FATE OF SELECTED PATHOGENS IN SPIKED «SALAME NOSTRANO» PRODUCED WITHOUT ADDED NITRATES FOLLOWING THE APPLICATION OF NONIT™ TECHNOLOGY

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Abstract

This study evaluated the effect of a novel formulation for starter culture associated with specific ripening conditions (NoNit™ technology) vs. a commercial» starter on the fate of selected pathogens and hygiene indicators during the fermentation and ripening of experimentally spiked salame nostrano (Italian dry sausage**)**. Selected strains of *Staphylococcus aureus* 27R, *Escherichia coli* CSH26 K 12, *Listeria innocua* ATCC 33090 and *Salmonella* Derby 27 were inoculated into salami batter and challenged with two formulations of starter cultures (a commercial formulation and the NoNit™ formulation, consisting of *Lactococcus lactis ssp. lactis*, strain 340; *L. lactis* ssp. *lactis*, strain 16; *Lactobacillus casei* ssp. *casei*, strain 208 and *Enterococcus faecium* strain 614) with ripening at a low temperature.

The proposed technology (NoNit™) performed better than the commercial formulation and limited the growth of spiked *Escherichia coli*, *Staphylococcus aureus* (including the production of enterotoxin), *Salmonella* Derby and *Listeria innocua*, yet maintained the basic product appearance and texture.

Key words: starter culture, dry salami, *Lactococcus lactis* ssp. *lactis*, *Lactobacillus casei* ssp. *casei*, *Enterococcus faecium*

1 Introduction

The fermentation of raw meat to improve the safety, shelf life and acceptability of certain foods has a long tradition. In fermented sausages produced from raw meat, the conditions arising from fermentation are generally sufficient to inhibit the growth and toxin production of most pathogens due to a combination of hurdles, such as pH, water activity, preservatives and the action of lactic acid bacteria, either added as starter cultures or naturally present (Cenci-Goga, Karama, Sechi, Iulietto, Novelli, Selvaggini, & Barbera, 2016; Cenci-Goga, Karama, Sechi, Iulietto, Novelli, Selvaggini, & Mattei, 2015; Leistner, 2000). However, studies have demonstrated that several bacteria, including *Staphylococcus aureus*, *Listeria* spp., *Escherichia coli* and *Salmonella* spp., can survive in the end products, and although fermented sausages have a well-founded reputation for safety, outbreaks of food poisonings do occur (D'Ostuni, Tristezza, De Giorgi, Rampino, Grieco, & Perrotta, 2016; Chajecka-Wierzchowska, Zadernowska, Nalepa, Sierpinska, & Laniewska-Trokenheim, 2015; Al-Mutairi, 2011). In Italy, for instance, salami has been implicated in several *E. coli* and *Salmonella* spp.-related family outbreaks (Conedera, Mattiazzi, Russo, Chiesa, Scorzato, Grandesso, Bessegato, Fioravanti, & Caprioli, 2007; Luzzi, Galetta, Massari, Rizzo, Dionisi, Filetici, Cawthorne, Tozzi, Argentieri, Bilei, Busani, Gnesivo, Pendenza, Piccoli, Napoli, Loffredo, Trinito, Santarelli, & Ciofi degli Atti, 2007). Outbreaks of *L. monocytogenes* linked to the consumption of pre-sliced, ready-to-eat foods have been described (Sartz, De Jong, Hjertqvist, Plym-Forshell, Alsterlund, Löfdahl, Osterman, Ståhl, Eriksson, Hansson, & Karpman, 2008; Sheen & Hwang, 2008; Anonymous, 2002). During the fermentation, ripening and drying of fermented sausages, levels of pathogens have been shown to drop with inactivation proportional to the pH, salt and nitrate/nitrite levels (Casey & Condon, 2000; Riordan, Duffy, Sheridan, Eblen, Whiting, Blair, & McDowell, 1998). In recent years, there has been growing consumer interest in ready-to- eat, fermented sausages, produced with

lower concentrations of additives. Many technologies have been proposed to limit their use, while maintaining the same level of safety (Cenci-Goga et al., 2016; Cenci-Goga et al., 2015; Cenci-Goga, Rossitto, Sechi, Parmegiani, Cambiotti, & Cullor, 2012; Zarringhalami, Sahari, & Hamidi-Esfehani, 2009). Moreover, further to the recommendation of the «International Agency for Research on Cancer» of the «World Health Organisation», based on epidemiological studies suggesting that small increases in the risk of several cancers may be associated with a high consumption of red or processed meat (Bouvard, Loomis, Guyton, Grosse, Ghissassi, Benbrahim-Tallaa, Guha, Mattock, & Straif, 2015), several agencies and the media have raised the question as to whether processed meat can be safely produced without added nitrates and nitrites, which are currently authorized as food additives.

Thus, consumer interest in nitrite-free products has increased and, as a consequence, locally hand-crafted products from small-scale plants are often marketed as nitrate and nitrite-free, in attempt to link locally made products to safer technologies (Cenci-Goga et al., 2016; Cenci-Goga et al., 2012; Zarringhalami et al., 2009). It is obvious that the manufacture of nitrite-free sausages poses two big problems to food business operators: first of all, the production of safe food, followed by colour formation and stability (Zarringhalami et al., 2009). Several alternatives to nitrates and nitrites have been tested for their antimicrobial action (Pegg & Shahidi, 2000), and many different methods have been proposed to enhance colour intensity and uniformity, including starter cultures based mainly on cold-adapted, lactic acid bacteria and natural colorant (Zarringhalami et al., 2009; Zhang, Kong, & Xiong, 2007).

The application of starter cultures in food production has a crucial aim: their activity is addressed to restraining indigenous microbiota, in order to control processing and promote food safety. Lactic acid bacteria and staphylococci are the most commonly used strains in cured meat production, and they have been directly applied to meat batter for several years. On the one hand, the application of staphylococci as starter cultures shows some limitations,

since pH tolerance and temperature tolerance are key factors to enable them to produce specific enzymes. Low temperatures can, in fact, affect their activity (Rai & Bai, 2014). On the other hand, lactic acid bacteria determine technological changes, which promote meat stability. They ferment the sugars available and produce organic acids: the acids produced determine a pH reduction, which contributes to the drying process by reducing the water holding capacity and to colour formation and inhibits undesired microbiota in the product (Bedia, Méndez, & Bañón, 2011).

To maximise the beneficial effect of the starter culture application, it is crucial, therefore, to clearly define the product characteristics and processing technology, and predict the end product.

During our studies on hand-crafted products made in small-scale plants in Umbria, Central Italy (Cenci-Goga et al., 2015; Cenci-Goga et al., 2012; Cenci-Goga, Ranucci, Miraglia, & Cioffi, 2008), we discovered that the fermentation and ripening temperatures were consistently below 12°C throughout the entire ripening process. In the area of food biotechnology, cold-adapted micro-organisms, i.e. psychrophilic, psychrotrophic and psychrotolerant microorganisms, have generally been regarded as food-spoilage organisms rather than as potentially useful. This view has grown, especially since the introduction of refrigerators for food storage. Thus, research into the mechanisms of control of the growth of cold-adapted micro-organisms and their enzyme activities has been very popular. Less attention has been paid to the fact that cold-adapted micro-organisms and their enzyme systems can actually be applied as a potential biocatalyst at low temperatures. Low temperature reactions using these biocatalysts have various advantages, e.g., low temperatures in food processing prevent contamination by means of mesophilic organisms (Margesin & Schinner, 2012).

Bearing this in mind, we reproduced the average conditions recorded during our studies in the ripening chamber of the pilot plant, and tested a new formulation of a cold-adapted micro-organism as opposed to a commercial formulation, to investigate its effect on selected, spiked pathogens throughout ripening.

2 Materials and methods

2.1 Definition of ripening conditions

Using several Testostor 175-2 (Testo, Lenzkirch, Germany) data loggers, we recorded the temperature and humidity conditions in small-scale plants, which produce fermented salami. The same conditions were used for this experiment (Table 1).

2.2 Selection of starter cultures

A selection of 138 lactic acid bacteria, isolated from meat and dairy products and identified in previous works (Cenci-Goga et al., 2016; Cenci-Goga et al., 2015; Sechi, Iulietto, Mattei, Traina, Codini, & Cenci-Goga, 2014; Cenci-Goga et al., 2012; Filipović, Cenci-Goga, Njari, Dobranić, Zdolec, & Kozačinski, 2012; Cenci-Goga et al., 2008; Clementi, Cenci-Goga, Trabalza-Marinucci, & Di Antonio, 1998), were tested for their ability to grow at a low temperature (10°C) in aerobic and anaerobic conditions. All strains, which showed growth at 10°C, both in aerobic and anaerobic conditions without gas production from dextrose, were tested for acidifying activity in Skim Milk (BD Difco, 232100). Then, based on the instantaneous acidification rate and its maximum value, a selection of four strains was made to be used as a cold adapted starter culture (NoNit™) (Cenci-Goga et al., 2016; Cenci-Goga et al., 2015).

Bacterial strains used in the NoNit™ formulation were: *Lactococcus lactis ssp. lactis*, strain 340; *L. lactis* ssp. *lactis*, strain 16; *Lactobacillus casei* ssp. *casei*, strain 208 and *Enterococcus faecium* UBEF-41. The morphological, biochemical and physiological characterisation, the growth curves at several temperatures, including refrigeration conditions, the acidifying activity of four bacterial strains and their ability to improve palatability of dry salami along with safety considerations for the commercial probiotic *E. faecium* UBEF-41 have been reported by the authors in previous papers (Cenci-Goga et al.,

2016; Cenci-Goga et al., 2015; Clementi et al., 1998; Cenci-Goga, Clementi, & Di Antonio, 1995). Before the challenge in 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 hrs. Each strain was then sub-cultured in Skim Milk (BD Difco, Franklin Lakes, NJ, USA, 232100) at 37°C for 24 hrs. The total viable cell (TVC) count (on Nutrient Agar, NA, Oxoid CM0003, incubated at 37°C in air for 24 hrs) at 24 hours was approximately 1 x 10^9 cfu ml^{-1}. For the tests (challenge in salami production), the strains were inoculated into salami batter to get an initial concentration of approximately 1 x 10^7 cfu g^{-1}, which mimics the initial starter concentration in salami production.

2.3 Selected spiked microorganisms.

Bacterial strains used as marker micro-organisms for inoculation were taken from the collection of the Laboratorio di Ispezione degli Alimenti di Origine Animale: *S. aureus* field strain (internal ref. # 27R *mec*A), resistant to 2 µg/ml of methicillin and producer of staphylococcal enterotoxin A (SEA); *E. coli*, strain CSH26 K-12, resistant to 200 µg/ml nalidixic acid; *Listeria innocua* ATCC 33090; *Salmonella* Derby field strain (internal ref. #27). The micro-organisms were adapted to the test conditions (pH, aw, salt concentration and temperature) before the challenge test (Spanu, Scarano, Ibba, Pala, Spanu, & De Santis, 2014; NACMCF, 2010; Anonymous, 2003). The total viable cell (TVC) count (on Nutrient Agar, NA, Oxoid CM0003, incubated at 37°C in air for 24 hours) at 24 hours was approximately 1 x 10^9 CFU/ml^{-1}. For the challenge test in salami, each strain was inoculated into salami batter, to achieve an initial concentration of approximately 1 x 10^6 cfu ml^{-1}.

2.4 Salami production, spiking and sampling

The study was performed in nine different replications on nine different days. Two batches were produced for each replication: with a «commercial starter culture formulation»

(Commercial) and with the «NoNit™ formulation» (NoNit™). Both batches were then spiked with the following strains: *S. aureus*, strain 27R *mec* (A), resistant to 2 µg/ml of methicillin; *E. coli*, strain CSH26 K-12, resistant to 200 µg/ml nalidixic acid; *Listeria innocua* ATCC 33090 and *Salmonella* Derby field strain (internal ref. #27). All batches were tested for the absence of *Listeria* spp. and *Salmonella* spp. before the intentional contamination with the selected microorganism, whereas for the antibiotic resistant strains of *S. aureus* and *E. coli*, the absence of contamination was ensured by using culture media with antibiotics.

Salami were prepared at the pilot plant of the Laboratorio di Ispezione degli Alimenti di Origine Animale according to a procedure handed down among butchers for centuries,. For each replication, the meat used came from the same farm and all the animals were "*suino pesante italiano tipico*" with a live weight of over 150 kg, and an age of over 9 months. Meat, shoulder and flank (70%) and hind fat (30%) was minced and blended with the ingredients (NaCl, 30 g kg^{-1}, pepper, 5 g kg^{-1}, garlic, 2 g kg^{-1}, dextrose 10 g kg^{-1} and starter cultures). All ingredients and salami batter were tested by an external ISO (International Organization for Standardization) 17025:2005 accredited laboratory for the absence of nitrates and nitrites using ionic chromatography (Lopez-Moreno, Perez, & Urbano, 2016). Starter cultures of the formulation NoNit™ were added at a concentration of 10^7 cfu g^{-1} of meat, with a cocci:bacilli:enterococci ratio of 2:1:1. The commercial starter contained a combination of *Lactobacillus paracasei* and *Lb. rhamnosus* with *Staphylococcus carnosus* and *S. xylosus* with a bacilli:cocci ratio of 1:1. According to the manufacturer's instructions, , the final concentration reached in the meat batter exceeded 10^7 cfu g^{-1} of meat.

Salami (30 mm by 10 cm) were then hoisted in a dry-curing hall. Sampling (three sausages per group, three subsamples per sausage) occurred at time 0 (ground meat), day 3 (middle

fermentation), day 7 (end-fermentation), day 13 (middle ripening), day 21 (end ripening process) and day 28 (final product).

2.5 Microbiological analysis

For each of the nine replications, 3 sausages per group at each sampling point and 3 subsamples per sausage were sampled.

For the analysis of salami (Cenci-Goga et al., 2012), approximately 25 g of sample were transferred aseptically to 225 ml of sterile, buffered, peptone water (Oxoid), and homogenised in a stomacher (PBI International). 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 total count and selective agar plates. Microbial analyses were conducted according to the methods described above (Cenci-Goga et al., 2012).

Briefly, an Oxacillin Resistance Screening Agar base (ORSAB, Oxoid CM1008) with ORSAB selective supplement (Oxoid, SR0195), incubated at 37°C for 24 h, was used for the *S. aureus* methicillin resistant strain. Enterotoxin production was determined using the method described previously (Cenci-Goga, Karama, Rossitto, Morgante, & Cullor, 2003) followed by an enzyme immunoassay for combined detection of *S. aureus* enterotoxins A, B, C, D and E (RIDASCREEN SET Total, R-Biopharm, Melegnano, Milan, IT). Violet Red Bile Glucose Agar (VRBG, Oxoid, CM0485), to which a solution of nalidixic acid at a final concentration of 200 µg/ml was added, was incubated at 37°C for 24h, and used for counts of the *E. coli* nalidixic acid resistant strain. A RAPID'Listeria spp. Agar Base (Biorad, USA, 3564744) with RAPID'Listeria spp. Supplement 1 (Biorad, 3564745) and RAPID'Listeria Supplement 2 (Biorad 3564746), incubated at 37°C for 24 h, was used for *Listeria innocua*. XLD AGAR (Oxoid CM0469), incubated at 37°C for 18-24 hours, was used for *Salmonella* Derby 27. Total aerobic mesophilic microbiota were determined on Plate Count Agar (Oxoid), at 30 °C for 72 h; *Lactococcus* spp. on M17 agar (Oxoid) 10% v/v lactose, at 37°C

for 48 h; *Lactobacillus* spp. on MRS Agar (Oxoid) pH 5.5, at 30°C for 72 h under anaerobic conditions (Gas generating kit, Oxoid); enterococci on Slanetz and Bartley (SB) Agar (Oxoid, CM0377), at 37 °C for 48 h. *Staphylococcus* spp. on Baird Parker agar (Oxoid CM 275) containing Egg Yolk Tellurite (Oxoid SR 54) at 37°C for 48 h, after replica-plating (Lederberg & Lederberg, 1952) on Oxacillin Resistance Screening Agar base (ORSAB, Oxoid CM1008) with ORSAB selective supplement (Oxoid, SR0195), incubated at 37°C for 24 h, to disregard spiked *S. aureus* methicillin resistant strain.

To differentiate the enterococci, lactococci and lactobacilli of the NoNit™ formulation from those of the spontaneous microbiota, a proportion of colonies grown on the specific culture media (5 colonies from plates with less than 100 cfu and 10 colonies for plates with a number of cfu between 100 and 300) were subjected to the determination of acidifying properties, as described previously (Cenci-Goga et al., 2015; Picque, Perret, Latrille, & Corrieu, 1992; Chamba & Prost, 1989). All isolates with a Vm (Δ pH) at 37°C higher than -1.5×10^{-3} at 37°C and higher than -2.5×10^{-3} at 10°C were confirmed as belonging to the NoNit™ group (Cenci-Goga et al., 2015).

Furthermore, to differentiate *E. faecium* of the NoNit™ formulation from those of the spontaneous microbiota, a proportion of colonies was confirmed by 16s rRNA sequencing (Cenci-Goga et al., 2016).

2.6 Physico-chemical and chemical analysis

With the sampling scheme used for the microbiological analysis, salami were macerated in a chopper and appropriate portions of the homogenised sample were used for the chemical analysis. A Double Pore F electrode (Hamilton Company, Reno, NV, USA) 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 dew-point hygrometer HygroLab 3 (Rotronic, Huntington, NY, USA). Five saturated solutions of

known a_w were used for calibration. The chemical composition and NaCl content were determined according to AOAC methods (AOAC, 2000). a_w, and pH was determined by means of ripening, whereas chemical composition and ash content were determined on the day of stuffing and at the end of the ripening process.

2.7 Sensory evaluation

One week after the conclusion of ripening, a descriptive sensory evaluation was performed. The panel consisted of 6 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 warned that some samples could be contaminated with pathogens and were asked to test the dry-cured sausages for the following characteristics: colour intensity, colour uniformity, fat/lean connection, fat/lean distribution, global odour, mould odour, elasticity, hardness, cohesiveness. Basic taste was, therefore, not evaluated for safety reasons. However, data on the effect of the NoNit™ starter on the full sensory attributes of nitrite-free, dry-cured, pork sausages has been reported elsewhere (Cenci-Goga et al., 2016). 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). Each tester wore personal protective equipment (protective glasses, disposable gloves and disposable lab cots). 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 a.m. To reduce fatigue, assessors conducted no more than three tests per day, lasting a maximum of 1 h.

2.8 Analysis of results.

The arithmetic mean within each sampling was computed and subsequently, all data (geometric mean for microbiology) was elaborated with GraphPad InStat, 3.0b and GraphPad Prism 6.0h for Mac OS X. For each of the nine replications, the $\log_{10}$ of the arithmetic mean for all the microbiological analyses was calculated, following which all $\log_{10}$ data was analysed using GraphPad InStat, version 3.0bfor Mac OS X, for the analysis of variance, followed by the Tukey-Kramer multiple comparisons test.

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 which fell 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. Samples showing at least one typical colony in the lowest dilution were defined as positive, otherwise the result was considered negative (Cenci-Goga, Ortenzi, Bartocci, Codega de Oliveira, Clementi, & Vizzani, 2005).

For the sensory evaluation, a t -student test was carried out to determine the effect of the starter formulation (Commercial vs. NoNit™) on the appearance of the salami.

3 Results

3.1 Salami challenge test

3.1.1 Pathogens

Neither *Listeria* spp. nor *Salmonella* spp. were detected before the challenge test on the raw materials, pork meat, pork fat and on natural casing and ingredients (NaCl, pepper, dextrose, garlic and starter cultures). Counts of *S. aureus*, strain 27R; *E. coli*, strain CSH26 K-12; *Listeria innocua* ATCC 33090 and *Salmonella* Derby (internal ref. #27) at time 0 (ground meat), day-3 (middle fermentation), day-7 (end-fermentation), day-13 (middle ripening), day-21 (end ripening process) and day-28 (final product) are displayed in Table 2. The initial inoculum levels (day-0) was always approx. 4 log_{10} cfu g^{-1}. During fermentation and ripening, with very little heterogeneity observed between batches, the *S. aureus* strain 27R reached levels of 6 log_{10} cfu g^{-1} from day-21 in the commercial batches, whereas it always remained below 6 log_{10} cfu g^{-1} in the NoNit™ batches. The *E. coli* strain CSH26 K-12 reached levels of 5.05 log_{10} cfu g^{-1} on day-7 and remained at approximately 4 log_{10} cfu g^{-1} throughout ripening in commercial batches, whereas it dropped to 1.93 log_{10} cfu g^{-1} in the NoNit™ batches, after a peak to 4.53 on day-3. *Listeria innocua* ATCC 33090 reached approximately 6 log_{10} cfu g^{-1} on day-3, with a plateau until the end of ripening in commercial batches, and reached 5.02 log_{10} cfu g^{-1} in NoNit™ on day-21, to drop below 5 log_{10} cfu g^{-1} at the end of ripening. *Salmonella* Derby reached levels of 5.42 log_{10} cfu g^{-1} on day-3 and stayed above 3 log_{10} cfu g^{-1} throughout fermentation and ripening in commercial batches, whereas it dropped below 3 from day-21 and to 1.79 log_{10} cfu g^{-1} at the end of ripening in the NoNit™ batches. SEA (staphylococcal enterotoxin A) (Table 2) was detected from day-3 only in commercial batches.

3.1.2 Growth of starter cultures.

Lactococcus spp. and *Lactobacillus* spp. counts were always above 6 $\log_{10}$ cfu g $^{-1}$ and reached values above 8 $\log_{10}$ cfu g $^{-1}$ from day-3 (for *Lactobacillus* spp.) and day-7 (for *Lactococcus* spp.) for both formulations (Table 3). *Enterococcus* spp. counts started from 4.14 $\log_{10}$ cfu g $^{-1}$ in the commercial batches and from 6.11 $\log_{10}$ cfu g $^{-1}$ in the NoNit™ batches, and remained above 6 $\log_{10}$ cfu g $^{-1}$ throughout fermentation and ripening in both batches. *Staphylococcus* spp. counts (this data is the difference between counts in the Baird Parker agar and counts in the Oxacillin Resistance Screening Agar base with ORSAB selective supplement, after replica plating), always exceeded 4 $\log_{10}$ cfu g $^{-1}$ and decreased to 3.25 $\log_{10}$ cfu g $^{-1}$ at the end of ripening in commercial batches, whereas in the NoNit™ batches, counts were always below 4 $\log_{10}$ cfu g $^{-1}$ and decreased below 2 $\log_{10}$ cfu g $^{-1}$ on day-21 (Table 3).

3.2 Physico-chemical and chemical analysis

Figures 1 and 2 show a_w and pH. a_w decreased below 0.90 on day-13 in the NoNit™ batches and on day-21 in the commercial batches. Fully ripened salami were between 0.82 in the NoNit™ batches and 0.84 in the commercial batches. Mean pH values on the day of stuffing were 6.10 and reached values below 6.0 on day-3 in the NoNit™ batches and on day-21 in the commercial batches. At the end of ripening, the NoNit™ batches reached pH values of 5.17 and the commercial batches of 5.67. Table 4 shows the chemical composition: on the day of stuffing, proteins were 40.44, fat 50.32, ash 8.88, (g 100 g^{-1} total solids), and similar data was obtained at the end of ripening. Moisture decreased from 58.67% on the day of stuffing to 28.22% (commercial) and 29.30 (NoNit™) in fully ripened salami. Neither nitrates nor nitrites were detected in the ingredients, meat or salami batter.

3.3 Sensory evaluation

Table 5 shows the results of sensory analysis, which was limited to appearance, odours and texture, because batches had been spiked with pathogens. Similar results were obtained for the two batch groups, with the exception of colour uniformity (3.38 for commercial batches and 5.00 for NoNit™ batches), fat/lean distribution (3.43 vs. 4.87), and hardness (2.50 vs. 3.87).

4. Discussion

4.1 Starter culture formulations (NoNit™ vs commercial)

Staphylococcus aureus 27R, *Escherichia coli* CSH26 K 12, *Listeria innocua* ATCC 33090 and *Salmonella* Derby 27 were spiked into salame nostrano (Italian dry sausage**)** to determine the impact of two different starter culture formulations, a commercial and an experimental one (Commercial vs. NoNit™) on their behaviour during manufacturing, fermentation and ripening. The commercial formulation is a common formulation, which is widely used by producers that require salami with the typical characteristics of home-made salami. The formulation contains *Lb. paracasei*, *Lb. rhamnosus, S. carnosus* and *S. xylosus.* According to the manufacturer, staphylococci enhance the formation of a stable colour, promote aroma formation and colour stability and prevent rancidity, whereas lactobacilli control the fermentation process and may result in medium acidity, depending on the amount of fermentable sugar. For producers that have opted for a production without added nitrates, this formulation is also active at low temperature. Producers, who have opted for the so-called "nitrate-free" production, commonly use a low temperature for fermentation and ripening to limit the growth of pathogens. A logical consequence of low fermentation and ripening conditions is the use of starter culture strains, which, at these temperatures, are still able to multiply and exert their activity. The experimental NoNit™ formulation, on the other hand, is

the result of several years of study on hand-crafted products made in small-scale plants in Umbria, Central Italy, during which we discovered that fermentation and ripening temperatures were consistently below 12°C throughout the entire ripening process and the majority of the microbiota was represented by *Lactobacillus* spp., *Lactococcus* spp. and *Enterococcus* spp. rather than *Staphylococcus* spp. and *Micrococcus* spp. (Cenci-Goga et al., 2015; Cenci-Goga et al., 2012; Cenci-Goga et al., 2008).

As a result of this new trend among manufacturers for the production of nitrate free salami, many culture starter companies are aggressively targeting new formulations for low temperature fermentation. However, many available, commercial, starter cultures, used in salami fermented and ripened at low temperatures, have sometimes been linked to a bitter taste and salami manufacturer are seeking better alternatives. It is well known that proteolysis and protein insolubility influence the flavour and texture of the end product and the release of free amino acids is closely correlated with the development of flavour (Cordoba, Antequera Rojas, García González, Ventanas Barroso, López Bote, & Asensio, 1994; Mc Lain, Blumer, Graig, & Stelel, 1968), and they have been reported as precursors of a sour, sweet and bitter taste (Kato, Rhue, & Nishimura, 1989). Aro Aro et al. (2010) demonstrated that staphylococci cultures, especially *S. xylosus*, increase free amino acid levels in salami, compared to *S. carnosus*, *L. sakei* + *S. carnosus* and *P. pentosaceus* + *S. xylosus*. In contrast, simple cultures with lactic acid bacteria do not affect proteolysis in salami made with beef (Candogan, Wardlaw, & Acton, 2009) and pork (Aro Aro et al., 2010).

The most common species traditionally added to the raw-meat sausages are *S. xylosus* and *S. carnosus* (Rai & Bai, 2014), such as those included in the commercial starter tested in this work . However, taking into account the main enzymatic activities provided by *Micrococcus* spp. and *Staphylococcus* spp., in Nonit™ technology, strains belonging to these species were not included, since their enzymatic makeup would be unnecessary. In fact, no nitrate-

reductase activities provided by these species is required, since there are no added nitrates to the mixture. In addition, the low temperature ripening condition is disadvantageous both for catalase activity (best enzyme temperature range conditions is between 25 – 55°C) and for lipolytic activities, which are mainly guaranteed by tissue enzyme activities (Molly, Demeyer, Johansson, Raemaekers, Ghistelinck, & Geenen, 1997; Zambonelli, Papa, Romano, Suzzi, & Grazia, 1992). Moreover, even though the proteolytic activities of *Micrococcus* spp. and *Staphylococcus* spp. generate appreciated profiles of aromatic compounds (Nazzaro, Di Luccia, Tremonte, Grazia, Sorrentino, Maurelli, & Coppola, 2004), the popular application among producers of selected strains as commercial starter cultures may determine a reduction in aroma variability in local productions. Thus, *Staphylococcus* spp. was replaced in the NoNit™ formulation by an *Enterococcus* spp. strain (*E. faecium*), which grows well at low temperatures and belongs to a species, capable of modulating the aroma by converting amino acids and free fatty acids (Corbiere Morot-Bizot, Leroy, & Talon, 2007; García Fontán, Lorenzo, Parada, Franco, & Carballo, 2007; González-Fernández, Santos, Rovira, & Jaime, 2006; Leroy, Verluyten, & De Vuyst, 2006). From a previous work (Cenci-Goga et al., 2015), 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, Rice, & Arias, 2014). The strain was indeed susceptible to glicopeptides and tetracycline, even though those antibiotics are frequently related to HGT. Some concerns can be raised to the resistance to ampicillin. However, it is known that *Enterococcus* spp. may present an intrinsic tolerance towards the achievable concentrations of beta-lactams commonly used. A key issue is the risk that enterococci can acquire antibiotic resistance genes. Thus, they are not included in the QPS (qualified presumption of safety) list of EFSA (European Food Safety Authority) or in the GRAS (Generally recognized as safe) designation of the FDA (American Food and Drug

Administration). On the other hand, several companies producing starter cultures for meat and dairy products include *E. faecium* in many of their formulations. Moreover, it must be stressed that a number of studies has attempted to compare the resistance spectra of different enterococci according to their human, animal or food origins. Although antibiotic resistant enterococci are isolated from foods, only a few are resistant to the clinically important antibiotics, ampicillin, penicillin, gentamicin and vancomycin. The literature concerning antibiotic resistance in enterococci is often limited to clinical enterococci and confuses the general characteristics of the genus Enterococcus with the characteristics of clinical, enterococcal isolates (Ogier & Serror, 2008; de Fátima Silva Lopes, Ribeiro, Abrantes, Figueiredo Marques, Tenreiro, & Crespo, 2005).

4.2 Salami challenge test

All testing demonstrated that the NoNit™ formulation is a promising candidate as a starter culture for salami produced at a low temperature throughout fermentation and ripening. Thus, the formulation was used in salami manufacture and compared to a commercial starter used for nitrate-free salami. Both formulations were able to control the growth of the three pathogens and of the surrogate for *L. monocytogene*s (*L. innocua*). However, counts in NoNit™ batters were statistically significant lower ($p < 0.001$), when compared to batters made with the commercial starter from day-3 for *S. aureus*, *L. innocua* and *S*. Derby and from day-7 for E. *coli*. In conclusion, the NoNit™ formulation performed better than the commercial formulation in any test.

A result of the utmost importance is the complete inhibition of SEA production by the NoNit™ formulation, when compared to commercial batches, where SEA was detected from day-3. The limiting factors for staphylococcal enterotoxin are the temperature, the number of cells (log cfu ml-1) and pH and a_w (Schelin, Wallin-Carlquist, Thorup Cohn, Lindqvist, Barker, & Rådström, 2011). In particular, the SEA gene is carried in the bacterial genome by

a polymorphic family of temperate bacteriophages, and temperature affects enterotoxin production more than growth (Schelin et al., 2011; Wallin-Carlquist, Cao, Márta, da Silva, Schelin, & Rådström, 2010). Validation of NoNit™ ripening conditions and physico-chemical parameters (i.e. temperature below 12°C, *S. aureus* counts below 10^4 cfu g^{-1} after day-3, pH below 5.5 after day-7 and a_w below 0.90 after day-7) were conducted, using both the formulas provided by Fujikawa and Morozumi (2006) for the temperature and by Soejima, Nagao, Yano, Yamagata, Kagi, and Shinagawa (2007) for the cell count and the classic ComBase Predictor (https://www.combase.cc). Moreover, we compared our data with the classic studies of Metaxopoulous et al. (Metaxopoulos, Genigeorgis, Fanelli, Franti, & Cosma, 1981a, 1981b), even though the authors had warned that their equations were to be used only with the specific sausage type and size, and with the manufacturing process used in their studies. Our data demonstrated that a temperature below 12°C alone is not sufficient to prevent SAE production by the strain of *S. aureus* spiked into salami batter. In fact, as observed also by Metaxopoulos et al. (1981b), the lack of enterotoxin production can be attributed to the successful fermentation attained in the salami batter.

As regards the validation for *Cl. botulinum* growth and toxin production, since the study also intended to assess the sensory properties of spiked salami and the assessor was not allowed to handle the salami spiked with *Cl. botulinum* spores, we compared the ripening conditions and physico-chemical parameters against the ComBase Predictor (https://www.combase.cc). This determined that proteolytic and non-proteolytic strains of *Cl. botulinum* cannot germinate or produce toxins.

4.3 Physico-chemical and chemical analysis

Aw development was similar in the two batches (NoNit™ and commercial) with values for fully ripened salami of 0.82 in NoNit™ batches and 0.84 in commercial batches. The pH drop was faster in the NoNit™ batches, which also reached slightly lower values at the end of

ripening. This demonstrates that the NoNit™ formulation did not cause a sudden drop in acidity, as observed in fast fermented sausages (Feiner, 2006). The addition of the proposed formulation, together with the specific ripening conditions, did not modify the characteristics of the end products, when compared to the characteristics of traditionally produced salami. Moisture decreased from 58.67% on the day of stuffing to 28.22% (commercial) and 29.30% (NoNit™) in fully ripened salami. Neither nitrates nor nitrites were detected in the ingredients, meat or salami batter. This finding is of the utmost importance for the accuracy of our experiment, since ingredients, such as plants and spices, contain nitrates, which are reduced to nitrites, and which in turn react and exert their activity on bacteria and on colour formation.

4.4 Sensory evaluation

Similar results were obtained for both groups of batches, except for colour uniformity, fat/lean distribution and hardness. We concluded that the NoNit™ batches were perceived as more uniform in colour and with a more uniform fat/lean distribution and slightly harder than the commercial batch.

Salami made with the NoNit™ formulation in previous experiments (Cenci-Goga et al., 2016; Cenci-Goga et al., 2015; Sechi et al., 2014; Cenci-Goga et al., 2012; Cenci-Goga et al., 2008) were perceived by panelists as slightly saltier, more cohesive, less bitter and generally more acceptable, when compared to commercial starters. This is possibly related to the incorporation of the *E. faecium* strain in the starter formulation. Enterococci are, in fact, capable of modulating the aroma by converting amino acids and free fatty acids (Corbiere Morot-Bizot et al., 2007; García Fontán et al., 2007; González-Fernández et al., 2006; Leroy et al., 2006).

Traditional starter cultures are considered necessary to achieve the desired fermentation parameters, specific for the product type. However, the implementation of other cultures with more significant pathogen inhibitory activities would contribute to increased product safety. In conclusion, this study reports that a formulation of cold-adapted LAB cultures, along with a probiotic, may be used to produce fermented sausages ripened at low temperature even without added nitrates and nitrites, leading to a potentially safer product with no adverse effect on the quality of Italian *salame nostrano*.

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Key to the figures and tables

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

Table 2. Microbiological counts for total *S. aureus*, *E. coli*, *L. innocua* spp. and *S.* Derby in salami produced with commercial starter vs. NoNit™ formulation (Log cfu g^{-1}).

Footnote for this table:
sd: standard deviation, T: SAE production
Different superscripts in the same row indicate significantly different means ($p<0.001$)
T: SAE production

Table 3. Microbiological counts for total mesophilic microbiota, *Lactococcus* spp., *Lactobacillus* spp. and *Enterococcus* spp. in salami produced with commercial starter vs. NoNit™ formulation (Log cfu g^{-1}).

Footnote for this table:
sd: standard deviation
Different superscripts in the same row indicate significantly different means ($p<0.001$)

Table 4. Chemical parameters on the day of stuffing and at the end of ripening1 of *salame nostrano*

Footnote for this table:
in brackets: standard deviation; n = 3

Table 5. Sensory analysis.

Footnote for this table:
sd: standard deviation
Different superscripts in the same row indicate significantly different means ($p<0.005$)

Figure 1. Development of a_w in *salame nostrano* during fermentation and ripening

Footnote for this figure:

Key: ███ commercial starter, ___ NoNit™ formulation

Figure 2. Development of pH in *salame nostrano* during fermentation and ripening

Footnote for this figure:

Key: ███ commercial starter, ___ NoNit™ formulation

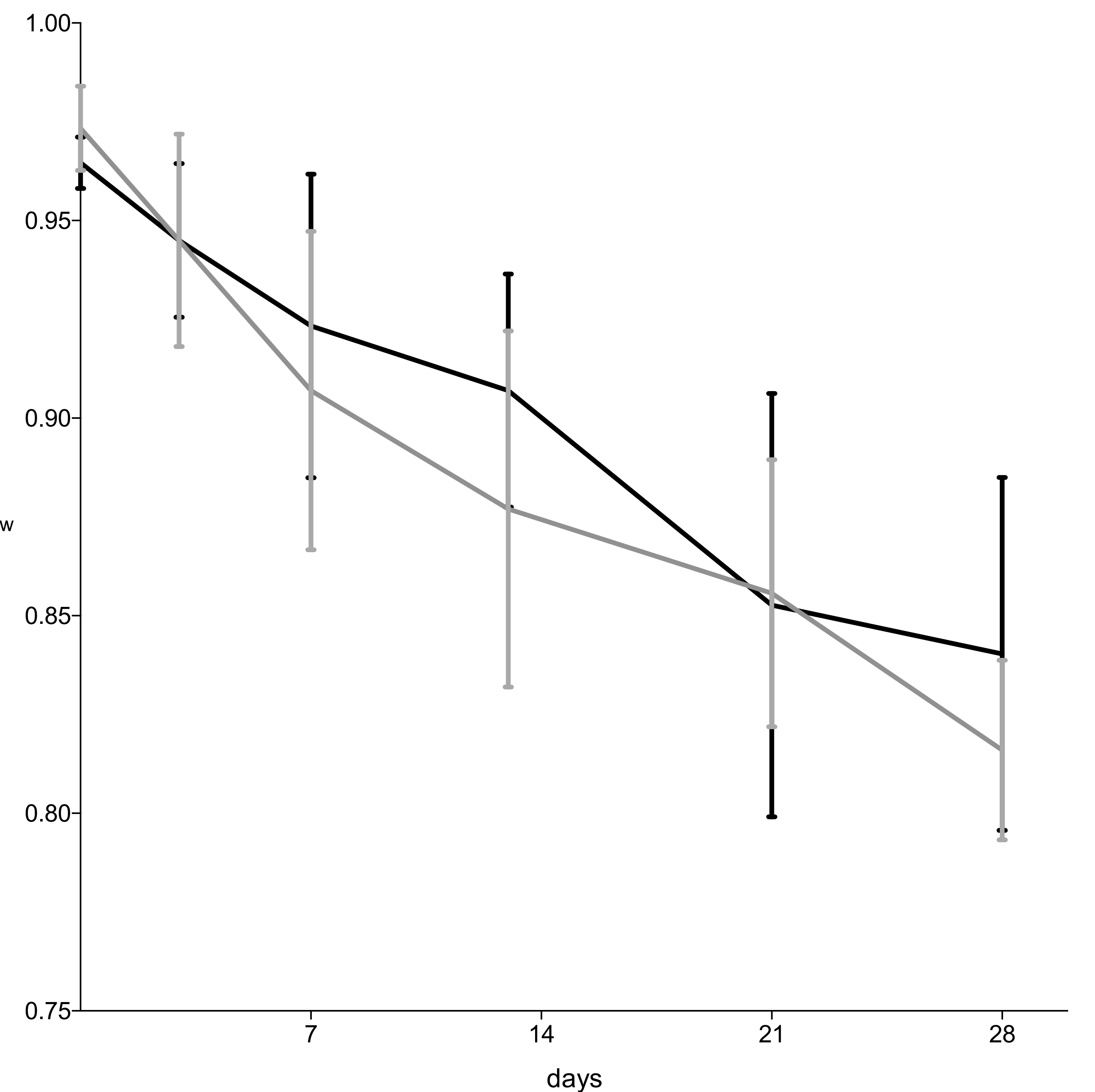

Figure 1

Figure 2

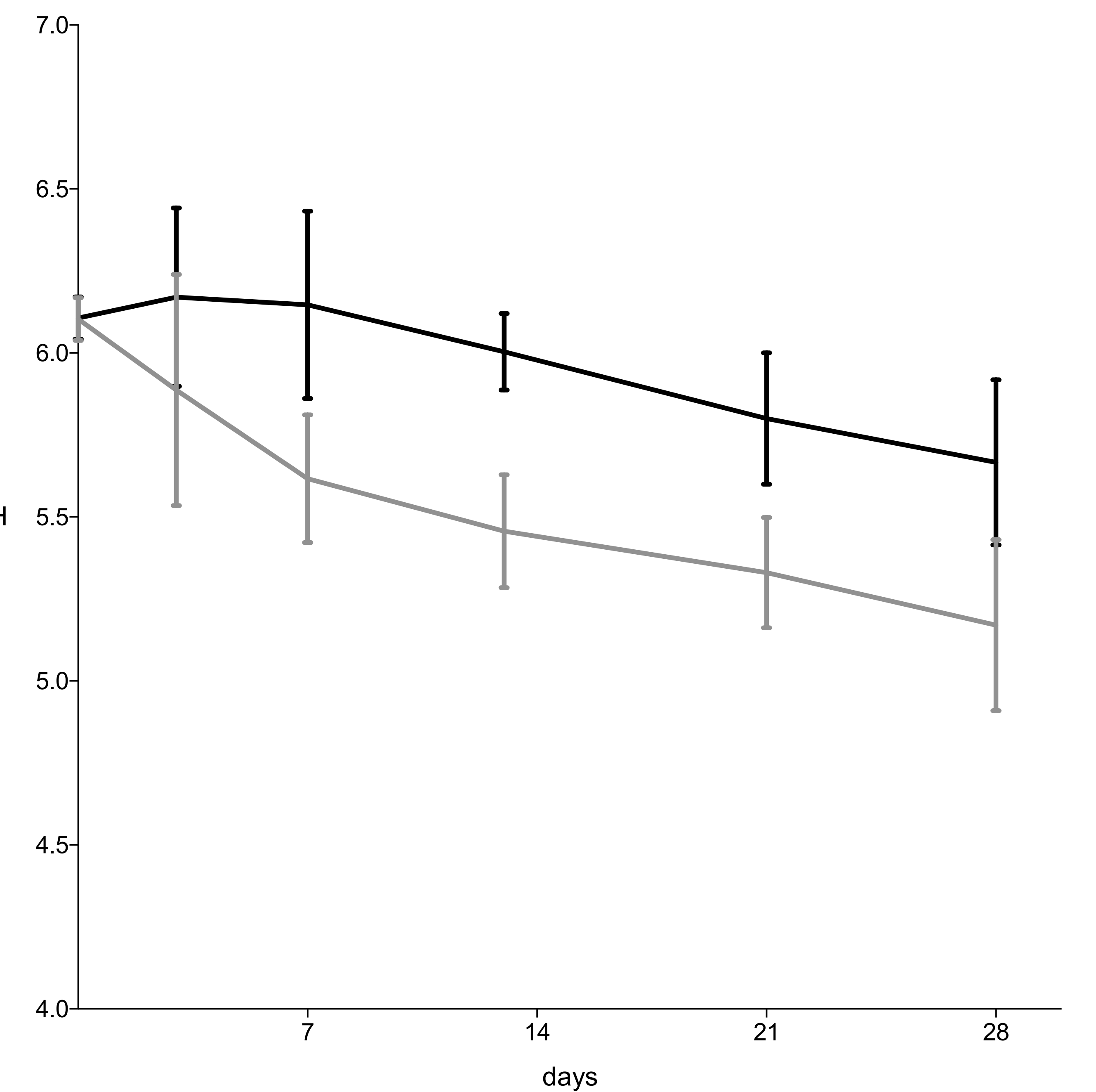

Table 1 Fermentation and ripening conditions used for *salame nostrano* production

Time	Temperature (°C)	Relative Humidity (%)
hour 0-5	6	65
hour 5-10	8	65
hour 10-15	10	70
hour 15-20	8	70
hour 20-24	10	80
day 1-10	14	85
day 10-28	10	85

Table 2. Microbiological counts for total *S. aureus*, *E. coli*, *L. innocua* spp. and *S.* Derby in salami produced with commercial starter vs. NoNit™ formulation (Log cfu g^{-1}).

	Day	Commercial		NoNit™	
		mean	sd	mean	sd
S. aureus 27R					
	0	4,19	0,17	4,26	0,15
	3	5,45[a] T	0,14	4,04[b]	0,47
	7	5,89 [a] T	0,10	3,69 [b]	0,50
	13	5,62 [a] T	0,48	3,87	0,59
	21	6,00 [a] T	0,12	3,85 [b]	0,15
	28	6,01 [a] T	0,33	3,82 [b]	0,16
E. coli CSH26 K-12					
	0	4,03	0,22	4,18	0,24
	3	4,54	0,08	4,53	0,20
	7	5,05 [a]	0,03	4,39 [b]	0,21
	13	4,62 [a]	0,14	3,75 [b]	0,17
	21	3,92 [a]	0,39	2,94 [b]	0,16
	28	3,54 [a]	0,32	1,93 [b]	0,63
L. innocua ATCC 330*90*					
	0	4,09	0,20	4,31	0,05
	3	5,99 [a]	0,08	4,73 [b]	0,12
	7	5,88 [a]	0,12	4,97 [b]	0,01
	13	5,89 [a]	0,11	4,98 [b]	0,05
	21	5,89 [a]	0,12	5,02 [b]	0,24
	28	5,80 [a]	0,06	4,93 [b]	0,05
S. Derby 27					
	0	3,69	0,16	3,90	0,13
	3	5,42 [a]	0,13	4,43 [b]	0,17
	7	4,97	0,10	4,35	0,24
	13	4,37	0,47	3,63	0,28
	21	3,41 [a]	0,20	2,55 [b]	0,42
	28	3,67 [a]	0,44	1,79 [b]	0,86

sd: standard deviation, T: SAE production

Different superscripts in the same row indicate significant different means ($p<0.001$)

T: SAE production

Table 3. Microbiological counts for total mesophilic microbiota, *Lactococcus* spp., *Lactobacillus* spp. and *Enterococcus* spp. in salami produced with commercial starter vs. NoNit™ formulation (Log cfu g^{-1}).

	Day	Commercial		NoNit™	
		mean	sd	mean	sd
Total mesophilic microbiota					
	0	6,69 [a]	0,02	6,34 [b]	0,06
	3	7,87	0,07	8,00	0,13
	7	8,50 [a]	0,11	8,23 [b]	0,08
	13	8,50 [a]	0,10	8,24 [b]	0,07
	21	8,40 [a]	0,10	8,10 [b]	0,07
	28	8,48 [a]	0,06	8,06 [b]	0,12
Lactococcus ssp.					
	0	6,34	0,15	6,16	0,07
	3	6,72 [a]	0,06	6,29 [b]	0,04
	7	6,94 [a]	0,16	6,35 [b]	0,04
	13	7,06 [a]	0,03	6,37 [b]	0,06
	21	7,05 [a]	0,05	6,49 [b]	0,35
	28	6,76 [a]	0,03	6,37 [b]	0,12
Lactobacillus spp.					
	0	6,66 [a]	0,06	6,20 [b]	0,05
	3	8,38 [a]	0,13	8,05 [b]	0,10
	7	8,21	0,10	8,12	0,06
	13	8,31	0,04	8,25	0,08
	21	8,25	0,12	8,08	0,06
	28	8,40 [a]	0,16	8,05 [b]	0,15
Enterococcus spp. (*E. faecium* for NoNit™)					
	0	4,14 [a]	0,23	6,11 [b]	0,04
	3	6,76	0,26	6,98	0,06
	7	6,64	0,39	6,82	0,20
	13	6,24	0,03	6,58	0,19
	21	6,49	0,31	6,75	0,15
	28	6,47	0,29	6,66	0,08
Staphylococcus spp.					
	0	4,03	0,22	3,06	0,23
	3	4,54	0,08	3,83	0,34
	7	5,05 [a]	0,03	3,24 [b]	0,10
	13	4,62 [a]	0,14	3,13 [b]	0,49
	21	3,92 [a]	0,39	1,59 [b]	1,20
	28	3,25 [a]	0,26	0,22 [b]	0,67

sd: standard deviation

Different superscripts in the same row indicate significant different means ($p<0.001$)

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

Property	Day of stuffing		End commercial		End NoNit™	
Protein	40.44	(0.35)	43.38	(4.11)	43.91	(4.72)
Fat	50.32	(2.56)	45.84	(6.02)	45.50	(4.13)
Ashes	8.88	(0.19)	9.51	(0.58)	9.26	(1.33)
Moisture	58.67	(1.77)	28.28	(1.40)	29.30	(0.72)

in brackets: standard deviation

Table 5. Sensory analysis.

	Commercial		NoNit™	
	mean	sd	mean	sd
colour intensity	4,38	0,92	4,53	1,81
colour uniformity	3,38[a]	1,30	5,00[b]	1,65
fat/lean connection	3,63	1,19	4,47	1,64
fat/lean distribution	3,43[a]	0,79	4,87[b]	1,69
odour (global)	3,88	0,83	4,53	1,36
mould odour	3,00	1,51	2,47	1,46
elasticity	3,88	1,81	3,27	1,62
hardness	2,50[a]	1,60	3,87[b]	1,41
cohesiveness	4,63	1,92	4,93	1,34

sd: standard deviation

Different superscripts in the same row indicate significant different means ($p<0.005$)