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SOURCES OF *LISTERIA MONOCYTOGENES* CONTAMINATION IN TRADITIONAL FERMENTED SAUSAGE PROCESSING PLANTS IN ITALY

D. MELONI*, F. PIRAS, A. MUREDDU, R. MAZZA, D. NUCERA[1] and R. MAZZETTE
Dipartimento di Biologia Animale, Facoltà di Medicina Veterinaria, Università di Sassari,
Via Vienna 2, 07100 Sassari, Italy
[1]Dipartimento di Patologia Animale, Facoltà di Medicina Veterinaria, Università di Torino,
Via L. Da Vinci 44, 10095 Grugliasco (TO), Italy
*Corresponding author: Tel. +39 079 229570, Fax +39 079 229458,
email: dmeloni@uniss.it

ABSTRACT

Environmental samples, raw materials and fermented sausages produced in Sardinia (Italy) were analysed in order to investigate the prevalence and enumeration of *L. monocytogenes*. Isolates were identified by single PCR and characterised by multiplex PCR-based serogrouping. The contamination routes of *L. monocytogenes* in the plants were traced using PFGE. In addition, a quantitative assessment of the *in vitro* biofilm formation was carried out. Fermented sausages seem to be regularly contaminated with *L. monocytogenes*: results showed the ability of the pathogen to overcome the hurdles of the manufacturing process and adapt to the processing plant environments, forming biofilms.

INTRODUCTION

Listeria monocytogenes is a ubiquitous organism, widely distributed in the environment. The principal reservoirs are soil, forage and water (EFSA, 2011). Other reservoirs include healthy humans and animals (ILSI, 2005) or infected domestic and wild animals (EFSA, 2011). *L. monocytogenes* has been isolated throughout the pork processing industry (NESBAKKEN *et al.*, 1996), with an increase of contamination along the production line (CHASSEIGNAUX *et al.*, 2002). One of the most recent listeriosis outbreaks (Canada 2008) was linked to the consumption of ready to eat (RTE) pork meat products, causing 22 deaths and 57 confirmed cases (PHAC, 2009). Fermented RTE pork meat products, such as dry and semi-dry sausages have rarely been implicated in food poisoning. Nevertheless, in the manufacturing of traditional fermented products, an empirical application of hurdle technologies often occurs and these products are regularly contaminated with *L. monocytogenes*. This fact may present a major public health concern if the pathogen is able to multiply and reach high levels of contamination (THEVENOT *et al.*, 2006[1]). Previous surveys carried out on traditional fermented products at the end of ripening showed a prevalence of 10% in France (THEVENOT *et al.*, 2005), 10.6% in Chile (CORDANO and ROCOURT, 2001) and 15.2% in Italy (DE CESARE *et al.*, 2007). In a former study, *L. monocytogenes* was found in 40% of "*Salsiccia Sarda*" at the end of ripening (MELONI *et al.*, 2009) with contamination levels always lower than 100 CFU/g. "*Salsiccia Sarda*" is the primary meat product (37% of consumption) of the meat supply chain in the Sardinia region (Italy) and is included on the national list of traditional food products. It is one of the typical Italian semi-dry sausages (a_w ranging from 0.90 to 0.95), naturally fermented (GRECO *et al.*, 2005) and marketed locally and regionally in Italy (COMI *et al.*, 2005). The main composition and characteristics of "*Salsiccia Sarda*" include: minced lean pork (87%); pork back fat (8%); salt (3%); a mixture of sugars and additives (0.8%); garlic (0.15%); ground pepper (0.25%); and a mixture of whole pepper, nutmeg, cloves and pimento (0.8%). The mixture, refrigerated overnight, is stuffed into natural pork casings. After they are first warmed up at 20°-22°C for 4-6 h, the products are dried for six days in a fermentation chamber. On the first day of drying, the products are stored at 20°-22°C and 60% relative humidity. Over the next five days of drying the temperature is gradually reduced to 15°C and the relative humidity is gradually increased to 70%. Ripening is then carried out for 15 days in store-rooms at 15°C and 70-75% relative humidity. The final products are cylindrical (about 30 mm Ø), about 40-45 cm long, shaped like horseshoes, and about half a

kg each in weight. The normal pH of "*Salsiccia Sarda*" at the end of ripening is 5.28 (GRECO *et al.*, 2005). *L. monocytogenes* may survive during the processing of the fermented meat products due to its high tolerance to low pH conditions and high salt concentrations (FARBER and PETERKIN, 1991) and if the standard hurdle technologies are ineffective. The main hurdles used during the processing of dry fermented products are nitrite and salt content, the decrease in redox potential and water activity (a_w) that inhibit many aerobic bacteria in the early stages of production (*Pseudomonas spp.* and other Gram-negative bacteria), and selecting the lactic acid bacteria (LAB) which cause the pH to decrease (BARBUTI and PAROLARI, 2002). These hurdles, with the coagulase negative *Staphylococci* development and the length of the ripening period, are essential for the microbial safety and stability of quick-ripened fermented sausages, which are not greatly dried. Pork meat used for the manufacturing of sausages may be contaminated from a wide variety of sources and *L. monocytogenes* once introduced in the processing plants can persist over time in the environment, contaminating food processing machines (LOPEZ *et al.*, 2008). *L. monocytogenes* forms assemblages of surface-associated microbial cells that are enclosed in hydrated extracellular polymeric substances and grow in biofilms on surfaces in contact or not with the food (GANDHI and CHIKINDAS, 2007), such as floor drains, storage tanks, hand trucks, conveyor belts and other food contact materials (MAFU *et al.*, 1990). The presence of the pathogen on surfaces in contact and without contact with food increases the food safety risk (KIM and FRANK, 1995). Thus, *L. monocytogenes* may become an important source of secondary contamination of food products, and without suitable sanitisation procedures, cross-contamination of the meat products may occur (SAMELIS and METAXOPOULOS, 1999). The main objective of the present study was to evaluate the occurrence of *L. monocytogenes* in the fermented sausage production of the Sardinia region (Italy). Isolates were identified by single PCR and further characterised by multiplex PCR-based serogrouping. The contamination routes of *L. monocytogenes* in the plants were traced using PFGE. In addition, a quantitative assessment of the *in vitro* biofilm formation was carried out in order to investigate the potential for persistence.

MATERIALS AND METHODS

Sampling

A total of 170 samples from environments, raw materials and final products from two large (A and D > 300 tons of "*Salsiccia Sarda*" per year)

and two small (B and C from 50 to 100 tons of "*Salsiccia Sarda*" per year) processing plants, located in different provinces of Sardinia (Italy), were sampled in order to investigate the prevalence, ecology and genetic profile of *L. monocytogenes*. All of the processing plants were representative of the regional fermented sausage production and some characteristics are summarised in Table 1. Three of the processing plants (A, B, D) were only sampled once (S1). In order to evaluate the role of swine carcasses as a possible source of plant contamination and the persistence of *L. monocytogenes*-adapted strains, in plant C (randomly chosen) sampling was repeated within three months (S2). Details on samples collected from each plant are provided in Table 2. A total of 132 environmental samples were collected: 96 swabbed surfaces without contact with meat (SWCM) and 36 surfaces in contact with meat (SCM). SWCM and SCM were sampled during the production stages by swabbing with 10 cm by 10 cm sterile gauze pads rehydrated with 10 mL of neutralising buffer (Solarcult sampling kit, Biogenetics, Padova, Italy) and using a sterile template to delineate the swabbed area of 100 cm². Sampling locations were chosen in order to represent those most likely to present *L. monocytogenes* contamination. Sampling sites for SWCM included walls and floor drains of the ground meat store-rooms, drying and ripening rooms, processing and packaging/ shipment rooms. Regarding SCM, the sampling sites included work tables, sausage trolleys, hooks, mincing, mixing and stuffing machines. A total of 16 samples from ground meat and 10 from fermented sausages at the end of ripening were collected. Only during the repeat sampling (S2) in plant C 12 samples from swine carcasses were collected: 6 of raw pork meat with and without rind and 6 swab samples of vertebral canal between the first thoracic vertebra and the seventh lumbar vertebra, in correspondence with the bone saw surface of the swine carcasses. The carcasses were stored at +4°C and were sampled before their entry in the processing line. Samples were collected by swabbing as previously described. All of the items were aseptically sampled, placed in sterile bags (kept in ice boxes at +3°C) during transport and were immediately analysed upon arrival at the laboratory.

Detection and enumeration of *L. monocytogenes*

Detection and enumeration of *L. monocytogenes* was carried out using the ISO 11290-1:1996 and 11290-2:1998 protocols, respectively. Samples of raw pork meat, ground meat, fermented sausages and swabbed samples were homogenised 1/10 with Fraser broth base (Biolife, Milan, Italy) in a Stomacher Lab Blender 400 (Seward Medical, London, UK) for 2 min. The homogenates were incubated at 20°C for 1 h, in order to resuscitate stressed microorganisms. For the enumeration of *L. monocytogenes*, 1 ml of each inoculum was distributed over the surface of three Aloa (Biolife) 90 mm plates using a sterile spreader. The plates were incubated at 37°C for 48 h. For detection of *L. monocytogenes*, the homogenates were supplemented by Fraser half-selective supplement (Biolife) and incubated at 30°C for 24 h. Afterwards, 0.1 mL of the primary enrichment was inoculated in 10 mL of Fraser broth supplemented (Biolife) by Fraser selective supplement (Biolife) and incubated at 37°C for 48 h. Cultures were streaked onto Oxford (Oxoid, Milan, Italy) and Aloa (Biolife) plates and incubated at 30° and 37°C for 48 h, respectively. From each plate of the primary and secondary enrichment, five colonies presumed to be *Listeria spp.* were streaked on Tsyea plates (Biolife) and incubated at 37°C for 24 h. Colonies were selected for typical appearance on Tsyea and submitted to Gram staining, catalase and oxidase tests. Haemolytic activity and CAMP tests on sheep blood agar were performed for confirmation of *L. monocytogenes*. Biochemical characterisation of all the isolates was performed using the API Listeria identification system (bioMérieux, Marcy l'Etoile, France).

Molecular identification and characterisation

Single PCR-based identification

The phenotypic identification of *L. monocytogenes* isolates was confirmed by a single PCR-based method (sPCR) aimed at *prfA* gene fragment detection. *L. monocytogenes* isolates were grown on BHI (Oxoid) at 37°C for 16-18 h. The cells were pelleted by centrifugation of 1 mL at

Table 1 - Some characteristics of the four Sardinian processing plants.

Plants	Tons/year	Processing line complexity	Origin of the groundmeat	Characteristics of the ground meat	Ripening period (fermented sausages)
A	300-500	+++	Domestic	Fresh	15 days
B	50-100	++	Domestic	Fresh	15 days
C	50-100	++	Domestic	Fresh	15 days
D	>500	++++	Domestic /European	Fresh	7-15 days

Processing line complexity: ++ (simple equipment); +++ (more equipment, more complex); ++++ (a lot of equipment, with a complex design).

12,000 rpm for 5 min at 4°C and washed twice in phosphate-buffered saline (PBS). DNA was extracted by suspending the pellet in 1 mL of PBS, which was boiled for 5 min and centrifuged at 10,000 rpm. The supernatant was quantified with a UV-1700 PharmaSpec spectrophotometer (Shimadzu, Kyoto, Japan) at OD_{260} and then stored at -20°C until use. Heterologous DNA of S. xylosus ATCC 29971 was used as a negative control and DNA of L. monocytogenes reference strain ATCC 19115 was used as a positive control. The sPCR was carried out with the primer set Lip1 and Lip2 (D'AGOSTINO et al., 2004; JOFRÉ et al., 2005) which produces a fragment of 274_{bp} (SIMON et al., 1996). All amplification reactions were performed in a final volume of 50 µL containing 5 µL of DNA, 5 µL of 10X PCR buffer (Invitrogen, Carlsbad, USA), 2.5 mM of $MgCl_2$, 0.3 mM each of dNTP, 0.3 mM each of primer and 1U of Platinum Taq DNA polymerase (Invitrogen). All amplification reactions were performed in a Gene-Amp 2700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) programmed as follows: denaturation at 94°C for 2 min, annealing at 55°C for 30 sec and elongation at 74°C for 1 min, followed by a final extension period at 74°C for 5 min. The amplified fragments were separated by 1.5% agarose gel electrophoresis (Roche diagnostics, Milan, Italy) in 1X Tris-acetate EDTA (TAE, Invitrogen) and stained with ethidium bromide (0.1 mg/mL) for 20 min. The gels were observed and digitalised by the Gel-Doc UV trans-illuminator (Bio-Rad, Hercules, CA, USA).

Multiplex PCR-based serogrouping

Multiplex PCR-based serogrouping was carried out using the target genes lmo0737, lmo1118, ORF2819, ORF2110 and prs (DOUMITH et al., 2004). The multiplex PCR products were resolved by electrophoresis on 1.5% agarose gel in 1X TAE (Invitrogen) and stained with ethidium bromide (0.1 mg/mL) for 20 min. The gel images were visualised and captured using the Gel-Doc UV trans-illuminator (Bio-Rad).

DNA macrorestriction and pulsed-field gel electrophoresis (PFGE)

Isolates were submitted to DNA macrorestriction with ApaI and AscI (New England Biolabs, Beverly, MA, USA). The separation of the restriction fragments was carried out by PFGE in a CHEF Mapper XA system (Bio-Rad) using the PFGE-PulseNet protocol (GRAVES and SWAMINATHAN, 2001). Gel images were visualised and captured using the Gel-Doc UV trans-illuminator (Bio-Rad). The banding patterns for each enzyme were assigned through visual analysis of the restriction profiles. Isolates were designated genetically indistinguishable (same pulsotype) when their restriction patterns had the same number of bands and the corresponding bands

were the same apparent size (TENOVER et al., 1995; GRAVES et al., 2005). AscI and ApaI macrorestriction patterns were analysed using BioNumerics software (Applied Maths, Sint-Martens-Platen, Belgium). The similarity between restriction patterns, based on bands position, was expressed as a Dice coefficient correlation. The position tolerance was optimal when set at 1.0 and 2.0% for AscI and ApaI, respectively. Clustering and construction of dendrograms were performed by the Unweighted Pair Group Method using arithmetic averages (UPGMA) combining both AscI and ApaI macrorestriction patterns into one unique PFGE profile.

Quantitative assessment of in vitro biofilm formation

Isolates were tested for their ability to attach to abiotic surfaces forming biofilm. The quantitative assessment of the in vitro biofilm formation was carried out on sterile 96-well polystyrene microtiter plates using the method described by STEPANOVIC et al. (2004), with some modifications. Isolates were grown for 24 h in 2 mL of BHI broth. All the wells of a polystyrene microtiter plate were filled with 230 µL of BHI broth. Afterwards, 21 wells per strain were filled with 20 µL of culture. Each plate included 12 wells of BHI broth without inoculum, as a negative control. Microtiter plates were incubated at 30° for 24 h. At the end of the incubation the content of the wells was removed and the microtiter plate washed three times with 300 mL of sterile, distilled water in order to remove loosely attached bacteria. The remaining attached bacteria were fixed with 250 µL of methanol per well, and after 15 min the wells were emptied and air-dried. Each well was stained with 250 µL of crystal violet for 5 min. After staining, the microtiter plates were washed under running tap water, then air-dried and the dye bound to the adherent cells was solubilised with 250 µL of 33% (v/v) glacial acetic acid per well. The microtiter plates were read spectrophotometrically (OD_{620}) using a Sunrise RC absorbance reader (Tecan, Maennedorf, Switzerland). The strains were divided into four categories: no biofilm producers (NP, OD=<0.5), weak producers (WP, OD= ≥0.5<1.0), moderate producers (MP, OD= ≥1.0<1.5) and strong producers (SP, OD= ≥1.5).

RESULTS

Detection and enumeration of L. monocytogenes

The results (Table 2) showed the presence of L. monocytogenes in all of the fermented sausage processing plants (overall prevalence in the environments: 15%). None of the samples from pork carcasses was positive for L. mono-

Table 2 - *Listeria monocytogenes* in environments, raw materials and final products of four fermented sausage processing plants.

Plants		SCM*** Positive/Total	%	SWCM**** Positive/Total	%	Pork carcasses Positive/Total	%	Ground meat Positive/Total	%	Fermented sausages Positive/Total	%
A	S1	0/6	-	2/18	11.11	-	-	2/4	50	2/2	100
B	S1	2/6	33.33	4/18	22.22	-	-	2/4	50	0/2	-
C	S1*	0/10	-	6/18	33.33	-	-	2/4	50	2/2	100
	S2**	0/8	-	2/20	10	0/12	-	-	-	-	-
D	S1	2/6	33	2/22	9	-	-	0/4	-	4/4	100
Grand total		4/36	11.11	16/96	16.66	0/12	-	6/16	37.50	8/10	80

*S1: first sampling; **S2: repeated sampling; ***SCM: Surfaces in contact with meat; ****SWCM: Surfaces without contact with meat.

cytogenes. The occurrence was 37% in ground meat and 80% in the fermented sausages. These products did show detectable levels always below 10 CFU/g, complying with the food safety criteria provided for RTE foods able to support the growth of *L. monocytogenes* (EFSA, 2005). According to THEVENOT *et al.* (2006[2]), several factors, such as the manufacturing process, the use of spices with antioxidative or antimicrobial properties, and the growth of the natural competitive microflora should significantly reduce the count of *L. monocytogenes* in fermented sausages. Altogether, 170 strains of *Listeria spp.* were isolated: 39% were *L. welshimeri*, 23% *L. monocytogenes*, 21% *L. innocua*, 10% *L. grayi*, 4% *L. ivanovii*, and 3% *L. seeligeri*. A subset of 20 *L. monocytogenes* isolates was selected and primarily identified by single PCR. From each processing plant, *L. monocytogenes* strains were selected in order to include almost 50% of each category of positive samples. Isolates were further characterised by multiplex PCR-based serogrouping, PFGE and *in vitro* biofilm formation. A summary of the phenotypic and genotypic characteristics of the 20 *L. monocytogenes* isolates is reported in Table 3.

Table 3 - Distribution of 20 *Listeria monocytogenes* isolates in the four processing plants, in relation to serotype, pulsotype, PFGE profile and *in vitro* biofilm production.

Plant and source of contamination		Strain code	Serotype	Pulsotypes AscI	ApaI	PFGE profile	Biofilm production
A	Ground meat store room: floor drains	A1	4b	I	I	9	NP
	Ground meat	A2	4b	II	II	11	MP
	Fermented Sausage	A3	1/2b	III	III	13	NP
B	Ground meat store room: floor drains	B1	1/2b	IV	IV	4	MP
	Ground meat store room: floor drains	B2	1/2b	IV	IV	4	NP
	Ground meat	B3	1/2b	V	V	2	WP
	Drying room: floor drains	B4	1/2b	V	V	2	WP
	Drying room: floor drains	B5	1/2b	VI	VI	7	NP
	Ripening room: hooks	B6	1/2b	VII	VII	1	WP
	Ripening room: hooks	B7	1/2b	VII	VII	1	WP
C	Drying room: floor drains	C1	1/2b	VIII	VIII	12	NP
	Ground meat	C2	1/2b	IX	IX	5	WP
	Fermented sausage	C3	1/2b	IX	IX	5	NP
	Shipment room: floor drains	C4	1/2b	X	X	8	WP
	Ripening room: floor drains	C5	1/2b	X	X	8	NP
	Ripening room: floor drains	C5bis	1/2b	X	X	8	MP
D	Fermented sausage	D1	1/2a	XI	XI	6	WP
	Fermented sausage	D2	1/2a	XII	XII	3	WP
	Processing room: work tables	D3	1/2a	XIII	XIII	10	WP
	Processing room: floor drains	D4	1/2a	XIV	n.t.*	n.t.*	NP

*n.t.: not typeable; NP: no biofilm producers; WP: weak producers; MP: moderate producer.

Molecular identification and characterisation

Single PCR-based identification

The amplification product of 274_{bp} was found in all the strains phenotypically identified as *L. monocytogenes*. The amplification products always exhibited the same electrophoretic pattern. These results confirm that the *prfA* gene is a reliable target gene for identification of the pathogen (VAZQUEZ-BOLAND *et al.*, 2001).

Multiplex PCR-based serogrouping

The prevalent serotype was 1/2b (70%), followed by 1/2a (20%) and 4b (10%). No specific serotype was recovered during the processing and ripening of the sausages (THEVENOT *et al.*, 2005).

DNA macrorestriction and pulsed-field gel electrophoresis (PFGE)

A high heterogeneity of pulsotypes (14 with *Asc*I and 13 with *Apa*I) occurred within the plants (Table 3). Restriction patterns were combined in 13 PFGE profiles appearing to be plant-specific. One strain from plant D was typeable only with *Asc*I (pulsotype XIV) and consequently was not assigned to any PFGE profile. Within each processing plant, particularly in the two large ones (A and D), a high heterogeneity distribution of PFGE profiles was observed. The presence of PFGE profile five both in ground meat and in the fermented sausages, may evidence the ability of specific strains of *L. monocytogenes* to survive during "*Salsiccia Sarda*" fermentation. Furthermore, the recovery of PFGE profile eight in floor drains from different rooms in both samplings (S1 and S2) showed the ability of the pathogen to adapt and persist in the different processing environments of plant C. The PFGE profiles were allotted into three major PFGE clus-

Fig. 1 - UPGMA clustering of the 19 *Listeria monocytogenes* PFGE profiles.

ters (similarity ≥ 70%) labelled A-B-C (Table 4 and Fig. 1). Cluster A included 9 isolates of serotype 1/2b (8) and 1/2a (1) from three processing plants (B-C-D). Cluster B included 6 isolates belonging to different serotypes: 1/2a (1), 1/2b
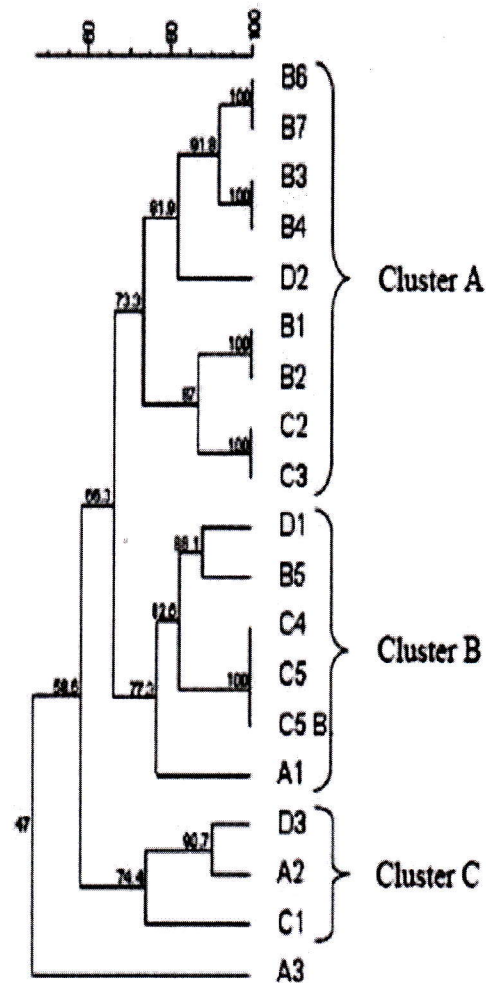
Table 4 - Clustering of 18 *Listeria monocytogenes* strains.

Cluster	Overall similarity	PFGE profile	N° of isolates	Serotype	Source of contamination in the plants
A	73.3%	1	2	*1/2b*	Hooks in the ripening room
		2	2	*1/2b*	Ground meat and floor drains in the drying room
		3	1	*1/2a*	Fermented sausage
		4	2	*1/2b*	Floor drains in the store room
		5	2	*1/2b*	Ground meat and Fermented sausage
B	77.3%	6	1	*1/2a*	Fermented sausage
		7	1	*1/2b*	Floor drains in the drying room
		8	3	*1/2b*	Floor drains in the shipping room (1) and ripening room (2)
		9	1	*4b*	Floor drains in the store room
C	74.4%	10	1	*1/2a*	Work tables in the processing room
		11	1	*4b*	Ground meat
		12	1	*1/2b*	Floor drains in the drying room

(4) and 4b (1) from all of the processing plants (A-B-C-D). Cluster C included three isolates of serotype 1/2a, 1/2b, and 4b from plants A-C-D. One strain (A3) of serotype 1/2b isolated from fermented sausage was an outlier, showing low similarity (47%) with the other clusters.

Quantitative assessment of *in vitro* biofilm formation

More than half (60%) of the strains previously identified and characterised by molecular methods were able to attach to abiotic surfaces forming biofilm. The results of this experiment are shown in Table 3 and Fig. 2. Serotypes 1/2a and 1/2b showed weak OD ($OD=\geq0.5<1.0$) or moderate OD ($OD=\geq1.0<1.5$) ability in biofilm formation. The microtiter plate assay is a useful method to assess the ability of *L. monocytogenes* strains to attach to abiotic surfaces (STEPANOVIC et al., 2004).

DISCUSSION AND CONCLUSION

In this study, the contamination of four "Salsiccia Sarda" processing plants representative of Sardinian fermented sausage production was investigated on samples from surfaces (with and without contact with meat), ground meat and fermented sausages. Our results show the overall presence of *L. monocytogenes* in environmental niches, raw materials and final products. *L. monocytogenes* was not detected in the pork carcasses prior to processing: contamination of the final products appears to be due to strains already present in the processing environments (LOPEZ et al., 2005), with all of the facilities serving as the source of product contamination (CHASSEIGNAUX et al., 2002). In the Sardinian processing plants and their products, *L. monocytogenes* serotype 1/2b predominated, followed by serotypes 1/2a and 4b. Previous studies have also reported the presence of these serotypes in meat processing environments (CHASSEIGNAUX et al., 2001; THEVENOT et al., 2006[1]). The recovery of serotypes frequently associated with epidemic or sporadic cases of listeriosis (McLAUCHLIN et al., 2004) is an interesting result in terms of public health protection. Previous surveys have shown that lineage I strains of serotype 4b belonging to a clonal group (DUP-ID 1038) linked to several listeriosis outbreaks (DE CESARE et al., 2001) were recently recovered in the same meat products (MELONI et al., 2009). It must be emphasised that the presence of such strains in the processing plants, and as a consequence in fermented sausages, may represent a hazard if the pathogen is able to multiply during the ripening of the product and reach levels higher than 1,000 CFU/g (ROSS et al., 2002; THEVENOT et al., 2006[1]). Thirteen different PFGE profiles were obtained indicating a great level of

diversity among the strains collected from the Sardinian processing plants and their products. The high number of PFGE profiles and their heterogeneous distribution within the plants is in agreement with the results of previous surveys carried out in meat processing plants (THEVENOT
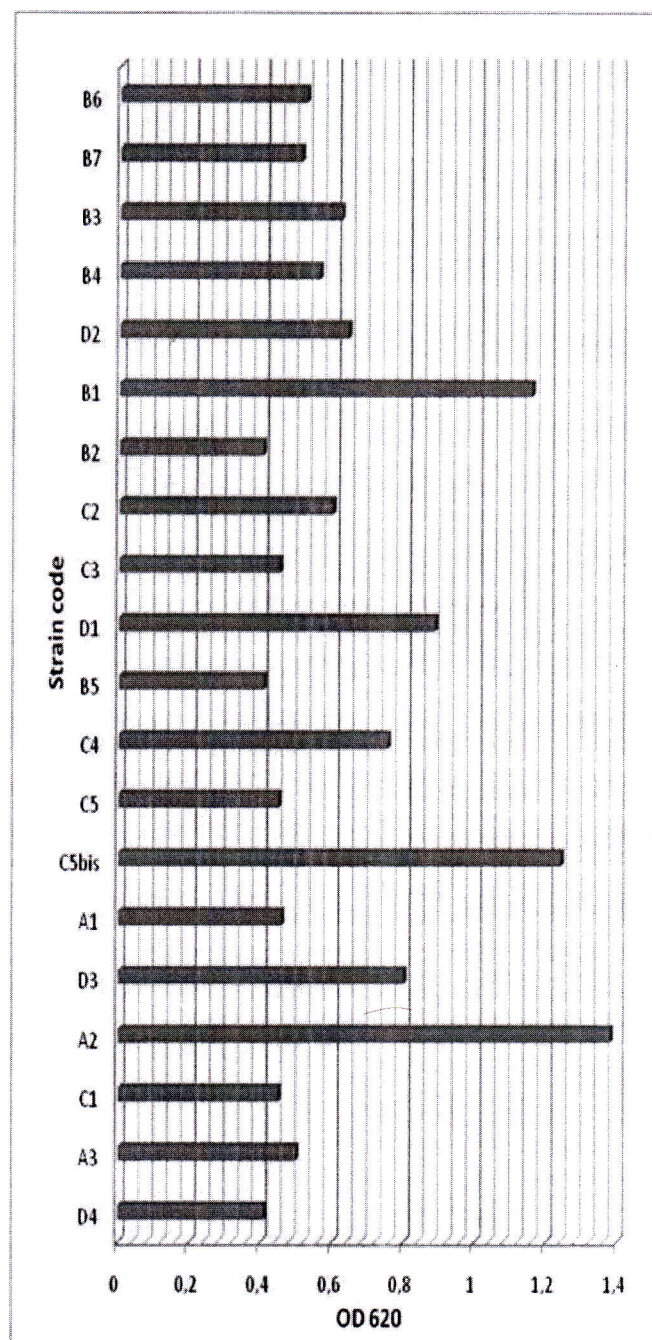

Fig. 2 - Biofilm production of the *Listeria monocytogenes* strains.
The quantitative assessment of the *in vitro* biofilm formation was evaluated spectrophotometrically (OD620). The strains were divided up into four categories: no biofilm producers (NP= OD <0.5), weak producers (WP= OD ≥0.5<1.0), moderate producers (MP= OD ≥1.0<1.5) and strong producers (SP= OD ≥1.5). Altogether, 60% of the strains were able to attach to abiotic surfaces forming biofilm, showing weak or moderate ability in biofilm formation.

et al., 2006[1]). Such subtypes appear to be unique to each processing plant (FUGETT *et al.*, 2007). These results may be due to the limited number of isolates included in this study or to the great diversity of the *L. monocytogenes* strains collected from ground meat, but also to the quantities used by each plant. The large plants exhibited the highest PFGE profile heterogeneity, since these plants also used the greatest amount of raw meat from several different sources (domestic and European). Numerical analysis of the PFGE profiles showed that the isolates included in the study could be allotted into three major PFGE clusters labelled A-B-C. Contrary to all expectations, these three genomic divisions were not linked with the flagellar antigen type, in spite of what was described by several authors (CHASSEIGNAUX *et al.*, 2001; AUTIO *et al.*, 2003; THEVENOT *et al.*, 2006[1]). In this study, *L. monocytogenes* strains were thought to be persistent when the same PFGE profile occurred in samples collected from the same plant equipment C after an interval of three months: such strains were found in several floor drains. The persistence of the same PFGE profile (eight) is probably due to two interacting factors. First of all, the nature of the strains themselves, which have a moderate ability to form biofilms and have adapted to environments where meat is processed. Several authors have shown that certain *L. monocytogenes* strains were more capable of causing persistent contamination of meat processing plants than others (AUTIO *et al.*, 2003; THEVENOT *et al.*, 2006[1]) probably due to their ability to form biofilms. The second factor affecting persistence may be the presence of ineffective cleaning and disinfection measures. Treatments need to reach the contamination site in sufficient quantities and duration in order to be effective (THEVENOT *et al.*, 2006[1]). This may not always occur with routine cleaning procedures because of the complexity of the processing line structure and because environmental niches, such as floor drains can be a site critical for controlling contamination of the processing plant environment and food products (TOMPKIN, 2002). Decontaminating floor drains is especially challenging because, when entrapped in a biofilm, *L. monocytogenes* is afforded unusual protection against available disinfectants and treatments (ZHAO *et al.*, 2004). *L. monocytogenes* strains can become well established in the floor drains and persist as resident microbial flora for up to several years. This study has shown the presence of adapted *L. monocytogenes* strains able to survive during sausage fermentation, overcoming the current hurdles of the "*Salsiccia Sarda*" manufacturing process. Our results highlighted that *L. monocytogenes* is able to survive in meat processing plants by forming biofilms on abiotic surfaces. The surface used for the *in vitro* experiment (polystyrene) approximately mimics the plastic material used in the plants (e.g.,

conveyor belts). Further testing with appropriate steel specimens of each plant is needed in order to understand better the mechanism of biofilm formation *in vivo*. To decrease the presence of *L. monocytogenes* in the traditional fermented sausages at the end of ripening, food business operators should adhere to accurate application of hurdle technologies. Products can also become contaminated through contact with work surfaces and equipment, even after routine cleaning and disinfecting operations (THEVENOT *et al.*, 2006[1]). More attention should be focused on respecting good manufacturing practices and the application of HACCP principles.

REFERENCES

Anonymous 1996. Microbiology of food and animal feeding stuffs - horizontal method for the detection and enumeration of *Listeria monocytogenes*. Part 1: Detection method, 1st Ed. ISO method 11290-1. International Organization for Standardization, Genève, Switzerland.

Anonymous 1998. Microbiology of food and animal feeding stuffs - horizontal method for the detection and enumeration of *Listeria monocytogenes*. Part 2: Enumeration method, 1st Ed. ISO method 11290-2. International Organization for Standardization, Genève, Switzerland.

Autio T., Keto-Timonen R., Biorkroth J. and Korkeala H. 2003. Characterization of persistent and sporadic *Listeria monocytogenes* strains by pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP). Syst. and Appl. Microbiol. 26, (4), 539.

Barbuti S. and Parolari G. 2002. Validation of manufacturing process to control pathogenic bacteria in typical dry fermented products. Meat. Sci. 62, (3), 323.

Chasseignaux E., Toquin M., Ragimbeau C., Salvat G., Colin P. and Ermel G. 2001. Molecular epidemiology of *Listeria monocytogenes* isolates collected from the environment, raw meat and raw products in two poultry and pork processing plants. J. of Appl. Microbiol. 91, (5), 888.

Chasseignaux E., Gerault P., Toquin M., Salvat G., Colin P. and Ermel G. 2002. Ecology of *Listeria monocytogenes* in the environment of raw poultry meat and raw pork meat processing plants. FEMS Microbiol. Lett. 210, (2), 271.

Comi G., Urso R., Iacumin L., Rantsiou K., Cattaneo P., Cantoni C. and Cocolin L. 2005. Characterisation of naturally fermented sausages produced in the North East of Italy. Meat Sci. 69, (3), 381.

Cordano A.M. and Rocourt J. 2001. Occurrence of *Listeria monocytogenes* in food in Chile. Int. J. of Food Microbiol. 70, (1-2), 175.

D'Agostino M., Wagner M., Vazquez-Boland J.A., Kutcha T., Karpiskova R., Hoorfar J., Novella S., Scortti A., Ellison J., Murray A., Fernandes I., Kuhn M., Pazlarova J., Heuvelink A. and Cook N. 2004. A validated PCR-based method to detect *Listeria monocytogenes* using raw milk as a food model-towards an international standard. J. of Food Prot. 67, (8), 1646.

De Cesare A., Bruce J.L., Dambaugh T.R., Guerzoni M.A. and Wiedmann M. 2001. Automated Ribotyping using different enzymes to improve discrimination of *Listeria monocytogenes* isolates with a particular focus on serotype 4b strains. J. of Clin. Microbiol. 39, (8), 3002.

De Cesare A., Mioni R. and Manfreda G. 2007. Prevalence of *Listeria monocytogenes* in fresh and fermented sausages and ribotyping of contaminating strains. Int. J. of Food Microbiol. 120, (1-2), 124.

Doumith M., Buchrieser C., Glaser P., Jacquet C. and Martin P. 2004. Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. J. of Clin. Microbiol. 42, (8), 3819.

European Food Safety Authority, European Centre for Disease Prevention and Control; The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2009. 2011. EFSA Journal 2011; 9 (3):2090. doi:10.2903/j.efsa.2011.2090. Available online: www.efsa.europa.eu/efsajournal.

European Commission, Regulation (EC) No. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. 2005. Off. J. Eur. U L 338, 1.

Farber J.M. and Peterkin P. 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol Mol. Biol. Rev. 55, (3), 476.

Fugett E.B., Schoonmaker-Bopp D., Dumas N.B., Corby J. and Wiedmann M. 2007. Pulsed-Field Gel Electrophoresis (PFGE) analysis of temporally matched *Listeria monocytogenes* isolates from human clinical cases, foods, ruminant farms, and urban and natural environments reveals source-associated as well as widely distributed PFGE types. J. of Clin. Microbiol. 45, (3), 865.

Gandhi M. and Chikindas M.L. 2007. *Listeria*: a foodborne pathogen that knows how to survive. Int. J. of Food Microbiol. 113, (1), 1.

Graves L.M. and Swaminathan B. 2001. PulseNet standardized protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulsed field gel electrophoresis. Int. J. of Food Microbiol. 65, (1-2), 55.

Graves L.M., Hunter S.B., Ong A.R., Schoonmaker-Bopp D., Hise K., Kornstein L., DeWitt W.E., Hayes P.S., Dunne E., Mead P. and Swaminathan B. 2005. Microbiological aspects of the investigation that traced the 1998 outbreak of listeriosis in the United States to contaminated hot dogs and establishment of molecular subtyping-based surveillance for *Listeria monocytogenes* in the PulseNet network. J. of Clin. Microbiol. 43, (5), 2350.

Greco M., Mazzette R., De Santis E.P.L., Corona A. and Cosseddu A.M. 2005. Evolution and identification of lactic acid bacteria isolated during the ripening of Sardinian sausages. Meat Sci. 69, (4), 733.

Ilsi Research Foundation-Risk Science Institute - Expert Panel on *Listeria monocytogenes* in foods, Achieving continuous improvement in reductions in foodborne listeriosis - a risk-based approach. 2005. J. of Food Prot. 68, (9), 1932.

Jofré A., Martin B., Garriga M., Hugas M., Pla M., Rodríguez-Lázaro D. and Aymerich T. 2005. Simultaneous detection of *Listeria monocytogenes* and *Salmonella* by multiplex PCR in cooked ham. Food Microbiol. 22, (1), 109.

Kim K.Y. and Frank J.F. 1995. Effect of nutrients on biofilm formation by *Listeria monocytogenes* on stainless steel. J. of Food Prot. 58, (1), 24.

López V., Villator D., Oertiz S., López P., Navas J., Dávila J.C. and Martínez-Suarez J.V. 2008. Molecular tracking of *Listeria monocytogenes* in an Iberian pig abattoir and processing plant. Meat Sci. 78, (1-2), 130.

Mafu A.A., Roy D., Goulet J. and Magny P. 1990. Attachment of *Listeria monocytogenes* to stainless steel, glass, polypropylene and rubber surfaces after short contact times. J. of Food Prot. 53, (9), 742.

Mc Lauchlin J., Mitchell R.T., Smerdon W.J. and Jewell K. 2004. *Listeria monocytogenes* and listeriosis: a review of hazard characterization for use in microbiological risk assessment of foods. Int. J. of Food Microbiol. 92, (1), 15.

Meloni D., Galluzzo P., Mureddu A., Piras F., Griffiths M. and Mazzette R. 2009. *Listeria monocytogenes* in RTE foods marketed in Italy: prevalence and automated EcoRI ribotyping of the isolates. Int. J. of Food Microbiol. 129, (2), 166.

Nesbakken T., Kapperud G. and Caugant D.A. 1996. Pathways of *Listeria monocytogenes* contamination in the meat processing industry. Int. J. of Food Microbiol. 31, (1-3), 161.

Public Health Agency Canada, Lessons Learned Report: The Canadian Food Inspection Agency's Recall Response to the 2008 Listeriosis Outbreak. 2009. Available online: http://www.phac-aspc.gc.ca/fs-sa/listeria/2008-lessons-lecons-eng.php.

Ross R.P., Morgan S. and Hill C. 2002. Preservation and fermentation: past, present and future. Int. J. of Food Microbiol.79, (1-2), 3.

Samelis J. and Metaxopoulos J. 1999. Incidence and principal sources of *Listeria* spp. and *Listeria monocytogenes* contamination in processed meats and a meat processing plant. Food Microbiol. 16, (5), 465.

Simon M.C., Gray D.I. and Cook N. 1996. DNA extraction and PCR methods for the detection of *Listeria monocytogenes* in cold-smoked salmon. Appl. Environ. Microbiol. (62), 822.

Stepanovic S., Cirkovic I., Ranin L. and Svabic-Vlahovic S. 2004. Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surfaces. Lett. in Appl. Microbiol. 38, (5), 428.

Tenover F.C., Arbeit R.D., Goering R.V., Mickelsen P.A., Murray B.E., Persing D.H. and Swaminathan B. 1995. Interpreting chromosomal DNA restriction patterns produced by Pulsed-Field Gel Electrophoresis: criteria for bacterial strain typing. J. of Clin. Microbiol. 33, (9), 2233.

Thévenot D., Delignette-Muller M.L., Christieans S. and Vernozy-Roland C. 2005. Prevalence of *Listeria monocytogenes* in 13 dried sausage processing plants and their products. Int. J. of Food Microbiol. 102, (1), 85.

Thévenot D., Delignette-Muller M.L., Christieans S., Leroy S., Kodjo A. and Vernozy-Roland C. 2006[1]. Serological and molecular ecology of *Listeria monocytogenes* isolates collected from 13 French pork meat salting-curing plants and their products. Int. J. of Food Microbiol. 112, (2), 153.

Thévenot D., Dernburg A., Christieans S. and Vernozy-Roland C. 2006[2]. An updated review of *Listeria monocytogenes* in the pork meat industry and its products. J. of Appl. Microbiol. 101, 7.

Tompkin R.B. 2002. Control of *Listeria monocytogenes* in the food-processing environment. J. of Food Prot. 65, (4), 709.

Vazquez-Boland J.A., Kuhn M., Berche P., Chakraborty T., Domingues-Bernal G., Goebel W., Gonzalez-Zorn B., Wehlamd J. and Kreft J. 2001. Listeria pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. 14, (3), 584.

Zhao T., Doyle M.P. and Zhao P. 2004. Control of *Listeria monocytogenes* in a biofilm by competitive-exclusion microorganisms. Appl. and Environ. Microbiol. (7), 3996.