Fate of selected pathogens in spiked «SALAME NOSTRANO» produced without added nitrates following the application of NONIT™ technology

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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^{TM} technology) vs. a commercial» starter on the fate selected pathogens and hygiene indicators during fermentation and ripening of experimentally spiked *salame nostrano* (italian dry salami**)**. 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 formulation of starter cultures (a commercial one 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) in specific ripening conditions.

The proposed formulation along with specific ripening conditions (NoNit™) limited the growth of spiked *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* Derby 27 and *Listeria innocua* yet maintaining the basic appearance and aroma and texture attributes of the products.

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

Introduction

Fermentation of raw meat to improve the safety, the shelf life and the acceptability of certain foods has a long tradition. In fermented sausages produced from raw meat the conditions that result from fermentation are in general sufficient to inhibit the growth and toxin production of most pathogens due to a combinations of hurdles, such as pH, water activity, preservatives and the action of lactic acid bacteria, either added as starter cultures or naturally present (B. Cenci-Goga et al., 2015; B. T. Cenci-Goga et al., 2016). However studies have demonstrated that several bacteria, including *Staphylococcus aureus*, *Listeria* spp., *Escherichia coli* and *Salmonella* spp. can survive in final products, and although fermented sausages have a well-founded reputation for safety, outbreaks of food poisonings occurs (Al-Mutairi, 2011; Chajecka-Wierzchowska, Zadernowska, Nalepa, Sierpinska, & Laniewska-Trokenheim, 2015; D'Ostuni et al., 2016). In Italy, for instance, salami have been implicated in several *E. coli* and *Salmonella* spp. related family outbreaks (Conedera et al., 2007; Luzzi et al., 2007). Outbreaks of *L. monocytogenes* linked to the consumption of pre-sliced ready to eat foods have been described (Anonymous, 2002; Sartz et al., 2008; Sheen & Hwang, 2008). During fermentation, ripening and drying of fermented sausages levels of pathogens have been shown to drop with the inactivation proportional to pH, salt and nitrates/nitrite levels (Casey & Condon, 2000; Riordan et al., 1998). Over the last years there has been a growing consumer interest in ready to eat fermented sausages produced with lower concentrations of additives and many technologies have been proposed to limit their use yet maintaining the same level of safety (Cenci Goga et al., 2012; B. Cenci-Goga et al., 2015; B. T. Cenci-Goga et al., 2016; Zarringhalami, Sahari, & Hamidi-Esfehani, 2009). Moreover, further to the recommendation of the «International agency for research on cancer» of the «World health organization» based on epidemiological studies suggesting that small increases in the risk of several cancers may be associated with high consumption of red meat or processed meat (Bouvard et al., 2015),

several agencies and the media have raised the question whether processed meat can be safely produced without added nitrates and nitrites. Processed meat refers to meat that has been transformed through salting, curing, fermentation, smoking, or other processes to enhance flavor or improve preservation, such as nitrates and nitrites addition. Nitrates and nitrites are currently authorized as food additives within the EU. These additives function as preservatives in food and they are both used extensively to enhance the color and extend the shelf life of processed meats. Nitrite is considered the active curing ingredient responsible for the preservation of the food in combination with other ingredients, like salt. Nitrate, when added to food, converts to nitrite before exerting a preservative function. The use of nitrates and nitrites in food products must comply with the provisions set out in Annex II part E of Regulation (EC) No.1333/2008 on food additives which is in force since 1 June 2013. The maximum levels at which nitrates and nitrites may be used, and also the specific foodstuffs in which they may be used as well as their conditions of use are also established by Regulation (EC) No. 1333/2008 as amended. These levels are set at values which ensure that a person consuming a typical diet would not exceed the Acceptable Daily Intake (ADI) established for these additives. Purity criteria have also been established for nitrates and nitrites via Regulation (EC) No. 231/2012 as amended. Two main nitrite and nitrate salt forms are allowed. These are sodium and potassium nitrite and potassium and sodium nitrate. These substances have been attributed E numbers (E250, E249, E251 and E252 respectively). In accordance with EU legislation, nitrates and nitrites are permitted for use in foods such as cheese, raw and processed meats, and processed fish and may only be sold in a mixture with salt or a salt substitute when labeled for food use. This is designed to limit the amount of nitrite that can be added and to prevent accidental poisoning through the addition of excessive quantities of nitrite to food (Anonymous, 2016).

A primary function of nitrite is the production of the characteristic red color of cured meats. Nitrite, in addition, impart antibacterial activity, particularly the inhibition of germination of spores and toxin formation by *Clostridium botulinum* (Adams & Moss, 2000; Mossel, Corry, Struijk, & Baird, 1995). Despite the desired properties, the safety of nitrite to human health has been questioned as they can cause the formation of carcinogenic N-nitrosamines. In foods, nitrosamines are produced from nitrites and secondary amines: their formation can occur only under certain conditions, including strongly acidic conditions such as that of the human stomach. Carcinogenic chemicals that form during meat processing include N-nitroso compounds and polycyclic aromatic hydrocarbons (Bouvard et al., 2015; Ozel, Gogus, Yagci, Hamilton, & Lewis, 2010).

For these reasons the consumer interest in nitrite-free products has raised and, as a consequence, hand-crafted products made locally in 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., 2012; B. T. Cenci-Goga et al., 2016; Zarringhalami et al., 2009). It is obvious that nitrite-free sausages manufacture pose two big problems to the food business operators: first of all the production of safe food (i.e. absence of *Clostridium botulinum* toxin) and then color formation and stability (Zarringhalami et al., 2009). Several alternatives to nitrates nitrites for their antimicrobial action have been tested (Pegg & Shahidi, 2000) and many different methods have been proposed to enhance color 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 restrain indigenous microbiota to control the processing and promote food safety. According to the *hurdle technology* (Leistner, 2000), every condition which make the pathogen or spoilers bacterial growth disadvantaged can be considered a hurdle; according to that,

beneficial bacterial strains can be applied to enhance shelf-stable cured meat products, guaranteeing safe productions. Lactic acid bacteria and staphylococci are the most used strains in cured meat production and they have been directly applied to the meat batter since several years. On one hand, the application of staphylococci as starter cultures shows some limitations since pH tolerance and temperature tolerance are key factor to enable them to produce elective enzymes; low temperatures, in fact, can affect their activity. Meat color formation and stability is enhanced by staphylococci while rancidity processes are slowed. Among staphylococci, the most frequently used are *Staphyloccous xylosus* and *S. carnosus* and the produced enzymes, in association with the enzymes in the meat, contribute to aroma definition. On the other hand, lactic acid bacteria determine technological changes, which promote meat stability. They ferment available sugars and produce organic acids: the produced acids determine pH reduction which contribute to drying process through the reduction of the water holding capacity, to color formation and to inhibit undesired microbiota occurred in the product.

Before defining which strains can be applied as starter cultures, it is required that the strains are homofermentative (to avoid unwanted gas production), producing mostly lactic acid or little amount of acetic acid, through the definition of the acidification profile. Among Lactic Acid Bacteria, *Lactobacillus* spp. and *Pediococcus* spp. are the most frequently applied in dry sausages production for the desired activities. Anyway, it has to be considered that according to each specific meat product raw composition and recipe, the acidification capability of indigenous microbiota may vary and influence the activities of the added strains. To maximize the beneficial effect of the starter culture application, therefore, it is crucial to well-define product characteristics, processing technology and predict the final product. In addition, processing parameters, mince composition, product diameter influence acidification speed which can affect the quality of the final product. In fact, fast reached low pH value affects

129 staphylococci enzymes activities with subsequent less effective color and aroma formation, as
130 required by the manufacturer to be satisfied.
131 During our studies on hand-crafted products made in small-scale plants in Umbria, central Italy
132 (Cenci Goga, Ranucci, Miraglia, & Cioffi, 2008; Cenci Goga et al., 2012; B. Cenci-Goga et
133 al., 2015) we discovered that fermentation and ripening temperature were consistently below
134 12°C throughout the whole ripening process. In the area of food biotechnology, cold-adapted
135 microorganisms, that is, psychrophilic, psychrotrophic and psychrotolerant microorganisms,
136 have generally been regarded as food-spoilage organisms rather than as potentially useful, an
137 outlook that has grown especially since the introduction of refrigerators for food storage. Thus,
138 research into the mechanisms of control of the growth of cold-adapted microorganisms and
139 their enzyme activities has been very popular. Less attention has been paid to the fact that cold-
140 adapted microorganisms and their enzyme systems can themselves be applied as potential
141 biocatalyst at low temperature. Low temperature reactions utilizing such biocatalysts have
142 various advantages, e.g., low temperatures in the processing of foods prevent contamination
143 by mesophilic organisms, and cold-adapted enzymes, due to their heat-lability, can be easily
144 inactivated by heating when unneeded after use.
145 With this in mind we reproduced in the ripening chamber of the pilot plant the average
146 conditions recorded during our studies and tested a new formulation of cold-adapted
147 microorganism against a commercial one to investigate their effect on selected spiked
148 pathogens throughout ripening.
149

Materials and methods

Definition of ripening conditions

During a survey on hand-crafted products made in small-scale plants in Umbria, central Italy (Cenci Goga et al., 2008; Cenci Goga et al., 2012; B. Cenci-Goga et al., 2015) we discovered that fermentation and ripening temperature for *salame nostrano*, a typical dry salami produced in central Italy, were consistently below 12°C throughout the whole ripening process. Using several Testostor 175-2 (Testo, Lenzkirch, Germany) data loggers we measured the temperature and humidity conditions in small-scale plants that produce fermented salami. The same conditions were used for this experiment (Figure 1).

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., 2008; Cenci Goga et al., 2012; B. Cenci-Goga et al., 2015; B. T. Cenci-Goga et al., 2016; Clementi, Cenci Goga, Trabalza-Marinucci, & Di Antonio, 1998; Filipović et al., 2012; Sechi et al., 2014) have been tested for ability to grow at low temperature (10°C) in aerobic and anaerobic conditions. All strains that 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) and then, based on the instantaneous acidification rate and its maximum value, a selection of four strain was made to be used as cold adapted starter culture (NoNit™) (B. Cenci-Goga et al., 2015; B. T. Cenci-Goga et al., 2016).

Starter cultures

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, physiological characterization, growth curves at several temperature, including refrigeration conditions, the acidifying activity of four

bacterial strains and their ability to improve palatability of dry salami have been reported by the authors in previous papers (Cenci Goga, Clementi, & Di Antonio, 1995; B. Cenci-Goga et al., 2015; B. T. Cenci-Goga et al., 2016; Clementi et al., 1998). Before further tests (acidification in milk, challenge in vitro and 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. Total viable cells (TVC) count (on Nutrient Agar, NA, Oxoid CM0003, incubated at 37°C on air for 24 hrs) at 24 hrs was approximately 1 x 10^9 cfu ml^{-1}. For the tests (acidification in milk, challenge in vitro and challenge in salami production) strains were inoculated into milk or salami batter to get an initial concentration of approximately 1 x 10^7 cfu ml^{-1} or g^{-1} which mimics the initial starter concentration in salami production.

Selected spiked microorganisms.

Bacterial strains used as marker micro-organisms for the inoculation were taken from the collection of the Laboratorio di Ispezione degli Alimenti di Origine Animale: *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; *Salmonella* Derby field strain (internal ref. #27). The micro-organisms were grown aerobically in Nutrient Broth (NB, Oxoid CM0001, Basingstoke, UK) at 37°C for 24 hrs. The total viable cells (TVC) count (on Nutrient Agar, NA, Oxoid CM0003, incubated at 37°C on air for 24 hrs) at 24 hrs was approximately 1 x 10^9 CFU/ml^{-1}. For the challenge test in milk and in salami each strain was inoculated in skim milk (BD Difco) or into salami batter to get an initial concentration of approximately 1 x 10^6 cfu ml^{-1} (1 x 10^4 cfu g^{-1} for the salami batter).

Characterization of NoNit™ starter cultures with acidification and challenge growth curves at 10°C

Acidification. Strains of the NoNit™ formulation (*Lactococcus lactis ssp. lactis*, strain 340; *L. lactis* ssp. *lactis*, strain 16; *Lactobacillus casei* ssp. *casei*, strain 208 and *Enterococcus faecium* UBEF-41) were inoculated as pure cultures into skim milk (BD Difco) at 37°C to get a final concentration of 10^9 ml^{-1} after 24 h of incubation. The association was then inoculated into skim milk (BD Difco) to get an initial cocci: bacilli:enterocci ratio of 2:1:1 and an initial concentration of about 10^7 cfu ml^{-1}. The association was incubated at 10°C and pH was measured with a Foodtrode electrode (Hamilton Company, Reno, NV, USA) hooked to an Eutech pH 2700 (Eutech Instrument Europe B.V. Nijkerk, Netherlands) which recorded pH values continuously with CyberComm 6000 (Eutech Instrument) every minute. To find mathematically the maximum instantaneous rate of acidification and the moment in which this is achieved, a fourth degree polynomial equation was used as an empirical model for fitting the experimental data collected for each microorganism and for each condition:

$$y= a + bx +cx^2 + dx^3 + ex^4 \quad \text{[equation1]}$$

In this equation y is the pH value, x is time (minutes) and a, b, c, d, and e are the regression coefficients of the independent variable x. The coefficients were determined by the statistical package STATGRAPHICS Centurion XVI version 16.2.04 (Statpoint Technologies, Inc., Warrenton, VA, USA). The first derivative of the equation gives the instantaneous acidification rate and its maximum value (V_m) corresponds to the inflection point of the acidification curve, whereas the second derivative gives the acceleration and one of its roots give the x value (t_m) of the inflection point. Substituting to x the t_m value, can be evaluated by the fourth degree equation the pH value corresponding to the inflection point.

Challenge test in vitro. Bacterial reduction for selected pathogens (*Escherichia coli*, strain CSH26 K-12, *Staphylococcus aureus*, strain 27R, *Salmonella* Derby n. 27) and *L.*

monocytogenes surrogate (*L. innocua* ATCC 33090) vs. the NoNit™ formulation were performed in six replicates. All strains for NoNit™ formulation were inoculated as pure cultures into skim milk (BD Difco) and incubated at 37°C to get a final concentration of about 10^9 cfu ml^{-1} after 24 h of incubation. The association was then inoculated into BHI broth (BD Difco) and skim milk (BD Difco) to get an initial cocci:bacilli:enterocci ratio of 2:1:1 and a concentration of approx 10^7 cfu ml^{-1} until challenge vs. selected pathogens and hygienic indicators.

Pathogens and hygiene indicators were inoculated as pure cultures into BHI (BD Difco) and incubated at 37°C to get final concentration of about 10^9 cfu ml^{-1} after 24 h of incubation. For the test each strain was then inoculated into skim milk (BD Difco) to get an initial concentration of approx 10^6 cfu ml^{-1}. The challenges were carried out in skim milk (BD Difco) at 10°C. Bacterial counts were recorded at time 0, 12 h, 24 h, 30 h, 48 h, 72 h, 120 h, 168 h, 240 h. Microbiological analysis were conducted using the methods described below (Microbiological analysis paragraph).

Salami production, spiking and sampling

The study was performed in nine different replications in nine diverse days. Two batches on each replication were produced: 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, before intentional contamination with the selected microorganism, were tested for the absence of *Listeria* spp. and *Salmonella* spp., while for antibiotic resistant strains of *S. aureus* and *E. coli* the absence of contamination was ensured by the use of culture media with antibiotics.

Salami were prepared with a procedure handed down among butchers for centuries, at the pilot plant of the Laboratorio di Ispezione degli Alimenti di Origine Animale. For each replication pork meat came form the same farm and all animals were "*suino pesante italiano tipico*" with a live weight of more than 150 kg, and more than 9 months of age. 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). 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 *S. carnosus* and *S. xylosus* with a bacilli:cocci ratio of 1:1, and according to manufacturer indications, the final concentration reached in meat batter is above 10^7 cfu g^{-1} of meat.

Salami (30 mm by 10 cm), were then hoisted in a dry-curing hall. Figure 1 shows fermentation and ripening parameters as logged by the chamber. Sampling (three sausages per group, three subsamples each 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).

Microbiological analysis

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

For the analysis of salami, *more solito* (Cenci Goga et al., 2012) about 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 analysis were conducted according to the methods described previously (Cenci Goga et al., 2012).

Briefly, Oxacillin Resistance Screening Agar base (ORSAB, Oxoid CM1008) with ORSAB selective supplement (Oxoid, SR0195), incubated at 37°C for 24 h was used for *S. aureus* methicillin resistant strain. 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, incubated at 37°C for 24h was used for counts of *E. coli* nalidixic acid resistant strain. 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*. X.L.D. AGAR (Oxoid CM0469), incubated at 37°C for 18-24 hours was used for Salmonella Derby 27. Total aerobic mesophilic microbiota 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), 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.

Physico-chemical and chemical analysis

With the sampling scheme used for microbiological analysis (nine replications, 3 sausages per group at each sampling point, 3 subsamples for sausage) 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, 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). Calibration was performed using five saturated

296 solutions of known a_w. Chemical composition and NaCl content were determined according to
297 AOAC methods (AOAC, 2000). a_w, and pH were determined through ripening, whereas
298 chemical composition and ashes content were determined on the day of stuffing and at the end
299 of the ripening process.

300 *Sensory evaluation*

301 Few days after the end of ripening, a descriptive sensory evaluation was performed. The panel
302 consisted of 6 assessors selected among the staff at the Laboratorio di Ispezione degli Alimenti
303 di Origine Animale, previously trained in descriptive analysis for cured meat products. The
304 tasters were warned that some samples could be contaminated with pathogens and asked to test
305 the dry-cured sausages for the following characteristics: colour intensity, colour uniformity,
306 fat/lean connection, fat/lean distribution, global odour, mould odour, elasticity, hardness,
307 cohesiveness. Each assessor was given sheets with a 7-point scale (non-numbered to avoid
308 biased assessment) for each characteristic: 7 = maximum intensity and 1 = minimum intensity.
309 The evaluations were held in individual booths, built according to the criteria of the
310 International Standards Organisation (ISO, 2003), each testers wore personal protective
311 equipment (protective glasses, disposable gloves and disposable lab cots). Samples were taken
312 from the middle of the sausage by cutting off 2 cm from each edge. The sausage slices were 4
313 mm thick and were immediately served on a plastic dish covered with plastic film and coded
314 with random, three-digit numbers. Assessments were carried out under natural light at a room
315 temperature of 20±2°C. The individual scores for each assessor were then averaged to give a
316 score for the taste panel as a whole. Three evaluations for each different sausage were made.
317 Each evaluation was carried out in different test sessions at the same time of day, between 10
318 and 12 a.m. To reduce fatigue, assessors conducted no more than three tests per day, lasting a
319 maximum of 1 h. The significance and the quantitative scale for each descriptor were discussed
320 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. Briefly, color intensity was defined as the characteristic red color of cured *salame nostrano*, color uniformity as the absence of a darker, external halo in the slice due to anomalous drying process, fat/lean connection as the degree of adherence of the product's principal ingredients (fat and lean), fat/lean distribution as the uniform distribution of lean and fat on the slice. Global odor is the global sensation of aroma, mold is the characteristic odor associated with the chemical compound 1-octen-3-ol and has a distinct mushroom odor. Elasticity is the rapidity of recovery from deforming force applied with forefinger and thumb, hardness is the force necessary to compress the sample between forefinger and thumb to achieve a given deformation; cohesiveness is the resistance of the sample before breaking when it is strained.

Analysis of results.

The arithmetic means within each sampling was computed, subsequently all data (geometric mean for microbiology) were 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 means for all microbiological analysis was calculated, 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.

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 included between 30 and 300 cfu were considered suitable for data analysis and 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 et al., 2005).

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

Results

Characterization of starter cultures with acidification and challenge growth curves at 10°C

Acidifying activity of selected starter cultures. Three species of lactic acid bacteria along with a commercial probiotic were chosen to be used as a starter. These strains had been previously identified by API 50 CHL and sequencing and a selection of them had already been used as a starter in the manufacturing of salami (Cenci Goga et al., 2008; Cenci Goga et al., 2012). The acidifying activity of the different species had been preliminarily tested (Cenci Goga et al., 1995; B. Cenci-Goga et al., 2015; B. T. Cenci-Goga et al., 2016; Clementi et al., 1998; Sechi et al., 2014) as pure cultures and as different associations in milk to assess their suitability to be used as a starter. The parameters describing the acidification kinetics for the test at 10°C are described in Table 1 together with the regression coefficients of the fourth degree polynomial [equation 1] which was used as an empirical model. This adequately fitted the experimental data, since the r^2 values varied from 0.998 to 0.999 and the actual values were almost exactly superimposed on the empirical model curves (Figure 2a and 2b). The values of the maximum instantaneous acidification rate [V_m] were of the same order for all strains, except for *Lc. lactis ssp. lactis* strain 340 which had the highest V_m [-2.35×10^{-4} ΔpH/min) and for *Lc. lactis ssp. lactis* strain 16 which had the lowest t_m [3754 min] values. After 12.000 min. all strains reached pH values below 5.5 (below 5.0 for strain 340). *E. faecium* gave a one fold pH decrease within about 2000 min, like some commercial cold adapted lactobacilli (Chamba & Prost, 1989). The association of the four strains performed reached a final pH of 4.20 and

showed the fastest pH drop in the first 24 hrs along with a steady descent subsequently. The association mimicked the behaviour of the two *Lc. lactis ssp. lactis* strains in the first 16 hrs and then, when the lactococci acidification activity decreased (steady pH values after 24 hrs), mirrored the *Lb. casei spp casei* performance for a continuous pH drop.

Challenge test. Results are shown in Figure 3 which display the different growth curves of pathogens or hygiene indicators alone or vs. the NoNit™ formulation. Figure 3a shows the evolution of *S. aureus*, strain 27R, 3b *E. coli* CSH26 K-12; 3c *L. innocua* ATCC 33090; 3d *S.* Derby strain 27. For the challenge *S. aureus* vs NoNit™, level of *S. aureus* dropped, after 240 hrs, below 10^5 cfu ml^{-1}. A similar behaviour was shown by *E. coli* with a slower drop (<10^7 cfu ml^{-1} after 120 hrs). *L. innocua* had a substantial reduction in total viable cells between 72 and 120 hrs (<10^5 cfu ml^{-1} after 24 hrs in BHI and at 240 hrs in milk). LAB had an effect on *Salmonella* Derby, with levels below 10^5 cfu ml^{-1} after 240 hrs. Table 2 shows the log reduction along with the statistics: after 240 hours of incubation with NoNit™ formulation, *S. aureus* had a 1.45 log reduction, *E. coli* 0.81 log, *L. innocua* 1.30 log and *S.* Derby 2.18 log.

Figure 4 show the correct evolution of the NoNit™ strains both in milk and in BHI alone and vs. selected pathogens.

Salami challenge test

Pathogens. No *Listeria* spp. nor *Salmonella* spp. were detected before the challenge testing in the raw materials, pork meat, pork fat and in 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 Figure 5 (5a *S. aurues*, *5b E. coli*, 5c *L. innocua*, 5d *S.* Derby) and Table 3. The initial inoculum levels (day-0) was always approx. 4 $\log_{10}$ cfu g^{-1}: for *S. aureus* it ranged from 4.19 (in commercial batch) to 4.26 (in NoNit™ batch), for *E. coli* form 4.03 to 4.18, for *L. innocua* from 4.09 to 4.18 and for *Salmonella* Derby from 3.69 to 3.90. During fermentation and ripening, with very little heterogeneity observed between batches, *S. aureus* strain 27R reached levels of 6 $\log_{10}$ cfu g^{-1} from day-21 in the commercial batches while remained always below 6 $\log_{10}$ cfu g^{-1} in the NoNit™ batches. *E. coli*, strain CSH26 K-12 reached levels of 5.05 $\log_{10}$ cfu g^{-1} on day-7 and remained at about 4 $\log_{10}$ cfu g^{-1} throughout ripening in commercial batches, while dropped to 1.93 $\log_{10}$ cfu g^{-1} in NoNit™batches, after a peak to 4.53 on day-3. *L. innocua* ATCC 33090 reached about 6 $\log_{10}$ cfu g^{-1} on day-3 with a plateau till 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 while dropped below 3 from day-21 and to 1.79 $\log_{10}$ cfu g^{-1} at the end of ripening in NoNit™ batches.

Evolution 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. *Enterococcus* spp. counts started from 4.14 $\log_{10}$ cfu g^{-1} in the commercial batches and from from 6.11 $\log_{10}$ cfu g^{-1} in the NoNit™ batches and remained above from 6 $\log_{10}$ cfu g^{-1} throughout fermentation and ripening in both batches. *Staphylococcus* spp. counts (these data are the difference between counts in Baird Parker agar and counts in Oxacillin Resistance Screening Agar base with

ORSAB selective supplement, after replica plating), were always above 4 log_{10} cfu g^{-1} and decreased to 3.25 log_{10} cfu g^{-1} at the end of ripening in commercial batches, while in NoNit™ batches counts were always below 4 log_{10} cfu g^{-1} and decreases below 2 log_{10} cfu g^{-1} on day-21 (Figure 6 and Table 4).

Physico-chemical and chemical analysis

Figures 7 and 8 show aw and pH. aw decreased below 0.90 on day-13 in NoNit™ batches and on day-21 in commercial batches. Fully ripened salami were between 0.82 in NoNit™ batchges and 0.84 in commercial batches. Mean pH values on the day of stuffing were 6.10 and reached values below 6.0 on day-3 in NoNit™ batches and on day-21 on commercial batches. At the end of ripening NoNit™ batches reached pH values of 5.17 and commercial batches of 5.67. Table 5 shows the chemical composition: on the day of stuffing, proteins were 40.44, fat 50.32, ashes 8.88, (g 100 g^{-1} total solids), and similar data were 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 full ripened salami.

Sensory evaluation

Figure 9 and Table 6 show the results of sensory analysis which was limited to appearance attributes, odors and texture attributes because batches had been spiked with pathogens. For the two group of batches similar results were obtained, except for color 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

Selected pathogens and hygiene indicators were spiked into salame nostrano (italian dry sausage**)** to determine the impact of two different starter cultures formulation, a commercial

435 one and an experimental one (Commercial vs NoNit™), on their behavior during
436 manufacturing, fermentation and ripening. The batters were spiked with three pathogens (*S.*
437 *aureus*, strain 27R *mec*(A), resistant to 2 µg/ml of methicillin; *E. coli*, strain CSH26 K-12,
438 resistant to 200 µg/ml nalidixic acid; and *Salmonella* Derby field strain, internal ref. #27), and
439 one surrogate for *L. monocytogenes* (*Listeria innocua* ATCC 33090).
440 The commercial formulation is a common formulation which is widely used by producer that
441 require salami with the typical characteristics of home made salami. The formulation contains
442 *Lb. paracasei*, *Lb. rhamnosus*, *S. carnosus* and *S. xylosus* and according to the manufacturer,
443 staphylococci enhance the formation of a stable color, promote aroma formation, color stability
444 and prevent rancidity, while lactobacilli control the fermentation process and may result in
445 medium acidity depending on the amount of fermentable sugar. For producers that have opted
446 for a production without added nitrates, this formulation is also active at low temperature. A
447 common trend among producers that have opted for the so-called nitrate free production, is in
448 fact the use low temperature for fermentation and ripening to limit the growth of pathogens. A
449 logic consequence of low fermentation and ripening conditions is the use of starter cultures
450 strains that at these temperatures are still able to multiply and to exert their activity. The
451 experimental NoNit™ formulation, instead, is the result of several years of study on hand-
452 crafted products made in small-scale plants in Umbria, central Italy, when we discovered that
453 fermentation and ripening temperature were consistently below 12°C throughout the whole
454 ripening process and that the majority of the microbiota was represented by *Lactobacillus* spp.,
455 *Lactococcus* spp. and *Enterococcus* spp. rather than *Staphylococcus* spp. and *Micrococcus* spp.
456 (Cenci Goga et al., 2008; Cenci Goga et al., 2012; B. Cenci-Goga et al., 2015).
457 The NoNit™ formulation, when tested for acidifying activity at 10°C performed well with a
458 final pH of 4.20 and showed the fastest pH drop, within the first 24 hrs along with a steady
459 descent subsequently. The association mimicked the behavior of the two *Lc. lactis ssp. lactis*

strains in the first 16 hrs and then, when the lactococci acidification activity decreased (steady pH values after 24 hrs), mirrored the *Lb. casei spp casei* performance for a continuous pH drop. This synergism is of the utmost importance in the production of fermented food such as dry salami because the activity of starter cultures is desirable throughout the whole fermentation process (Cenci Goga et al., 2012; Cizeikiene, Juodeikiene, Paskevicius, & Bartkiene, 2013). There is a difference in fermented sausage technology between the United States and the European countries. American methods rely on rapid acid production (lowering pH) through a fast fermentation in order to stabilize the sausage against spoilage bacteria. Fast acting starter cultures such as *Lactobacillus plantarum* and *Pediococus acidilactici* are used at high temperatures up to 40º C. As a result pH drops to 4.6, the sausage is stable but the flavor suffers and the product is sour and tangy. In European countries, the temperatures of 22-26ºC are used and the drying, instead of the acidity (pH) is the main hurdle against spoilage bacteria which favors better flavor development. The final acidity of a traditionally made salami is low (high pH) and the sourly taste is not present. The new trend among manufacturers is the production of nitrate free salami and many culture starter companies are aggressively targeting new formulation 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 bitterness taint and salami manufacturer are seeking for better alternatives. It well known that proteolysis and protein insolubility influence the flavor and texture of the final product and the release of free amino acids are highly correlated with flavor development (Cordoba et al., 1994; Mc Lain, Blumer, Graig, & Stelel, 1968) and they have been reported as precursors of sour, sweet, and bitter taste (Kato, Rhue, & Nishimura, 1989). Aro Aro et al. (2010) demonstrated that Staphylococci cultures, especially *S. xilosus*, increase free aminoacid levels in salami compared with *S. carnosus*, *L. sakei* associated with *S. carnosus* and *P. pentosaceus* associated with *S. xilosus*. 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 commercial starter tested in this work contained a combination of *Lb. paracasei* and *Lb. rhamnosus* with *S. carnosus* and *S. xylosus*. Indeed, the most common species traditionally added to the raw-meat sausages are *S. xylosus* and *S. carnosus* (Rai & Bai, 2014). 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 not be necessary. In fact, no nitrate-reductase activities provided by these species is required since there are no added nitrates to the mixture, the low temperature ripening condition is disadvantageous both for the catalase activity (best enzyme temperature range conditions is between 25 – 55°C) and the lipolytic activities which is mainly guaranteed by tissues enzymes activities (Molly et al., 1997; Zambonelli, Papa, Romano, Suzzi, & Grazia, 1992). Moreover, even if the proteolytic activities of *Micrococcus* spp. and *Staphylococcus* spp. generate appreciated profiles of aromatic compounds (Nazzaro et al., 2004), the popular application among producers of selected strains as commercial starter cultures may determine a reduction in aroma variability in local productions. For these reasons in the NoNit™ formulation *Staphylococcus* spp. was replaced by an *Enterococcus* spp. strain (*E. faecium*) which grows well at low temperature and belong to a species which is capable of modulating the aroma by means of the conversion of 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).

The NoNit™ formulation had already been tested in an in vitro set up and challenged vs selected pathogens and hygiene indicators at 37°C (B. Cenci-Goga et al., 2015): *E. coli* in milk dropped, after 48 hrs, below 10^4 cfu ml^{-1}, and was no longer detectable at 72 hrs, while in BHI

the growth curve of *E. coli* was parallel to control, indicating an effect of pH rather than a direct activity of starter cultures and/or bacteriocins. A similar behavior was shown by *S.* Derby 27, with an even faster drop ($<10^4$ cfu ml^{-1} after 30 hrs). *S. aureus* proved to be more resilient and a substantial reduction in total viable cells was observed between 72 and 120 hrs ($<10^3$ cfu ml^{-1} after 120 hrs) with a complete elimination after 168 hrs. *L. innocua* was no longer detectable in milk after 120 hrs, with a steady drop after 24 hrs ($<10^4$ cfu ml^{-1} after 72 hrs). LAB had an effect on *L. innocua*, also in BHI, with levels below 10^4 cfu ml^{-1} just after 30 hrs. The direct effect of certain LAB strains on *Listeria* spp. rather than the indirect effect of pH drop has been described (Winkelströter & De Martinis, 2015). Salami made with the NoNit™ formulation in previous experiments (Cenci Goga et al., 2008; Cenci Goga et al., 2012; B. Cenci-Goga et al., 2015; B. T. Cenci-Goga et al., 2016; Sechi et al., 2014) were perceived by panelists as slightly saltier, more cohesive and generally more acceptable. 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.

Before challenging the NoNit™ formulation vs selected pathogens in salami, an in vitro set up was arranged in the present study with incubation temperature lowered to 10°C. Even a low temperature level of *S. aureus* in milk and in BHI dropped, after 240 hrs, below 10^5 cfu ml^{-1}. *E. coli* showed a similar behavior with a slower drop ($<10^7$ cfu ml^{-1} after 120 hrs). *L. innocua* had a substantial reduction in total viable cells between 72 and 120 hrs ($<10^5$ cfu ml^{-1} after 24 hrs in BHI and at 240 hrs in milk). The NoNit™ formulation had an effect on *Salmonella* Derby, in milk, with levels below 10^5 cfu ml^{-1} after 240 hrs. These results, even if the inoculum used in this challenge was very high (10^6 cfu ml-^{1}) and not representative of the natural contamination commonly encountered in the raw material for salami production, demonstrates that the NoNit™ formulation is able to prevent the growth of *S. aureus* and *E. coli* yet exerting

535 a bactericidal activity on *L. innocua* and *S.* Derby. All testing demonstrated that the NoNit™
536 formulation is a promising candidate as starter culture for salami produced a low temperature
537 throughout fermentation and ripening, therefore the formulation was used in salami
538 manufacture and compared to a commercial starter used for nitrate-free salami. Both
539 formulation were able to control the growth of the three pathogens and of the surrogate for *L.*
540 *monocytogene*s (*L. innocua*), however from day-3 for *S. aureus*, *L. innocua* and *S.* Derby and
541 from day-7 for E. *coli*, counts in NoNit™ batters were statistically significant lower ($p < 0.001$)
542 when compared to batters made with the commercial starter. In conclusion the NoNit™
543 formulation performed better than the commercial formulation in any test.
544

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691

692 **Figures and table legends**

693

694 **Table 1**. Acidifying activity of the starter cultures in sterile skim milk at 10°C as pure cultures
695 or in association. Kinetic parameters and regression coefficients (+/- se) of the acidification
696 curves as determined by equation 1

697

698 **Table 2**. Growth and bacterial reduction at 10°C for selected pathogens challenged with the
699 NoNit™ formulation in milk

700

701 **Table 3**. Microbiological counts for total *S. aureus*, *E. coli*, *L. innocua* spp. and *S.* Derby in
702 salami produced with commercial starter vs. NoNit™ formulation (Log cfu g^{-1}). Different
703 superscripts in the same row indicate significant different means ($p<0,001$)

704

705 **Table 4**. Microbiological counts for total mesophilic microbiota, *Lactococcus* spp.,
706 *Lactobacillus* spp. and *Enterococcus* spp. in salami produced with commercial starter vs.
707 NoNit™ formulation (Log cfu g^{-1}). Different superscripts in the same row indicate significant
708 different means ($p<0,001$)

709

710 **Table 5**. Chemical parameters on the day of stuffing and at the end of ripening *salame nostrano*

711

712 **Table 6**. Sensory analysis. Different superscripts in the same row indicate significant different
713 means ($p<0,005$)

714

715 **Figure 1**. Temperature (---) and relative humidity (—) recorded throughout fermentation and
716 ripening of *salame nostrano*

717

718 **Figure 2**. Empirical model curves for acidifying curves at 10°C for LAB and association of
719 NoNJit™ + pathogens

720

721 **Figure 3**. Growth curves for the challenge in BHI and in milk: NoNit™ formulation vs
722 pathogens at 10°C (3a: *S. aureus* 27R, 3b: *E. coli* CSH26 K-12, 3c: *L. innocua* ATCC 33090,
723 3d: *S.* Derby 27), ___□□□□ pathogens alone in BHI, ---□--- pathogens vs NoNit™

formulation in BHI, ___❒□□□ pathogens alone in milk, ---❒--- pathogens vs NoNit™ formulation in milk

Figure 4. Evolution of NoNit™ formulation in BHI and in milk at 10°C (4a: NoNit™ formulation in BHI, 4b: NoNit™ formulation in milk, 4c: *E. faecium* in BHI, 4c: *E. faecium* in milk), ___□□□ LAB alone, ---O--- NoNit™ formulation vs *S. aureus* 27R, ---ᘓ--- NoNit™ formulation vs *E. coli* CSH26 K-12, ---□--- NoNit™ formulation vs *L. innocua* ATCC 33090, ---ᘐ--- NoNit™ formulation vs S. Derby 27

Figure 5. Growth curves for pathogens in the challenge in *salame nostrano* (5a *S. aureus* 27R, *5b E. coli* CSH26 K-12, 5c *L. innocua* ATCC 33090, 5d *S*. Derby 27), ___ commercial starter, --- NoNit™formulation

Figure 6. Growth curves for starter in the challenge in *salame nostrano* (6a: mesophilic microbiota, 6b: lactococci, 6c: lactobacilli, 6d: enterococci, 6e: staphylococci), ___ commercial starter, --- NoNit™formulation

Figure 7. Development of a_w in *salame nostrano* during fermentation and ripening, ___ commercial starter, --- NoNit™formulation

Figure 8. Development of pH in *salame nostrano* during fermentation and ripening, ___ commercial starter, --- NoNit™formulation

Figure 9. Sensory descriptive analysis of *salame nostrano* (9a: appearance attributes, 9b: basic aromas and texture attributes), ___ commercial starter, --- NoNit™formulation