate, starter, classic ripening, · · ·■· · ·: no nitrate, starter, specific ripening).

Table 4. Chemical parameters on the day of stuffing and at the end of ripening *salame nostrano*.

Day of stuffing	Mean	SE	*n*
Proteins g 100 g^{-1} total solids	39.73	0.66	9
Fat g/100 g^{-1} total solids	48.64	1.51	9
Ashes g/100 g^{-1} total solids	8.92	0.10	9
Moisture	60.68	0.40	9
End of ripening			
Proteins g/100 g^{-1} total solids	42.67	1.17	9
Fat g/100 g^{-1} total solids	47.34	1.56	9
Ashes g/100 g^{-1} total solids	9.23	0.26	9
Moisture	29.68	0.72	9

Average values of nine replicates sampled in triplicate and standard error (SE).

Table 5. Sensory analysis, triangle test for *salame nostrano*.

	α: 0.01
STA NO SPEC *vs* NO STA NO SPEC	s
STA NO SPEC *vs* STA SPEC	ns
NO STA NO SPEC *vs* NO STA SPEC	ns
STA SPEC *vs* NO STA SPEC	ns

Alpha (α): probability of concluding that a perceptible difference exists when one does not; Beta (β): probability of concluding that no perceptible difference exists when one does; pd: the proportion of assessments in which a perceptible difference is detected between the two products. β = 0.10, pd = 50%; s: statistically significant ($p < 0.05$); ns: statistically nonsignificant.

stuffing) inside the meat decreased to 5.36 in fully ripened salami. Desirable colour formation was observed, and the cohesiveness of the meat was slightly higher in the NO NIT–STA-SPEC group when compared to NO NIT–NO STA-SPEC. A decline in numbers of *Enterobacteriaceae* was observed at the end of ripening (day-21) for all groups of salami made with the addition of starter cultures. At the end of ripening, in fact, the counts of *Enterobacteriaceae* were lower than 10 cfu g^{-1} in all groups made with starter cultures, whereas counts were consistently higher than 10^2 cfu g^{-1} for the groups made without starter cultures. A more dramatic drop was observed for coliform organisms, given that they were always undetectable in all groups of salami made with starter addition, whereas counts reached values up to 2.64 in all groups of salami made without starter.

A similar pattern was observed for *Pseudomonas* spp., when all groups of salami made without starter had counts higher than 10^4 cfu g^{-1} and all groups made with starter were below 10^3 cfu g^{-1}.

At the end of ripening, *S. aureus* and *Listeria* spp. were only isolated from sausages made without nitrates, with the exception of the batches made with starter and ripened under specific conditions.

At the end of ripening (day 21), counts for lactic microbiota, total mesophilic microbiota and enterococci in all salami made with starter, without nitrate and ripened under specific ripening conditions were higher than any count in the groups made with added nitrates, even in the two groups to which the starter cultures had been added. This might be linked to a known and described sensitivity of certain strains of lactic acid bacteria to nitrite or nitrate (Verluyten et al. 2003). On the other hand, given that our strains had been selected on the basis of their growth at low temperature (Cenci-Goga et al. 2015), generally, salami ripened under specific ripening conditions had counts consistently higher than their counterparts ripened under classic ripening conditions.

Statistically significant differences for *Staphylococcus* spp., *Pseudomonas* spp., *Enterobacteriaceae* and coliform organisms were less evident, given that a natural decline for these microbial groups is usually also observed in home-made salami, produced neither with a starter culture nor additives (Cenci Goga et al. 2008; Al-Zeyara et al. 2011; Forzale et al. 2011; Tasic et al. 2012; Cenci Goga et al. 2012).

The final pH for all groups of salami was similar, ranging from 5.32 to 5.47 at day21, with a similar decrease pattern for all groups. This demonstrates that the formulation used as starter does not cause a sudden drop in acidity, as observed in fast-fermented sausages (Feiner 2006). A similar pattern was recorded for a_w drop, although in this case, a different decrease was observed, especially at the end of fermentation, when all groups of salami ripened under specific ripening conditions had slightly higher values, due to the higher fermentation humidity of the protocol. At the end of ripening (day21), however, all groups reached very similar values, ranging between 0.79 and 0.82.

The starter formulation used in this experiment, together with the ripening conditions, was able to maintain pH just above 5, which is a good compromise for the hygienic and the organoleptic characteristics (Cizeikiene et al. 2013). The addition of the proposed formulation, together with the specific ripening conditions, did not modify the characteristics of the final products, when compared with the characteristics of salami produced according to the traditional procedure. Moreover, salami of the groups made without nitrates were less rancid and all groups made with starter had a lesser bitter flavour (Table 6). This is possibly related to the incorporation of the *Enterococcus faecium* strain in the starter formulation. In fact, throughout fermentation and ripening, counts of enterococci were consistently higher in salami made with the addition of starter cultures, when compared to salami produced without starter addition. Enterococci are, in fact, capable of modulating the aroma by converting amino acids and free fatty acids (González-

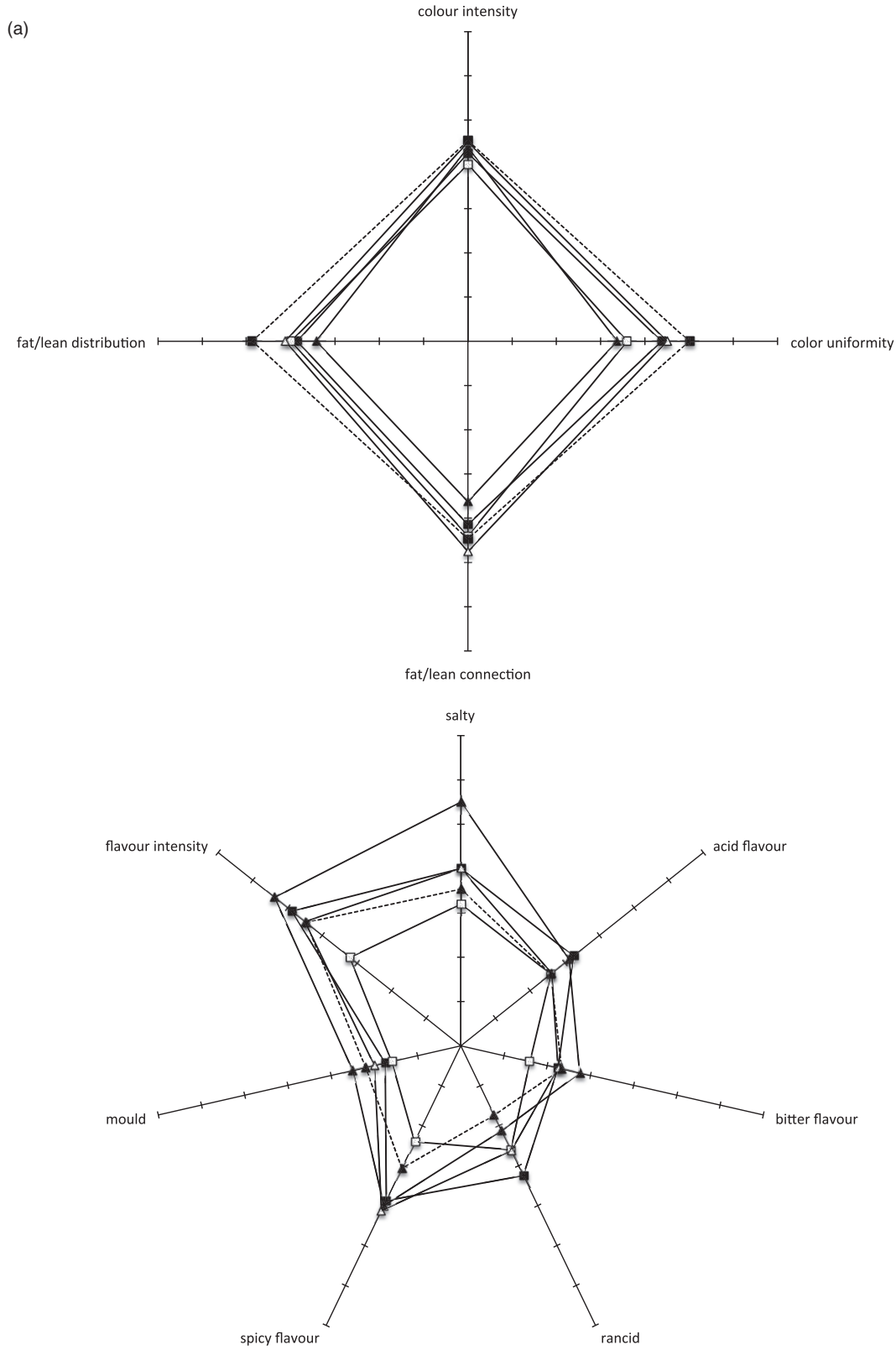

Figure 6. Sensory descriptive analysis of *salame nostrano* (a: appearance attributes; b: basic tastes; c: texture attributes). (—△—: nitrate, no starter, classic ripening; —▲—: nitrate, no starter, specific ripening; —□—: nitrate, starter, classic ripening; —■—: nitrate, starter, specific ripening; · · ·■· · ·: no nitrate, starter, specific ripening).

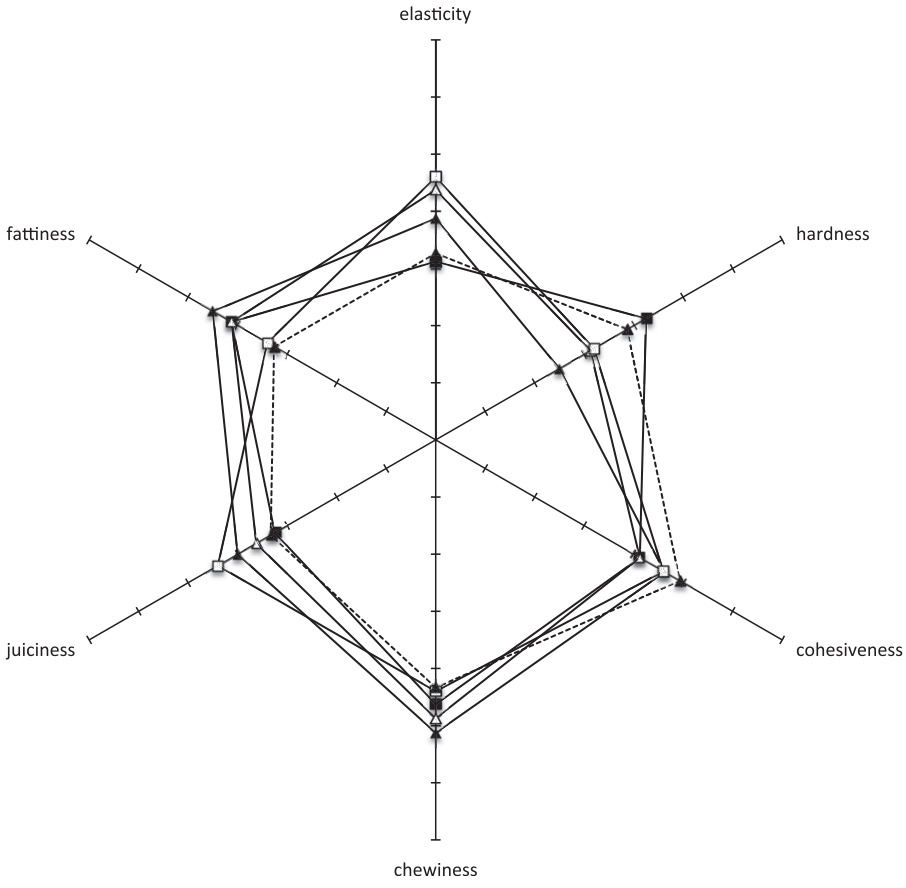

Figure 6. Continued.

Fernández et al. 2006; Leroy et al. 2006; Corbiere Morot-Bizot et al. 2007; García Fontán et al. 2007). The *E. faecium* strain used in this study showed resistance to ampicillin, quinolones (nalidixic acid and ciprofloxacin) and erythromycin, which are known to be transmissible through the so-called horizontal gene transfer (HGT) (Kristich et al. 2014). The strain was indeed susceptible to glicopeptides and tetracycline, even if those antibiotics are frequently related to HGT. Some concerns can be raised to the resistance to ampicillin, but it is known that *Enterococcus* spp. may present an intrinsic tolerance towards the achievable concentrations of beta-lactams commonly used.

The use of the starter strains described in this study, together with the specific ripening conditions applied mainly based on lower temperatures, is a great tool for limiting the growth and survival of undesirable microbiota, yet maintaining sensory properties of dry salami produced in Umbria, Italy. The use of cold-adapted microorganisms in food biotechnology has been very limited. In the area of food biotechnology, cold-adapted microorganisms, i.e. psychrophilic, psychrotrophic and psychrotolerant microorganisms, have generally been regarded as food-spoilage rather than as potentially useful organisms, although several cold-active enzymes have been isolated from cold-adapted microorganisms. These enzymes include lipases, proteases, galactosidases, alkaline phosphatases, triosephosphate isomerase and amylase (Okuyama et al. 1999).

This research proved that the use of cold-adapted strains (a commercial strain of *E. faecium* and lactococci and lactobacilli of dairy origin), together with fermentation and ripening based on low temperatures, achieve a statistically significant inhibition of pathogens without any detrimental effect on sensory assets of salami. Several techniques have been proposed to obtain low-acid salami, yet preserving the safety of these meat products, including low-temperature fermentation, but none of them proved to be worthwhile when applied alone (Moore 2004). The formulation of selected LAB used in this study is the result of previous investigations carried out on farm-manufactured cheeses and on the properties of autochthonous LAB. This formulation has been chosen on the basis of growth at low temperatures and acidifying activity tests, previously conducted on several different associations of LAB, using a fourth degree polynomial as an empirical model to fit the experimental data so that the formulation of choice was the association with the

Table 6. Sensory descriptive analysis of *salame nostrano*.

	NIT–NO STA–NO SPEC			NIT–NO STA–SPEC			NIT–STA–NO SPEC			NIT–STA–SPEC			NO NIT–STA–SPEC		
	Mean	SEM	*n*	Mean	SEM	*n*	Mean	SEM	*n*	Mean	SEM	*n*	Mean	SEM	*n*
Colour intensity	4.5	0.5	8	4.38	0.32	8	4	0.71	5	4.25	0.56	8	4.53	0.47	15
Colour uniformity	4.5	0.57	8	3.38	0.46	8	3.6	0.68	5	4.38	0.26	8	5	0.43	15
Fat/lean connection	4.75	0.59	8	3.63	0.42	8	4.4	0.51	5	4.14	0.59	7	4.47	0.42	15
Fat/lean distribution	4.13	0.58	8	3.43	0.3	7	4	0.55	5	3.86	0.67	7	4.87	0.43	15
Odour (overall intensity)	3.38	0.5	8	3.88	0.3	8	3.6	0.81	5	3.5	0.6	8	4.53	0.35	15
Mould odour	1.75	0.25	8	3	0.53	8	2	0.32	5	2	0.5	8	2.47	0.38	15
Salty taste	4	0.5	8	5.5	0.27	8	3.2	0.8	5	4	0.53	8	3.53	0.32	15
Acid flavour	2.63	0.6	8	3.13	0.58	8	2.6	0.4	5	3.25	0.53	8	2.6	0.35	15
Bitter flavour	2.25	0.41	8	2.75	0.53	8	1.6	0.4	5	2.25	0.37	8	2.33	0.3	15
Rancid	2.63	0.65	8	2.13	0.35	8	2.6	0.81	5	3.25	0.7	8	1.73	0.21	15
Spicy flavour	4.13	0.3	8	4	0.53	8	2.4	0.6	5	3.88	0.55	8	3.07	0.4	15
Mould taste	2	0.5	8	2.5	0.6	8	1.6	0.24	5	1.75	0.25	8	2.2	0.34	15
Flavour intensity	4.5	0.33	8	5.38	0.32	8	3.2	0.66	5	4.88	0.23	8	4.47	0.4	15
Elasticity	4.38	0.32	8	3.88	0.64	8	4.6	0.68	5	3.13	0.3	8	3.27	0.42	15
Hardness	3.13	0.48	8	2.5	0.57	8	3.2	0.58	5	4.25	0.49	8	3.87	0.36	15
Cohesiveness	4.13	0.35	8	4.63	0.68	8	4.6	0.4	5	4.13	0.52	8	4.93	0.34	15
Chewiness	4.88	0.23	8	5.13	0.52	8	4.4	0.81	5	4.63	0.5	8	4.33	0.39	15
Juiciness	3.63	0.42	8	4	0.27	8	4.4	0.68	5	3.25	0.59	8	3.33	0.37	15
Fattiness	4.13	0.35	8	4.5	0.53	8	3.4	0.51	5	4.13	0.35	8	3.27	0.3	15
Overall acceptability	5.38	0.42	8	5.29	0.42	7	3.6	0.51	5	4.63	0.42	8	4.87	0.35	15

SEM: standard error. *n* is the samples tested.

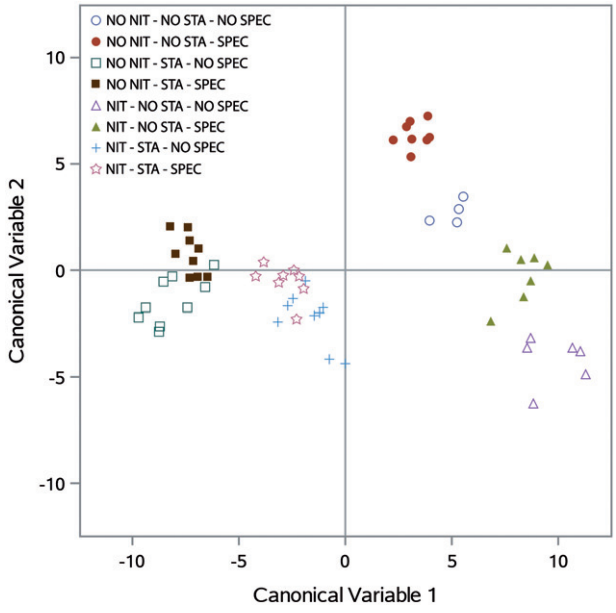

Figure 7. Axis 1-2 by canonical discriminant analysis for use of starters, nitrites and type of ripening at the end of ripening.

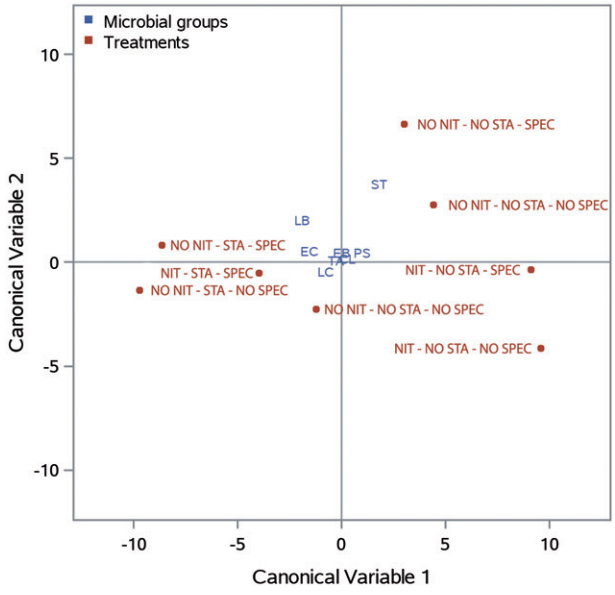

Figure 8. Axis 1-2 by canonical discriminant analysis with the means of treatments and microbial groups. (ST: *Staphylococcus* spp.; LB: lactobacilli; EC: enterococci; LC: lactic acid cocci; TA: total aerobic; CL: coliform organisms; EB: *Enterobacteriaceae*; PS: *Pseudomonas* spp.).

maximum acceleration and instantaneous acidification rate (Clementi et al. 1998; Cenci-Goga et al. 2015). The original formulation (without the probiotic strain) has already proven to be effective in reducing *Enterobacteriaceae*, *S. aureus*, *Listeria* spp. and *Salmonella* spp., both in cheeses and in fermented sausages (Cenci Goga et al. 1995; Cenci Goga & Vizzani, 1998; Clementi et al. 1998; Cenci Goga et al. 2008; Cenci Goga et al. 2012). Previous studies conducted by the authors demonstrated that this starter formulation greatly inhibited the growth of *S. aureus* together with enterotoxin and thermonuclease production, in both field trials and in controlled *in vitro* conditions. Results were demonstrated on both selective and non-selective media (Cenci Goga et al. 1994; Cenci Goga & Vizzani 1998; Miraglia et al. 2002). With this research the experience was further developed with the inclusion of the probiotic strain and with the use of low temperature throughout fermentation and ripening.

The combination of the two hurdles described in this study is an additional tool for preventing the growth and survival of potentially pathogenic bacteria, and contribute to sensory qualities of low acid, nitrite and nitrate-free, fermented sausages.

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