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

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ABSTRACT

The inactivation of *Salmonella* during curing of Italian traditional pork salami was investigated. A total of 150 batches of ground raw meat (GRM) used for salami manufacturing by four producers were tested for *Salmonella* by real-time PCR followed by ISO 6579 cultural confirmation and MPN enumeration. Salami produced with *Salmonella* positive GRMs were re-tested at the end of their curing period. Aw, pH and NaCl content were also measured. Detection of *Salmonella* was performed testing both 25 and 50 g of the samples.

By Real-Time PCR 37% of the GRMs resulted positive, but cultural detection of *Salmonella* was obtained in 14% of the samples only. *Salmonella* enumeration ranged from 31 MPN/g to < 1.3 MPN/g. The difference between testing 50 g and 25 g of the samples was statistically significant (p value ≤ 0.01). In particular, ISO-50 g detected *Salmonella* in 100% of all positive samples, *vs.* 62% of ISO-25 g. Salami made of the contaminated GRMs were 29% *Salmonella*-positive, as most batches of salami produced with *Salmonella*-positive GRMs resulted negative after regular curing (20–48 days). Overall, 13% of salami produced with *Salmonella*-contaminated GRMs were positive. They belonged to six batches, which turned out negative after prolonged curing ranging between 49 and 86 days. *Salmonella* enumeration in salami ranged from 8.7 MPN/g to < 1.3 MPN/g. Unlike GRMs, no significant difference was observed between the ISO-50 g and the ISO-25 g in detecting *Salmonella* in cured salami (p value: > 0.05).

The most common *Salmonella* serovars in GRMs were Derby (52%), Typhimurium monophasic variant 4, (Barbuti et al., 1993), 12:i:- (19%) and Stanley (10%). *Salmonella* Derby (56%), London, Branderup, Panama (13%, respectively) and Goldcoast (6%) were most frequent in cured salami. The study showed negative correlation between real-time CT values and cultural confirmation of *Salmonella*, as well as the importance of sample size for *Salmonella* detection. Among considered factors with possible effect on the occurrence of *Salmonella* in salami, statistical analysis revealed a role for aw in salami and for *Salmonella* load in GRMs, while pH and NaCl content did not significantly affect the probability of finding *Salmonella* in dry-cured salami in the context of this study. In particular the lower aw values due to longer curing were associated with lower *Salmonella* presence in traditional dry-cured salami.

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 and 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

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(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., 2015a, 2015b).

Salmonella ranks second among pathogens reported in the European Union (EU) as causative agents of human 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 ECDC, 2015). In Italy, consumption of salami was recently associated with both clustered and sporadic cases of salmonellosis due to S. Goaldcost (Scavia et al., 2013), *S.* Manhattan (Scaltriti et al., 2015), *S.* Typhimurium and *S.* Typhimurium monophasic variant (Andreoli et al., 2017; Luzzi et al., 2007). Other EU countries confirmed *Salmonella* outbreaks linked to salami, like Sweden in 2005, Norway in 2006 and Denmark in 2010 (Emberland et al., 2006; Hjertqvist et al., 2006; Kuhn et al., 2011).

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

In the EU, microbiological food-safety criteria are set by the Regulation EC 2073/2005 (European Commission, 2005), which identifies culture-based ISO methods as the analytical reference methods. ISO 6579 is the standard for *Salmonella* detection in foods. This method relies on several cultural steps and requires > 5 days for conclusive results in case of positive samples. This is why, to meet the needs of the food industry, the same Regulation allows the use of alternative methods, generally more convenient and faster, under specified conditions. PCR-based methods are among available alternatives and several studies have been performed to assess real-time PCR protocols for the rapid and sensitive detection of *Salmonella* in foods in < 24 h (Delibato et al., 2014; Rodriguez-Lazaro et al., 2014).

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

2. Material and methods

2.1. Sample collection

From April to December 2015, 150 samples of ground raw mixtures (GRM) made of ground pork and fat, collected from 150 different batches of starting material for salami processing, were tested for *Salmonella.* Only pig meat and pig backfat were used for the salami manufacturing. The samples were collected in four production plants, here identified as A (47 samples), B (23 samples), C (11 samples), D (69 samples) located in Emilia-Romagna region, Northern Italy. The plants were included in the study based on their willingness to take part to the study (five were asked to participate) and the different number of samples collected from each plant was proportional to its production capacity. Meat and fat suppliers of the four plants were many and, often, meat and fat from more than one supplier were mixed in the same batch of GRM. In our study the GRM samples of the four companies were collected before addition of other ingredients and additives (salt, nitrites, nitrates, ascorbates, black pepper) to avoid potential interference with *Salmonella* detection. Nitrates and nitrates were added in compliance with the limits set by Regulation EU No 1129/2011 on food additives. Curing was performed at temperatures below 20 °C, specifically in the range of 12–18 °C.

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

2.2. Salmonella detection in ground raw mixture (GRM) and salami

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

Table 1
Salami samples culture-positive for *Salmonella* at different curing time.

Producer	Batch No. of *Salmonella*-positive salami	MPN/g in GRM samples	Days of curing before testing		
			First curing period (no. of positive sampling units/5)	Second curing period[a] (no. of positive sampling units/5)	Third curing period[b] (no. of positive sampling units/5)
A	15	31	33 (3/5)	54 (1/5)	62 (0/5)
D	25	< 1.3	34 (0/5)		
D	26	< 1.3	34 (0/5)		
A	47	4.1	47 (0/5)		
A	50	< 1.3	42 (0/5)		
A	57	< 1.3	40 (0/5)		
A	59	< 1.3	40 (0/5)		
D	67	< 1.3	34 (0/5)		
A	73	< 1.3	40 (0/5)		
A	78	12	36 (1/5)	86 (0/5)	
A	79	29	36 (2/5)	86 (0/5)	
B	82	< 1.3	28 (0/5)		
D	84	< 1.3	38 (0/5)		
B	96	< 1.3	21 (4/5)	54 (0/5)	
D	98	< 1.3	47 (0/5)		
D	107	< 1.3	44 (0/5)		
C	112	< 1.3	49 (0/5)		
B	121	< 1.3	20 (3/5)	49 (0/5)	
D	140	< 1.3	40 (1/5)	54 (0/5)	
D	143	< 1.3	43 (0/5)		
A	147	< 1.3	28 (0/5)		
No of positive salami in the different curing periods (%)			14/105 (13%)	1/30 (3%)	0/5 (0%)
No of positive salami of the study (%)			15/140 (11%)		

[a] It includes the first curing period.
[b] It includes the first and the second curing period.

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 *Xba*I (Roche Italia, Milan, Italy) restriction of DNA.

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

2.5. Measure of pH, a_w and NaCl content

The pH value was measured on 5 g of salami homogenized in distilled water (10/1 water/sample, w/w) with a Crison micro pH 2001 instrument equipped with a Xerolyt 52-02 electrode (Crison Instruments, Barcelona, Spain). The a_W value was measured at 25 °C by an AquaLab series 4TE a_W meter (Decagon Devices, Inc., Pullman 99,163, WA).

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

2.6. Statistical analyses

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

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

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

Salmonella ~ pH + aw + NaCl + MPN + (1 | *plant*).

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

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

Table 2
Comparison of positive samples by real-time PCR and confirmed positive samples by cultural-based methods.

Type of samples	N. of real-time PCR-positive samples (%)	N. of PCR-positive samples confirmed by cultural methods (%)	N. of PCR-positive samples confirmed by testing 25 g (%)	N. of PCR-positive samples confirmed by testing 50 g (%)	N. of culture-confirmed samples (%)
GRM[a]	56/150 (39%)	21/56 (38%)	13/21 (62%)	21/21 (100%)	21/150 (14%)
Cured salami	55/140 (39%)	15/55 (27%)	11/15 (73%)	13/15 (87%)	15/140 (11%)

[a] GRM (ground raw mixture).

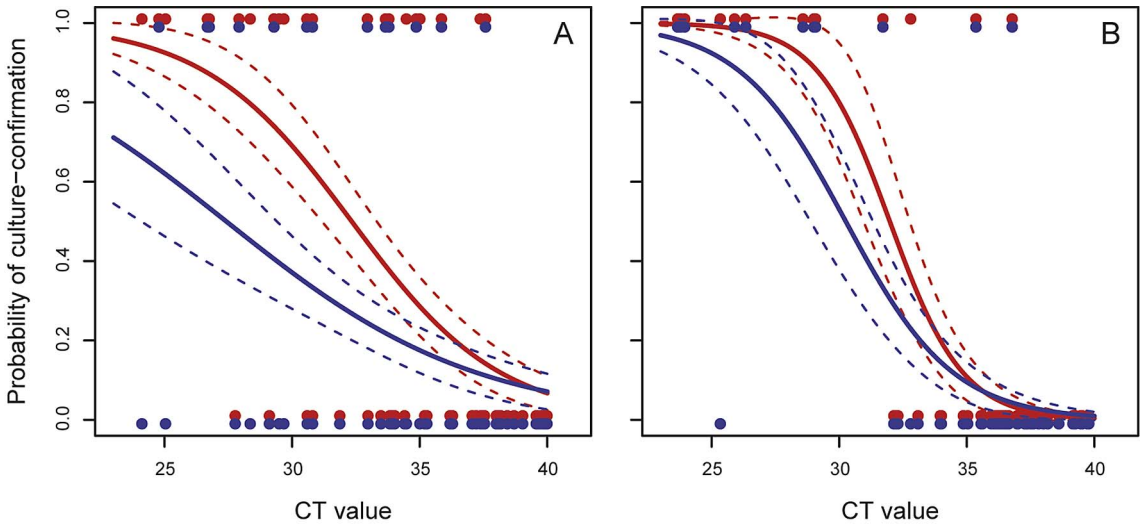

Fig. 1. Probability of culture-confirmation of real time PCR positive samples as a function of the CT-value of PCR for GRM (panel A) and cured salami (panel B). Blue and red lines represent the probability of culture-confirmation for sampling aliquots of 25 g and 50 g, respectively. Solid lines represent 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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) (Fig. 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 (Fig. 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 serovars, *i.e.* six among *S.* Derby isolates (D1-D6) and four among the monophasic variant of *S. enterica*

Table 3
Salmonella serovars, pulsotypes and MPN enumeration of the ground raw mixture (GRM) samples and the positive dry-cured salami thereof. Other GRMs were found to be positive for *Salmonella* but are not shown in this table, which refers only to the raw materials that ended up as contaminated salami. All positive salami of the study are shown in this table. Physico-chemical parameters of *Salmonella*-positive salami are shown.

GRM		Salami					
Batch no (producer's code)	*Salmonella* serovar, PFGE type (MPN values)	Batch n°/sampling unit	*Salmonella* serovar, PFGE type	MPN values	pH	a_w	NaCl %
15 (A)	London, L1 (31/g)	15/1	Goldcoast	8.7 MPN/g	6.25	0.914	4.21
		15/2	Derby, D8	1.4 MPN/g	6.01	0.916	4.28
		15/3	London, L1	< 1.3 MPN/g	6.19	0.918	3.66
		15 II/1	London, L1	< 1.3 MPN/g	6.29	0.896	3.89
78 (A)	Stanley, S1 (12/g)	78/4	Panama	< 1.3 MPN/g	5.98	0.902	3.72
79 (A)	Stanley, S1 (29/g)	79/3	Panama Branderup, B1	8.7 MPN/g	5.85	0.904	3.35
		79/4	Branderup, B2	1.3 MPN/g	5.99	0.902	3.31
96 (B)	Derby, D4 (< 1.3/g)	96/1	Derby, D4	< 1.3 MPN/g	5.78	0.946	2.86
		96/3	Derby, D4	< 1.3 MPN/g	6.03	0.926	3.64
		96/4	Derby, D4	1.3 MPN/g	5.84	0.948	2.94
		96/5	Derby, D4	1.3 MPN/g	5.63	0.951	2.98
121 (B)	Derby, D6 (< 1.3/g)	121/1	Derby, D4	< 1.3 MPN/g	5.93	0.920	3.44
		121/4	Derby, D7	< 1.3 MPN/g	5.79	0.908	3.47
		121/5	Derby, D7	< 1.3 MPN/g	5.56	0.920	3.49
140 (D)	Goettingen, G1 (< 1.3/g)	140/2	Derby, D1	2.7 MPN/g	5.59	0.918	3.24

Table 4
Forward stepwise model selection for *Salmonella* occurrence in salami obtained from a GLMM with binomial error distribution. Models were compared using log-likelihood ratio test. The best models for *n* fixed effects are shown, with the log-likelihood (loglik), number of degree of freedom (Df) and the p-value of the comparison with the *n*-1 variables best model (p-value tests: < 0.05 as the inclusion criterion and > 0.10 as the exclusion criterion). Full model: *Salmonella* ~ pH + aw + NaCl + MPN + (1 | *plant*).

Best *n* fixed effects models	loglik	Df	p-Value
~1[a] + (1 | *plant*)	− 48.19	2	–
~a_w + (1 | *plant*)	− 43.20	3	0.0016
~a_w + MPN[b] + (1 | *plant*)	− 34.50	4	$3 * 10^{-5}$
~a_w + MPN + pH + (1 | *plant*)	− 33.12	5	0.096

[a] Null model.
[b] Enumeration of samples below the lowest countable: 1.3/sqrt(2) MPN/g.

4,[5],12:i:- (MT1-MT4). One PFGE type was identified for *S.* Stanley (S1) (Table 3).

3.2. Detection and enumeration of Salmonella in cured salami

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

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

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

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

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

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

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

3.3. Physicochemical parameters: pH, a_w and NaCl content

The pH values of salami prepared from culture-positive GRMs ranged from 5.30 to 6.48 (average 5.94). The pH values of the *Salmonella*-positive salami ranged from 5.56 to 6.29 (average 5.91). There were not observed pH differences between the samples with the highest *Salmonella* levels (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). The water activity (a_w) values of salami ranged from 0.822 to 0.951 (average 0.903). The a_w values of the *Salmonella*-positive salami ranged from 0.896 to 0.951 (average 0.919). The NaCl content of cured salami ranged from 2.25% to 4.80% (average 3.67%). The NaCl content of the *Salmonella*-positive salami ranged from 2.86% to 4.28% (average 3.50%). The physicochemical parameters of *Salmonella*-positive salami are shown in Table 3.

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

4. Discussion

In Italy the proportion of human cases of salmonellosis attributed to pork is high compared with the other European Union Member States, being 73.2% (95% Credibility Interval 71.0–75.4) in 2009–2011, *vs.* 2.3% attributed to broilers, 2.1% to laying hens and 5.3% to turkeys. Only in Belgium the proportion was similar to Italy (74%), while in the other reporting countries it ranged from 4.9% (Finland and Sweden) to 53% (Cyprus) (Pires et al., 2011). Another study attributed to pork 59.9% to 53.9% of the human cases reported in Italy, whereas poultry meat and table eggs were considered less important *Salmonella* sources, responsible for 24.4% to 31.0% and for 15.1% to 15.7% of the cases, respectively (de Knegt et al., 2015). Survival of *Salmonella* in dry-cured pork salami can contribute to the exposure of the Italian consumer and the results of our study are consistent with this hypothesis.

4.1. Presence of Salmonella in GRM samples and dry-cured salami

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

A limited proportion (ranging from 27% for salami to 37% for GRMs) of PCR-positive samples were culture-confirmed. Among the possible explanations could be the high sensitivity of real-time PCR that

has been demonstrated by several studies. In this respect, Rodriguez-Lazaro et al. (2014) observed that a real-time PCR protocol was able to detect down to 2–4 *Salmonella* CFU in 25 g of different samples, including raw pork. That protocol was validated by Delibato et al. (2014), showing that it was an excellent alternative to the ISO 6579:2002 standard with a limit of detection down to 10 CFU per 25 g.

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

As regards the size of sampling aliquots for microbiological testing, the increased probability of confirming PCR-positive GRMs starting from 50 g instead of 25 g shows how critical the sample size can be in low-contamination samples (Fig. 1a). On the other hand, the probability of confirming PCR-positive samples in cured salami was similar starting from 25 g and 50 g (Fig. 1b). The difference between GRMs and salami as regards the effect of sample size on the probability of confirmation could hypothetically be due to a more homogeneous distribution of the viable pathogen in salami compared to GRMs as a possible consequence of bacterial diffusion in the first hours of salami curing when mobile bacteria can swarm in the food matrix. Regulation (EC) 2073/2005 defines the food safety criterion for *Salmonella* in meat products intended to be eaten raw as “absence in 25 g in five sampling units”. Our results highlight that a larger sampling unit could be considered for higher sensitivity at unit level. For example, this could increase the confirmation rate of a screening PCR when the CT values are high. *Salmonella* regulations setting larger sample sizes exist outside Europe, as is the case of USA standards (USDA/FSIS, 2014), which require a sample portion of 325 ± 6 g for *Salmonella* detection in raw meat and ready to eat foods. In particular, a larger sample size should be considered when the CT values of a screening PCR are high.

4.2. *Salmonella* serovars in GRMs and salami

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

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 a_w lowering was the only factor statistically related to the reduction of *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%.

The influence of a_w on the probability to obtain *Salmonella*-negative salami at the end of curing indicates that this parameter is critical for the safety of this type of dry-cured products fermented at temperature below 20 °C, as is the case of our study, whereas pH did not appear to influence the probability to obtain contaminated salami at the end of curing in the context of our study. Previous studies by Drosinos et al. (2006) and Gounadaki et al. (2007) showed that reduction of a_w was associated with decline of *Listeria monocytogenes* in dry-cured sausages fermented at low temperature (below 20 °C), while pH reduction during early fermentation had no effect in the same temperature conditions. Conversely, according to the same studies, pH effect on *Listeria monocytogenes* reduction was significant at higher fermentation temperature. A recent study on *Salmonella* in Italian salami, fermented at low temperature (Mataragas et al., 2015a), evidenced a rapid decrease of pH associated with a small change of a_w and a limited decline of *Salmonella*. These authors hypothesized that the observed limited decline could be explained with the dominant effect of a_w at low fermentation temperature as previously described for *Listeria monocytogenes*. Our results on *Salmonella* decline in field conditions are consistent with these studies as far as the effect of a_w and pH are concerned.

Another study on dry fermented sausages proposed $a_w \leq 0.90$ in the final product as a target value for *Salmonella* control (Lucke, 2000). The lowest value in a positive salami of our study was 0.896 and none of the salami produced from contaminated GRMs and having a_w lower than 0.896 was positive (28 salami). These results are in line with the proposed threshold of 0.90. Nevertheless, such threshold seems difficult to reach in traditional Italian pork salami because of market constraints that require soft products (Mataragas et al., 2015b). The influence of curing duration on *Salmonella* survival is apparent in our study as is its reverse correlation to a_w, the only physicochemical parameter showing influence on *Salmonella* survival. In particular, extending curing by additional 21–38 days after the standard period of 20–48 days, the prevalence of positive salami dropped from 13.3% to 3.3%. All initially positive batches turned out negative after an overall duration of curing varying between 49 and 86 days. Therefore, reduced curing time appears to be one of the main risk factors in salami production, compromising the inhibition of *Salmonella*. For this reason, curing duration should be clearly considered by manufacturers of traditional dry-cured salami as a critical safety factor.

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

Different experimental studies on Italian pork salami were carried

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

5. Conclusions

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

Among the physicochemical parameters considered, in the study conditions, only a_w showed an effect in reducing the probability of contamination at the end of curing, suggesting that production of soft salami characterized by high a_w values appears to negatively impact their microbiological safety. Nevertheless, the addition of preservatives, such as nitrites and nitrates (maximum level 150 ppm each according to EU Regulation 1129/2011), may contribute to control *Salmonella* growth in dried fermented sausages even when pH and a_w values are permissive (Hospital et al., 2014). Finally, the observed increased probability of detecting *Salmonella* by ISO 6579:2002 starting from larger samples of GRMs (50 g of sample *vs.* 25 g) should be carefully considered in its potential impact on microbiological verification procedures performed by food producers on food safety outcomes of both industry and competent authorities through their own-checks and official controls, respectively.

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