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# FATE OF SELECTED PATHOGENS IN SPIKED «SALAME NOSTRANO» PRODUCED WITHOUT ADDED NITRATES FOLLOWING THE APPLICATION OF NONIT<sup>TM</sup> TECHNOLOGY 4

5 B. T. Cenci-Goga<sup>1</sup>, M. Karama<sup>2</sup>, P. Sechi, M.F. Iulietto, L. Grispoldi, R. Selvaggini<sup>3</sup>, S.
6 Barbera<sup>4</sup>

7

8 Medicina Veterinaria, Laboratorio di Ispezione degli Alimenti di Origine Animale, Università

- 9 degli Studi di Perugia, 06126 Perugia, Italy
- 10 <sup>3</sup> Dipartimento di Scienze agrarie, alimentari e ambientali Università di Perugia
- <sup>4</sup> Dipartimento di Scienze Agrarie, Forestali e Alimentari DISAFA Università di Torino

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<sup>&</sup>lt;sup>1</sup> Address and correspondence to: B. T. Cenci-Goga, Dipartimento di Medicina Veterinaria, Università degli Studi di Perugia, via San Costanzo, 06126 Perugia, Italy, **Telephone**: +39 075 585 7929, **Telefax**: +39 075 585 7976, **E-mail**: beniamino.cencigoga@unipg.it.

<sup>&</sup>lt;sup>2</sup> Present address: University of Pretoria, Faculty of Veterinary Science, Department of Paraclinical Sciences, Onderstepoort, South Africa

#### 14 Abstract

15 This study evaluated the effect of a novel formulation for starter culture associated with specific ripening conditions (NoNit<sup>TM</sup> technology) vs. a commercial» starter on the fate selected 16 pathogens and hygiene indicators during fermentation and ripening of experimentally spiked 17 18 salame nostrano (italian dry salami). Selected strains of Staphylococcus aureus 27R, 19 Escherichia coli CSH26 K 12, Listeria innocua ATCC 33090 and Salmonella Derby 27 were 20 inoculated into salami batter and challenged with two formulation of starter cultures (a 21 commercial one and the NoNit<sup>™</sup> formulation consisting of *Lactococcus lactis ssp. lactis*, strain 22 340; L. lactis ssp. lactis, strain 16; Lactobacillus casei ssp. casei, strain 208 and Enterococcus 23 faecium strain 614) in specific ripening conditions. 24 The proposed formulation along with specific ripening conditions (NoNit<sup>TM</sup>) limited the growth of spiked Escherichia coli, Staphylococcus aureus, Salmonella Derby 27 and Listeria 25 26 innocua yet maintaining the basic appearance and aroma and texture attributes of the products. 27

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

#### 31 Introduction

32 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 33 result from fermentation are in general sufficient to inhibit the growth and toxin production of 34 35 most pathogens due to a combinations of hurdles, such as pH, water activity, preservatives and 36 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 37 several bacteria, including Staphylococcus aureus, Listeria spp., Escherichia coli and 38 39 Salmonella spp. can survive in final products, and although fermented sausages have a well-40 founded reputation for safety, outbreaks of food poisonings occurs (Al-Mutairi, 2011; 41 Chajecka-Wierzchowska, Zadernowska, Nalepa, Sierpinska, & Laniewska-Trokenheim, 2015; 42 D'Ostuni et al., 2016). In Italy, for instance, salami have been implicated in several E. coli and 43 Salmonella spp. related family outbreaks (Conedera et al., 2007; Luzzi et al., 2007). Outbreaks 44 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, 45 46 ripening and drying of fermented sausages levels of pathogens have been shown to drop with 47 the inactivation proportional to pH, salt and nitrates/nitrite levels (Casey & Condon, 2000; 48 Riordan et al., 1998). Over the last years there has been a growing consumer interest in ready 49 to eat fermented sausages produced with lower concentrations of additives and many 50 technologies have been proposed to limit their use yet maintaining the same level of safety 51 (Cenci Goga et al., 2012; B. Cenci-Goga et al., 2015; B. T. Cenci-Goga et al., 2016; 52 Zarringhalami, Sahari, & Hamidi-Esfehani, 2009). Moreover, further to the recommendation 53 of the «International agency for research on cancer» of the «World health organization» based 54 on epidemiological studies suggesting that small increases in the risk of several cancers may 55 be associated with high consumption of red meat or processed meat (Bouvard et al., 2015),

56 several agencies and the media have raised the question whether processed meat can be safely 57 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 58 59 flavor or improve preservation, such as nitrates and nitrites addition. Nitrates and nitrites are 60 currently authorized as food additives within the EU. These additives function as preservatives 61 in food and they are both used extensively to enhance the color and extend the shelf life of 62 processed meats. Nitrite is considered the active curing ingredient responsible for the 63 preservation of the food in combination with other ingredients, like salt. Nitrate, when added 64 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 65 Regulation (EC) No.1333/2008 on food additives which is in force since 1 June 2013. The 66 67 maximum levels at which nitrates and nitrites may be used, and also the specific foodstuffs in 68 which they may be used as well as their conditions of use are also established by Regulation 69 (EC) No. 1333/2008 as amended. These levels are set at values which ensure that a person 70 consuming a typical diet would not exceed the Acceptable Daily Intake (ADI) established for 71 these additives. Purity criteria have also been established for nitrates and nitrites via Regulation 72 (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 73 74 attributed E numbers (E250, E249, E251 and E252 respectively). In accordance with EU 75 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 76 labeled for food use. This is designed to limit the amount of nitrite that can be added and to 77 78 prevent accidental poisoning through the addition of excessive quantities of nitrite to food 79 (Anonymous, 2016).

80 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 81 spores and toxin formation by Clostridium botulinum (Adams & Moss, 2000; Mossel, Corry, 82 83 Struijk, & Baird, 1995). Despite the desired properties, the safety of nitrite to human health has 84 been questioned as they can cause the formation of carcinogenic N-nitrosamines. In foods, 85 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 86 87 stomach. Carcinogenic chemicals that form during meat processing include N-nitroso 88 compounds and polycyclic aromatic hydrocarbons (Bouvard et al., 2015; Ozel, Gogus, Yagci, 89 Hamilton, & Lewis, 2010).

90 For these reasons the consumer interest in nitrite-free products has raised and, as a 91 consequence, hand-crafted products made locally in small-scale plants are often marketed as 92 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 93 94 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 95 96 formation and stability (Zarringhalami et al., 2009). Several alternatives to nitrates nitrites for 97 their antimicrobial action have been tested (Pegg & Shahidi, 2000) and many different methods 98 have been proposed to enhance color intensity and uniformity, including starter cultures based 99 mainly on cold adapted lactic acid bacteria and natural colorant (Zarringhalami et al., 2009; 100 Zhang, Kong, & Xiong, 2007).

101 The application of starter cultures in food production has a crucial aim: their activity is 102 addressed to restrain indigenous microbiota to control the processing and promote food safety. 103 According to the *hurdle technology* (Leistner, 2000), every condition which make the pathogen 104 or spoilers bacterial growth disadvantaged can be considered a hurdle; according to that,

105 beneficial bacterial strains can be applied to enhance shelf-stable cured meat products, 106 guaranteeing safe productions. Lactic acid bacteria and staphylococci are the most used strains 107 in cured meat production and they have been directly applied to the meat batter since several 108 years. On one hand, the application of staphylococci as starter cultures shows some limitations 109 since pH tolerance and temperature tolerance are key factor to enable them to produce elective 110 enzymes; low temperatures, in fact, can affect their activity. Meat color formation and stability 111 is enhanced by staphylococci while rancidity processes are slowed. Among staphylococci, the 112 most frequently used are *Staphyloccous xylosus* and *S. carnosus* and the produced enzymes, in 113 association with the enzymes in the meat, contribute to aroma definition. On the other hand, 114 lactic acid bacteria determine technological changes, which promote meat stability. They 115 ferment available sugars and produce organic acids: the produced acids determine pH reduction 116 which contribute to drying process through the reduction of the water holding capacity, to color 117 formation and to inhibit undesired microbiota occurred in the product.

118 Before defining which strains can be applied as starter cultures, it is required that the strains 119 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 120 121 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 122 to each specific meat product raw composition and recipe, the acidification capability of 123 124 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 125 product characteristics, processing technology and predict the final product. In addition, 126 127 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 128

staphylococci enzymes activities with subsequent less effective color and aroma formation, asrequired 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 al., 2015) we discovered that fermentation and ripening temperature were consistently below 133 134 12°C throughout the whole ripening process. In the area of food biotechnology, cold-adapted microorganisms, that is, psychrophilic, psychrotrophic and psychrotolerant microorganisms, 135 136 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 biocatalyst at low temperature. Low temperature reactions utilizing such biocatalysts have 141 142 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.

With this in mind we reproduced in the ripening chamber of the pilot plant the average conditions recorded during our studies and tested a new formulation of cold-adapted microorganism against a commercial one to investigate their effect on selected spiked pathogens throughout ripening.

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#### 150 Materials and methods

#### 151 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).

#### 159 Selection of starter cultures

160 A selection of 138 lactic acid bacteria isolated from meat and dairy products and identified in 161 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, 162 1998; Filipović et al., 2012; Sechi et al., 2014) have been tested for ability to grow at low 163 164 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 165 166 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 167 starter culture (NoNit<sup>™</sup>) (B. Cenci-Goga et al., 2015; B. T. Cenci-Goga et al., 2016). 168

169 Starter cultures

Bacterial strains used in the NoNit<sup>™</sup>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

174 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 175 al., 2015; B. T. Cenci-Goga et al., 2016; Clementi et al., 1998). Before further tests 176 177 (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, 178 179 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 180 (on Nutrient Agar, NA, Oxoid CM0003, incubated at 37°C on air for 24 hrs) at 24 hrs was 181 approximately 1 x 10<sup>9</sup> cfu ml<sup>-1</sup>. For the tests (acidification in milk, challenge in vitro and 182 challenge in salami production) strains were inoculated into milk or salami batter to get an 183 184 initial concentration of approximately  $1 \times 10^7$  cfu ml<sup>-1</sup> or g<sup>-1</sup> which mimics the initial starter 185 concentration in salami production.

#### 186 Selected spiked microorganisms.

187 Bacterial strains used as marker micro-organisms for the inoculation were taken from the 188 collection of the Laboratorio di Ispezione degli Alimenti di Origine Animale: S. aureus, strain 189 27R mec(A), resistant to 2 µg/ml of methicillin; E. coli, strain CSH26 K-12, resistant to 200 190 µg/ml nalidixic acid; Listeria innocua ATCC 33090; Salmonella Derby field strain (internal 191 ref. #27). The micro-organisms were grown aerobically in Nutrient Broth (NB, Oxoid 192 CM0001, Basingstoke, UK) at 37°C for 24 hrs. The total viable cells (TVC) count (on Nutrient 193 Agar, NA, Oxoid CM0003, incubated at 37°C on air for 24 hrs) at 24 hrs was approximately 1 x 10<sup>9</sup> CFU/ml<sup>-1</sup>. For the challenge test in milk and in salami each strain was inoculated in skim 194 195 milk (BD Difco) or into salami batter to get an initial concentration of approximately 1 x 10<sup>6</sup> cfu ml<sup>-1</sup> (1 x  $10^4$  cfu g<sup>-1</sup> for the salami batter). 196

197 Characterization of NoNit<sup>™</sup> starter cultures with acidification and challenge growth curves
198 at 10°C

Acidification. Strains of the NoNit<sup>™</sup> formulation (*Lactococcus lactis ssp. lactis*, strain 340: 199 200 L. lactis ssp. lactis, strain 16; Lactobacillus casei ssp. casei, strain 208 and Enterococcus 201 faecium UBEF-41) were inoculated as pure cultures into skim milk (BD Difco) at 37°C to get a final concentration of 10<sup>9</sup> ml<sup>-1</sup> after 24 h of incubation. The association was then inoculated 202 203 into skim milk (BD Difco) to get an initial cocci: bacilli:enterocci ratio of 2:1:1 and an initial concentration of about 10<sup>7</sup> cfu ml<sup>-1</sup>. The association was incubated at 10°C and pH was 204 205 measured with a Foodtrode electrode (Hamilton Company, Reno, NV, USA) hooked to an 206 Eutech pH 2700 (Eutech Instrument Europe B.V. Nijkerk, Netherlands) which recorded pH values continuously with CyberComm 6000 (Eutech Instrument) every minute. To find 207 mathematically the maximum instantaneous rate of acidification and the moment in which this 208 209 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: 210

211 
$$y=a+bx+cx^2+dx^3+ex^4$$
 [equation1]

212 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 213 214 package STATGRAPHICS Centurion XVI version 16.2.04 (Statpoint Technologies, Inc., Warrenton, VA, USA). The first derivative of the equation gives the instantaneous acidification 215 216 rate and its maximum value (V<sub>m</sub>) 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  $(t_m)$ 217 of the inflection point. Substituting to x the  $t_m$  value, can be evaluated by the fourth degree 218 219 equation the pH value corresponding to the inflection point.

220 Challenge test in vitro. Bacterial reduction for selected pathogens (*Escherichia coli*, strain

221 CSH26 K-12, Staphylococcus aureus, strain 27R, Salmonella Derby n. 27) and L.

222 *monocytogenes* surrogate (*L. innocua* ATCC 33090) vs. the NoNit<sup>TM</sup> formulation were 223 performed in six replicates. All strains for NoNit<sup>TM</sup> formulation were inoculated as pure 224 cultures into skim milk (BD Difco) and incubated at 37°C to get a final concentration of about 225  $10^9$  cfu ml<sup>-1</sup> after 24 h of incubation. The association was then inoculated into BHI broth (BD 226 Difco) and skim milk (BD Difco) to get an initial cocci:bacilli:enterocci ratio of 2:1:1 and a 227 concentration of approx  $10^7$  cfu ml<sup>-1</sup> until challenge vs. selected pathogens and hygienic 228 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<sup>9</sup> cfu ml<sup>-1</sup> 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<sup>6</sup> cfu ml<sup>-1</sup>. 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).

#### 236 Salami production, spiking and sampling

237 The study was performed in nine different replications in nine diverse days. Two batches on 238 each replication were produced: with a «commercial starter culture formulation» (Commercial) 239 and with the «NoNit<sup>TM</sup> formulation» (NoNit<sup>TM</sup>). Both batches were then spiked with the following strains: S. aureus, strain 27R mec(A), resistant to 2 µg/ml of methicillin; E. coli, 240 strain CSH26 K-12, resistant to 200 µg/ml nalidixic acid; Listeria innocua ATCC 33090 and 241 242 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* 243 244 spp., while for antibiotic resistant strains of S. aureus and E. coli the absence of contamination 245 was ensured by the use of culture media with antibiotics.

246 Salami were prepared with a procedure handed down among butchers for centuries, at the pilot 247 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 248 249 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<sup>-1</sup>, 250 pepper, 5 g kg<sup>-1</sup>, garlic, 2 g kg<sup>-1</sup>, dextrose 10 g kg<sup>-1</sup> and starter cultures). Starter cultures of the 251 formulation NoNit<sup>TM</sup> were added at a concentration of 10<sup>7</sup> cfu g<sup>-1</sup> of meat, with a 252 cocci:bacilli:enterococci ratio of 2:1:1. The commercial starter contained a combination of 253 254 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 255 256 batter is above  $10^7$  cfu g<sup>-1</sup> 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).

#### 262 Microbiological analysis

For each of the nine replications, 3 sausages per group at each sampling point and 3 subsamplesfor 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).

271 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 272 methicillin resistant strain. Violet Red Bile Glucose Agar (VRBG, Oxoid, CM0485), to which 273 274 a solution of nalidixic acid at a final concentration of 200 µg/ml was added, incubated at 37°C 275 for 24h was used for counts of E. coli nalidixic acid resistant strain. RAPID'Listeria spp. Agar 276 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 277 278 Listeria innocua. X.L.D. AGAR (Oxoid CM0469), incubated at 37°C for 18-24 hours was used 279 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; 280 281 Lactobacillus spp. on MRS Agar (Oxoid) pH 5.5, at 30 °C for 72 h under anaerobic conditions 282 (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 283 284 Tellurite (Oxoid SR 54) at 37°C for 48 h, after replica-plating (Lederberg & Lederberg, 1952) 285 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 286 287 methicillin resistant strain.

#### 288 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<sub>w</sub>) was measured with a dew-point hygrometer HygroLab 3 (Rotronic, Huntington, NY, USA). Calibration was performed using five saturated

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

300 Sensory evaluation

Few days after the end of ripening, a descriptive sensory evaluation was performed. The panel 301 302 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 tasters were warned that some samples could be contaminated with pathogens and asked to test 304 305 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 biased assessment) for each characteristic: 7 = maximum intensity and 1 = minimum intensity. 308 309 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 equipment (protective glasses, disposable gloves and disposable lab cots). Samples were taken 311 312 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

321 assessor could thoroughly discuss and clarify each attribute to be evaluated. Briefly, color 322 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 323 connection as the degree of adherence of the product's principal ingredients (fat and lean), 324 fat/lean distribution as the uniform distribution of lean and fat on the slice. Global odor is the 325 326 global sensation of aroma, mold is the characteristic odor associated with the chemical 327 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 328 329 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. 330

#### 331 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<sub>10</sub> of the arithmetic means for all microbiological analysis was calculated, following which all log<sub>10</sub> 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<sup>-1</sup> for spread plate and 10 cfu g<sup>-1</sup> 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).

344 For sensory evaluation a t -student test was carried out to determine the effect of the starter

345 formulation (Commecial vs. NoNit<sup>TM</sup>) on the appearance salami.

347 **Results** 

*Characterization of starter cultures with acidification and challenge growth curves at 10°C* 348 349 Acidifying activity of selected starter cultures. Three species of lactic acid bacteria along 350 with a commercial probiotic were chosen to be used as a starter. These strains had been 351 previously identified by API 50 CHL and sequencing and a selection of them had already been 352 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 353 354 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 355 356 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 357 degree polynomial [equation 1] which was used as an empirical model. This adequately fitted 358 the experimental data, since the  $r^2$  values varied from 0.998 to 0.999 and the actual values were 359 360 almost exactly superimposed on the empirical model curves (Figure 2a and 2b). The values of 361 the maximum instantaneous acidification rate [V<sub>m</sub>] were of the same order for all strains, except for *Lc. lactis ssp. lactis* strain 340 which had the highest  $V_m$  [-2.35 x 10<sup>-4</sup>  $\Delta pH/min$ ) and for *Lc.* 362 363 lactis ssp. lactis strain 16 which had the lowest t<sub>m</sub> [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 364 365 decrease within about 2000 min, like some commercial cold adapted lactobacilli (Chamba & 366 Prost, 1989). The association of the four strains performed reached a final pH of 4.20 and

367	showed the fastest pH drop in the first 24 hrs along with a steady descent subsequently. The
368	association mimicked the behaviour of the two Lc. lactis ssp. lactis strains in the first 16 hrs
369	and then, when the lactococci acidification activity decreased (steady pH values after 24 hrs),
370	mirrored the Lb. casei spp casei performance for a continuous pH drop.
371	Challenge test. Results are shown in Figure 3 which display the different growth curves of
372	pathogens or hygiene indicators alone or vs. the NoNit <sup>TM</sup> formulation. Figure 3a shows the
373	evolution of S. aureus, strain 27R, 3b E. coli CSH26 K-12; 3c L. innocua ATCC 33090; 3d S.
374	Derby strain 27. For the challenge S. aureus vs NoNit <sup>TM</sup> , level of S. aureus dropped, after 240
375	hrs, below $10^5$ cfu ml <sup>-1</sup> . A similar behaviour was shown by <i>E. coli</i> with a slower drop (< $10^7$
376	cfu ml <sup>-1</sup> after 120 hrs). <i>L. innocua</i> had a substantial reduction in total viable cells between 72
377	and 120 hrs (<10 <sup>5</sup> cfu ml <sup>-1</sup> after 24 hrs in BHI and at 240 hrs in milk). LAB had an effect on
378	Salmonella Derby, with levels below 10 <sup>5</sup> cfu ml <sup>-1</sup> after 240 hrs. Table 2 shows the log reduction
379	along with the statistics: after 240 hours of incubation with NoNit <sup>TM</sup> formulation, S. aureus
380	had a 1.45 log reduction, E. coli 0.81 log, L. innocua 1.30 log and S. Derby 2.18 log.
381	Figure 4 show the correct evolution of the NoNit <sup>TM</sup> strains both in milk and in BHI alone and
382	vs. selected pathogens.

383 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

388 (ground meat), day-3 (middle fermentation), day-7 (end-fermentation), day-13 (middle 389 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 390 (day-0) was always approx. 4 log<sub>10</sub> cfu g<sup>-1</sup>: for *S. aureus* it ranged from 4.19 (in commercial 391 batch) to 4.26 (in NoNit<sup>™</sup> batch), for *E. coli* form 4.03 to 4.18, for *L. innocua* from 4.09 to 392 393 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 log10 394 cfu g<sup>-1</sup> from day-21 in the commercial batches while remained always below 6  $\log_{10}$  cfu g<sup>-1</sup> 395 in the NoNit<sup>TM</sup> batches. E. coli, strain CSH26 K-12 reached levels of 5.05 log<sub>10</sub> cfu g<sup>-1</sup> on day-396 397 7 and remained at about 4 log<sub>10</sub> cfu g<sup>-1</sup> throughout ripening in commercial batches, while 398 dropped to 1.93 log<sub>10</sub> cfu g<sup>-1</sup> in NoNit<sup>™</sup> batches, after a peak to 4.53 on day-3. *L. innocua* ATCC 33090 reached about 6 log<sub>10</sub> cfu g<sup>-1</sup> on day-3 with a plateau till the end of ripening in 399 commercial batches and reached 5.02 log<sub>10</sub> cfu g<sup>-1</sup> in NoNit<sup>™</sup> on day-21 to drop below 5 log<sub>10</sub> 400 cfu g<sup>-1</sup> at the end of ripening. Salmonella Derby reached levels of 5.42 log<sub>10</sub> cfu g<sup>-1</sup> on day-3 401 and stayed above 3 log<sub>10</sub> cfu g<sup>-1</sup> throughout fermentation and ripening in commercial batches 402 while dropped below 3 from day-21 and to 1.79 log<sub>10</sub> cfu g<sup>-1</sup> at the end of ripening in NoNit<sup>TM</sup> 403 404 batches.

**Evolution of starter cultures**. *Lactococcus* spp. and *Lactobacillus* spp. counts were always above 6  $\log_{10}$  cfu g <sup>-1</sup> and reached values above 8  $\log_{10}$  cfu g <sup>-1</sup> 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 <sup>-1</sup> in the commercial batches and from from 6.11  $\log_{10}$  cfu g <sup>-1</sup> in the NoNit<sup>TM</sup> batches and remained above from 6  $\log_{10}$  cfu g <sup>-1</sup> 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

412 ORSAB selective supplement, after replica plating), were always above 4  $\log_{10}$  cfu g <sup>-1</sup> and 413 decreased to 3.25  $\log_{10}$  cfu g <sup>-1</sup> at the end of ripening in commercial batches, while in NoNit<sup>TM</sup> 414 batches counts were always below 4  $\log_{10}$  cfu g <sup>-1</sup> and decreases below 2  $\log_{10}$  cfu g <sup>-1</sup> on day-415 21 (Figure 6 and Table 4).

#### 416 Physico-chemical and chemical analysis

417 Figures 7 and 8 show aw and pH. aw decreased below 0.90 on day-13 in NoNit<sup>™</sup> batches and on day-21 in commercial batches. Fully ripened salami were between 0.82 in NoNit<sup>™</sup> batchges 418 and 0.84 in commercial batches. Mean pH values on the day of stuffing were 6.10 and reached 419 420 values below 6.0 on day-3 in NoNit<sup>TM</sup> batches and on day-21 on commercial batches. At the end of ripening NoNit<sup>™</sup> batches reached pH values of 5.17 and commercial batches of 5.67. 421 422 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. 423 424 Moisture decreased from 58.67% on the day of stuffing to 28.22% (commercial) and 29.30 425 (NoNit<sup>TM</sup>) in full ripened salami.

#### 426 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<sup>TM</sup> batches), fat/lean distribution (3.43 vs. 4.87), and hardness (2.50 vs. 3.87).

#### 432 **4. Discussion**

433 Selected pathogens and hygiene indicators were spiked into salame nostrano (italian dry434 sausage) to determine the impact of two different starter cultures formulation, a commercial

one and an experimental one (Commercial vs NoNit<sup>TM</sup>), on their behavior during manufacturing, fermentation and ripening. The batters were spiked with three pathogens (*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; and *Salmonella* Derby field strain, internal ref. #27), and one surrogate for *L. monocytogenes* (*Listeria innocua* ATCC 33090).

440 The commercial formulation is a common formulation which is widely used by producer that require salami with the typical characteristics of home made salami. The formulation contains 441 442 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 and prevent rancidity, while lactobacilli control the fermentation process and may result in 444 445 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 common trend among producers that have opted for the so-called nitrate free production, is in 447 448 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 strains that at these temperatures are still able to multiply and to exert their activity. The 450 experimental NoNit<sup>TM</sup> formulation, instead, is the result of several years of study on hand-451 crafted products made in small-scale plants in Umbria, central Italy, when we discovered that 452 453 fermentation and ripening temperature were consistently below 12°C throughout the whole ripening process and that the majority of the microbiota was represented by Lactobacillus spp., 454 *Lactococcus* spp. and *Enterococcus* spp. rather than *Staphylococcus* spp. and *Micrococcus* spp. 455 (Cenci Goga et al., 2008; Cenci Goga et al., 2012; B. Cenci-Goga et al., 2015). 456

The NoNit<sup>TM</sup> formulation, when tested for acidifying activity at  $10^{\circ}$ C performed well with a final pH of 4.20 and showed the fastest pH drop, within the first 24 hrs along with a steady descent subsequently. The association mimicked the behavior of the two *Lc. lactis ssp. lactis* 

460 strains in the first 16 hrs and then, when the lactococci acidification activity decreased (steady 461 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 462 463 salami because the activity of starter cultures is desirable throughout the whole fermentation process (Cenci Goga et al., 2012; Cizeikiene, Juodeikiene, Paskevicius, & Bartkiene, 2013). 464 465 There is a difference in fermented sausage technology between the United States and the 466 European countries. American methods rely on rapid acid production (lowering pH) through a 467 fast fermentation in order to stabilize the sausage against spoilage bacteria. Fast acting starter 468 cultures such as Lactobacillus plantarum and Pediococus acidilactici are used at high temperatures up to 40° C. As a result pH drops to 4.6, the sausage is stable but the flavor suffers 469 470 and the product is sour and tangy. In European countries, the temperatures of 22-26°C are used 471 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 472 473 pH) and the sourly taste is not present. The new trend among manufacturers is the production 474 of nitrate free salami and many culture starter companies are aggressively targeting new formulation for low temperature fermentation. However, many available commercial starter 475 476 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 477 478 that proteolysis and protein insolubility influence the flavor and texture of the final product and 479 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 480 sour, sweet, and bitter taste (Kato, Rhue, & Nishimura, 1989). Aro Aro et al. (2010) 481 482 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 483 associated with S. xilosus. In contrast, simple cultures with lactic acid bacteria do not affect 484

485 proteolysis in salami made with beef (Candogan, Wardlaw, & Acton, 2009) and pork (Aro Aro
486 et al., 2010).

487 The commercial starter tested in this work contained a combination of *Lb. paracasei* and *Lb.* 488 rhamnosus with S. carnosus and S. xylosus. Indeed, the most common species traditionally 489 added to the raw-meat sausages are S. xylosus and S. carnosus (Rai & Bai, 2014). However 490 taking into account the main enzymatic activities provided by Micrococcus spp. and Staphylococcus spp., in Nonit<sup>TM</sup> technology, strains belonging to these species were not 491 included, since their enzymatic makeup would not be necessary. In fact, no nitrate-reductase 492 493 activities provided by these species is required since there are no added nitrates to the mixture, 494 the low temperature ripening condition is disadvantageous both for the catalase activity (best 495 enzyme temperature range conditions is between  $25 - 55^{\circ}$ C) and the lipolytic activities which 496 is mainly guaranteed by tissues enzymes activities (Molly et al., 1997; Zambonelli, Papa, 497 Romano, Suzzi, & Grazia, 1992). Moreover, even if the proteolytic activities of *Micrococcus* 498 spp. and Staphylococcus spp. generate appreciated profiles of aromatic compounds (Nazzaro 499 et al., 2004), the popular application among producers of selected strains as commercial starter 500 cultures may determine a reduction in aroma variability in local productions. For these reasons 501 in the NoNit<sup>TM</sup> formulation *Staphylococcus* spp. was replaced by an *Enterococcus* spp. strain 502 (E. faecium) which grows well at low temperature and belong to a species which is capable of 503 modulating the aroma by means of the conversion of amino acids and free fatty acids (Corbiere 504 Morot-Bizot, Leroy, & Talon, 2007; García Fontán, Lorenzo, Parada, Franco, & Carballo, 505 2007; González-Fernández, Santos, Rovira, & Jaime, 2006; Leroy, Verluyten, & De Vuyst, 2006). 506

507 The NoNit<sup>™</sup> formulation had already been tested in an in vitro set up and challenged vs 508 selected pathogens and hygiene indicators at 37°C (B. Cenci-Goga et al., 2015): *E. coli* in milk 509 dropped, after 48 hrs, below 10<sup>4</sup> cfu ml<sup>-1</sup>, and was no longer detectable at 72 hrs, while in BHI

510 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, 511 with an even faster drop ( $<10^4$  cfu ml<sup>-1</sup> after 30 hrs). S. aureus proved to be more resilient and 512 a substantial reduction in total viable cells was observed between 72 and 120 hrs (<10<sup>3</sup> cfu ml<sup>-1</sup> 513 after 120 hrs) with a complete elimination after 168 hrs. L. innocua was no longer detectable 514 in milk after 120 hrs, with a steady drop after 24 hrs (<10<sup>4</sup> cfu ml<sup>-1</sup> after 72 hrs). LAB had an 515 effect on *L. innocua*, also in BHI, with levels below 10<sup>4</sup> cfu ml<sup>-1</sup> just after 30 hrs. The direct 516 effect of certain LAB strains on *Listeria* spp. rather than the indirect effect of pH drop has been 517 described (Winkelströter & De Martinis, 2015). Salami made with the NoNit<sup>™</sup> formulation in 518 519 previous experiments (Cenci Goga et al., 2008; Cenci Goga et al., 2012; B. Cenci-Goga et al., 520 2015; B. T. Cenci-Goga et al., 2016; Sechi et al., 2014) were perceived by panelists as slightly 521 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 522 523 fermentation and ripening, counts of enterococci were consistently higher in salami made with 524 the addition of starter cultures, when compared to salami produced without starter addition.

Before challenging the NoNit<sup>TM</sup> formulation vs selected pathogens in salami, an in vitro set up 525 was arranged in the present study with incubation temperature lowered to 10°C. Even a low 526 527 temperature level of S. aureus in milk and in BHI dropped, after 240 hrs, below 10<sup>5</sup> cfu ml<sup>-1</sup>. *E. coli* showed a similar behavior with a slower drop ( $<10^7$  cfu ml<sup>-1</sup> after 120 hrs). *L. innocua* 528 had a substantial reduction in total viable cells between 72 and 120 hrs (<10<sup>5</sup> cfu ml<sup>-1</sup> after 24 529 530 hrs in BHI and at 240 hrs in milk). The NoNit<sup>TM</sup> formulation had an effect on Salmonella Derby, in milk, with levels below 10<sup>5</sup> cfu ml<sup>-1</sup> after 240 hrs. These results, even if the inoculum 531 used in this challenge was very high (10<sup>6</sup> cfu ml-<sup>1</sup>) and not representative of the natural 532 contamination commonly encountered in the raw material for salami production, demonstrates 533 that the NoNit<sup>™</sup> formulation is able to prevent the growth of *S. aureus* and *E. coli* yet exerting 534

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

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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 <sup>TM</sup> 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 <sup>TM</sup> formulation (Log cfu g <sup>-1</sup> ). 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 <sup>TM</sup> formulation (Log cfu g <sup>-1</sup> ). Different superscripts in the same row indicate significant
708	different means (p<0,001)
709	
710	<b>Table 5</b> . Chemical parameters on the day of stuffing and at the end of ripening salame nostrano
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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
/1/	
/18	Figure 2. Empirical model curves for acidifying curves at 10°C for LAB and association of
/19	NoNJit <sup>1M</sup> + pathogens
720	Figure 2. Crowsthe surgest for the shellower in DIU and in wills. NoNitTM formulation as
721 722	<b>Figure 5.</b> Growin curves for the chancinge in BETI and in milk: Normulation VS pathogens at $10^{\circ}C$ (3a; S <i>aurous</i> 27 <b>R</b> 3b; E coli CSU26 K 12 3a; L <i>innocura</i> ATCC 22000
722 723	parrogens at 10 C (sa. 5. $uureus 27R$ , 50. $E$ . $cou CS1120 R-12$ , 50. $E$ . $uurocuu ATCC 55090$ , 3d: S Derby 27) $\square \square \square \square$ pathogens alone in BHI $\square$ pathogens vs NoNitTM
143	Ju. J. Derby 27), Dubu paulogens alone in Diff, D paulogens vs NolNit***

724	formulation in BHI,□□□ □ pathogens alone in milk,□ pathogens vs NoNit™
725	formulation in milk
726	
727	Figure 4. Evolution of NoNit <sup>TM</sup> formulation in BHI and in milk at 10°C (4a: NoNit <sup>TM</sup>
728	formulation in BHI, 4b: NoNit <sup>™</sup> formulation in milk, 4c: <i>E. faecium</i> in BHI, 4c: <i>E. faecium</i> in
729	milk), $\Box \Box \Box \Box LAB$ alone, $\mathbf{O}$ NoNit <sup>TM</sup> formulation vs <i>S. aureus</i> 27R, $\mathfrak{R}$ NoNit <sup>TM</sup>
730	formulation vs <i>E. coli</i> CSH26 K-12,□ NoNit <sup>™</sup> formulation vs <i>L. innocua</i> ATCC 33090,
731	20 NoNit <sup>TM</sup> formulation vs S. Derby 27
732	
733	Figure 5. Growth curves for pathogens in the challenge in salame nostrano (5a S. aureus 27R,
734	5b E. coli CSH26 K-12, 5c L. innocua ATCC 33090, 5d S. Derby 27), commercial starter,
735	NoNit <sup>TM</sup> formulation
736	
737	Figure 6. Growth curves for starter in the challenge in salame nostrano (6a: mesophilic
738	microbiota, 6b: lactococci, 6c: lactobacilli, 6d: enterococci, 6e: staphylococci),
739	commercial starter, NoNit <sup>™</sup> formulation
740	
741	Figure 7. Development of aw in salame nostrano during fermentation and ripening,
742	commercial starter, NoNit <sup>™</sup> formulation
743	
744	Figure 8. Development of pH in salame nostrano during fermentation and ripening,
745	commercial starter, NoNit <sup>™</sup> formulation
746	
747	Figure 9. Sensory descriptive analysis of salame nostrano (9a: appearance attributes, 9b: basic
748	aromas and texture attributes), commercial starter, NoNit <sup>TM</sup> formulation
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