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*Original*

Assessment of Salmonella survival in dry-cured Italian salami / Bonardi, Silvia; Bruini, Ilaria; Bolzoni, L.; Cozzolino, P.; Pierantoni, M.; Brindani, Franco; Bellotti, P.; Renzi, Marco; Pongolini, S.. - In: INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY. - ISSN 0168-1605. - 262:(2017), pp. 99-106. [10.1016/j.ijfoodmicro.2017.09.016]

*Availability:*

This version is available at: 11381/2830905 since: 2021-10-11T11:54:13Z

*Publisher:*

Elsevier B.V.

*Published*

DOI:10.1016/j.ijfoodmicro.2017.09.016

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note finali coverpage

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1    **Assessment of *Salmonella* survival in dry-cured Italian salami**

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Key words: *Salmonella*, pork, dry-curing, real-time PCR, MPN enumeration

## 1. Introduction

Italian salami are dry fermented sausages which have been produced for centuries with a variety of ingredients and manufacturing processes. Dry fermented sausages from Mediterranean countries are usually air dried, due to the favourable climate, and rarely smoked. Pork meat is the main ingredient and fungal starter cultures may be used on the external surface imparting a complexity of flavours to the product (Talon et al., 2004).

The use of sodium chloride (NaCl) is essential in dry fermented sausages to solubilize proteins and emulsify fat. Furthermore, NaCl can control the growth of undesirable bacteria responsible for spoilage of meat and pathogenic bacteria (King et al., 2016). The usual amount of added salt is generally between 2-4% by weight (Ockerman and Basu, 2007) but its concentration increases in final products due to the drying process (Zanardi et al., 2010). In addition to NaCl, other salts are generally added to the pork and fat mixture, namely nitrates (maximum 150 mg/kg) and nitrites (maximum 150 mg/kg) to inhibit *Clostridium botulinum* (Hospital et al., 2016), enterobacteriaceae and enterococci (Coloretti et al., 2008) and to favour the red colour of cured meat (Villaverde et al., 2014).

Salami manufactured in the Emilia-Romagna region of Northern Italy, where this study was conducted, are generally made with pork only, and they have coarsely ground meat and 3-4 mm size cubes of fat. Fresh meat is obtained from shoulder and belly and fat is normally pork backfat. After grounding, salt, whole peppercorns and garlic are added in traditional products. Some formulations include sugars and starter bacterial cultures. Salami are then stuffed into a pork casing and commonly aged for 20 to 40 days according to their size (Mataragas et al., 2015).

*Salmonella* ranks second among pathogens reported in the European Union (EU) as causative agents of human [foodborne-zoonotic](#) diseases (EFSA and ECDC, 2016). In 2014, pork meat and products thereof were responsible for 9.3% of 225 foodborne outbreaks caused by *Salmonella*, thus representing the type of meat most frequently associated with salmonellosis in humans (EFSA and

59 ECDC, 2015). In Italy, consumption of salami was recently associated with both clustered and  
60 sporadic cases of salmonellosis due to *S. Goaldcost* (Scavia et al., 2013), *S. Manhattan* (Scaltriti et  
61 al., 2015), *S. Typhimurium* and *S. Typhimurium* monophasic variant ( Andreoli et al., 2017; Luzzi  
62 et al., 2007). Other EU countries confirmed *Salmonella* outbreaks linked to salami, like Sweden in  
63 2005, Norway in 2006 and Denmark in 2010 (Emberland et al., 2006; Hjertqvist et al., 2006; Kuhn  
64 et al., 2011).

65 *Salmonella* survival in cured meat products depends on dry-curing and physicochemical conditions  
66 created by several parameters, which interact all together, like salt, nitrite, pH, water activity and  
67 temperature. For example, water activity decrease is a key factor for *Salmonella* inactivation, but its  
68 effect depends also on contemporary pH decrease, as well as salt and nitrite concentration (Messier  
69 et al., 1989). Different studies were performed on Italian traditional salami, which addressed the  
70 fate of *Salmonella* under experimental conditions through artificial contamination (Mataragas et al.,  
71 2015; Nightingale et al., 2006). In these studies, composition and physicochemical conditions of  
72 salami were specified. On the contrary, our study aimed to assess the behavior of *Salmonella* in  
73 traditional salami, characterized by low standardization of production parameters, in field  
74 conditions. In this context, to cover the variability of the production process, a large number of  
75 batches distributed among different producers were included in the study with the purpose of  
76 assessing the effects of the basic physicochemical parameters (pH,  $a_w$ , NaCl) regardless of the  
77 heterogeneity of the actual products on the market.

78 In the EU, microbiological food-safety criteria are set by the Regulation EC 2073/2005 (European  
79 Commission, 2005), which identifies culture-based ISO methods as the analytical reference  
80 methods. ISO 6579 is the standard for *Salmonella* detection in foods. This method relies on several  
81 cultural steps and requires more than 5 days for conclusive results in case of positive samples. This  
82 is why, to meet the needs of the food industry, the same Regulation allows the use of alternative  
83 methods, generally more convenient and faster, under specified conditions. PCR-based methods are

84 among available alternatives and several studies have been performed to assess real-time PCR  
85 protocols for the rapid and sensitive detection of *Salmonella* in foods in less than 24 h (Delibato et  
86 al., 2014; Rodriguez-Lazaro et al., 2014).

87 Based on the above considerations, our study focus<sup>es</sup> on characteristic salami from Emilia  
88 Romagna and the aims were: *i*) to determine the prevalence of *Salmonella* in the mixtures of minced  
89 raw pork and fat used for salami production, *ii*) to assess the effect of curing on the fate of  
90 *Salmonella* in the end product by testing the batches of salami manufactured with the *Salmonella*-  
91 positive raw mixtures, *iii*) to investigate the influence of physicochemical parameters on *Salmonella*  
92 contamination in dry-cured salami, *iv*) to measure the load of *Salmonella* in dry-cured salami, *v*) to  
93 assess the effect of sample size on the probability of *Salmonella* detection, and *vi*) to investigate the  
94 proportion of real-time PCR positive samples confirmed by ISO 6579.

95

## 96 **2. Material and methods**

### 97 **2.1 Sample collection**

98 From April to December 2015, 150 samples of ground raw mixtures (GRM) made of ground pork  
99 and fat, collected from 150 different batches of starting material for salami processing, were tested  
100 for *Salmonella*. Only pig meat and pig backfat were used for the salami manufacturing. The  
101 samples were collected in four production plants, here identified as A (47 samples), B (23 samples),  
102 C (11 samples), D (69 samples) located in Emilia-Romagna region, Northern Italy. The plants were  
103 included in the study based on their willingness to take part to the study (five were asked to  
104 participate) and the different number of samples collected from each plant was proportional to its  
105 production capacity. Meat and fat suppliers of the four plants were many and, often, meat and fat  
106 from more than one supplier were mixed in the same batch of GRM. In our study the GRM samples  
107 of the four companies were collected before addition of other ingredients and additives (salt,

108 nitrites, nitrates, ascorbates, black pepper) to avoid potential interference with *Salmonella* detection.  
109 Nitrates and nitrates were added in compliance with the limits set by Regulation EU No 1129/2011  
110 on food additives.

111 Whenever a GRM was positive for *Salmonella*, the derived batch of salami was tested for the  
112 pathogen at the end of its curing period which ranged from 20 to 48 days according to the different  
113 producers' protocols. Five salami (5 sampling units) per batch were tested. Since 21 GRM samples  
114 were positive for *Salmonella*, 21 batches of salami (for a total of 105 salami) were analyzed at the  
115 end of their curing. The total number of tested salami was 140, because 6 batches of salami out of  
116 21 resulted contaminated by *Salmonella*, and were re-tested after a prolonged curing period of 21-  
117 38 days (for an additional 5 salami per batch). Furthermore, since one batch was still positive, 5  
118 more salami were analyzed after an additional curing of 8 more days (total curing duration: 62 days)  
119 (Table 1). The values of pH and  $a_w$  and the content of NaCl were determined in the 140 tested  
120 salami.

121

## 122 2.2 *Salmonella* detection in ground raw mixture (GRM) and salami

123 Detection of *Salmonella* in both GRM and salami was performed by real-time PCR followed by  
124 microbiological confirmation. A pre-enrichment broth was prepared suspending 25 g of sample in  
125 225 ml of Buffered Peptone Water (BPW, Oxoid, Basingstoke, UK) and homogenizing for 2  
126 minutes in a Stomacher blender. After  $18 \pm 2$  h at  $37 \pm 1^\circ\text{C}$  DNA was extracted from 1 ml of the  
127 pre-enrichment culture using SureFood PREP *Salmonella* Kit (R-Biopharm, Darmstadt, Germany)  
128 and PCR master-mix was prepared with SureFast *Salmonella* ONE Kit (R-Biopharm) for a final  
129 volume of 25  $\mu\text{l}$  containing 5  $\mu\text{l}$  of template DNA. PCR reactions were run on a Mx3005P QPCR  
130 System (Agilent Technologies, Italy) with the following thermal program: a cycle of DNA  
131 polymerase activation of 5 min at  $95^\circ\text{C}$  followed by 45 amplification cycles of 15 s at  $95^\circ\text{C}$  and

30 s at 60 °C (annealing-extension step). The samples with a cycle threshold (CT) value lower than 40 were considered positive. The other samples were considered negative for *Salmonella*. PCR positive samples underwent microbiological testing by ISO 6579:2002 starting from aliquots of 25 g and 50 g, the latter being resampled from the meat matrix, the former coinciding with the pre-enrichment step used for PCR. All meat samples were stored at 3°C (± 1°C) for up to 24 h before resampling. Presumptive isolates of *Salmonella* were assayed with O-omnivalent *Salmonella* serum by slide agglutination (Denka Seiken, Tokyo, Japan). Biochemical identification to the genus level was carried out with API® 20E system (bioMérieux, Marcy l'Etoile, France).

140

141

## 2.3 Typing of *Salmonella* isolates

Serotyping of isolates was performed following the White-Kauffmann-Le Minor scheme by slide agglutination with O and H antigen specific sera (DID, Milan, Italy; Biogenetics, Padua, Italy). Discrimination of *S. enterica* 4,[5],12:i:- from *S. Typhimurium* was done phenotypically by repeating phase inversion at least three times without evidence of expression of phase-two flagellar antigens and genotypically by PCR (Barco *et al.*, 2011). PFGE was performed according to standard methods (PulseNet, 2010) with *XbaI* (Roche Italia, Milan, Italy) restriction of DNA.

149

## 2.4 *Salmonella* enumeration

The miniaturized Most Probable Number technique according to ISO 6579-2:2012 was used for *Salmonella* enumeration in all PCR positive GRM and salami samples. Fifty grams of the sample were diluted 10<sup>-1</sup> in BPW and 2.5 ml of the initial dilution were inoculated in triplicate in the first column of a 12 multi-well microtiter plate. Two ml of sterile BPW were distributed in the wells of the remaining columns (three wells per column). Further dilutions of the samples were performed by sequentially transferring 0.5 ml from the wells of the first column to the wells of successive

157 columns. The plates were incubated at  $37 \pm 1^\circ\text{C}$  for 16-20 h. Thereafter, a 20  $\mu\text{l}$ -aliquot from each  
158 well was inoculated in a 12 multi-well microtiter plate containing 2 ml of Modified Semi-Solid  
159 Rappaport-Vassiliadis (MSRV) per well. After incubation at  $41.5 \pm 1^\circ\text{C}$  for  $24 \pm 3\text{h}$ , the wells  
160 showing a grey-white zone extending out of the inoculum drop were further tested by streaking a 10  
161  $\mu\text{l}$ -loopful of the bacterial growth onto XLD (Oxoid) agar plates. Microtiter plates not showing  
162 bacterial growth were incubated for further  $24 \text{ h} \pm 3 \text{ h}$  and were considered negative if bacterial  
163 growth was still absent. XLD agar plates were incubated at  $37 \pm 1^\circ\text{C}$  for 24 h and suspect colonies  
164 were subjected to ISO 6579 confirmation tests. Confirmed *Salmonella* colonies were used to  
165 estimate the MPN *Salmonella*/g.

166

## 167 2.5 Measure of pH, $a_w$ and NaCl content

168 The pH value was measured on 5 g of salami homogenized in distilled water (10/1 water/sample,  
169 w/w) with a Crison micro pH 2001 instrument equipped with a Xerolyt 52-02 electrode (Crison  
170 Instruments, Barcelona, Spain). The  $a_w$  value was measured at  $25^\circ\text{C}$  by an AquaLab series 4TE  $a_w$   
171 meter (Decagon Devices, Inc., Pullman 99163, WA).

172 Sodium chloride (NaCl) content was measured following the ISO 1841-1:1996 method for the  
173 determination of the chloride content of meat and meat products with NaCl contents equal to or  
174 greater than 1.0%.

175

## 176 2.6 Statistical analyses

177 Statistical differences in the probability of cultural confirmation of PCR positive samples using  
178 different sampling aliquots (namely, 25 g and 50 g) were evaluated through Fisher's exact tests.



179 We estimated the probability of detecting *Salmonella* by culture in PCR positive samples, starting  
180 from aliquots of 25 g and 50 g, as a function of the PCR CT value. Specifically, two sets of  
181 generalised linear models (GLM) with binomial error distribution and logit link function were built,  
182 one for GRM and one for salami. The GLMs were built by using *Salmonella* detection by  
183 microbiological assay as response variable, and the PCR CT value as explanatory variable. Each set  
184 of GLMs included a model where the microbiological assays were carried out from sampling  
185 aliquots of 25 g and a model with sampling aliquots of 50 g.

186 We assessed whether the detection of *Salmonella* in salami prepared from *Salmonella*-positive  
187 GRMs was related to the physicochemical parameters of the salami – namely pH,  $a_w$ , and NaCl  
188 content – and the enumeration of *Salmonella* (MPN) in GRM. Specifically, a generalised linear  
189 mixed model (GLMM) with binomial error distribution and logit link function was built by using  
190 the detection of *Salmonella* by microbiological assay in salami, (starting from aliquots of 50 g) as  
191 response variable (defined *Salmonella*), the physicochemical parameters (pH,  $a_w$ , NaCl) and most  
192 probable number of *S. enterica* in GRM (MPN) as fixed effects, and the production plant (called  
193 *plant*) as random effect. The full GLMM can be written as follows:

194  $Salmonella \sim pH + a_w + NaCl + MPN + (1 | plant)$

195 The response variable was modelled for dependence on predictor variables (fixed effects) using a  
196 forward stepwise selection procedure with log-likelihood ratio test to define the model providing  
197 the best prediction (Venables and Ripley, 2002). In addition, we tested through linear models  
198 whether physicochemical parameters of the salami were related to the curing time. Statistical  
199 analysis was carried out in the R 3.2.0 environment (R Development Core Team 2015), with  
200 “MASS”, “lme4”, and “lmerTest” packages.

201

### 3. Results

#### 3.1 Detection and enumeration of *Salmonella* in ground raw mixture (GRM) samples

The real-time PCR CT values were lower than 40.0 in 56 out of 150 (37%) GRM samples, ranging from 24.8 to 39.9. *Salmonella* was isolated from 21/56 (38%) of the PCR-positive samples. Therefore, the testing procedure used provided a prevalence of *Salmonella* in GRMs of 14% (21/150) (95% CI: 9–21) (Table 2).

As regards the effect of using 25 g or 50 g of GRM on cultural detection of *Salmonella* in PCR-positive samples, the ISO-25 g method detected *Salmonella* in 13/21 (62 %) of all culture-confirmed samples, while the ISO-50 g procedure was positive in 21/21 (100 %) of the confirmed samples (Table 2). The difference between the two procedures (ISO-50 g vs. ISO-25 g) was statistically significant (Fisher exact's test, p-value < 0.01). In addition, the statistical analysis showed that the proportion of culture-confirmed samples in PCR-positive GRMs significantly decreased with increasing PCR CT values both for sampling aliquots of 25 g (p-value < 0.01) and 50 g (p-value < 0.0001) (Figure 1a). Moreover, the proportion of culture-confirmed samples in PCR-positive GRMs was larger with sampling aliquots of 50 g than with aliquots of 25 g also for low PCR CT-values (Figure 1a).

The MPN enumeration in the PCR-positive samples ranged from 31 MPN/g to < 1.3 MPN/g (Table 3), being <1.3 MPN/g in 17/21 (81 %) samples, corresponding to the limit of quantification of the technique.

Seven *Salmonella* serovars were identified, with *S. Derby* (11/21; 52 %) as the most common, followed by *S. Typhimurium* monophasic variant (antigenic formula 4,[5],12:i:-) (4/21; 19 %) and *S. Stanley* (2/21; 10 %). *S. London*, *S. Brandenburg*, *S. Goettingen* and *S. Rissen* were identified in one sample each (1/21; 5 %). Different *Xba*I PFGE profiles were found among the most common

225 serovars, *i.e.* six among *S. Derby* isolates (D1-D6) and four among the monophasic variant of *S.*  
226 *enterica* 4,[5],12:i:- (MT1-MT4). One PFGE type was identified for *S. Stanley* (S1) (Table 3).

227

228 3.2 Detection and enumeration of *Salmonella* in cured salami

229 The 21 batches of dry-cured salami manufactured with the *Salmonella*-contaminated GRMs were  
230 tested at the end of their regular curing (20- 48 days) (Table 1). Five sampling units per batch,  
231 corresponding to a total of 105 salami, were tested. The real-time PCR CT values were lower than  
232 40.0 for 41/105 (39.0%) salami, ranging from 23.7 to 39.3. The ISO-25 g and ISO-50 g methods  
233 detected *Salmonella* in 10 and 12 samples respectively, giving an overall proportion of 12 out of 41  
234 (29 %) culture-confirmed samples. Notably, two more culture-positive salami were found by the  
235 MPN method; they were characterized by high CT-values (36.1 and 39.3).

236 The culture-confirmed salami were 14/105 (13 %) (95% CI 8 -21), belonging to six different  
237 batches, and the proportion of *Salmonella*-positive GRMs which ended up as contaminated batches  
238 of salami was 6/21 (29%). The 6 positive batches were re-tested (5 salami per batch, for a total of  
239 30 salami) after a prolonged curing time varying from 21 to 38 days, according to the different  
240 manufacturers. The total curing period of these products ranged from 49 to 86 days. Re-testing  
241 ended up in 11/30 (37 %) PCR-positive salami, but only one was confirmed by the ISO method  
242 with a prevalence of culture-positive salami after prolonged curing of 1/30 (3 %) (details in Table  
243 1). Confirmation rate of PCR-positive samples by cultural detection was 9% (1/11).

244 Since 1/30 salami (3 %) was still positive, 5 salami of the batch were re-tested after 8 more days  
245 (overall curing time of the batch: 62 days). Three sampling units/5 (60 %) were positive by real-  
246 time PCR (CT-values from 37.4 to 39.5), but *Salmonella* could not be detected by culture. The  
247 salami tested were 140 in total and were manufactured by three producers out of four (A, B and D)

248 (Table 1).

249 Overall, 55 out of 140 salami (39 %) were positive by real-time PCR. The statistical analysis  
250 showed that the proportion of culture-confirmed samples in PCR-positive salami significantly  
251 decreased with the increase of CT values obtained in PCR both for aliquots of 25 g (p-value = <  
252 0.001) and 50 g (p-value = < 0.001) (Figure 1b). Figure 1b showed that, unlike GRM samples, the  
253 proportion of culture-confirmed samples in PCR-positive salami was similar with sampling aliquots  
254 of 50 g and 25 g also for low PCR CT-values.

255 The ISO-25 g method detected *Salmonella* in 11/15 (73%) of all culture-positive salami of the study  
256 while ISO-50 g reached 87% (13/15). The difference between the two procedures was not  
257 statistically significant (Fisher exact's test, p-value > 0.05). Two samples out of 15 (13 %) were  
258 negative with both ISO-50 g and ISO-25 g, but positive by MPN enumeration.

259 The enumeration of *Salmonella* ranged from 8.7 MPN/g to 1.3 MPN/g in seven samples, and was <  
260 1.3 MPN/g in the remaining eight. *Salmonella* isolates detected in the 15 positive salami were 16, as  
261 one sample was contaminated by two different serovars. Sero- and PFGE typing results are shown  
262 in Table 3.

263 Five *Salmonella* serovars were identified and Derby was the most prevalent (9/16; 56 %), followed  
264 by London (2/16; 13 %), Panama (2/12; 13 %), Branderup (2/16; 13 %) and Goldcoast (1/16; 6 %)  
265 Four PFGE profiles were identified among *S. Derby* isolates (D1, D4, D7, D8) and one of them  
266 (D4) was found in the corresponding GRM sample. Two PFGE profiles were found for *S.*  
267 Branderup (B1, B2). One PFGE type was identified for *S. London*, identical to the genotype  
268 detected in the corresponding GRM sample. PFGE analysis of *S. Panama* and *S. Goldcoast* could  
269 not identify a genomic profile, because of self-degrading DNA (Table 3).

270

### 271 3.3 Physicochemical parameters: pH, $a_w$ and NaCl content

272 The pH values of salami prepared from culture-positive GRMs ranged from 5.30 to 6.48 (average  
273 5.94). The pH values of the *Salmonella*-positive salami ranged from 5.56 to 6.29 (average 5.91).  
274 There were not observed pH differences between the samples with the highest *Salmonella* levels  
275 (8.7 MPN/g, 2.7 MPN/g and 1.4 MPN/g) and the lowest (1.3 MPN/g and < 1.3 MPN/g) (Table 3).  
276 The water activity ( $a_w$ ) values of salami ranged from 0.822 to 0.951 (average 0.903). The  $a_w$  values  
277 of the *Salmonella*-positive salami ranged from 0.896 to 0.951 (average 0.919). The NaCl content of  
278 cured salami ranged from 2.25% to 4.80% (average 3.67%). The NaCl content of the *Salmonella*-  
279 positive salami ranged from 2.86% to 4.28% (average 3.50%). The physicochemical parameters of  
280 *Salmonella*-positive salami are shown in Table 3.

281 Statistical analysis through GLMM of factors affecting the occurrence of *Salmonella* in salami  
282 revealed that water activity ( $a_w$ ) and *Salmonella* count (MPN) in GRM used for the salami  
283 production were included in the best model from forward stepwise selection (Table 4), while values  
284 of pH and NaCl content did not significantly affect the probability to find *Salmonella* in salami  
285 samples. Analyses through linear models showed that the pH values in salami were not significantly  
286 affected by the curing time (slope = 0.0003, p-value > 0.05), while the values of  $a_w$  (slope = 0.001,  
287 p-value < 0.001) and NaCl (slope = 0.01, p-value < 0.001) were negatively and positively affected  
288 by the curing time, respectively.

289

## 290 4. Discussion

291 In Italy the proportion of human cases of salmonellosis attributed to pork is high compared with the  
292 other European Union Member States, being 73.2% (95% Credibility Interval 71.0 – 75.4) in 2009-  
293 2011, vs. 2.3% attributed to broilers, 2.1% to laying hens and 5.3% to turkeys. Only in Belgium the  
294 proportion was similar to Italy (74%), while in the other reporting countries it ranged from 4.9%

295 (Finland and Sweden) to 53% (Cyprus) (Pires *et al.*, 2011). Another study attributed to pork 59.9%  
296 to 53.9% of the human cases reported in Italy, whereas poultry meat and table eggs were considered  
297 less important *Salmonella* sources, responsible for 24.4% to 31.0% and for 15.1% to 15.7% of the  
298 cases, respectively (de Knecht *et al.*, 2015). Survival of *Salmonella* in dry-cured pork salami can  
299 contribute to the exposure of the Italian consumer and the results of our study are consistent with  
300 this hypothesis.

301

#### 302 4.1 Presence of *Salmonella* in GRM samples and dry-cured salami

303 *Salmonella* was isolated from 14 % of the GRM batches and a significant relationship was shown  
304 between initial *Salmonella* load of GRMs and presence of the pathogen in salami at the end of  
305 curing. These findings underline that hygienic conditions of raw materials are of great importance  
306 for microbiological risk mitigation and that curing does not always ensure safe productions.

307 A limited proportion (ranging from 27% for salami to 37% for GRMs) of PCR-positive samples  
308 were culture-confirmed. Among the possible explanations could be the high sensitivity of real-time  
309 PCR that has been demonstrated by several studies. In this respect, Rodriguez-Lazaro *et al.* (2014)  
310 observed that a real-time PCR protocol was able to detect down to 2-4 *Salmonella* CFU in 25 g of  
311 different samples, including raw pork. That protocol was validated by Delibato *et al.* (2014),  
312 showing that it was an excellent alternative to the ISO 6579:2002 standard with a limit of detection  
313 down to 10 CFU per 25 g.

314 Other possible explanations could be the ability of PCR to amplify DNA from dead bacteria  
315 (Barbau-Piednoir *et al.*, 2014; Li *et al.*, 2013; Wolffs *et al.*, 2005), as could be the case in cured  
316 salami, and the overgrowth of commensal flora in culture media, inhibiting or masking *Salmonella*  
317 colonies in presence of low *Salmonella* loads, like those observed in this study.

318 As regards the size of sampling aliquots for microbiological testing, the increased probability of

319 confirming PCR-positive GRMs starting from 50 g instead of 25 g shows how critical the sample  
320 size can be in low-contamination samples (Figure 1a). On the other hand, the probability of  
321 confirming PCR-positive samples in cured salami was similar starting from 25 g and 50 g (Figure  
322 1b). The different effect of sample size on the probability of confirmation observed between GRMs  
323 and salami could be hypothetically due to a more homogeneous distribution of the viable pathogen  
324 in salami compared to GRMs as a possible consequence of bacterial diffusion in the first hours of  
325 salami curing when bacteria can swarm in the food matrix. transition from GRM to salami.  
326 Although Regulation (EC) 2073/2005 defines the food safety criterion for *Salmonella* in minced  
327 salami meat and (food category 1.8 - meat products intended to be eaten raw), as “absence in 25 g  
328 in five sampling units”. Our ~~our~~ results highlight that a larger sample sampling size unit could be  
329 reconsidered- for higher sensitivity at unit level. to improve food safety. For example, this could  
330 increase the confirmation rate of a screening PCR when the CT values are high. *Salmonella*  
331 regulations setting larger sample sizes exist outside Europe, as is the case of USA standards  
332 (USDA/FSIS, 2014), which require a sample portion of  $325 \pm 6$  g for *Salmonella* detection in raw  
333 meat and ready to eat foods. In particular, a larger sample size should be considered when the CT  
334 values of a screening PCR are high.

335

#### 336 4.2 *Salmonella* serovars in GRMs and salami

337 Most samples of GRMs were contaminated by *S. Derby* and *S. Typhimurium* monophasic variant  
338 (antigenic formula 4,[5],12:i: -) and *S. Derby* was also prevalent in dry-cured salami. This result is  
339 consistent with the host-adapted nature of *S. Derby* known to be associated with pigs (Uzzau et al.,  
340 2000) and most common in Italian slaughter pigs (Bonardi et al., 2003; 2016; Piras et al., 2011). At  
341 the same time, this serotype is not devoid of pathogenic potential for humans, as foodborne  
342 infections by *S. Derby* have been recently reported in Spain (Armedo-Pena et al., 2016), Germany  
343 (Frank et al., 2014), France (Kerouanton et al., 2013) and Italy (Enternet Reference Laboratory of

Emilia-Romagna - surveillance data). *S. Typhimurium* monophasic variant also has been increasingly reported in both pig populations and humans affected by salmonellosis in several European countries (Argüello et al., 2014; Bonardi et al., 2016; EFSA and ECDC, 2016; Gossner et al., 2012) ~~and it~~ and it was responsible for 46% of the human salmonellosis cases notified in Emilia-Romagna region in 2015 (Enternet Reference Laboratory of Emilia-Romagna - surveillance data).

Serotyping and PFGE of isolates showed a variety of strains inside positive batches of cured salami sometimes confirming the presence of the types detected in the corresponding GRMs and sometimes not. This could be mostly the effect of typing limited numbers of colonies, normally ranging from two to three per sample. A larger variety of serovars was detected in cured salami than in GRMs. The larger sample portion tested for each salami batch (375 g; i.e. 25 g + 50 g times 5 sampling units) than for GRMs (75 g; i.e. 25 g + 50 g) could explain this difference. At the same time, the presence of mixed populations of *Salmonella* in GRMs and their final products is suggestive of a diverse and complex origin of the contamination, which could have been originated from raw meat as well as from other ingredients, equipment and workers. For instance, pepper added to GRMs has been demonstrated as a source of contamination in different outbreaks (Gieraltowski et al., 2013; Jernberg et al., 2015). In addition, the role of the environment as contamination source for pig meat was reported both in pig slaughterhouses (Andreoli et al., 2017; Gomes-Neves et al., 2012;) and in salami producing plants (Andreoli et al., 2017).

362

#### 4.3 Influence of physicochemical parameters

Reduction of GRM contamination by *Salmonella* should be seen as a multicomponent action along the pork production chain, including lowering of infection prevalence in swine population and adoption of proper hygiene procedures during slaughter and meat processing. In our study, The the  
aw lowering was the only factor able statistically correlated to the reduction of to reduce Salmonella



**persistence** in cured salami, and this effect was related to the curing time. Nevertheless, standard curing, as applied by the producers involved in the study, did not satisfactorily mitigate the risk of *Salmonella* being present in the end product, as about 30% of the batches and 13% of the salami produced from contaminated GRMs were still positive after curing. Conversely, additional curing was effective in reducing *Salmonella* survival to below 1%.

*Salmonella*-negative salami at the end of curing confirms that this parameter is critical for the safety of dry-cured products (Lucke, 2000). Regarding *Salmonella* inactivation because of  $a_w$  lowering, our data show a positive correlation between  $a_w$  reduction and *Salmonella*-negative results in contaminated batches of salami. Comparing our results to other studies on dry fermented Italian style salami, we found the lowest  $a_w$  value (0.986) ever reported in *Salmonella*-positive salami. Other studies on Cacciatore, Milano and Felino salami reported  $a_w$  reduction values not lower than 0.930-0.940 in salami at the end of curing (Mataragas et al., 2015a; 2015b) and observed that *Salmonella* inactivation in these products was a multifactorial process influenced by pH and  $a_w$  lowering, together with fermentation temperature. „Those studies were performed inoculating *Salmonella* (ranging from  $10^4$  –  $10^5$  CFU/g to  $10^5$  –  $10^6$  CFU/g.) and monitoring its progression in the final products (Mataragas et al., 2015a, 2015b). On the contrary, we tested natural contaminated salami and followed *Salmonella* decrease during curing up to negativity of the samples, regardless of the standard curing time proposed by the different producers. „Our results highlight that *Salmonella* was still not inactivated at very low  $a_w$  values (0.896-0.926) and this finding is important for safety assurance of fermented products. Rispetto all'inattivazione di *Salmonella* conseguente

all'abbassamento dell'aw. I nostri risultati indicano una correlazione tra l'abbassamento dell'aw e la negativizzazione dei salami. Comunque abbiamo trovato salami positive fino a 0,896, più basso di valori considerati in altri studi su salami italiani (dry fermented Italian style salami). Il limite di inattivazione di *S.* conseguente all'abbassamento dell'aw è certamente inferiore a 0,896. Come esiste dai due Mataragas sul controllo di salmonella occorre considerare l'interazione tra diversi fattori. Sottolineare che i livelli

393 ~~decontaminazione rano quelli naturali!!~~ The influence of  $a_w$  on the probability to obtain *Salmonella*  
394 ~~negative salami at the end of curing confirms that this parameter is critical for the safety of dry-~~  
395 ~~cured products (Lucke, 2000). Water activity threshold values for Italian dry-cured salami have~~  
396 ~~been reported, indicating that *Salmonella* should be inhibited below 0.94 (Barbuti et al., 1993).~~  
397 ~~Nevertheless, our results show that a large proportion (80%) of *Salmonella* positive salami had~~  
398 ~~values lower than 0.94 (0.896-0.926). Consistent with our results, a study on Italian Cacciatore and~~  
399 ~~Felino salami reported that the drop of  $a_w$  to 0.928-0.936 values during curing did not affect~~  
400 ~~*Salmonella* survival in the final products (Mataragas et al., 2015). Another study on dry fermented~~  
401 ~~sausages proposed  $a_w$  target values  $\leq 0.90$  to inhibit *Salmonella* (Lucke, 2000), which seem difficult~~  
402 ~~to reach in traditional Italian pork salami because of technological constraints.~~

403 The influence of curing duration on *Salmonella* survival is apparent in our study as is its reverse  
404 correlation to  $a_w$ , the only physicochemical parameter showing influence on *Salmonella* survival. In  
405 particular, extending curing by additional 21-38 days after the standard period of 20-48 days, the  
406 prevalence of positive salami dropped from 13.3% to 3.3%. All initially positive batches turned out  
407 negative after an overall duration of curing varying between 49 and 86 days. Therefore, reduced  
408 curing time appears to be one of the main risk factors in salami production, compromising the  
409 inhibition of *Salmonella*. For this reason, curing duration should be clearly considered by  
410 manufacturers of traditional dry-cured salami as a critical safety factor.

411 In our study, NaCl and pH did not appear to influence the probability to obtain contaminated salami  
412 at the end of curing in the context of our study. This could be due to values of those parameters far  
413 from inhibiting levels for *Salmonella*. In this respect, Barbuti et al. (1993) reported an inhibitory  
414 level for *Salmonella* of 6% NaCl, far above the highest value observed in a positive salami in our  
415 study (i.e. 4.28%).

416 Different experimental studies on Italian pork salami were carried out, where the production

417 conditions, ingredients and additives concentration were kept under control, highlighting the role of  
418 starter culture, temperature, relative humidity, pH and  $a_w$  on *Salmonella* survival (Barbuti and  
419 Parolari, 2002; Mataragas et al., 2015; Pisacane et al., 2015;). On the contrary, our study  
420 investigated *Salmonella* contamination of salami in field conditions, consequently many processing  
421 details (such as temperature and relative humidity) and the concentration of preservatives (nitrites,  
422 nitrates, ascorbate) were not communicated by the manufactures. Unlike experimental studies, field  
423 studies cannot control ~~the majority of~~most of processing conditions, but they have the advantage of  
424 referring to the products actually placed on the market, therefore representing the real consumer  
425 exposure to *Salmonella*.

426

## 427 5. Conclusions

428 Although different pork products or infection routes could be involved in the transmission of  
429 *Salmonella* from pigs to humans, our results highlight that production of traditional salami does not  
430 always ensures safe cured products. This appears to be linked to excessively short curing, as  
431 currently applied by the industry. This factor, associated with the considerable proportion of  
432 contaminated meat mixtures used as starting raw material in salami processing translates into a non-  
433 negligible risk to the consumers. Not surprisingly, we found that the probability of putting  
434 contaminated products on the market was correlated to the contamination level of the raw material,  
435 asking for more efforts to reduce the starting contamination. Therefore, the selection hygiene level  
436 of raw meat is critical for safe production of ready-to-eat fermented products, together with the  
437 improvement and maintenance of appropriate Good Hygienic Practices and Good Manufacturing  
438 Practices to reduce environmental contamination.

439 Among the physicochemical parameters considered, in the study conditions, only  $a_w$  showed an  
440 effect in reducing the probability of contamination at the end of curing, suggesting that production

441 of soft salami characterized by high  $a_w$  values appears to negatively impact their microbiological  
442 safety. Nevertheless, the addition of preservatives, such as nitrites and nitrates (maximum level 150  
443 ppm each according to EU Regulation 1129/2011), may contribute to control *Salmonella* growth in  
444 dried fermented sausages even when pH and  $a_w$  values are permissive (Hospital et al., 2014).  
445 Finally, the observed increased probability of detecting *Salmonella* by ISO 6579:2002 starting from  
446 larger samples of GRMs (50g of sample vs. 25g) should be carefully considered in its potential  
447 impact on food safety outcomes of both industry and competent authorities through their own-  
448 checks and official controls, respectively.

449

#### 450 **Acknowledgements**

451 The authors gratefully acknowledge Dott. Gisella Pizzin and Mrs. Ida Poli of the University of  
452 Parma for their technical support and R-Biopharm for real-time PCR equipment.

**Figure 1**

Probability of culture-confirmation of real time PCR positive samples as a function of the CT-value of PCR for GRM (panel A) and cured salami (panel B). Blue and red lines represent the probability of culture-confirmation for sampling aliquots of 25 g and 50 g, respectively. Solid lines represent the best model fits; dashed lines represent the 95% confidence intervals. Blue and red dots represent the occurrence (top) or not (bottom) of a culture-confirmation for sampling aliquots of 25 g and 50 g, respectively.

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