

Occurrence and traceability of *Salmonella* spp. in five Sardinian fermented sausage facilities

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Abstract

The aims of the present study were to evaluate the presence of *Salmonella* in five fermented sausage processing plants and their products during the production process, and to trace the possible sources of contamination. A total of 270 samples were collected: mixture of ground pork meat and fat, products at the end of acidification, sausages at the end of ripening and, during production stages, surfaces in contact with meat and surfaces not in contact with meat. For samples of ground meat, product at the end of acidification and sausages at the end of ripening, the pH and water activity (a_w), were determined. All the samples were tested for the presence of *Salmonella*. Thirty-two *Salmonella* isolates were obtained, subjected to serotyping and PFGE. The sausages at the end of ripening pH and a_w mean values were 5.39 ± 0.24 and 0.91 ± 0.03 , respectively. *Salmonella* was detected in three processing plants with an overall prevalence of 16.7% in food samples and 5.8% in environmental samples. *Salmonella* prevalence was 24% in ground meat and products at the end of acidification and was also detected in a sample of sausage at the end of ripening (2%). In environmental samples, *Salmonella* was detected in 6.6% of surfaces in contact with meat and 5% of surfaces not in contact with meat. Five serotypes were identified among 32 isolates: *S. Derby* (37.5%), *S. Typhimurium* and *S. Rissen* (both 25%), *S. Give* and monophasic *S. Typhimurium* (both 6.25%). Six different pulsotypes were obtained with PFGE. The serotypes and the PFGE pattern of the strains were specific for each facility with no overlapping between different processing plants. The same observation can be pointed out considering different sampling days for the same processing plants, thus presumably indicating the raw material (ground pork meat and fat) as the source of contamination. The detection of *Salmonella* in a sample of sausage at the end of ripening highlights the ability of the pathogen to survive during manufacturing

process.

Introduction

Salmonella is a zoonotic pathogen present in the intestinal tract of a wide range of domestic and wild animals which may be recovered in a variety of foodstuffs, of both animal and plant origin, as consequence of either direct or indirect contamination. In 2016, 94,530 confirmed cases of human salmonellosis were reported in the European Union (EFSA and ECDC, 2017). Although a statistically significant decreasing trend of salmonellosis has been observed between 2008 and 2016, in recent years (2012-2016) the trend has stabilized and the number of reported cases and EU notification has slightly increased. Among the different food categories, a high proportion of *Salmonella*-positive units were reported for meat products intended to be eaten raw posing a serious risk for human health (EFSA and ECDC, 2017). In 2014, pork meat and products thereof were responsible for 9.3% of foodborne outbreaks caused by *Salmonella*, representing the type of meat most frequently associated with salmonellosis in humans (EFSA and ECDC, 2015). In Southern Europe countries pigs account for 43.6% of all salmonellosis cases (Pires *et al.*, 2011).

Ready-to-eat (RTE) pork meat products have been linked to several *Salmonella* outbreaks: salami and sausages in Italy (Pontello *et al.*, 1998; Luzzi *et al.*, 2007; Scavia *et al.*, 2013; Scaltriti *et al.*, 2015; Andreoli *et al.*, 2017) and north-European countries (Emberland *et al.*, 2006; Hjertqvist *et al.*, 2006; Kuhn *et al.*, 2011), chorizo in Spain (Hernandez-Arribita *et al.*, 2015) and Denmark (Nygard *et al.*, 2007). In 2015, an outbreak of monophasic and biphasic *S. Typhimurium* and *S. Derby* associated with the consumption of dried pork sausage was reported in Spain (Arnedo-Pena *et al.*, 2016).

The raw materials used for the production of fermented meat products may be contaminated by *Salmonella* at the slaughterhouse (Pearce *et al.*, 2004; Piras *et al.*, 2011; Gomes-Neves *et al.*, 2012; Bonardi *et al.*, 2013; Fois *et al.*, 2017), during the production process (Delhalle *et al.*, 2009; Manios *et al.*, 2015; D'Ostuni *et al.*, 2016) and the post-processing stages (Barbuti and Parolari, 2002; Ferreira *et al.*, 2009).

Traditional ripened Sardinian sausage is a typical Mediterranean-style, semi-dry, naturally fermented (without use of starter cultures) pork meat product. It is the main meat product of the meat supply chain in Sardinia (Italy), included in the national list of traditional food products (Ministerial Decree 8 September 1999, n.350; Italian Republic, 1999). Ground pork meat and pork fat are the main ingredients. The safety

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of fermented sausages is ensured by the combined effect of different factors, known as "hurdles" (Leistner, 2000). In fact, for the manufacturing of dry fermented sausages, carbohydrates, starter cultures and curing salts (NaCl, nitrates and nitrites) are added. The fermentation of the carbohydrates by lactic acid starter cultures leads to pH decrease to values of ca. 5.3, and the drying step decreases the water activity (a_w) to values of ca. 0.920 (Christieans *et al.*, 2018). The use of sodium chloride (NaCl) is essential, other than to solubilize proteins and emulsify fat, to control spoilage and pathogenic bacteria (Bonardi *et al.*, 2017). Also nitrates and nitrites can be added, at maximum concentrations of 150 mg/kg each (Reg. EC 1129/2011). These additives are used due to their important role on color and flavor development and their antimicrobial activity against pathogenic bacteria such as *Clostridium botulinum* and *Enterobacteriaceae* (Hospital *et al.*, 2014). Nevertheless, in case of initial contamination of the raw materials with high levels of pathogenic bacteria and/or insufficient control of the antimicrobial factors, the safety of these products can be compromised (Ducic *et al.*, 2016). Survival of *Salmonella* in fermented meat products depends upon dry-curing and physicochemical conditions created by the hurdles. 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).

In Sardinian sausages mean values of pH between 5.3-5.5 and a_w between 0.90-0.92 at the end of ripening indicate a correct acidification and drying processes that allow obtaining safe and stable products (Mazzette *et al.*, 1998; Greco *et al.*, 2005; Mangia *et al.*, 2007). To our knowledge, there are no published data on the occurrence of *Salmonella* in Sardinian sausages. Therefore, the objective of the present study was to evaluate the presence of *Salmonella* in five fermented sausage processing plants and their products.

Materials and Methods

Features of the processing plants

The survey was conducted in five sausage processing plants (S1, S2, S3, S4 e S5) located in Sardinia (Italy) during one year. The processing plants included in the survey were representative of the regional fermented sausage production characterized by similar sausage manufacturing process (Figure 1). All the processing plants processed raw materials (fresh meat) supplied by Italian regions (Northern Italy) other than Sardinia. S1, S2 and S4 had an average production of 300-500 tons meat/year, while S3 and S5 of 50-100 tons meat/year. S5 declared to use microbial starter, consisting of lactic bacteria or staphylococci coagulase negative (MI100, Briostarter CAP, dried SH101 Texel, SA301 Milan). All plants used natural bowel (mutton or beef). The ripening step had an average duration of 16.2 days (range 9-20). Table 1 shows some of the features of the processing plants included in the study.

Samples collection

Each plant was visited twice within one month, collecting a total of 270 samples. Overall, 150 raw materials, semi-finished and finished products were collected and analyzed: n. 50 mixture of raw ground pork meat and fat (RM), n. 50 products at the end of acidification (PA, two days after stuffing) and n.50 sausages at the end of ripening (SR, n. days after stuffing variable accord-

ing to the processing plant). Moreover, n. 120 environmental samples were collected by means of hydrated-sponges pre-moistened with 10 ml Buffered Peptone Water Broth (BPW, 3M Health Care, Milan, Italy): a) 60 samples from surfaces in contact with meat (SCM): work tables, trolleys, hooks, meat grinder machine, mixer, stuffing machine; b) 60 samples from surfaces not in

contact with meat (SNCM): drains and a pool of walls+floor of ground meat store room, drying and ripening room. Environmental sampling was performed during the production stages. All samples were transferred to the laboratory under aseptic and refrigerated conditions in portable insulated cold-boxes. Samples were kept at 4°C and analyzed within 24h.

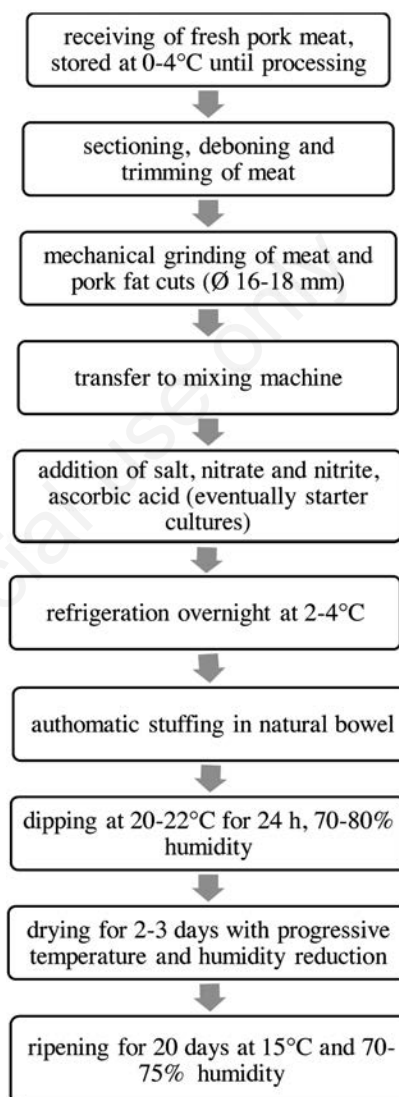


Figure 1. Process flow diagram of Sardinian sausage.

Table 1. Characteristics of the processing plants included in the study.

	Production tons meat/year	Origin of the raw meat	Characteristics of the raw meat	Use of starter culture	Ripening duration (days)
S1	300-500	National	Fresh	No	20
S2	300-500	National	Fresh	No	18
S3	50-100	National	Fresh	No	15
S4	300-500	National	Fresh	No	15
S5	50-100	National	Fresh	Yes	18

Chemical-physical analysis

For samples of RM, PA and SR, the pH and water activity (a_w), were determined. The potentiometric measurement of pH was carried out with 10 g of sample in 1:1 ratio with sterile distilled water using a pH meter GLP 22 (Crison Instruments SA, Barcelona, Spain) directly. Water activity (a_w) was determined at 25°C, using an Aqualab CX3 (Decagon, Pullman, Washington, USA).

Salmonella detection, serotyping and phage typing

All the samples were tested for the presence of *Salmonella* according to EN ISO 6579-1:2017. *Salmonella* suspect colonies were tested for biochemical properties with API® 20E micro-substrate system (bioMérieux, Marcy l'Etoile, France). A single confirmed *Salmonella* isolate from each positive sample was serotyped according to Kauffmann-Le Minor scheme by slide agglutination with O and H antigen specific sera (Staten Serum Institute, Copenhagen, Denmark). Definitive identification of *S. enterica* ser. 4,[5],12:i:- was obtained by a previously described PCR protocol (Barco *et al.*, 2011). Isolates identified as *S. Typhimurium* and the monophasic variant of *S. Typhimurium* were phage-typed according to standard methods (Anderson *et al.*, 1977) and following the interpretative guidelines defined for *S. Typhimurium* by the international federa-

tion for enteric phage typing (IFEPT, International Federation for Enteric Phage Typing, Colindale, London, United Kingdom).

Pulsed field gel electrophoresis

Pulsed Field Gel Electrophoresis PFGE typing was performed according SalmGene protocol by Peters *et al.* (2003) with *Xba*I (Invitrogen, Carlsbad, CA, USA) restriction of DNA. The comparison of PFGE patterns was made using the BioNumerics software v7.1 (Applied Maths, Saint-Martens-Platen, Belgium). Cluster analysis was performed using the Dice similarity coefficient, with 0.5% optimization and 1.5% tolerance, and the unweighted pair group method with arithmetic mean (UPRMA).

Results

Chemical-physical analysis

The pH values (mean±s.d.) of RM, PA and SR were 5.79±0.16, 5.57±0.28 and 5.39±0.24, respectively. The a_w values (mean±s.d.) were 0.97±0.02, 0.95±0.01 and 0.91±0.03 in RM, PA and SR, respectively.

Table 2 shows the physico-chemical parameters of sausages at the end of ripening and ripening duration (days) in the different facilities.

Salmonella prevalence

Salmonella was detected in three out of five processing plants (S1, S3 and S4), with an overall prevalence of 16.7% (25/150) in semi-finished and finished samples and 5.8% (7/120) in environmental samples (Table 3). Different processing plants showed different prevalence: S1 showed the highest prevalence (16/54; 29.6%), followed by S3 (10/54; 18.5%) and S4 (6/54; 11.1%). Between food samples, RM and PA, showed the highest prevalence (both 12/50; 24%) followed by SR (1/50; 2%). Among environmental samples, prevalence of 6.6% (4/60) and 5% (3/60) were detected, respectively in SCM and SNCM. At S1, *Salmonella* was detected from RM (60%; 6/10) and PA (60%; 6/10) during both sampling days. As regard to environmental samples, at S1 *Salmonella* was detected during the first sampling day from SCM (2/5; 40%: trolley and work table), and during the second sampling day from SNCM (2/6; 33%: drain and pool floor + walls of drying room). At S3, *Salmonella* was detected only during the second sampling day, from RM (20%; 2/10), PA (40%; 4/10) and also from a sample of SR (10%; 1/10). Moreover, during the same sampling day, *Salmonella* was detected from SCM (2/5; 40%: work table and meat grinder machine) and SNCM (20%; 1/6: floor + walls of ripening room). Finally, at S4 *Salmonella* was detected during both sampling days, from samples of RM, (40%; 4/10) and PA (20%; 2/10).

Table 2. Distribution of Salmonella serotypes and pulsotypes in samples of ground pork meat and fat, product at the end of acidification, sausages at the end of ripening and environmental samples of the processing plants. For samples of sausages at the end of ripening physico-chemical parameters and ripening duration (days) are shown.

Plant	Sampling day	GM <i>Salmonella</i> serotype/ pulsotype	PA <i>Salmonella</i> serotype/ pulsotype	SR <i>Salmonella</i> serotype/ pulsotype	pH mean (range)	a_w mean (range)	Ripening duration, days	SCM <i>Salmonella</i> serotype/ pulsotype	SNCM <i>Salmonella</i> serotype/ pulsotype
S1	1	Typhimurium /Typh1	Typhimurium /Typh1	-	5.46 (5.38-5.56)	0.895 (0.890-0.893)	20	Typhimurium /Typh1	-
	2	Rissen/Riss1	Rissen/Riss1	-	5.44 (5.38-5.51)	0.902 (0.899-0.909)	19	-	Rissen/Riss1
S2	1	-	-	-	5.70 (5.49-6.20)	0.901 (0.897-0.900)	20	-	-
	2	-	-	-	5.32 (5.10-5.43)	0.909 (0.901-0.919)	14	-	-
S3	1	-	-	-	5.44 (5.39-5.47)	0.902 (0.900-0.905)	18	-	-
	2	Give/Give1	Derby/Der1	Derby /Der1	5.39 (5.10-5.49)	0.922 (0.914-0.941)	14	Derby /Der1	Derby/Der1
S4	1	4,[5],12:i:- /Monof1	4,[5],12:i:- /Monof1	-	5.25 (5.05-5.82)	0.945 (0.939-0.950)	9	-	-
	2	Derby/Der2	Derby/Der2	-	4.94 (4.91-4.96)	0.945 (0.938-0.950)	13	-	-
S5	1	-	-	-	5.63 (5.50-5.81)	0.860 (0.818-0.889)	21	-	-
	2	-	-	-	5.39 (5.29-5.53)	0.918 (0.901-0.927)	14	-	-

RM: mixture of ground pork meat and fat; PA: product at the end of acidification; SR: sausages at the end of ripening; SC: surfaces in contact with meat; SNC: surfaces not in contact with meat.

Salmonella serotyping and phage typing

Five different serotypes were identified among 32 *S. enterica* isolates (Table 4). *S. enterica* ser. Derby was the most prevalent (37.5%; 12/32), followed by *S. Typhimurium* and *S. Rissen* (both 25%; 8/32). Finally, *S. Give* and monophasic variant of *S. Typhimurium* (antigenic formula 4, [5], 12:i-) accounted both for two isolates (6.25%).

Phage typing of *S. Typhimurium* isolates resulted into DT193, while *S. Typhimurium* monophasic variant were untypable.

In samples of RM, the following serotypes were identified: *S. Derby* (n.3), *S. Typhimurium* (n.3), *S. Rissen* (n.3), *S. Give* (n.2) and *S. Typhimurium* monophasic variant (n.1). In PA *S. Derby* (n.5), *S. Typhimurium* (n.3), *S. Rissen* (n.3) and *S. Typhimurium* monophasic variant (n.1) were identified. In SR, only *S. Derby* was identified. As regard to environmental samples, in SCM *S. Derby* (n.2) and *S. Typhimurium* (n.2) were detected and the same serotypes were identified in SNCM (n.1 for *S. Derby* and n.2 for *S. Typhimurium*).

Pulsed field gel electrophoresis

Six different pulsotypes were identified. Among *S. Derby* isolates, it was possible to identify two different pulsotypes (Der1 and Der2). Among the other serotypes, only one pulsotype was detected.

Table 2 shows the distribution of *Salmonella* serotypes and pulsotypes in food and environmental samples.

Discussion

This study showed the overall prevalence of *Salmonella enterica* in different samples of Sardinian fermented sausages and processing plants environment.

Our results on *Salmonella* prevalence in samples of raw ground pork meat and fat (24%) were higher than those reported by other authors that observed a prevalence between 4.3% (Delhalle *et al.*, 2009) and 14% (Bonardi *et al.*, 2017). Moreover, this prevalence was higher than those detected in pig carcasses at slaughterhouse where prevalence between 7 and 16% were detected (Pearce *et al.*, 2004; Vieira-Pinto *et al.*, 2005; De Busser *et al.*, 2011), and fresh pig meat where mean prevalence of 3-5% were observed (Zhao *et al.*, 2001; Busani *et al.*, 2005; Hansen *et al.*, 2010).

S. Derby was the most prevalent serotype (37.4%). This result was expected as this serotype is well known to be adapted to pigs (Matiasovic *et al.*, 2014). In fact, it is one of the most common in slaughter pigs in Italy (Bonardi *et al.*, 2003 and 2016; Piras *et al.*, 2011 and 2014; Fois *et al.*, 2017; Pesciaroli *et al.*, 2017) and other European countries (Nollet *et al.*, 2004; Hauser *et al.*, 2011; Gomes-Neves *et al.*,

2012; Kerouanton *et al.*, 2013; Sanchez-Rodriguez *et al.*, 2018). Moreover, *S. Derby* is between the top five most commonly reported serovars in human salmonellosis acquired in EU during 2016 (EFSA and ECDC, 2017) and it has also been recently associated to outbreaks linked to pork meat products (Arnedo-Pena *et al.*, 2016).

S. Typhimurium has been identified in samples of ground meat, sausages at the end of acidification and environmental samples (SCM). Also this serotype, which is the second most reported in human salmonellosis, has been increasingly described both in slaughter pigs (Arguello, *et al.*, 2013; Van Damme *et al.*, 2018; Morales-Partera *et al.*, 2018) and in cases of human salmonellosis attributed to pork meat and meat products (Luzzi *et al.*, 2007; Andreoli *et al.*, 2017).

Serotyping and PFGE allowed to presumably indicate the raw materials (pork meat and fat) as the primary source of contamination of Sardinian sausages. In fact, the serovar and the PFGE patterns of the strains were specific for each facility with no exchange between different abattoirs. The same observation can be pointed out considering different sampling days for the same slaughterhouse. This result seems to demonstrate the effective application of cleaning and disinfection procedures that do not allow to the pathogen to become part of the resident flora in the investigated processing plants.

The evolution of pH and a_w detected in our survey during the process was similar to

Table 3. Prevalence of *Salmonella* in mixture of ground pork meat and fat, product at the end of acidification, sausages at the end of ripening and environments of sausages processing plants.

Plant	RM		PA		SR		SCM		SNCM	
	Positive/total	%	Positive/total	%	Positive/total	%	Positive/total	%	Positive/total	%
S1	6/10	60	6/10	60	0/10	0	2/12	16.7	2/12	16.7
S2	0/10	0	0/10	0	0/10	0	0/12	0	0/12	0
S3	2/10	20	4/10	40	1/10	10	2/12	16.7	1/12	8.3
S4	4/10	40	2/10	20	0/10	0	0/12	0	0/12	0
S5	0/10	0	0/10	0	0/10	0	0/12	0	0/12	0
Total	12/50	24	12/50	24	1/50	2	4/60	6.6	3/60	5

RM: mixture of ground pork meat and fat; PA: product at the end of acidification; SR: sausages at the end of ripening; SC: surfaces in contact with meat; SNC: surfaces not in contact with meat.

Table 4. Prevalence of *Salmonella* serotypes in samples of ground pork meat and fat, product at the end of acidification, sausages at the end of ripening and environmental samples.

<i>Salmonella</i> serovars	RM			PA			SR			SCM			SNCM			Total (%)
	S1	S3	S4	S1	S3	S4	S1	S3	S4	S1	S3	S4	S1	S3	S4	
Derby			3		4	1		1			2			1		12 (37.4)
Typhimurium DT 193	3			3						2						8 (25)
Rissen	3			3									2			8 (25)
Give		2														2 (6.3)
4,[5], 12:i- (untypable)			1			1										2 (6.3)
Total	6	2	4	6	4	2		1		2	2		2	1		32 (100)

RM: mixture of ground pork meat and fat; PA: product at the end of acidification; SR: sausages at the end of ripening; SC: surfaces in contact with meat; SNC: surfaces not in contact with meat.

that described for other Italian typical fermented meat products (Bonardi *et al.*, 2017; Montanari *et al.*, 2018). A_w mean values between 0.89 and 0.91 in Sardinian semi-dry fermented sausage are usually detected after 20 days of ripening (Mazzette *et al.*, 1998; Meloni *et al.*, 2014). In the same conditions, pH at the end of ripening is between 5.03 and 5.28 (Greco *et al.*, 2005; Mangia *et al.*, 2007). These conditions, along with NaCl concentration, should allow to obtain shelf-stable and safe meat products.

It is known that *Salmonella* is able to growth within a broad range of pH (4.1-9) and with minimum a_w value of 0.95 (Cox *et al.*, 2000). Messier *et al.* (1989) showed that in fermented meat products with NaCl % between 2 and 3.3 and pH < 4.9 *Salmonella* survival is inhibited with a_w between 0.93 and 0.96.

We isolated *Salmonella* in a sample of sausage at the end of ripening that had been dried for 13 days and showed a pH of 4.92 and an a_w value of 0.950, thus sufficient to allow *Salmonella* survival and growth. This can be the consequence of an empirical application of the hurdle's technology in the manufacturing of traditional products like Sardinian sausages that, most often when consumers demand increases (e.g. during Christmas or Easter time), leads to a shortage of the processing steps, particularly of the ripening, not allowing to obtain a correct drying of the product.

Conclusions

Reduction of *Salmonella* contamination of raw materials used for sausage production 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. The presence of *Salmonella* in the ripened Sardinian sausages confirms that the hurdles of microbial growth (mainly the a_w decrease) should be properly applied during the ripening in order to prevent the survival of *Salmonella* that can contribute to the consumer exposure.

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