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

4

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14 **Abstract**

15 This study evaluated the effect of a novel formulation for starter culture associated with specific
16 ripening conditions (NoNit™ technology) vs. a commercial» starter on the fate selected
17 pathogens and hygiene indicators during fermentation and ripening of experimentally spiked
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™ 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™) limited the
25 growth of spiked *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* Derby 27 and *Listeria*
26 *innocua* yet maintaining the basic appearance and aroma and texture attributes of the products.

27

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

30

31 **Introduction**

32 Fermentation of raw meat to improve the safety, the shelf life and the acceptability of certain
33 foods has a long tradition. In fermented sausages produced from raw meat the conditions that
34 result from fermentation are in general sufficient to inhibit the growth and toxin production of
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-
37 Goga et al., 2015; B. T. Cenci-Goga et al., 2016). However studies have demonstrated that
38 several bacteria, including *Staphylococcus aureus*, *Listeria* spp., *Escherichia coli* and
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
45 described (Anonymous, 2002; Sartz et al., 2008; Sheen & Hwang, 2008). During fermentation,
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
58 transformed through salting, curing, fermentation, smoking, or other processes to enhance
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
65 nitrites in food products must comply with the provisions set out in Annex II part E of
66 Regulation (EC) No.1333/2008 on food additives which is in force since 1 June 2013. The
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
73 sodium and potassium nitrite and potassium and sodium nitrate. These substances have been
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
76 meats, and processed fish and may only be sold in a mixture with salt or a salt substitute when
77 labeled for food use. This is designed to limit the amount of nitrite that can be added and to
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.
81 Nitrite, in addition, impart antibacterial activity, particularly the inhibition of germination of
82 spores and toxin formation by *Clostridium botulinum* (Adams & Moss, 2000; Mossel, Corry,
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
86 under certain conditions, including strongly acidic conditions such as that of the human
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
93 Goga et al., 2012; B. T. Cenci-Goga et al., 2016; Zarringhalami et al., 2009). It is obvious that
94 nitrite-free sausages manufacture pose two big problems to the food business operators: first
95 of all the production of safe food (i.e. absence of *Clostridium botulinum* toxin) and then color
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
120 amount of acetic acid, through the definition of the acidification profile. Among Lactic Acid
121 Bacteria, *Lactobacillus* spp. and *Pediococcus* spp. are the most frequently applied in dry
122 sausages production for the desired activities. Anyway, it has to be considered that according
123 to each specific meat product raw composition and recipe, the acidification capability of
124 indigenous microbiota may vary and influence the activities of the added strains. To maximize
125 the beneficial effect of the starter culture application, therefore, it is crucial to well-define
126 product characteristics, processing technology and predict the final product. In addition,
127 processing parameters, mince composition, product diameter influence acidification speed
128 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

150 **Materials and methods**

151 *Definition of ripening conditions*

152 During a survey on hand-crafted products made in small-scale plants in Umbria, central Italy
153 (Cenci Goga et al., 2008; Cenci Goga et al., 2012; B. Cenci-Goga et al., 2015) we discovered
154 that fermentation and ripening temperature for *salame nostrano*, a typical dry salami produced
155 in central Italy, were consistently below 12°C throughout the whole ripening process. Using
156 several Testostor 175-2 (Testo, Lenzkirch, Germany) data loggers we measured the
157 temperature and humidity conditions in small-scale plants that produce fermented salami. The
158 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;
162 B. T. Cenci-Goga et al., 2016; Clementi, Cenci Goga, Trabalza-Marinucci, & Di Antonio,
163 1998; Filipović et al., 2012; Sechi et al., 2014) have been tested for ability to grow at low
164 temperature (10°C) in aerobic and anaerobic conditions. All strains that showed growth at 10°C
165 both in aerobic and anaerobic conditions without gas production from dextrose were tested for
166 acidifying activity in Skim Milk (BD Difco) and then, based on the instantaneous acidification
167 rate and its maximum value, a selection of four strain was made to be used as cold adapted
168 starter culture (NoNit™) (B. Cenci-Goga et al., 2015; B. T. Cenci-Goga et al., 2016).

169 *Starter cultures*

170 Bacterial strains used in the NoNit™formulation were: *Lactococcus lactis ssp. lactis*, strain
171 340; *L. lactis* ssp. *lactis*, strain 16; *Lactobacillus casei* ssp. *casei*, strain 208 and *Enterococcus*
172 *faecium* UBEF-41. The morphological, biochemical, physiological characterization, growth
173 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
175 the authors in previous papers (Cenci Goga, Clementi, & Di Antonio, 1995; B. Cenci-Goga et
176 al., 2015; B. T. Cenci-Goga et al., 2016; Clementi et al., 1998). Before further tests
177 (acidification in milk, challenge in vitro and challenge in salami production), freeze-dried
178 strains of the starter cultures were grown aerobically in Nutrient Broth (NB, Oxoid CM0001,
179 Basingstoke, UK) at 37°C for 24 hrs. Each strain was then sub-cultured in Skim Milk (BD
180 Difco, Franklin Lakes, NJ, USA, 232100) at 37°C for 24 hrs. Total viable cells (TVC) count
181 (on Nutrient Agar, NA, Oxoid CM0003, incubated at 37°C on air for 24 hrs) at 24 hrs was
182 approximately 1×10^9 cfu ml^{-1}. For the tests (acidification in milk, challenge in vitro and
183 challenge in salami production) strains were inoculated into milk or salami batter to get an
184 initial concentration of approximately 1×10^7 cfu ml^{-1} or g^{-1} 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
194 $\times 10^9$ CFU/ml^{-1}. For the challenge test in milk and in salami each strain was inoculated in skim
195 milk (BD Difco) or into salami batter to get an initial concentration of approximately 1×10^6
196 cfu ml^{-1} (1×10^4 cfu g^{-1} for the salami batter).

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

199 **Acidification**. Strains of the NoNit™ formulation (*Lactococcus lactis ssp. lactis*, strain 340;
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
202 a final concentration of 10^9 ml^{-1} after 24 h of incubation. The association was then inoculated
203 into skim milk (BD Difco) to get an initial cocci: bacilli:enterocci ratio of 2:1:1 and an initial
204 concentration of about 10^7 cfu ml^{-1}. The association was incubated at 10°C and pH was
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
207 values continuously with CyberComm 6000 (Eutech Instrument) every minute. To find
208 mathematically the maximum instantaneous rate of acidification and the moment in which this
209 is achieved, a fourth degree polynomial equation was used as an empirical model for fitting the
210 experimental data collected for each microorganism and for each condition:

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

212 In this equation y is the pH value, x is time (minutes) and a, b, c, d, and e are the regression
213 coefficients of the independent variable x. The coefficients were determined by the statistical
214 package STATGRAPHICS Centurion XVI version 16.2.04 (Statpoint Technologies, Inc.,
215 Warrenton, VA, USA). The first derivative of the equation gives the instantaneous acidification
216 rate and its maximum value (V_m) corresponds to the inflection point of the acidification curve,
217 whereas the second derivative gives the acceleration and one of its roots give the x value (t_m)
218 of the inflection point. Substituting to x the t_m value, can be evaluated by the fourth degree
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™ formulation were
223 performed in six replicates. All strains for NoNit™ 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^{-1} 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^{-1} until challenge vs. selected pathogens and hygienic
228 indicators.
229 Pathogens and hygiene indicators were inoculated as pure cultures into BHI (BD Difco) and
230 incubated at 37°C to get final concentration of about 10^9 cfu ml^{-1} after 24 h of incubation. For
231 the test each strain was then inoculated into skim milk (BD Difco) to get an initial concentration
232 of approx 10^6 cfu ml^{-1}. The challenges were carried out in skim milk (BD Difco) at 10°C.
233 Bacterial counts were recorded at time 0, 12 h, 24 h, 30 h, 48 h, 72 h, 120 h, 168 h, 240 h.
234 Microbiological analysis were conducted using the methods described below (Microbiological
235 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™ formulation» (NoNit™). Both batches were then spiked with the
240 following strains: *S. aureus*, strain 27R *mec*(A), resistant to 2 µg/ml of methicillin; *E. coli*,
241 strain CSH26 K-12, resistant to 200 µg/ml nalidixic acid; *Listeria innocua* ATCC 33090 and
242 *Salmonella* Derby field strain (internal ref. #27). All batches, before intentional contamination
243 with the selected microorganism, were tested for the absence of *Listeria* spp. and *Salmonella*
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
248 pork meat came form the same farm and all animals were "*suino pesante italiano tipico*" with
249 a live weight of more than 150 kg, and more than 9 months of age. Meat, shoulder and flank
250 (70%) and hind fat (30%), was minced and blended with the ingredients (NaCl, 30 g kg^{-1},
251 pepper, 5 g kg^{-1}, garlic, 2 g kg^{-1}, dextrose 10 g kg^{-1} and starter cultures). Starter cultures of the
252 formulation NoNit™ were added at a concentration of 10^7 cfu g^{-1} of meat, with a
253 cocci:bacilli:enterococci ratio of 2:1:1. The commercial starter contained a combination of
254 *Lactobacillus paracasei* and *Lb. rhamnosus* with *S. carnosus* and *S. xylosus* with a bacilli:cocci
255 ratio of 1:1, and according to manufacturer indications, the final concentration reached in meat
256 batter is above 10^7 cfu g^{-1} of meat.
257 Salami (30 mm by 10 cm), were then hoisted in a dry-curing hall. Figure 1 shows fermentation
258 and ripening parameters as logged by the chamber. Sampling (three sausages per group, three
259 subsamples each sausage) occurred at time 0 (ground meat), day 3 (middle fermentation), day
260 7 (end-fermentation), day 13 (middle ripening), day 21 (end ripening process) and day 28 (final
261 product).

262 *Microbiological analysis*

263 For each of the nine replications, 3 sausages per group at each sampling point and 3 subsamples
264 for sausage were sampled.
265 For the analysis of salami, *more solito* (Cenci Goga et al., 2012) about 25 g of sample were
266 transferred aseptically to 225 ml of sterile, buffered, peptone water (Oxoid), and homogenised
267 in a stomacher (PBI International). Serial decimal dilutions in buffered peptone water were
268 prepared and triplicate 1 ml or 0.1 ml samples of appropriate dilutions were poured or spread
269 on total count and selective agar plates. Microbial analysis were conducted according to the
270 methods described previously (Cenci Goga et al., 2012).

271 Briefly, Oxacillin Resistance Screening Agar base (ORSAB, Oxoid CM1008) with ORSAB
272 selective supplement (Oxoid, SR0195), incubated at 37°C for 24 h was used for *S. aureus*
273 methicillin resistant strain. Violet Red Bile Glucose Agar (VRBG, Oxoid, CM0485), to which
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
277 RAPID'Listeria Supplement 2 (Biorad 3564746), incubated at 37°C for 24 h, was used for
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),
280 at 30 °C for 72 h; *Lactococcus* spp. on M17 agar (Oxoid) 10% v/v lactose, at 37°C for 48 h;
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
283 for 48 h. *Staphylococcus* spp. on Baird Parker agar (Oxoid CM 275) containing Egg Yolk
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
286 selective supplement (Oxoid, SR0195), incubated at 37°C for 24 h to disregard spiked *S. aureus*
287 methicillin resistant strain.

288 *Physico-chemical and chemical analysis*

289 With the sampling scheme used for microbiological analysis (nine replications, 3 sausages per
290 group at each sampling point, 3 subsamples for sausage) salami were macerated in a chopper
291 and appropriate portions of the homogenised sample were used for chemical analysis. A
292 Double Pore F electrode (Hamilton Company, Reno, NV, USA) hooked to an Eutech pH 2700
293 (Eutech Instrument Europe B.V.) was used to measure the pH by mixing 10 g of sausage with
294 90 ml of distilled water. Water activity (a_w) was measured with a dew-point hygrometer
295 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

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
323 as the absence of a darker, external halo in the slice due to anomalous drying process, fat/lean
324 connection as the degree of adherence of the product's principal ingredients (fat and lean),
325 fat/lean distribution as the uniform distribution of lean and fat on the slice. Global odor is the
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
328 from deforming force applied with forefinger and thumb, hardness is the force necessary to
329 compress the sample between forefinger and thumb to achieve a given deformation;
330 cohesiveness is the resistance of the sample before breaking when it is strained.

331 *Analysis of results.*

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

338 The detectable colony limit was 10^2 cfu g^{-1} for spread plate and 10 cfu g^{-1} for pour plate and
339 the confidence limit 95%, according to the classic formula $2s = 2\sqrt{x}$. Only values included
340 between 30 and 300 cfu were considered suitable for data analysis and when the count revealed
341 lower values at the lowest dilution, the results were reported as <300 for pour plate and <3000
342 for spread plate. Samples showing at least one typical colony in the lowest dilution were
343 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™) on the appearance salami.

346

347 **Results**

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

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.,
353 2012). The acidifying activity of the different species had been preliminarily tested (Cenci
354 Goga et al., 1995; B. Cenci-Goga et al., 2015; B. T. Cenci-Goga et al., 2016; Clementi et al.,
355 1998; Sechi et al., 2014) as pure cultures and as different associations in milk to assess their
356 suitability to be used as a starter. The parameters describing the acidification kinetics for the
357 test at 10°C are described in Table 1 together with the regression coefficients of the fourth
358 degree polynomial [equation 1] which was used as an empirical model. This adequately fitted
359 the experimental data, since the r^2 values varied from 0.998 to 0.999 and the actual values were
360 almost exactly superimposed on the empirical model curves (Figure 2a and 2b). The values of
361 the maximum instantaneous acidification rate [V_m] were of the same order for all strains, except
362 for *Lc. lactis ssp. lactis* strain 340 which had the highest V_m [-2.35 x 10^{-4} ΔpH/min) and for *Lc.*
363 *lactis ssp. lactis* strain 16 which had the lowest t_m [3754 min] values. After 12.000 min. all
364 strains reached pH values below 5.5 (below 5.0 for strain 340). *E. faecium* gave a one fold pH
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™ 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™, level of *S. aureus* dropped, after 240
375 hrs, below 10^5 cfu ml^{-1}. A similar behaviour was shown by *E. coli* with a slower drop (<10^7
376 cfu ml^{-1} after 120 hrs). *L. innocua* had a substantial reduction in total viable cells between 72
377 and 120 hrs (<10^5 cfu ml^{-1} after 24 hrs in BHI and at 240 hrs in milk). LAB had an effect on
378 *Salmonella* Derby, with levels below 10^5 cfu ml^{-1} after 240 hrs. Table 2 shows the log reduction
379 along with the statistics: after 240 hours of incubation with NoNit™ 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™ strains both in milk and in BHI alone and
382 vs. selected pathogens.

383 *Salami challenge test*

384 **Pathogens**. No *Listeria* spp. nor *Salmonella* spp. were detected before the challenge testing in
385 the raw materials, pork meat, pork fat and in natural casing and ingredients (NaCl, pepper,
386 dextrose, garlic and starter cultures). Counts of *S. aureus*, strain 27R; *E. coli*, strain CSH26
387 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
390 (5a *S. aurues*, *5b E. coli*, 5c *L. innocua*, 5d *S.* Derby) and Table 3. The initial inoculum levels
391 (day-0) was always approx. 4 $\log_{10}$ cfu g^{-1}: for *S. aureus* it ranged from 4.19 (in commercial
392 batch) to 4.26 (in NoNit™ batch), for *E. coli* form 4.03 to 4.18, for *L. innocua* from 4.09 to
393 4.18 and for *Salmonella* Derby from 3.69 to 3.90. During fermentation and ripening, with very
394 little heterogeneity observed between batches, *S. aureus* strain 27R reached levels of 6 $\log_{10}$
395 cfu g^{-1} from day-21 in the commercial batches while remained always below 6 $\log_{10}$ cfu g^{-1}
396 in the NoNit™ batches. *E. coli*, strain CSH26 K-12 reached levels of 5.05 $\log_{10}$ cfu g^{-1} on day-
397 7 and remained at about 4 $\log_{10}$ cfu g^{-1} throughout ripening in commercial batches, while
398 dropped to 1.93 $\log_{10}$ cfu g^{-1} in NoNit™batches, after a peak to 4.53 on day-3. *L. innocua*
399 ATCC 33090 reached about 6 $\log_{10}$ cfu g^{-1} on day-3 with a plateau till the end of ripening in
400 commercial batches and reached 5.02 $\log_{10}$ cfu g^{-1} in NoNit™ on day-21 to drop below 5 $\log_{10}$
401 cfu g^{-1} at the end of ripening. *Salmonella* Derby reached levels of 5.42 $\log_{10}$ cfu g^{-1} on day-3
402 and stayed above 3 $\log_{10}$ cfu g^{-1} throughout fermentation and ripening in commercial batches
403 while dropped below 3 from day-21 and to 1.79 $\log_{10}$ cfu g^{-1} at the end of ripening in NoNit™
404 batches.

405 **Evolution of starter cultures**. *Lactococcus* spp. and *Lactobacillus* spp. counts were always
406 above 6 $\log_{10}$ cfu g^{-1} and reached values above 8 $\log_{10}$ cfu g^{-1} from day-3 (for *Lactobacillus*
407 spp.) and day-7 (for *Lactococcus* spp.) for both formulations. *Enterococcus* spp. counts started
408 from 4.14 $\log_{10}$ cfu g^{-1} in the commercial batches and from from 6.11 $\log_{10}$ cfu g^{-1} in the
409 NoNit™ batches and remained above from 6 $\log_{10}$ cfu g^{-1} throughout fermentation and
410 ripening in both batches. *Staphylococcus* spp. counts (these data are the difference between
411 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 $^{-1}$ and
413 decreased to 3.25 log_{10} cfu g $^{-1}$ at the end of ripening in commercial batches, while in NoNit™
414 batches counts were always below 4 log_{10} cfu g $^{-1}$ and decreases below 2 log_{10} cfu g $^{-1}$ 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™ batches and
418 on day-21 in commercial batches. Fully ripened salami were between 0.82 in NoNit™ batchges
419 and 0.84 in commercial batches. Mean pH values on the day of stuffing were 6.10 and reached
420 values below 6.0 on day-3 in NoNit™ batches and on day-21 on commercial batches. At the
421 end of ripening NoNit™ batches reached pH values of 5.17 and commercial batches of 5.67.
422 Table 5 shows the chemical composition: on the day of stuffing, proteins were 40.44, fat 50.32,
423 ashes 8.88, (g 100 g^{-1} total solids), and similar data were obtained at the end of ripening.
424 Moisture decreased from 58.67% on the day of stuffing to 28.22% (commercial) and 29.30
425 (NoNit™) in full ripened salami.

426 *Sensory evaluation*

427 Figure 9 and Table 6 show the results of sensory analysis which was limited to appearance
428 attributes, odors and texture attributes because batches had been spiked with pathogens. For
429 the two group of batches similar results were obtained, except for color uniformity (3.38 for
430 commercial batches and 5.00 for NoNit™ batches), fat/lean distribution (3.43 vs. 4.87), and
431 hardness (2.50 vs. 3.87).

432 **4. Discussion**

433 Selected pathogens and hygiene indicators were spiked into salame nostrano (italian dry
434 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*

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.
462 This synergism is of the utmost importance in the production of fermented food such as dry
463 salami because the activity of starter cultures is desirable throughout the whole fermentation
464 process (Cenci Goga et al., 2012; Cizeikiene, Juodeikiene, Paskevicius, & Bartkiene, 2013).
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
469 temperatures up to 40º C. As a result pH drops to 4.6, the sausage is stable but the flavor suffers
470 and the product is sour and tangy. In European countries, the temperatures of 22-26ºC are used
471 and the drying, instead of the acidity (pH) is the main hurdle against spoilage bacteria which
472 favors better flavor development. The final acidity of a traditionally made salami is low (high
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
475 formulation for low temperature fermentation. However, many available commercial starter
476 cultures used in salami fermented and ripened at low temperatures have sometimes been linked
477 to a bitterness taint and salami manufacturer are seeking for better alternatives. It well known
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.,
480 1994; Mc Lain, Blumer, Graig, & Stelel, 1968) and they have been reported as precursors of
481 sour, sweet, and bitter taste (Kato, Rhue, & Nishimura, 1989). Aro Aro et al. (2010)
482 demonstrated that Staphylococci cultures, especially *S. xilosus*, increase free aminoacid levels
483 in salami compared with *S. carnosus*, *L. sakei* associated with *S. carnosus* and *P. pentosaceus*
484 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

510 the growth curve of *E. coli* was parallel to control, indicating an effect of pH rather than a direct
511 activity of starter cultures and/or bacteriocins. A similar behavior was shown by *S.* Derby 27,
512 with an even faster drop (<10^4 cfu ml^{-1} after 30 hrs). *S. aureus* proved to be more resilient and
513 a substantial reduction in total viable cells was observed between 72 and 120 hrs (<10^3 cfu ml^{-1}
514 after 120 hrs) with a complete elimination after 168 hrs. *L. innocua* was no longer detectable
515 in milk after 120 hrs, with a steady drop after 24 hrs (<10^4 cfu ml^{-1} after 72 hrs). LAB had an
516 effect on *L. innocua*, also in BHI, with levels below 10^4 cfu ml^{-1} just after 30 hrs. The direct
517 effect of certain LAB strains on *Listeria* spp. rather than the indirect effect of pH drop has been
518 described (Winkelströter & De Martinis, 2015). Salami made with the NoNit™ formulation in
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
522 incorporation of the *Enterococcus faecium* strain in the starter formulation. In fact, throughout
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.
525 Before challenging the NoNit™ formulation vs selected pathogens in salami, an in vitro set up
526 was arranged in the present study with incubation temperature lowered to 10°C. Even a low
527 temperature level of *S. aureus* in milk and in BHI dropped, after 240 hrs, below 10^5 cfu ml^{-1}.
528 *E. coli* showed a similar behavior with a slower drop (<10^7 cfu ml^{-1} after 120 hrs). *L. innocua*
529 had a substantial reduction in total viable cells between 72 and 120 hrs (<10^5 cfu ml^{-1} after 24
530 hrs in BHI and at 240 hrs in milk). The NoNit™ formulation had an effect on *Salmonella*
531 Derby, in milk, with levels below 10^5 cfu ml^{-1} after 240 hrs. These results, even if the inoculum
532 used in this challenge was very high (10^6 cfu ml-^{1}) and not representative of the natural
533 contamination commonly encountered in the raw material for salami production, demonstrates
534 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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548 **References**

549 Adams, M., & Moss, M. (2000). *Food microbiology*. Cambridge: Royal Society of Chemistry.

550 Al-Mutairi, M. F. (2011). The incidence of enterobacteriaceae causing food poisoning in some
551 meat products. [Article]. *Advance Journal of Food Science and Technology, 3*(2), 116-
552 121.

553 Anonymous. (2002). Public health dispatch: outbreak of listeriosis—Northeastern United
554 States. *CDC (Centers for Disease Control and Prevention)*
555 *Morbidity and Mortality Weekly Report, 51*, 951-951.

556 Anonymous. (2016). Use and Removal of Nitrite in Meat Products Retrieved February 2016,
557 2016, from https://www.fsai.ie/faq/use_and_removal_of_nitrite.html

558 AOAC. (2000). *Official Methods of Analysis of AOAC International (OMA), 17th Edition* (17
559 ed.). Wasington, DC, USA: Association of Official Analytical Chemists.

560 Aro Aro, J. M., Nyam-Osor, P., Tsuji, K., Shimada, K. i., Fukushima, M., & Sekikawa, M.
561 (2010). The effect of starter cultures on proteolytic changes and amino acid content in
562 fermented sausages. [Article]. *Food Chemistry, 119*(1), 279-285. doi:
563 10.1016/j.foodchem.2009.06.025

564 Bouvard, V., Loomis, D., Guyton, K. Z., Grosse, Y., Ghissassi, F. E., Benbrahim-Tallaa, L., .
565 . . Straif, K. (2015). Carcinogenicity of consumption of red and processed meat. *The*
566 *Lancet Oncology, 16*(16), 1599-1600. doi: http://dx.doi.org/10.1016/S1470-
567 2045(15)00444-1

568 Candogan, K., Wardlaw, F. B., & Acton, J. C. (2009). Effect of starter culture on proteolytic
569 changes during processing of fermented beef sausages. [Article]. *Food Chemistry,*
570 *116*(3), 731-737. doi: 10.1016/j.foodchem.2009.03.065

571 Casey, P., & Condon, S. (2000). Synergistic lethal combination of nitrite and acid pH on a
572 verotoxin-negative strain of Escherichia coli O157. [Conference Paper]. *International*

573 *Journal of Food Microbiology, 55*(1-3), 255-258. doi: 10.1016/S0168-1605(00)00199-
574 9
575 Cenci Goga, B. T., Clementi, F., & Di Antonio, E. (1995). Behaviour of lactic and non lactic
576 microflora during production and ripening of farm manufactured Pecorino cheese.
577 *Annals of Microbiology, 45*(2), 219-236.
578 Cenci Goga, B. T., Ortenzi, R., Bartocci, E., Codega de Oliveira, A., Clementi, F., & Vizzani,
579 A. (2005). Effect of the implementation of HACCP on the microbiological quality of
580 meals at a university restaurant. *Foodborne Pathog Dis, 2*(2), 138-145.
581 Cenci Goga, B. T., Ranucci, D., Miraglia, D., & Cioffi, A. (2008). Use of starter cultures of
582 dairy origin in the production of Salame nostrano, an Italian dry-cured sausage. *Meat*
583 *Science, 78*(4), 381-390. doi: 10.1016/j.meatsci.2007.07.001
584 Cenci Goga, B. T., Rossitto, P. V., Sechi, P., Parmegiani, S., Cambiotti, V., & Cullor, J. S.
585 (2012). Effect of selected dairy starter cultures on microbiological, chemical and
586 sensory characteristics of swine and venison (Dama dama) nitrite-free dry-cured
587 sausages. *Meat Sci., 90*(3), 599-606. doi: 510.1016/j.meatsci.2011.1009.1022. Epub
588 2011 Oct 1016.
589 Cenci-Goga, B., Karama, M., Sechi, P., Iulietto, M. F., Novelli, S., Selvaggini, R., & Mattei,
590 S. (2015). Growth inhibition of selected microorganisms by an association of dairy
591 starter cultures and probiotics. *Italian Journal of Animal Science, 14*(2), 246-250.
592 Cenci-Goga, B. T., Karama, M., Sechi, P., Iulietto, M. F., Novelli, S., Selvaggini, R., &
593 Barbera, S. (2016). Effect of a novel starter culture and specific ripening conditions on
594 microbiological characteristics of nitrate-free dry-cured pork sausages. *Italian Journal*
595 *of Animal Science, 15*(3), 358-374. doi: 10.1080/1828051X.2016.1204633
596 Chajecka-Wierzchowska, W., Zadernowska, A., Nalepa, B., Sierpinska, M., & Laniewska-
597 Trokenheim, T. (2015). Coagulase-negative staphylococci (CoNS) isolated from ready-

598 to-eat food of animal origin - Phenotypic and genotypic antibiotic resistance. [Article]. 599 *Food Microbiology, 46*, 222-226. doi: 10.1016/j.fm.2014.08.001

600 Chamba, J. F., & Prost, F. (1989). Mesure de l'activité acidifiante des bactéries lactiques 601 thermophiles utilizées pour la fabrication des fromages à pâte cuite. *Lait, 69*, 417-431.

602 Cizeikiene, D., Juodeikiene, G., Paskevicius, A., & Bartkiene, E. (2013). Antimicrobial activity 603 of lactic acid bacteria against pathogenic and spoilage microorganism isolated from 604 food and their control in wheat bread. *Food Control, 31*(2), 539-545.

605 Clementi, F., Cenci Goga, B. T., Trabalza-Marinucci, M., & Di Antonio, E. (1998). Use of 606 selected starter cultures in the production of farm manufactured goat cheese from 607 thermized milk. *Italian Journal of Food Science, 10*(1), 41-56. doi: IDS Number: 608 ZH874

609 Conedera, G., Mattiazzi, E., Russo, F., Chiesa, E., Scorzato, I., Grandesso, S., . . . Caprioli, A. 610 (2007). A family outbreak of Escherichia coli O157 haemorrhagic colitis caused by 611 pork meat salami. [Article]. *Epidemiology and Infection, 135*(2), 311-314. doi: 612 10.1017/S0950268806006807

613 Corbiere Morot-Bizot, S., Leroy, S., & Talon, R. (2007). Monitoring of staphylococcal starters 614 in two French processing plants manufacturing dry fermented sausages. *Journal of* 615 *Applied Microbiology, 102*(1), 238-244. doi: doi:10.1111/j.1365-2672.2006.03041.x

616 Cordoba, J. J., Antequera Rojas, T., García González, C., Ventanas Barroso, J., López Bote, 617 C., & Asensio, M. A. (1994). Evolution of free amino acids and amines during ripening 618 of Iberian cured ham. [Article]. *Journal of Agricultural and Food Chemistry, 42*(10), 619 2296-2301.

620 D'Ostuni, V., Tristezza, M., De Giorgi, M. G., Rampino, P., Grieco, F., & Perrotta, C. (2016). 621 Occurrence of Listeria monocytogenes and Salmonella spp. in meat processed products

622 from industrial plants in Southern Italy. [Article]. *Food Control, 62*, 104-109. doi:
623 10.1016/j.foodcont.2015.10.025
624 Filipović, I., Cenci Goga, B., Njari, B., Dobranić, V., Zdolec, N., & Kozačinski, L. (2012).
625 *Safety and quality of traditional croatian product Meat from tiblica.* Paper presented at
626 the Proceedings of 6th Central European Congress on Food - CEFood Congress,
627 University of Novi Sad, Institute of Food Technology.
628 García Fontán, M. C., Lorenzo, J. M., Parada, A., Franco, I., & Carballo, J. (2007).
629 Microbiological characteristics of "androlla", a Spanish traditional pork sausage. *Food*
630 *Microbiology, 24*(1), 52-58.
631 González-Fernández, C., Santos, E. M., Rovira, J., & Jaime, I. (2006). The effect of sugar
632 concentration and starter culture on instrumental and sensory textural properties of
633 chorizo-Spanish dry-cured sausage. *Meat Science, 74*(3), 467-475.
634 ISO. (2003). Sensory analysis - Guidelines for the use of quantitative response scales, ISO
635 4121:2003. *International Organization for Standardization, Geneva, Switzerland*.
636 Kato, H., Rhue, M. R., & Nishimura, T. (1989). Role of free amino acids and peptides in food
637 taste. *Flavor Chemistry. Trends and Developments, In R. T. Teranishi, R. G. Buttery,*
638 *F. Shahidi, Washington, DC: American Chemical Society*, 158-174.
639 Lederberg, J., & Lederberg, E. M. (1952). Replica plating and indirect selection of bacterial
640 mutants. *Journal of Bacteriology, 63*, 399-406.
641 Leroy, F., Verluyten, J., & De Vuyst, L. (2006). Functional meat starter cultures for improved
642 sausage fermentation. *International Journal of Food Microbiology, 106*(3), 270-285.
643 Luzzi, I., Galetta, P., Massari, M., Rizzo, C., Dionisi, A. M., Filetici, E., . . . Ciofi degli Atti,
644 M. L. (2007). An Easter outbreak of Salmonella Typhimurium DT 104A associated
645 with traditional pork salami in Italy. *Euro Surveill, 12*(4), E11-12.

646 Mc Lain, G. R., Blumer, T. N., Graig, H. B., & Stelel, R. G. (1968). Free amino acids in ham
647 muscle during successive aging periods. *Journal of Food Science, 33*, 142-146.
648 Molly, K., Demeyer, D., Johansson, G., Raemaekers, M., Ghistelinck, M., & Geenen, I. (1997).
649 The importance of meat enzymes in ripening and flavour generation in dry fermented
650 sausages. First results of a European project. *Food Chemistry, 59*(4), 539-545. doi:
651 http://dx.doi.org/10.1016/S0308-8146(97)00004-6
652 Mossel, D. A. A., Corry, J. E. L., Struijk, C. B., & Baird, R. M. (1995). *Essentials of the*
653 *microbiology of food*. Chichester, UK: John Wiley & Sons Ltd.
654 Nazzaro, F., Di Luccia, A., Tremonte, P., Grazia, L., Sorrentino, E., Maurelli, L., & Coppola,
655 R. (2004). Evaluation of proteolytic activity of Staphylococcus xylosus strains in
656 Soppressata Molisana, a typical Southern Italy fermented sausage. *Annals of*
657 *Microbiology 54*(3), 269-281
658 Ozel, M. Z., Gogus, F., Yagci, S., Hamilton, J. F., & Lewis, A. C. (2010). Determination of
659 volatile nitrosamines in various meat products using comprehensive gas
660 chromatography-nitrogen chemiluminescence detection. *Food and Chemical*
661 *Toxicology, 48*(11), 3268-3273. doi: 10.1016/j.fct.2010.08.036
662 Pegg, R., & Shahidi, F. (2000). *Nitrite curing of meat. The N-Nitrosamine Problem and Nitrite*
663 *Alternatives*: Food & nutrition press, Inc. Trumbull, Connexticut 06611 USA.
664 Rai, R., & Bai, J. A. (2014). *Beneficial Microbes in Fermented and Functional Foods*: CRC
665 Press.
666 Riordan, D. C. R., Duffy, G., Sheridan, J. J., Eblen, B. S., Whiting, R. C., Blair, I. S., &
667 McDowell, D. A. (1998). Survival of Escherichia coli O157:H7 during the manufacture
668 of pepperoni. [Article]. *Journal of Food Protection, 61*(2), 146-151.
669 Sartz, L., De Jong, B., Hjertqvist, M., Plym-Forshell, L., Alsterlund, R., Löfdahl, S., . . .
670 Karpman, D. (2008). An outbreak of Escherichia coli O157:H7 infection in southern

671 Sweden associated with consumption of fermented sausage; aspects of sausage
672 production that increase the risk of contamination. [Article]. *Epidemiology and*
673 *Infection, 136*(3), 370-380. doi: 10.1017/S0950268807008473
674 Sechi, P., Iulietto, M. F., Mattei, S., Traina, G., Codini, M., & Cenci Goga, B. (2014). Effect
675 of a formulation of selected dairy starter cultures and probiotics on microbiological,
676 chemical and sensory characteristics of swine dry-cured sausages. *Journal of*
677 *Biotechnology, 185*(S83). doi: 10.1016/j.jbiotec.2014.07.283
678 Sheen, S., & Hwang, C. A. (2008). Modeling transfer of Listeria monocytogenes from slicer
679 to deli meat during mechanical slicing. [Article]. *Foodborne Pathogens and Disease,*
680 *5*(2), 135-146. doi: 10.1089/fpd.2007.0049
681 Winkelströter, L. K., & De Martinis, E. C. P. (2015). In vitro protective effect of lactic acid
682 bacteria on Listeria monocytogenes adhesion and invasion of Caco-2 cells. *Beneficial*
683 *Microbes, 11*, 1-8. doi: 10.3920/BM2013.0091
684 Zambonelli, C., Papa, F., Romano, P., Suzzi, G., & Grazia, L. (1992). *Microbiologia dei Salumi*
685 (Edagricole-Edizioni Agricole ed.).
686 Zarringhalami, S., Sahari, M. A., & Hamidi-Esfehani, Z. (2009). Partial replacement of nitrite
687 by annatto as a colour additive in sausage. *Meat Science, 81*(1), 281-284.
688 Zhang, X., Kong, B., & Xiong, Y. L. (2007). Production of cured meat color in nitrite-free
689 Harbin red sausage by Lactobacillus fermentum fermentation. *Meat Science, 77*(4),
690 593-598.
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™

724 formulation in BHI, ___❒ pathogens alone in milk, ---❒--- pathogens vs NoNit™
725 formulation in milk
726
727 **Figure 4**. Evolution of NoNit™ formulation in BHI and in milk at 10°C (4a: NoNit™
728 formulation in BHI, 4b: NoNit™ formulation in milk, 4c: *E. faecium* in BHI, 4c: *E. faecium* in
729 milk), ___ LAB alone, ---O--- NoNit™ formulation vs *S. aureus* 27R, ------ NoNit™
730 formulation vs *E. coli* CSH26 K-12, ---☐--- NoNit™ formulation vs *L. innocua* ATCC 33090,
731 ------ NoNit™ 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™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™formulation
740
741 **Figure 7**. Development of a_w in *salame nostrano* during fermentation and ripening, ___
742 commercial starter, --- NoNit™formulation
743
744 **Figure 8**. Development of pH in *salame nostrano* during fermentation and ripening, ___
745 commercial starter, --- NoNit™formulation
746
747 **Figure 9**. Sensory descriptive analysis of *salame nostrano* (9a: appearance attributes, 9b: basic
748 aromas and texture attributes), ___ commercial starter, --- NoNit™formulation
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