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Evaluation of *Listeria monocytogenes* contamination in sheep's milk cheese-making plants

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CONTENTS

Abstract

The general aim of the thesis was to evaluate the pattern *Listeria monocytogenes* contamination in sheep's milk industrial cheese-making plants operating in the regional territory of Sardinian (Italy). Chapter 3 describes a longitudinal study conducted in 2 cheese-making plants over one year period. The objective of the study was to identify sources, sites of persistence and route of contamination within premises. Contamination mostly occurred in salting, product washing, packaging, ricotta salata storage and cheese ripening areas. The greater persistence of contamination over time was observed in washing, salting and cheese ripening areas. In Chapter 4 is presented a cross-sectional study on *Listeria* spp environmental contamination in 13 sheep cheese-making plants. The objective was to investigate the genetic diversity (population study) and the route of contamination (traceability) of *L. monocytogenes* strains. Strains originating from the processing environment were disseminated to other sites and food samples within and among facilities. In chapter 5 the potential pathogenicity of environmental and food isolates collected in cheese-making plants was studied by DNA sequencing. In particular the modification in nucleotide sequence of virulence factors hly, InlA and InlJ was investigated. *L. monocytogenes* environmental strains showed 90-100% of identity with reference sequences, suggesting their potential pathogenicity.

CHAPTER 1

Introduction

1.1 Taxonomy *Listeria spp*

1.1.1 The *Listeria* genus

The genus *Listeria* was first placed in the *Corynebacteriaceae* family, but today, thanks to the sequencing of ribosomal RNA (rRNA) it has been positioned between the *Bacillus* and the *Lactobacillus/Streptococcus* groups, within the *Clostridium-Lactobacillus-Bacillus* branch (Jay *et al.*, 2009).

The members of the genus are Gram-positive rods, anaerobic facultative, not sporulated and not encapsulated bacteria. They are catalase positive, oxidase negative and beta-hemolytic. *Listeria* is motile if cultured between 20°C and 25°C due to the presence of peritrichous flagella. The *Listeria* species grow at temperature ranging between 0-45 °C (Halter *et al.*, 2013). Although it is not able to grow below -1.5 °C, *Listeria* can survive at lower temperature. Optimal range for growth is between 30-37°C, while temperature >50°C is lethal (Rocourt and Buchrieser, 2007).

The genus *Listeria* comprises ten species: *Listeria monocytogenes, L. innocua, L. welshimeri, L. seeligeri, L. ivanovii* and *L. murray (subsp. grayi e subsp. murray)* (Rocourt, J. and Buchrieser, C., 2007), species which have long been known, and four new species that have been reported in the last years, *L. rocourtiae* (Leclercq *et al.*, 2010), *L. marthii*, (Graves *et al.*, 2010*), L. fleischmannii* (Bertsch *et al.*, 2012) and *L. weihenstephanensis* (Halter *et. al*, 2013). Of these species only *L. innocua* and *Listeria monocytogenes* are considered to be pathogenic. *L. innocua* is generally associated with cerebral infection in ewe and in cattle (Rocha *et al.,* 2013), even if a case of fatal bacteraemia in an old man caused by this specie was reported in 2003 (Perrin *et al.*, 2003). One case of human infection due to *L. seeligeri* and two cases due to *L. ivanovii*

(Rocourt *et al.*, 1987; Cummins *et al.*, 1994; Lessing *et al.*, 1994) have been reported in literature.

Listeria spp are widely distributed in natural environment (Sauders and Wiedmann, 2007) and can be recovered from different sources: soil, plants, animal feed, faces of animal ad humans, water surface, effluents, food processing environment, processed food (Gravani, 1999; McLauchlin *et al.*, 2004).

1.1.2 *Listeria monocytogenes*

According to the serotyping method developed by Seeliger and Höhne (Seeliger and Jones, 1986), based on the reaction of somatic (O) and flagellar (H) antigens with a series of antisera, *L. monocytogenes* can be differentiates into 13 serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b,3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7 (table n.1.1). Generally only serovars 1/2a, 1/2b and 4b are involved in human cases of listeriosis (Wiedmann *et al.*, 1997). The serotype 4b strains are generally linked with epidemic outbreaks while 1/2a and 1/2b are commonly associated to sporadic cases (McLauchlin, 1997).

Listeria monocytogenes consist of 4 evolutionary lineages: I, II, III, IV (Orsi *et. al.*, 2011). Strains can be ascribed to the lineages through different types of genotyping such as Pulsed-field gel electrophoresis (PFGE), rybotyping and multilocus sequence typing (MLST) Fugett *et al.*, 2006; Nadon *et al.*, 2001; Wiedmann *et al.,* 1997), and phenotypic approach, such as API *Listeria* profiles (; however to assign more accurately isolates to lineages it is advisable the use of DNA-sequencing method (Orsi *et. al*, 2011). The majority of the strains isolated from human disease are included in the lineage I (serotypes 1/2b and 4b) and II (serotype 1/2a). Human listeriosis outbreaks are generally associated with serotypes included in the lineage I while sporadic clinical cases seem to be more related with strains belonging to lineage II. Also strains that are

habitually isolated from food and environment are included in the lineage II. Strains belonging to lineage III and IV are rare and frequently isolated from animal sources (Orsi *et. al*, 2011).

1.2 Ecology of *Listeria monocytogenes*

1.2.1 Growth limits

Listeria monocytogenes is a ubiquitous pathogen widespread in the environment (Ragon *et al.,* 2008) that can be isolated from different sources: silage, vegetation, soil, sewage, stream water, mud, slaughter-house waste, milk of normal and mastitic cows, and faeces of animals and healthy humans (Farber and Losos, 1988; Farber and Peterkin, 1991).

The ecological characteristics of *L. monocytogenes* allow survival and growth under extreme environmental conditions that are generally hostile and lethal to other foodborne pathogens (Zarei *et al.*, 2012; Sleator *et al.,* 2003).

The optimum temperature for the growth of *Listeria monocytogenes* range between 30°C and 37°C, but in presence of nutrients it can growth at temperature between 0°C and 45°C (Farber and Losos, 1988). The ability to grow at low temperature allows the multiplication of the microorganism in foods kept refrigerated at 0-4°C (Chan and Wiedmann, 2009; Sergelidis and Abrahim, 2009). Furthermore, even if it cannot multiply at temperature below -1.5°C, the microorganism is able to survive at freezing temperature. Heat treatments applied in food processing using temperature over 50°C, such as high-temperature short-time (HTST) pasteurization, are effective in eliminating the presence of *L.monocytogenes* (Cava-Roda *et al*., 2012). However, in literature are reported cases of listeriosis outbreaks linked with milk correctly

pasteurized (Fleming *et al.*, 1985) and evidence of heat resistance to some extent of *L. monocytogenes* has been described (Sergelidis and Abrahim, 2009; Lin and Chou, 2004; Lou and Yousef, 1997). Several studies have demonstrated that the microorganism increases its resistance to heat treatments if previously exposed to temperatures above its maximum for growth (Hassani *et al.*, 2007; Lin and Chou, 2004; Pagán *et al.*, 1997).

Listeria monocytogenes can grow at values of pH ranging between 4.0 and 9.5 (Liu *et. al*, 2005). It seems to be moderately acid-tolerant (Lado and Yousef, 2007); this condition allows the resistance to the acid pH of the gastric content, permitting the oral transmission of *L. monocytogenes* (Wiedmann *et al.*, 1998). The resistance to low pH value decreases with the increase of the temperature (Lado and Yousef, 2007).

It was demonstrated that *L. monocytogenes* can survive for long time at water activity (a_W) values ranging from 0.790 to 0.860 (Johnson *et al.* 1988), and it is able to grow at a_w value of 0.900 (Lado and Yousef, 2007). However, the optimal growth is at $a_W \geq 0.970$ (Ryser and Marth, 1999).

With regard to tolerance to NaCl it is widely demonstrated that *L. monocytogenes* can multiply at NaCl concentration up to 10 % and can survive at values of 20% (Sutherland *et al.*, 2003; Seeliger, 1987; Mc.Loure, 1989). In a study of Liu *et al.* (2005) was observed a higher tolerance to NaCl; *L. monocytogenes* strains were submitted to incubation for 20h in a solution saturated at 40% and they were still able to grow. This tolerance seems to be related to the ability of the organism to adapt to osmotic stress accumulating intracellular solutes such as: glutamate, glutamine, aspartate, alanine and proline (Liu *et al.*, 2005; Patchett *et al.*, 1992).

Listeria monocytogenes is a facultative anaerobe; even if it is predominantly aerobic, it is also able to growth under microaerobic and aerobic conditions (Lungu *et al.*, 2009).

1.2.2 Biofilms and Niches

Biofilm is a complex organization of microorganisms attached to surfaces and protected by a matrix of their own synthesis that can be found in several natural and artificial environments (Sutherland, 2001). This matrix, called ―extracellular polymeric substance‖ (EPS), consist of a mix of polysaccharides, nucleic acid and proteins (Sauer *et al.,* 2007); EPS allows the adhesion of microorganisms and creates a protective structure around them. Throughout biofilms are present water channels by which nutrients, metabolites and waste products are interchanging (Sauer *et al.* 2007; Sutherland, 2001). Biofilms can harbour one or more species of bacteria (Shi and Zhu, 2009).

The formation of biofilm is a complex process that is conditioned by several factors, such as properties of adhesion surfaces (texture or roughness, hydrophobicity), cells surface (extracellular appendages and polymeric substance), environmental conditions (pH, temperature, nutrient components) and bacterial genetic regulation (Simões *et al*., 2010; Shi and Zhu, 2009; Donlan, 2002). According to Vlková *et al.* (2009) the formation process can be dived in three major steps: adherence of free planktonic microbial cells; colonization of the preconditioned surfaces; release of microbial cells from the biofilm structures or from the surface (figure 1.1).

Microorganisms growing in biofilms are physiologically and phenotypically different from planktonic cells of the same organism, being cells in biofilm more resistant to adverse environmental conditions (Sauer *et al.* 2007). In fact, biofilm provides protection from different factors such as nutrient deprivation, acidity, oxygen radicals, heat, drying, salinity, disinfectants and antimicrobial agents (Silva Meira *et al.,* 2011; Jefferson, 2004; Møretrø and Langsrud; 2004; Spoering and Lewis, 2001;

Mittelman, 1998) and encourage the colonization of niches (Jefferson, 2004). The increased resistance of bacterial community as consequence of microorganism interaction has been demonstrated (Morton and Gaylarde, 2001). This phenomenon is called "*quorum sensing*": biofilm population modulates its density and its gene expression accordingly; also the production of EPS seems to be related to the "*quorum sensing*" (Morton and Gaylarde, 2001).

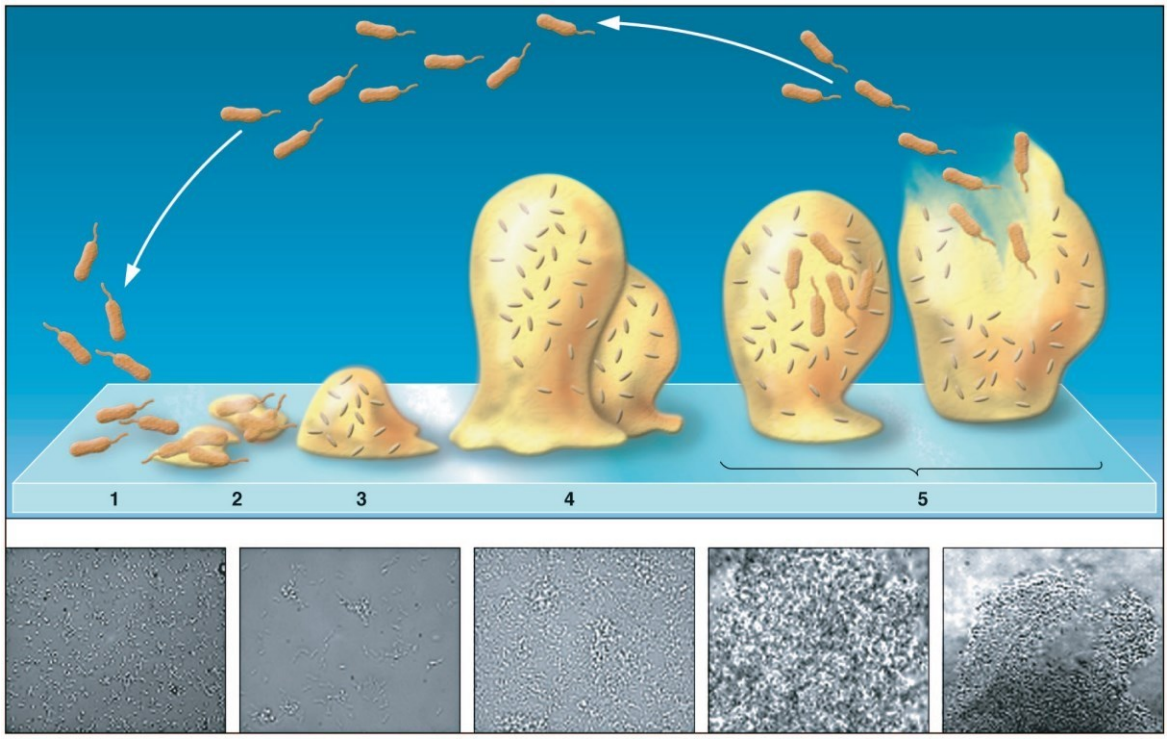

Figure n.1.1. Stages of biofilm development process: (1) reversible attachment, (2) irreversible attachment, (3) maturation-1, (4) maturation-2, and (5) dispersion (Sauer *et al*., 2007).

The presence of microorganism in biofilms formed on food-contact surface represents one of the main sources of contamination in food processing plants and the major cause of transmission of food-borne diseases (Bonsaglia *et al.*, 2014; Shi and Zhu, 2009).

The ability of *L. monocytogenes* to form biofilms has already been established (Mariani *et al.*, 2011; Chae *et al.*, 2006; Beresford *et al.*, 2001).

Listeria monocytogenes is able to adhere on most of the surface present in the food processing environment (Rieue *et al*., 2008): stainless steels, rubber and polymers (Beresford *et al.*, 2001),Teflon®, nylon, and polyester floor sealant (Blackman and Frank, 1996), wood (Mariani *et al.*, 2011) and glass (Chae *et al.*, 2006). The areas most frequently associated with *Listeria* biofilms are wet surfaces such as floor, floor drains, conveyer belts (Wong, 1998), storage tanks, trucks (Shi and Zhu, 2009) and parts that are difficult to clean such as joints, crevices and gaskets (Wong, 1998).

Several authors demonstrated that the presence or the absence of *Listeria* in the biofilm is conditioned by the resident microbial flora (Tompkin, 2002); for example it was demonstrated that the presence of strains of the genus of *Pseudomonas* (Sasahara and Zottola, 1993) and *Flavobacterium* strains (Bremer *et al.*, 2001) can increases the attachment of surfaces by *L. monocytogenes*, while Mariani *et al.* (2011) demonstrated that the presence in wooden shelves of an active resident microbial biofilm formed by *Lactococcus lactis* and hetero-fermentative lactobacilli have an anti-*Listeria* action. Also Zhao *et al.* (2006) and Loessner *et al.* (2002) showed how competitive bacteria (*Lactococcus lactis* and *Enterococcus durans* or *Lactobacillus plantarum*) could considerably decrease the contamination of *Listeria* spp. in the environment.

The adhesion of *L. monocytogenes* on the surface is also correlated with the serotype of the strains (Folsom *et al.* 2006). *Listeria monocytogenes* serotypes 1/2a and 1/2c strains belonging to the *Lineage II,* are generally considered more efficient in biofilm formation as compared to strains of serotypes 4b and 1/2b, belonging to the *Lineage I* (Nilsson *et al.*, 2011; Harvey *et al.*, 2007; Borucki *et al.*, 2003). However, other authors report opposite results (Norwood and Gilmour, 1999; Djordjevic *et al.*,

2002). Some studies highlight inter-strains variation which is not correlated with lineage or serotype but rather related to intrinsic properties of the single strain. Differences in adherence to surface were detected between persistent and non-persistent strains: persistent isolates from food processing environments exhibited enhanced ability to adhere and to form biofilm (Folsom *et al.*, 2006; Lundén *et al.*, 2000; Norwood and Gilmour, 1999).

Biofilm formation permits the survival of *L. monocytogenes* under various chemical and physical stresses (Harvey *et al.*, 2007; Chae *et al.*, 2006) and improves the opportunity of persistence in the environment (Di Bonaventura *et al.*, 2008; Chae *et al.*, 2006).

The ability of *L. monocytogenes* to attach to different materials has led some authors to consider the microorganism as able to populate and colonize environmental niches (Beresford, 2001). Niches are sites in the working areas difficult to clean and to disinfect with normal procedures (Carpentier and Chief, 2011; Tompkin, 2002), generally characterized by humidity, brines and low temperatures. These conditions are ideal for the growth of the microorganism. Niches, or harbourage sites, serve as reservoir sites from which *Listeria* can be transmitted from the environment to the finished products (Tompkin, 2002). Examples of harbourage sites are: floor drains, equipment sites difficult to access and to clean such as conveyors, cracks, junctions between equipment components, forklifts, equipment cleaning tools, on-off valves and switches for equipment (Tompkin, 2002). Within niches *L. monocytogenes* can survive and growth for months or even for several years (Unnerstad *et al.*, 1996).

1.2.3 Persistent contamination

The presence of *L. monocytogenes* in foods is not due to the survival of the microorganism to listericidal treatments but instead to a recontamination of the products along the post-processing equipment and environment (Senczek *et al*., 2000). It is well known that *Listeria* is widely distributed in food premises and that can be isolated from the majority of food processing environments (Kovacevic *et al.*, 2011). Even if hygienic conditions in industrial food production are developing and improving (e.g. high-risk design, cleaning and disinfection systems, employee food hygiene training) outbreaks of listeriosis due to contaminated foods are still an important issue at international level for consumers health (Harvey *et al.*, 2006; Holah *et al.*, 2004). One of the main reasons that make *Listeria* hard to eradicate is its ability to persist in the food processing environment, allowing the pathogen to survive within the production areas over time (Holah *et al.*, 2004; Tompkin, 2002). The evidence of *L. monocytogenes* persistence has been widely reported and authors demonstrated that the microorganism can resist for periods of time that raging from few months up to several years in different kind of food premises: cheese, raw and smoked fish, mussels, pate, fresh, cooked and fermented meats, pesto sauce etc. (Unnerstad *et al.*, 1996; Holah *et al.*, 2004; Tomking, 2002; Senczek *et al.*, 2000; Keto-Timonen *et al.*, 2007). *L. monocytogenes* strains are considered to be persistent when isolated several times in the same processing plants in several sampling visit (Carpentier and Cerf, 2011). There is discordance between authors in the definition of number of visit, isolation and period that are necessary to indicate a strain as persistent. In their review Carpentier and Cerf (2011), according with data showed by Ragimbeau (2002), defined persistence a strain ―isolated on at least three sampling dates in a one-year period‖. However Pan *et al.* (2006) pointed that in some circumstances the isolation from the environment of the same strains over time

can be caused by a continuous introduction of the contamination in the plants from outsides sources. It is not fully clear the mechanisms that permit a strain to became persistent; properties that influence the possibility of *L. monocytogenes* to persist are: the ability to form biofilm, to establish into niches, to grow at low temperatures and to resist to sanitizers (Pan *et al.*, 2006). It is important to underline that the relationship between the presence of persistent strains in food processing plants and listeriosis outbreaks has already been documented (McLauchlin *et al.*, 2004; Tompkin, 2002).

1.3 Infection and Disease

1.3.1 Listeriosis

Listeria monocytogenes is the causative agent of a foodborne illness referred to as listeriosis. Listeriosis is one of the most important foodborne disease (Schneider *et al.*, 2009) and the Centers for Disease Control and Prevention (CDC) has stated that it is the 3rd leading cause of death from food poisoning.

It is generally considered a sporadic disease (Allerberger and Wagner, 2010; Swaminathan and Gerner-Smidt, 2007). However some authors agree that in some countries (especially in Asia) its incidence might be underestimated. This may be due to the fact that the cases of listeriosis are underreported because of lacks in the surveillance system, for a deficiency of the mandatory notification system and for the absence of diagnostic test capable to diagnose the illness (Barbuddhe *et al.*, 2004; Siegman-Igra *et al.,* 2000).

Listeriosis has an incidence that can vary between 0.1 and 11.3 cases for 1,000,000 populations per year (WHO/FAO 2004; EFSA, 2014; Mead *et al.*, 1999). It is responsible of an average case-fatality rate of 20-30% (Farber and Peterkin 1991; Mead

et al., 1999; WHO/FAO 2004) and shows the highest hospitalisation rates (91%) compared to other food-borne pathogens (EFSA, 2014; CDC, 2013; Jemmi and Stephane, 2006; WHO, 2004). From 1996 to 2001 in USA was reported a decrease of Listeriosis cases by 24%, after which a relatively stable incidence rate was observed (CDC, 2013). A total of 1,642 cases of listeriosis have been reported from 26 European Union Member States in 2012 , showing an increased rate (10,5%) compare to 2011 (EFSA, 2013). Several studies and reports indicate that this increase is due mainly to a rise of cases in adults aged ≥65, regardless other predisposing conditions (EFSA 2014; CDC, 2013; Goulet, 2008).

Listeriosis is universally recognized as a foodborne disease (Rocourt and Buchrieser, 2007). However cases of human listeriosis occurred in farmers and veterinarians (cutaneous *Listeriosis*) due to direct transmission from animals to humans have been reported (Bortolussi and Mailman, 2010; Posfay-Barbe and Wald, 2009); vertical transmission from mother to foetus (Allerberger and Wagner, 2010) and also nosocomial cases were reported (Graham *et al*., 2002; Simmons *et al*., 1986).The first documented case of nosocomial outbreak was a cross-infection occurred in a neonatal unit due to contact with contaminated mineral oil used to bath infants (Schuchat, 1991).

The importance of the disease is also related to the population at risk; in fact listeriosis affects primary the elderly, neonates, pregnant women and people with pre-existing medical conditions such as immunocompromised (i.e. individual infected with the human immunodeficiency virus, HIV) or those receiving immunosuppressive treatments (e.g. cancer, transplantation) or affected by other underlying diseases (CDC; Doganay, 2003). Other predisposing conditions for listeriosis are represented by diabetes, alcoholism, liver and kidney diseases, drug addiction (Bortolussi and Mailman, 2010; Schlech, 2000). Listeriosis can also occur in healthy patients (30% of

adults and 54% of children and young adults) not showing such risk factors (Doganay, 2003). In 2006 Kalyani *et al.* presented a case of meningitis caused by *Listeria monocytogenes* in a 17-years-old immunocompetent patient. In pregnant women the possibility of contracting the disease after consumption of food is 12 times higher as compared to the general population (Hof, 2003). Generally the mother present mild flu-like illness (Swaminathan and Gerner-Smidt, 2007) but the bacteria can induce a placentitis and infects the foetus because its immune system is still not sufficiently developed (Hof, 2003). The foetus can contract the infection in utero for the passage of *Listeria* through the placental barrier or, alternatively, the mother can contaminate the respiratory tract of the baby during the passage through an infected birth canal (Allerberger and Wagner, 2010; Schlech, 2000). The results of the infection can be foetal distress, miscarriage, death or premature birth of a severely ill infant and in the case of contamination during the childbirth, infants can present meningitis up to 2 or 3 weeks after exposure (Schlech, 2000). The CDC (2013) reported that in the USA one-third of reported human listeriosis cases occur during pregnancy, while McLauchlin *et al*. (2004) indicated that pregnancy and neonatal *Listeria* disease account for 10–20% of UK cases. It estimated that 22% of pregnancy-related cases of listeriosis lead to the death of the baby (NHS, 2013).

1.3.2 Pathogenesis and virulence factors

Listeria monocytogenes is a facultative intracellular pathogen and its virulence depends on the ability to adhere and enter into host cells. The processes of infection can be divided in few crucial steps: a) entry of the bacterium into the host; b) adhesion and invasion of host cells; c) lysis of the phagosomal vacuole; d) multiplication in the

cytosol; e) direct cell-to-cell spread using actin-based motility (Jemmi and Stephane, 2006;Vazquez-Boland *et al*, 2001).

Human mainly contract the infection through the ingestion of contaminated food, therefore the entry of *L. monocytogenes* in the host is generally represented by the gastrointestinal tract (Vazquez-Boland *et al*., 2001). In the stomach the bacteria is subjected to the action of proteolytic enzymes, hydrochloric acid and bile salts (Liu *et al*, 2007). Due to the action of stress- response genes that are under the control of the sigma factor, *sigma B* (σ^B) (Abram *et al.*, 2008), *L. monocytogenes* is able to survive. As consequence of the acid environment of the stomach, the concentration of the ingested microorganisms can be partially reduced (McLauchlin *et al,* 2004; Vazquez-Boland *et al*., 2001). Several authors reported that treatments with antiacids which neutralize the stomach acidity increased the susceptibility to the infection (Cobb *et al.*, 1996; Schuchat *et al,* 1992).

After passing through the stomach *L. monocytogenes* invades the small intestine (Vazquez-Boland *et al*., 2001). Once in the intestine, the entry into intestine epithelial cells is mediated by the interaction of *L. monocytogenes* surface proteins with host cells receptors. The surface proteins are named internalines and are encoded by different internaline genes (*inl*), (Bortolussi and Mailman, 2010; Jemmi and Stephane, 2006). Internalines are important virulent factors taking part in the invasion and colonization of intestinal epithelium; *L. monocytogenes* have 25 genes encoding for putative surface-associated internalines (Bierne *et al.*, 2007). InlAB, InlC, InlD, InlE, InlF, InlG, InlH and InlJ are the more important virulence factors (Vazquez-Boland *et al*., 2001). InlA and InlB are the best characterised and the ones that show the main role with internalization in the intestinal fase (Bierne *et al*., 2007; Liu *et al*, 2007). The InlA and InlB (encoded by the *inlAB* operon) interact respectively with the E-cadherin receptors

and with Met receptors present on human epithelial cells. The interaction internaline/receptor determines an invading strategy typically referred as –zipper mechanism'' (Disson and Lecuit, 2013; Vazquez-Boland *et al*., 2001). Another important internalin is the InlC that is one of the most important targets of the humoral immune response against *L.monocytogenes* in humans (Bierne *et al.*, 2007). The full function of the InlC is not clear but it seems to play a key role when the microorganism is already inside the host cell and during the spread from one cell to another. The InlJ instead has an important role in crossing the intestinal barrier and is involved in the successive stages of the infection (Sabet *et al*., 2005). The InlF increases the host cell binding and entry in cells such as fibroblasts and hepatocytes (Kirchner and Higgins, 2008).

Once inside the host cell the microorganism is entrapped into a vacuole (or phagosomes), which *Listeria* is able to escape from. Then it can replicate in the cytosol of the host cell (Disson and Lecuit, 2013; Schnupf and Portnoy, 2007; Vazquez-Boland *et al*., 2001). Disruption of phagosomes is mediated by the secretion of pore-forming proteins listeriolysin O (LLO, a haemolysin encoded by the gene *hlyA*), and two phospholipase C (PlcA and PlcB, encoded respectively by the gene *plcA* and the gene *plcB*). These proteins are essential for the survival and the intracellular growth of *Listeria* (Jemmi and Stephane, 2006; Hof, 2003). Once in the cytosol *Listeria* induces the production of actin and the formation of protrusion in the apical part of the host cells; this extrusion penetrates the neighbouring cells allowing the passage of the bacteria from infection to uninfected neighbour cells (Cossart and Toledo-Arana, 2008; Hof, 2003; Vazquez-Boland *et al.,* 2001). The direct cell-to-cell spreading allows the dissemination of avoiding the host cells defences (Disson and Lecuit, 2013; Cossart and Toledo-Arana, 2008; Portnoy *et al.*, 2002). The genes encoding for virulence factors of

L. monocytogenes are regulated by a transcriptional regulatory factor, the positive regulatory factor A (PrfA), and they are physically located in a chromosomal island (Vazquez-Boland *et al.,* 2001) called Virulence Gene Clusters (pVGC) or *Listeria* pathogenicity island 1 (LIP 1). Strains that present deletion or absence of the PrfA are considered avirulent (Posfay-Barbe and Wald, 2009).

Listeria monocytogenes, after the invasion of the enteric cells, reaches mesenteric lymph nodes, the spleen, and the liver through the lymphatic system and the bloodstream (Vazquez-Boland *et al,* 2001). At this time in the liver polymorphonuclear neutrophils are recruited to eliminate the pathogens and the infected cells, causing micro abscess formation (Vazquez-Boland *et al,* 2001); after some days neutrophils are replaced by monocytes together with lymphocytes to form granulomas. Since most of the microorganisms are internalized into the hepatocytes, in the attempt to eliminate the infection, these cells undergo autophagy to destroy internalised *L. monocytogenes* (Sleator *et al.,* 2009). Generally in the majority of the cases, such as in healthy individuals, the infection is resolved in the initial phase of invasion (McLauchlin, 2004) with the presence of only gastrointestinal symptoms (Schneider, 2009). If the infection is not properly counteracted at liver and spleen level, as in the case of immunocompromised individuals, the microorganism can cause a bacteraemia leading to the invasion of other organs. The target organs are the central nervous system (CNS) and the placenta (Lecuit, 2007; McLauchlin, 2004; Vazquez-Boland *et al*., 2001). *Listeria monocytogenes* is able to enter and proliferate not only in enterocytes and hepatocytes, but also in the phagocytes cells, i.e. polymorphonuclear, granulocytes and macrophages (Orndorff, 2006); with this mechanism *Listeria* is able to avoid antibodies, complement, or neutrophils and to be disseminated throughout the body with the bloodstream (Vazquez-Boland *et al*., 2001).

1.3.3 Listeriosis signs and symptoms

There are two forms of listeriosis: a non-invasive gastrointestinal listeriosis and an invasive listeriosis. The gastrointestinal form is more frequent in immunocompetent individuals and generally causes a self-limiting febrile gastroenteritis (Allerberger and Wagner, 2010; Vazquez-Boland *et al*., 2001) with nausea, vomiting and diarrhoea being the more common symptoms, occurring about 24-48 h after the ingestion of the contaminated food (Doganay, 2003). Manifestation of the invasive listeriosis, usually associated with the population at risk, can include septicaemia or meningitis and encephalitis in immunocompromised and old individuals, abortion in pregnant women and generalized infections in infants (Allerberger and Wagner, 2009; Barbuddhe *et al.*, 2004). The population at risk of *Listeria* infection presents the symptoms after 3-70 days from the exposure (Lecuit, 2007). Meningoencephalitis symptoms are fever, intense headache, nausea, vomiting and signs of meningeal irritation such as nuchal rigidity, movement disorders such as tremor, ataxia and seizures disorders (Allerberger and Wagner, 2009; Doganay, 2003). Among bacterial meningitis *Listeria* account for 11% of all cases and has the highest mortality rate, close to 22% (Lecuit, 2007).

Infection in pregnant women occurs mainly in the third trimester of pregnancy when the immune defences are low, with the mother presenting non-specific symptoms (Allerberger and Wagner, 2009), very similar to a flu: fever (often above 39°C), headache, myalgia, arthralgia and malaise (DiMaio, 2000); in some cases the infection can be asymptomatic (Doganay, 2003). Infection with *L. monocytogenes* during pregnancy can result in miscarriage or stillbirth (40-50%), preterm birth (less than 35 weeks in approximately 70% of cases) and neonatal infection (Golos *et al.,* 2013; Bortolussi and Mailman, 2010). The placenta presents chorioamnionitis, villitis,

vasculitis, fibrinoid necrosis, thrombosis and placental abscesses (Golos *et al.,* 2013; Lecuit, 2007). In the foetus *L. monocytogenes* infection (or granulomatosis infantiseptica) microabscesses and granulomas are detected in various organs such as liver, spleen, brain and skin (Hof, 2003; Lecuit, 2007).

In newborns two clinical forms are distinguished: an early-onset form that occurs within the first week of life, and a late-onset form that can appear from several days to weeks after birth (Delgado, 2008). The early-onset form infants present mainly sepsis-like manifestations but can also show respiratory distress, pneumonia and, more rarely, meningitis or myocarditis, while meningitis is more common in the late-onset forms (Posfay-Barbe and Wald, 2009).

Cases of local infection are reported especially in farmers and veterinary after contact with infected animals: papules and pustules appear on the arms and the hands with associated general symptoms such as fever, myalgia and headache (Regan *et al.,* 2005; Posfay-Barbe and Wald, 2009). Generally the symptoms are successfully resolved, but sometimes the infection can generalize and led to invasive listeriosis (McLauchlin and Low, 1994).

Other clinical manifestations of listeriosis have been reported in literature: aortic aneurysms, prosthetic joints and brain abscesses (Cone *et al*., 2008), endocarditis and myocarditis (Lecuit, 2007), conjunctivitis, lymphadenitis, cholecystitis, peritonitis, pleuropulmonary infection, joint infection, osteomyelitis, arteritis, necrotizing fasciitis and endophthalmitis (Allerberger and Wagner, 2009).

The incubation period is variable: for serious forms of listeriosis ranges from a few days up to three weeks, while for gastrointestinal forms in healthy people ranges from 12 hours to two days (CFSPH, 2005; FDA, 2012).

1.3.4 Infective dose

The dose necessary to cause the invasive listeriosis depends on a number of factors, including the virulence of the microorganism, the number of cells ingested, the host susceptibility and its immune status, and the characteristics of the food matrix (FDA, 2003).

The minimum dose required to cause clinical infection in humans has not been exactly determined. The long incubation period that occurs in listeriosis cases rarely allows the testing of the food responsible for the infection (CFSPH, 2005). However, several studies indicate that it is necessary a high dose to cause infection: microbiological test in foods implicated in epidemic and sporadic cases detected levels of *L. monocytogenes* between 10^2 to 10^6 cfu/g of ingested product (Posfay-Barbe and Wald, 2009; Jemmi and Stephane, 2006; Vazquez-Boland *et al*, 2001).

Foods in which the levels do not exceed 100 cfu/g seems to have no effect on healthy people, but in people with associated risk factors a dose of 1000 cells can be enough to cause illness (CFSPH, 2005; FDA, 2003).

1.4 *Listeria monocytogenes* in foods

1.4.1 International microbiological food safety criteria for *Listeria monocytogenes*

In the European Union the microbiological food safety criteria concerning *L. monocytogenes* are provide by Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. The microbiological criteria define the acceptability of foodstuff products placed on the market; if the criteria are not met, products must be withdrawn or recalled.

According to Article 4 of Regulation (EC) No 852/2004, food business operators are responsible for the compliance with microbiological criteria. Food safety criteria for *L. monocytogenes* are indicated in the Annex I of the Regulation where are also indicated the food category, sampling plan, microbiological limits, analytical methods and stage where the microbiological food safety criteria is to be applied.

Ready-to-eat (RTE) foods are the food category that should be tested for *L. monocytogenes* as indicated in the annex. Among RTE foods the Regulation distinguish in three categories:

- RTE foods intended for infants and ready-to-eat foods for special medical purposes: the criteria is applied for products placed on the market during their shelf-life and contemplates the absence in 25 g (n = 10, c = 0; where n = number of units comprising the sample; c = number of sample units giving values between m and M). The reference analysis method indicated by the Regulation is the EN / ISO 11290-1.

- RTE foods able to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes**:** for products placed on the market during their shelf-life is contemplated a limit of 100 ufc/g (n = 5, c = 0), if the manufacturer is able to demonstrate that the product will not exceed the limit of 100 cfu/g throughout the shelf-life. The operator may fix intermediate limits during the process that should be low enough to guarantee that the limit of 100 cfu/g is not exceeded at the end of the shelf-life. The reference analysis method indicated by the Regulation is the EN / ISO 11290-2.

Before products have left the immediate control of the food business operator when no evidence can be provided to demonstrate that the product will not exceed the limit of 100 cfu/g throughout the shelf-life, the absence criteria in 25 g (n = 5, c = 0) is applied. The reference analysis method indicated by the Regulation is the EN / ISO 11290-1.

- Ready-to-eat foods unable to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes**:** foods in this category include products with pH ≤ 4.4 or $a_W \leq 0.92$, or pH ≤ 5.0 and $a_W \leq 0.94$ and those with a shelf-life of less than 5 days. If they are placed on the market during their shelf-life is set a limit of 100 ufc/g (n = 5, c = 0). For these products, placed on the market during their shelf-life is expected a limit of 100 cfu/g (n = 5, c = 0). The reference method of analysis of is the EN / ISO 11290-2.

In the United States the responsibility for food regulation are shared by the Food and Drug Administration (FDA) and United States Department of Agriculture (USDA). Food Drug Administration (FDA) applies a "*zero-tolerance*" policy for *L. monocytogenes* in RTE foods. Since 1995 with a sentence of United States District court decision, *United States vs. Union Cheese Co.*, the detection of any *Listeria monocytogenes* in either of two 25g samples of a food renders the food adulterated (Federal Food, Drug, and Cosmetic Act. 21 U.S.C. 342). Similar policy is applied by the Food Safety and Inspection Service (FSIS) branch of the USDA responsible for controls on meat and poultry products and certain egg products (Federal Meat Inspection Act and the Poultry Inspection Act, 21 U.S.C).

In Canada the *Listeria* policy for RTE foods is based on the application of Good Manufacturing Practices (GMP) and on the HACCP principle. The control of the risk for *Listeria* contamination was developed using a health risk assessment approach, taking into account the combination of several factors such as inspection, environmental sampling and finished products testing. The Canada's authority classified RTE foods in two categories based on the health risk (Canadian Food Inspection Service, 2011):

- Category 1: RTE products in which the growth of *L. monocytogenes* can occur. These should receive the highest priority for industry control, as well as regulatory inspection and compliance activities. The presence of *L. monocytogenes* in these Category 1 ready-to-eat foods will likely trigger a Health Risk 1 concern. Limit expected: absence in 125 g on 5 sample units of 25 g each;

- Category 2: include two subgroups: 2a) RTE products in which limited growth of *L. monocytogenes* to levels not greater than 100 CFU/g can occur before the end of the stated shelf-life; and 2b) RTE food in which the growth of *L. monocytogenes* cannot occur throughout the expected shelf life. These products should receive a lower priority with regards to industry control, as well as regulatory inspection and compliance action. Limit expected: 100 cfu/g enumeration in 50 g on 5 sample units of 10 g each.

1.4.2 Ready-to-eat foods

The Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) in 1999 published data that supported the idea that *L. monocytogenes* can contaminate almost all food categories. Listeriosis infection are associated with meat

products (Selby *et al.,* 2006), dairy products (Leite *et al.*, 2005), fish products (Jallewar *et al.*, 2007) and vegetables (Crepet *et al.*, 2007).

In the last years many categories of food have been linked with listeriosis; however the majority of the cases were due to the consumption of RTE (Garrido *et al.,* 2010; Uyttendaele *et al.,* 2009). RTE foods are defined by the EC Regulation No. 2073/2005 as ―products that are intended by the producer for direct consumption by humans without the need for thermal treatment such as cooking that will eliminate or reduce to acceptable level micro-organisms of concern‖. Some examples of RTE foods include: soft cheeses such as ricotta, brie, feta, blue-veined (e.g. Gorgonzola) and Mexican-style soft cheeses such as queso fresco; hot dogs, luncheon meats, cold cuts; sandwiches; pâtés/meat spreads; refrigerated smoked seafood products such as smoked salmon; deli-type salads such as coleslaw, macaroni, tuna; pre-packed raw vegetables and mixed raw vegetable salad and pre-cut fresh fruits. The application of heat treatment during food processing can reduce the risk of *L. monocytogenes* contamination in the finished product. However, the re-contamination of RTE cooked can occur during post-processing steps (Zhu *et al.*, 2005). After recontamination, in the presence of favourable conditions, *Listeria* can multiply in the product to level representing a potential risk to human health (EFSA, 2013).

A survey on the prevalence of *L. monocytogenes* in RTE at the end of their shelf-life from retail markets, conducted by the European Food Safety Authority (EFSA) during 2010-2011 in 26 EU states members, showed a prevalence of contamination of 10.3 % in fishery products, of 2.07% in meat products and of 0.47% in cheese samples. The rate of samples exceeding the 100 cfu/g food safety limit imposed by the European Commission was 1.7 %, 0.43 % and 0.06 % for fishery products, for cooked meat and soft and semi-soft cheeses samples, respectively. Although the rate of

samples exceeding 100 cfu/g was very low, they still represent a serious concern for public health due to the large distribution of such products in the market.

The Food Drug Administration (FDA, 2003) reported that in the United State 90% of the listeriosis cases were linked to meat and dairy RTE products. Gombas *et al.* (2003) in a survey conducted in retails markets in the USA found prevalence of *L. monocytogenes* ranging from 0.17 to 4.7% in eight RTE food categories and levels of contamination from <0.3 MPN (most probable number) per g to 1.5 x 10^5 cfu/g.

1.5 *Listeria monocytogenes* in the dairy sector

Milk and dairy products are largely consumed in Europe (about 132 kg per person annually) and are consumed by all categories of people (Lunden *et al.*, 2004), including high-risk groups (European Commission, 2000).

In Europe dairy products have been implicated in approximately half of all cases of outbreaks and of large part of the sporadic cases of listeriosis (Lunden *et al.,* 2004). Most of the cases are linked to the consumption of raw milk and products made with unpasterizated milk. Cases of listeriosis have also been reported after the ingestion of dairy products obtained from pasteurised milk as consequence of post process contamination (Cumming *et al.*, 2008; Fleming *et al*., 1985).

1.5.1 Milk

The shedding of *L. monocytogenes* with milk of infected dairy ruminants is considered a rare finding. The sanitary state of the animals (encephalitis, abortions, mastitis) usually makes them unsuitable for milking. Level of the microorganism up to 10^4 cfu/ml in milk of infected animals have been reported; in some circumstances the

microorganism can be eliminated with milk of healthy animals for several months representing a great concern for public health (Ryser, 2011). The pasteurization applied for fluid milk, which has become a routine practice in USA and in Europe since the 1950s ensure the total destruction of *L. monocytogenes*. In the last 10 years an increase in the trade and consumption of raw (unpasteurized) milk was registered (Van Kessel *et al.,* 2004). The prevalence of *L. monocytogenes* contamination in raw milk is generally low and can vary from 0% to 6.5% (D'Amico and Donnelly, 2010; Vilar *et al*., 2007; Van Kessel *et al.*, 2004; Meyer-broseta *et al.,* 2003). Some authors (Hassan *et al.*, 2000) in a survey conducted in New York showed higher prevalence of contamination (12.7%). Contamination levels are generally below 0.1cfu/ml (Meyer-Broseta *et al*., 2003).

Two studies conducted on *L. monocytogenes* prevalence in sheep's milk showed a prevalence of contamination between 3.6 % (Rahim *et al.*, 2014) and 10-12.7% (Al-Tahiri *et al.,* 2008). MacDonald and Sutherland (1993) demonstrated that in whole sheep milk inoculated at concentration of 10^6 cfu/ml *Listeria* spp. can survive to heath treatment at 65°C for 15 min. Finding that suggest a protective effect of sheep's milk fat which was not reported in cow's and goat's milk. However *Listeria* is effectively killed by other treatments, e.g. high temperature short time pasteurization (72 °C for 15 s). Contamination of raw milk with *L. monocytogenes* is rarely due to mastitis or to faecal contamination, but it is generally caused by environmental contaminants such as feeds, e.g. spoiled silage, (Vazquez-Boland *et al*, 2001) and drinking water provided to the animals where the bacterium can proliferate (Venegas *et al.*, 2004).

Because of the ubiquitous nature of *L. monocytogenes* may be found in natural and urban environments; however it seems to be considerably more frequently isolated from farm environments (Sauders *et al.,* 2002).

Listeria monocytogenes strains isolated from raw milk belong to serotypes 1/2a, 1/2b, 4b, 3b and 4c (Van Kessel *et al.,* 2004).

1.5.2 Dairy products

Listeria monocytogenes in dairy products rarely presents level greater than 100 cfu/g (EFSA, 2010), however they have been linked with several cases of listerioris outbreaks reported worldwide (Almeida *et al.,* 2013), accounting for 35% of the all *Listeria monocytogenes* outbreaks (FDA, 2003). For this reason cheeses are included in the ―FDA Top Ten‖ riskiest foods report (CSPI, 2009). In table n. 1.2 are reported the outbreaks liked with *L. monocytogenes* in dairy products from 1983 to date (Health Canada, 2011; Marler Clark, 2014). It is included a recent outbreak occurred in USA caused by Ricotta salata cheese produced by a Sardinian dairy company.

The risk of *L. monocytogenes* contamination could involve both raw-milk cheeses and those made with pasteurised and micro filtered milk because post-process contamination is equally possible in these products (Rudolf and Scherer, 2001). Some authors reported that *L. monocytogenes* is most often detected in cheeses made from raw milk (Loncarevic *et al.*, 1995) while others observed an higher prevalence in cheeses made from pasteurized milk (Rudolf and Scherer, 2001), data confirmed by the EFSA report on *L.monocytogenes* contamination in cheeses (EFSA, 2013).

Soft-cheeses are frequently associated with listeriosis outbreaks; due to their characteristics of high water activity (> 0.920) and pH they are an excellent substrate for *Listeria* growth (Rudolf and Scherer, 2001). In particular mould-ripened (e.g. Stilton, Gorgonzola, Roquefort) and blue-veined (e.g. Brie and Camembert cheeses) are at risk because of the rise of pH during ripening. The pH of these cheeses arrive close the neutrality during ripening, enhancing the possibilities of survival and growth of *L.*

monocytogenes (Carminati *et al.*, 2004). Conversely fermented lactic cheeses seems to be less favourable for the microorganism since during the production the combination of competition with the starter culture, low water activity, and a low pH prevent to a certain level the survival and the growth of *L. monocytogenes* (Morgan *et al.,* 2001). The prevalence of *L. monocytogenes* reported for cheeses made from cows, sheep and goat milk is very similar comparing whole wheels or cheese cut into smaller wedges (Rudolf and Scherer, 2001; Loncarevic *et al.,* 1995). Data on the prevalence of *L. monocytogenes* contamination in cheeses reported in bibliography differs considerably depending on the cheese type. Rudolf and Scherer (2001) in a survey conducted on European red-smear cheeses and Loncaveric *et al.* (1995) in a survey conducted on various types of cheeses have reported a prevalence of contamination of 6.4% and 6%, respectively. In traditional soft cheeses made from raw ewe's milk in Portugal, Pintado *et al.* (2005) showed a prevalence of *L. monocytogenes* of 29% while Silva *et al.* (2003) demonstrated an occurrence of 26.7% of Minas-type cheeses. In blue-veined cheese rinds Bernini *et al.* (2013) revealed a prevalence of 55% with an increase in presence and levels associated with a longer ripening time.

1.5.3 Dairy processing environments

Outbreak investigations provide evidence indicating that *L. monocytogenes* contamination of pre-packaged industrial food products is not a result of the survival of the organism to processing operation, instead is due to recontamination originating from the processing environment (Widemann, 2003). In dairy product processing plants the use of heat treatments (pasteurization, thermization) on raw milk guarantee an inactivation of ca. 3-6 log cfu/ml (ICMSF. 1996).

The environmental contamination of the product is supported by the fact that even if the raw milk is found to be positive for the presence of *L. monocytogenes* the curd samples are usually negative (Cagri-Mehmetoglu *et al.,* 2011) and by the fact that the strains recovered in raw milk are not usually recovered in the environment or in the products (Pak *et al.,* 2002).

Listeria monocytogenes is widespread in several kind of environment, including natural and urban environment. The microorganism can be introduced into a food processing environment in a number of different ways and in any point of the processing chain (Ryser, 1999). The main sources of contamination are: raw materials, equipment and people. *Listeria* can be introduced in the food processing environment through the raw materials and subsequently contaminate foods and equipment if appropriate control measures are not in place (Almeida *et al.*, 2013). Employees or other people visiting food facilities can introduce *Listeria* into the processing environment via shoes, clothing and personal items. Authors showed levels of *L. monocytogenes* contamination of about 7% in the hands of food production workers (Kells and Gilmour, 2004). Lomonaco *et al.* (2009) detected *L. monocytogenes* contamination in the toilet and in the changing rooms, supporting the idea that the employers play an important role in the *Listeria* dissemination in the environment. Interestingly, the dairy processing plants that are associated with other processing plants (e.g. shared personnel, exchange of products) show a higher prevalence of positive samples respect to independent facilities (Fox *et al.*, 2011; Pak *et al.*, 2002).

Listeria monocytogenes presents characteristics that allow the survival in food processing plants: ability to grow at low temperature, adaptability to stress conditions (e.g. acidity, alkalinity and high salt concentration) and competition with other environmental microorganisms (Lou and Yousef, 1999). The dairy processing plants

environment is characterised by conditions which are particularly favourable to *L. monocytogenes* growth, e.g. refrigeration temperatures, moisture and humidity and presence of nutrition for the microorganism (Tompkin, 2002; Unnerstad *et al*., 1996).

Longitudinal studies demonstrated that *Listeria* contamination in food processing plants is related both to transient and persistent strains (Wiedmann, 2003). The persistence of *L. monocytogenes* it is of particular concern in the food industry (Kells and Gilmour, 2004). Various studies indicated that just a limited number of strains with similar genetic profile are found within the same food processing plant for several months or years (Keto-Timonen *et al*., 2007; Møretrø and Langsrud, 2004). Persistent strains in the environment can act as reservoir, providing a continual source for products contamination (Kells and Gilmour, 2004); this is demonstrated by the fact that persistent strains generally are the main cause for finished products contamination (Wiedmann, 2003). Niches that can harbour *L. monocytogenes* are sites generally hard to reach and clean (e.g. hollows part, cracks in flooring, worn gaskets) with the routine cleaning and sanitizing procedures.

Once *L. monocytogenes* become established in a niche the routine sanitizing operations are ineffective in eliminating the microorganism from the environment (Tomking, 1999), even though routinely cleaning and disinfection are implemented (Carpentier and Cerf, 2011); for this reason it is important to control the predisposing condition that allowed the creation of niches or that facilitate the diffusion of *Listeria*, as moisture, presence of nutrients, areas not accessible to cleaning (Kornacki, 2006). Fox *et al.* (2011) in a study on the prevalence of *L. monocytogenes* cheese making plants found that in 5 out 16 of the facilities the microorganism was not detected, even if the cleaning and sanitation procedures were the same applied by premises where *Listeria* was detected.

Studies on the prevalence of *Listeria* in cow's and sheep's milk cheese making plants showed a prevalence of contaminated sites ranging from 20% to 90% (Pritchard *et al*., 1995; Pilo *et al*., 2008; Parisi *et al.*, 2010; Ibba *et al*., 2013; Spanu *et al*., 2015) and a period of persistence up to 7 years (Unnerstad *et al.*, 1996). Several areas can harbour the microorganism, but the sites with the higher risk of contamination are those where the unpackaged food is exposed to direct food contact (e.g. conveyor belts, equipment and utensils), indirect contact (e.g. ceiling) or non-food contact surfaces (e.g. floors, drains, walls) sites between the lethality treatment and packaging areas (Tompkin, 1999). The sites more exposed to *L. monocytogenes* contamination are: floors, drains, walls, trolleys (Tompkin, 2002). According to several studies the site with the higher prevalence of contamination is the floor drain (Tompkin, 2002; Pilo *et al*., 2008; Parisi *et al.*, 2012; Cagri-Mehmetoglu *et al.,* 2011). For this reason they can be used as indicator site for the presence of *L. monocytogenes*, in food processing plant. Floor drains in fact represent an important source of dissemination of the contamination to other sites in the processing environment. For instance, during cleaning procedures (use of pressurized water) the microorganism could be spread on the equipment present in adjacent areas (Kells and Gilmour, 2004; Almeida *et al*, 2013). Another site that is frequently contaminated in cheese making plants and that can serve as an important source of contamination is the equipment used for cheese brushing and washing (Jaquet *et al.,* 1996; Almeida *et al*, 2013). The presence of *Listeria monocytogenes* in sites so close to the end of the production line gives further support to the post-processing contamination theory (Pritchard *et al*., 1995).

The use of molecular typing methods, such as pulsed-field gel electrophoresis (PFGE) and fluorescent amplied fragment length polymorfism (fAFLP), is important to identify the source of contamination, the niches of persistence and to trace

contamination patterns within premises (Parisi *et al.*, 2012). Knowledge on the pathways of contamination is an essential part in tracking action for controlling *L. monocytogenes* environmental contamination (Jadhav *et al*., 2012). Well-designed control and sanitation procedures, as sampling and cleaning programs, are important to decrease the incidence of *L. monocytogenes* and to avoid the diffusion of the contamination in the environment and in the products (Ryser *et al.*, 2011). While the harbourage sites are often recognised in the floor, to avoid cross contamination of the adjacent equipment, authors suggest that floor should be cleaned and disinfected before proceeding with the other sites (Carpentier and Cerf, 2011).

It is important to underline that processing plants in which the *L. monocytogenes* contamination is ascertained, also for long period, not always are implicated in listeriosis cases. The probability that cases of listeriosis occurs seems to be related to food contamination with more virulent strains (Tompkin, 2001).

The ubiquitous nature allows constant reintroduction of *L. monocytogenes* in the plant environment. Despite it is not realistic to expect that food premises can be continuously maintained free of *Listeria* contamination (Swaminathan and Gerner-Smidt, 2007), it is possible to control and prevent the food contamination avoiding the establishment of the microorganism in the environments and prevent the diffusion of the pathogen to other areas and sites. Tompkin (2002) suggests a *L. monocytogenes* control program for food processing environments consisting in six strategies: 1) prevent the formation of niches, 2) implement sampling program to assess the operation of the control program, 3) rapid and effective response when the results of the sampling are positive, 4) use of follow-up sampling to check the effective detection and elimination of the source of contamination, 5) short- term assessment to early detection of problems

and trends (last 4-8 sampling), 6) long-term assessment to evaluate improvement in the processing plants.

Sampling programs are the key to provide a continuous evaluation of the environmental contamination and for its control. Food business operator should establish their own *L. monocytogenes* monitoring program according with the plant, the products made, the processes which take place in and previous experience (Tompkin, 2001). Sampling operation should be performed during the processing operation, at least 2 hours after the beginning of the production, or at the end of the production before the cleaning procedure (Carpentier and Barre, 2012). In this manner the possibility to detect *Listeria* is increased. In fact, during the production activities, movements and vibrations of the tools and the equipment cause the detachment of *Listeria* cells present in biofilms and niches; in this way the microorganism is more accessible to sampling and it is possible to detect the presence of niches and biofilm (Tompkin, 2004). Another reason of the sampling during the processing phases is that after the cleaning procedure is possible to collect cells that are injured; they are still alive but non-culturable and then it is possible to have false negative sample (Carpentier and Barre, 2012). However, pre-operational samplings should be conducted since they are useful to verify the effective action of the cleaning and sanitation programs (Kornacki, 2012).

Understanding where *L. monocytogenes* and its niches are settled help to apply appropriate interventions aimed to prevent and to eliminate *Listeria* contamination. *L. monocytogenes* is the only species of the genus *Listeria* that has been involved in food-borne outbreaks, however the presence of any *Listeria species* in food premises is considered as a useful indicator of a decline of process hygiene conditions during food production (McLauchlin, 1997). Since all *Listeria* spp share the same ecological niches, the recovery of any *Listeria* species, also in absence of *L. monocytogenes*, is indicative

of the presence of favourable conditions that increase the risk of contamination with *L. monocytogenes* (Tompkin, 1999; Fox *et al.*, 2011). When environmental monitoring is conducted, it is strategic to find any type of *Listeria* as these act as markers for the likelihood of the presence of *Listeria monocytogenes* and allow taking preventive action for its establishment in the environment (Lakićević *et al.,* 2010).

The site of sampling has to be chosen according to historical data collected by the premises; it should include points that have been found to be good indicators for *Listeria* contamination and those that are known to harbour most frequently the microorganism (e.g. floor drains). The selection of areas and sites to be sampled should be risk based and should identify potential growth niches and points where there is a high potential for transfer *Listeria* contamination from one area to another (Carpentier and Barre, 2012; Kornachi, 2012). It is useful to use the zoning concept in order to track environmental contamination. Zones are defined based on the probability of product contamination if the microorganism is present in the zone. Based on the risk it is possible to divide the sampling area into 4 zones (Kornacki, 2012; FDA, 2014). The zone one includes direct and indirect product contact areas; these are areas of high risks in which are present favourable conditions for the growth of *Listeria* in surface close to the production line. Surfaces that can be include in the zone 1 are conveyors, sliders, utensils, racks, work table, valves, pipes that transports food, packaging material, hands, gloves. Contamination of zone 1 areas means that some product contamination is likely to have occurred. The zone two includes areas adjacent to Zone 1 where the product is processed and handled (equipment guard and framework). Zone 3 includes other surfaces within the production line, as floors, drains and walls that if contaminated with the pathogen, could lead to contamination of zone 1 and 2 via actions of employers or movement of equipment. Zone 4 includes remote areas not involved in processing as the

warehouse, locker room and break rooms etc. Most of the environmental samples collected during the sampling should be taken from zone 1-2, and to a lesser degree zone 3. Very few, if any, environmental samples should be taken from zone 4.

The number of sampling points and the sampling may be decided according to the data collected over time by the single company. The number is related to the complexity of the plant, of the production process and the food produced. Subsequently to repeat negative results the frequency of sampling for the site and area can be decreased (Tompkin, 1999).

A good sampling program should allow the assessment of *Listeria* contamination sites and to implement control strategies aimed to prevent potential contamination of the product before it becomes a problem for the human health (Tompkin, 2004).

TABLES

Table 1.1 Compositions of somatic (O) and flagellar (H) antigens in *Listeria monocytogenes* serotypes based on Seeliger & Jones (1986).

Serotype	O antigens	H antigens
1/2a	I, II	A, B
1/2b	I, II	A, B, C
1/2c	I, II	B, D
3a	II, IV	A, B
3b	II, IV	A, B, C
3c	II, IV	B, D
4a	(V), VII, IX	A, B, C
4b	V, VI	A, B, C
4c	V, VII	A, B, C
4d	(V), VI, VIII	A, B, C
4e	V, VI, (VIII), (IX)	A, B, C
7	XII, XIII	A, B, C
5	(V), VI, (VIII), X	A, B, C
6a	V, (VI), (VII), (IX), XV	A, B, C
6b	(V), (VI), (VII), IX, X, XI	A, B, C

Table 1.2: Listeriosis Outbreaks Related to Dairy Products

Year	Location	Invasive/ Non-invasive	Number of cases (deaths)	Foods	References
1983	U.S.A.	Invasive	49 (14)	Pasteurized milk	Fleming *et al.*, 1985
1983-1987	Switzerland	Invasive	122 (31)	Soft cheese	Bille, 1990; Büla *et al.*, 1995; Farber and Peterkin 1991
1985	U.S.A.	Invasive	142 (48)	Mexican-style fresh cheese	Anonymous, 1985; Linnan *et al.*, 1988
1989-1990	Denmark	Invasive	26 (6)	Blue mould cheese or hard cheese	Jensen *et al.*, 1994
1994	U.S.A.	Invasive	45	Chocolate milk	Proctor *et al.*, 1995; Dalton *et al.*, 1997
1995	France	Invasive	37 (11)	Raw milk soft cheese	Goulet *et al.*, 1995; Rocourt *et al.*, 1997; Lundén *et al.*, 2004
1997	France	Invasive	14	Soft cheeses	Jacquet *et al.*, 1998
1998-1999	Finland	Invasive	25 (6)	Butter from pasteurized milk	Lyytikäinen *et al.*, 2000
2000	Canada (MB)	Invasive	7	Flat whipping cream	Pagotto *et al.*, 2006; Clark *et al.*, 2010
2000-2001	U.S.A.	Invasive	13	Mexican-style fresh cheese	Boggs *et al.*, 2001; MacDonald *et al.*, 2005
2001	Sweden	Non-invasive	> 120	Raw milk fresh cheese made	Carrique-Mas *et al.*, 2003; Danielsson-Tham*et al.*, 2004
2001	Japan	Non-invasive	38	Washed-type cheese	Makino *et al.*, 2005
2002	Canada (BC)	Invasive	47	Cheese	Pagotto *et al.*, 2006
2002	Canada (PQ)	Invasive	17	Soft and semi-hard raw milk cheese	Gaulin *et al.*, 2003; Pagotto *et al.*, 2006
2002	Canada (BC)	Non-invasive	86	Cheese from pasteurized milk	Pagotto *et al.*, 2006
2003	U.S.A.	Invasive	13 (2)	Mexican-style fresh cheese	Carriedo, 2003; Swaminathan and Gerner-Smidt, 2007
2005	Switzerland	Invasive	10 (3)	Soft cheese	Bille *et al.*, 2006
2007	U.S.A.	Invasive	5 (3)	Pasteurized flavoured and non-flavoured milk	Cumming *et al.*, 2008
2008	Canada (PQ)	Invasive	40 (2)	Cheeses	Gaulin and Ramsay, 2010
2009-2010	Austria, Germany and Czech Republic	Invasive	34 (8)	Acid curd cheese	Fretz *et al.*, 2010a; Fretz *et al.*, 2010b
2008	U.S.A	Invasive	38	Pasteurized Cheese	CDC, 2013
2010	U.S.A	Invasive	5	Queso Fresco cheese	CDC, 2014
2012	Spain	Invasive	11 (1)	Cheese	Efsa, 2014
2012	U.S.A	Invasive	20 (4)	Ricotta Salata	CDC, 2014
2013	U.S.A	Invasive	6 (1)	Les Freres Cheese	CDC, 2014
2013	U.S.A	Invasive	8 (1)	soft or semi-soft cheese	CDC, 2014

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CHAPTER 2

Thesis project

Listeria monocytogenes is the causative agent of human listeriosis, a severe foodborne disease associated with a high case fatality rate. Listeriosis is the third leading cause of death from food poisoning (CDC, 2011). Listeriosis outbreaks are often associated with ready-to-eat foods that are generally consumed without any heat treatment (European Safety Authority, 2011). Dairy products and in particular cheeses have been often associated with outbreaks and sporadic cases of listeriosis. Despite the application of heat treatment during dairy product manufacturing (e.g. pasteurization), which have been proven to be effective to inactivate *L. monocytogenes*, the presence of the pathogen in the final product is not a rare finding. Contamination of pasteurized dairy products is mainly due to post process contamination (Silva *et al.*, 2003).

In Sardinia (Italy) one of the most important economic resources is the production and export of various type of cheeses made from sheep milk. Among these, Pecorino Romano protected designation of origin (PDO) and Ricotta salata are widely exported to many European and third countries, mainly in North America.

In the last years several recalls due to *L. monocytogenes* contamination of Ricotta salata, a whey protein cheese traditionally made in Sardinia (Italy) from sheep's milk, have been reported by USA and European authorities (RASFF, 2008), and in 2012 an important multistate outbreak in USA led to the hospitalization of 20 persons and to the death of 4 patients (FDA, 2012).

To prevent product contamination with *L. monocytogenes* it is crucial to understand *Listeria* contamination pattern in the cheese making processing environment.

The general aim of the thesis project was to investigate the pattern of *Listeria monocytogenes* contamination in sheep's milk industrial cheese-making plants operating in the regional territory of Sardinian (Italy), in particular with regard to

prevalence, sources, sites of persistence and route of contamination within cheese-making plants. A depth characterization of *L. monocytogenes* strains isolated from the cheese making plants environment and food products was conducted in order to investigate the relationship among strains (population study). Finally, the potential pathogenicity of isolates was studied by sequencing: alteration in nucleotide sequence of virulence factors was investigated.

The first contribution of the thesis (Chapter 3) is a study already published in the *Italian Journal of Food Safety* 2013, Volume 2 (2):109-112 entitled: ―Evaluation of *Listeria monocytogenes* environmental contamination in two sheep's milk cheese-making plants‖ which the candidate is first author. The present is a longitudinal study conducted on two Sardinian sheep's milk cheese-making plants over one year period. During this period both plants were visited 7 times to collect environmental samples from processing areas. The main objective of the study was to evaluate the prevalence of *L. monocytogenes* strains in each facility. Once identified the sites of *L. monocytogenes* contamination and the main contamination sources, the attention was focused on the identification of potential niches in the processing environment. With this aim the collection of environmental samples was conducted during the period that best represents the production fluctuation during the year. This allowed identifying areas with favourable conditions for *L. monocytogenes* growth and persistence assessing the relationship between *L. monocytogenes* strains persistence niches and dairy products contamination during the cheese making season. As expected the persistence of the contamination was observed in sites difficult to reach or to clean (e.g. brushes of cheese washing machine, wooden shelves of ripening rooms) or where conditions favourable to *Listeria* survival and growth persist such as humidity (e.g. floor drains in product washing areas), presence of salt (shelves and floor drains in salting areas), low

temperatures (Ricotta cold rooms) or presence of food debris. However, further investigation on strains isolated from sites with persistent contamination shall be conducted to differentiate whether contamination is due to the presence of persistent strains or a continuous reintroduction of *L. monocytogenes* from outer sources.

The second contribution to the thesis project (chapter 4) published in the scientific journal Food Control (2015) Volume 47: 318-325 entitled: ‒Occurrence and traceability of *Listeria monocytogenes* strains isolated from sheep's milk cheese-making plants environment‖ the candidate have co-authored. The present is a cross-sectional study (or prevalence study) conducted on 13 industrial cheese making plants representative of great part of sheep milk cheese production of Sardinia. Each cheese making plant was visited once to collect environmental and food sample to detect the presence of *L. monocytogenes*. The aims of the study were to investigate within each facility the occurrence of contamination (prevalence) by *Listeria* spp. and *L. monocytogenes* in the main niches in the processing environment and to identify the environmental contamination pathways of the microorganism (traceability). With this aim *L. monocytogenes* isolates were subtyped by PFGE allowing identify the possible routes of contamination. Restriction profiles of *L. monocytogenes* were also used to investigate genetic diversity of strains between and within cheese making plants (population study). Raw milk was excluded as possible source of contamination, confirming the processing environment as the origin of *L. monocytogenes* contamination. It was also possible to identify the main niches (floor drains), and cross contamination sites (cheese washing machine) in the processing environment and to trace the contamination downstream the processing line. The population study showed a wide variability of strains both within and among facilities. Plants characterized by

introduction of products from other processing facilities showed a grater variability of *L. monocytogenes* strains circulating in the processing environments.

The third contribution to this thesis (Chapter 5) is a genetic investigation of the potential pathogenic traits of *L. monocytogenes* strains. A selection of 100 strains isolated from Sardinian cheese making plants environment and cheeses available from the strains collection of the Department of Veterinary Medicine, Food Hygiene Sector, of the University of Sassari (Italy) was used for genetic analysis.

In order to assess the potential virulence of environmental *L. monocytogenes* isolates, strains were submitted to fluorescence amplified fragment length polymorphism (fAFLP) and the profiles obtained were compared with identical profiles of *L. monocytogenes* strains isolated from clinical cases of listeriosis provided by the foodborne pathogens reference services of the Public Health of England (UK). Overall 20 strains of environmental origin showed the same fAFLP pattern as strains of human listeriosis. Next Generation Sequencing (NGS) was carried out on the selected strains and the sequences obtained used to perform a preliminary investigation of virulence-related genes, i.e. internalin A (*inlA*), internalin J (*inlJ*) and hemolysin (*hly*). Sequences were aligned with *L. monocytogenes* references strains using BioEdit software version 7.2.5 (http://www.mbio.ncsu.edu/bioedit/bioedit.html**).**

Comparison of sequences showed that strains isolated from cheese making plants environment and from cheeses showed 90-100% of identity after alignment with reference sequences. Despite in literature is often indicated that strains of environmental and food origin could be characterized by low-virulence, the results of the present study indicate their potential pathogenicity. However, in order to better characterize the virulence pattern a wider range of virulence genes (i.e. *Acta, prfA, inlC, inlF* and *InlB)* should be investigated.

Due to time constraints the majority of the sequencing data obtained during the project research has not been included in the thesis as they need further analysis.

In a second phase all the 100 strains selected for the third contribution were sequenced. Also the human clinical strains with identic fAFLP, selected from the PHE archive, were sequenced.

Further prospective of the study is to use NGS data for the construction of phylogenetic trees (SNPs analysis) in order to better analyse the relationship and the genetic diversity within strains isolated from cheese making plant environmental. The data may be used to verify the potential use of NGS for tracking *Listeria monocytogenes* environmental contamination and to compare it to the typing methods used in the project (PFGE and fAFLP).

The sequences of strains from food and environmental samples will be compared with those isolated from human clinical cases.

In order to characterize the potential pathogenic power of the isolates, a panel of virulence genes will be investigated in strains from cheese making plants and from human clinical strains. The comparison of nucleic sequence of the virulence genes will allow assessing difference in virulence proprieties within clinical and non-clinical strains.

CHAPTER 3

Listeria monocytogenes contamination in dairy plants: evaluation of *Listeria monocytogenes* environmental contamination in two cheese-making plants using sheeps milk

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The contribution of the doctoral candidate to the paper presented in this chapter concerned several aspects of the experiment, from samples collection at the cheese-making plants and to the execution of the microbiological and molecular analysis, to the statistical analysis of the data. The candidate contributed also in manuscript writing.

***Listeria monocytogenes* contamination in dairy plants**

Evaluation of *Listeria monocytogenes* environmental contamination in two sheep's milk cheese-making plants

3.1 Abstract

Listeria monocytogenes harbouring niches established in the processing plant support post-process contamination of dairy products made from pasteurized or thermized milk. The present study investigated *L. monocytogenes* environmental contamination in two sheep's milk cheese-making plants. Persistence of contamination in the area at higher risk was also investigated. During a one year survey 7 samplings were carried out in each dairy plant, along the production lines of Pecorino Romano and ricotta salata cheese. A total of 613 environmental samples collected from food contact and non-food contact surfaces were analysed according to ISO 11290-1:2005 standard method. Identification of the isolated strains was carried out by PCR. *Listeria monocytogenes* prevalence was 23.2% in dairy A and 13.1% in dairy B, respectively. The higher prevalence rate was found in the following areas: salting, products washing, packaging, ricotta salata storage and Pecorino Romano ripening rooms. *Listeria monocytogenes* was never found in the cheese-making area. The probability of observing samples positive for the presence of *L. monocytogenes* was associated with dairy plant, sampling area and the period of cheese-making ($P<0.001$). The greater persistence of contamination over time was observed in the washing, salting, and Pecorino Romano ripening areas. The control of persistent environmental contamination relies on the identification of niches within the processing environment and the prevention of harbourage sites formation. The importance of strict cleaning and

sanitizing procedure in controlling environmental contamination is confirmed by the lower level of contamination observed after these procedures were correctly implemented.

3.2 Introduction

Raw milk contaminated with *L. monocytogenes* could represent a potential source for the introduction of the pathogen in dairy products processing plant environment. The prevalence of positive raw milk samples ranges between 3.0 and 6.5% (Vitas *et al*., 2004; Al-Tahiri and Rewashdeh, 2008), with estimated most probable concentration of 0.1 colony forming units/mL (Meyer-Broseta *et al*., 2003). The application of thermization and pasteurization processes in sheep milk cheese-making plants results in a 3 to 6 log reduction of the number of viable *Listeria monocytogenes* cells present in raw milk (ICMSF, 1996, Pearce *et al*., 2012).Therefore, the presence of *L. monocytogenes* in dairy products is mainly ascribed to a post-process contamination originating from the processing environment (Tompkin, 2002; Unnerstad *et al*., 1996). Contamination is generally associated with the presence of resident strains that colonize specific niches in processing plant environments (Blackman and Frank, 1996). Niches are represented by areas with favourable conditions for *L. monocytogenes* growth and persistence, such as moisture and condenses, low temperatures and high NaCl concentrations (Tompkin, 2002). Moreover, the ability of *L. monocytogenes* to adhere on surfaces and form biofilms allows the microorganism to adapt and resist against detergents and sanitizers (Chavant *et al*., 2004; Pan *et al*., 2006). The evaluation of *L. monocytogenes* environmental contamination is essential, especially in cheese-making plants producing foods able to support *L. monocytogenes* growth or that are exposed to the environment after lethality treatments are applied (EC, 2005;

USDA/FSIS, 2006; Health Canada, 2011). In Sardinia milk production and cheese-making are seasonal and gathered during the winter-spring period, which is referred to as cheese-making season. The aim of the present research was to evaluate the prevalence of *L. monocytogenes* environmental contamination over the cheese-making season in two Sardinian sheep's milk cheese-making plants. The relationship between *L. monocytogenes* persistence niches and dairy products contamination during the dairy season was also assessed.

3.3 Materials and methods

Environmental samples were collected from processing areas and equipment in two sheep milk cheese-making plants located in Sardinia. In order to take into account seasonal variations each dairy plant was visited 7 times during the cheese-making season. Three visits were conducted during the peak of production (January-June), 2 visits during the break in cheese-making production (September-October) and the other 2 at the beginning of cheese-making season (November-December). Overall were collected 613 environmental samples. During each visit environmental samples were collected along production lines of ricotta cheese, Pecorino Romano and in environments shared by these products, such as areas of coagulation, ricotta production, moulding, salting, washing, ripening and packaging. Environmental sampling included food contact surfaces (tables, carts, shelves, moulds, machinery) and non-food contact surfaces (outer surfaces of equipment, walls, floors and floor drains). Sampling was conducted during processing operation using sterile sponges pre-moistened with neutralizing buffer (3M, St. Paul, Minnesota, USA.). In order to increase the likelihood of detecting no delimitation of sampling area was defined. After sampling each sponge was placed into a sterile bag and kept refrigerated until analysis. The detection of

Listeria monocytogenes was performed according to standard method UNI EN ISO 11290-1:2005. In the present study a persistent contamination site was defined as the recovery of *L. monocytogenes* from a defined site, in two or more sampling time during the observation period. All the isolates were identified as by PCR and major serotype were also detected using primers *lmo0737*, *lmo1118*, *ORF2819* and *ORF2110* (Doumith *et al*., 2004). Differences in *L. monocytogenes* prevalence between the two cheese-making plants were compared using chi-square test ($\chi 2$). The probability of recovering *Listeria monocytogenes* was investigated using logistic regression (Statgraphics Plus software vers.16.0.09, Centurion). The probability of observing samples positive for *L. monocytogenes* was the binary response variable (presence, absence). The effect of the dairy (A and B), production phase (peak, break and resume of production), production line (Pecorino Romano, ricotta, common line), areas (cheese curdling, whey floating, salting, seasoning Pecorino Romano, ricotta storage, cheese washing, packaging, other), sampling site (shelves, carts, tables, cheeses washing machines, floors, drains, moulds, other), type of surface (food contact and non-food contact) were included in the model.

3.4 Results

Listeria monocytogenes was detected in 109 (17.8%) out of the 613 collected samples. Overall the prevalence was 23.2% for dairy A and 13.1% for dairy B. In dairy A was detected in 29 (26.4%) out of 110 food contact surfaces and in 37 (21.1%) out of 175 non-food contact surfaces. In dairy B was detected in 15 (10.9%) out of 138 food contact surfaces and in 28 (14.7%) out of 190 non-food contact surfaces.

Listeria monocytogenes prevalence reported for the two cheese-making plants in the three main production phases are showed in table 3.1. In table 3.2 is reported the prevalence of *L. monocytogenes* along the production lines of Pecorino Romano, ricotta

salata and in the common production areas. The production period influenced *L. monocytogenes* prevalence only for Pecorino Romano's ripening rooms. The probability (*odds ratio*) to detect *L. monocytogenes* in Pecorino Romano's ripening rooms was ca. 6 times lower in the resume production period as compared to the peak of production and break periods (table 3.3). Comparison between the cheese-making plants showed that the probability of contamination was 2 times greater in dairy A than in dairy B (P <0.01). The highest rate of contamination was observed for cheese washing rooms (44.5%), packaging area (36.8%), salting room (33.3%), ricotta storage room (25.6%) and Pecorino Romano ripening area (13.9%). *L. monocytogenes* was detected in 3.3% of the samples collected from whey floating area, while, in curdling area the microorganism was never detected. In the areas common to the two main production lines, *L. monocytogenes* prevalence was 5.9%. Logistic regression analysis showed that the probability of detecting *L. monocytogenes* in samples was significantly affected by the following factors: cheese making plant, production phases and production areas (P <0.001). No significant effect of production line, site and surface type was observed. *Listeria monocytogenes* persistent contamination was detected in cheese washing rooms of both cheese-making plants, in the following sites: washing machine, floors and drains. Pecorino Romano production line was characterized by persistent contamination in the ripening rooms (shelves, drains and floors) and salting room (drains). Ricotta salata storage rooms showed persistent contamination of drains and floors. PCR results on serotyping showed that all the 43 strains isolated from dairy B were serotype 1/2a. On the other hand, dairy A showed a wide strains variability with 17 isolates (25.8%) belonging to serotype 1/2a, 27 (40.9%) and 22 (33.0%) to serotypes1/2b and 4b, respectively.

3.5 Discussion and conclusion

The present study was aimed to investigate the prevalence and the persistence of environmental contamination in two sheep milk cheese-making plants. The probability of detecting *L. monocytogenes* positive samples was significantly affected by the plants, processing areas and production phases during the cheese-making season (P <0.001). *Listeria monocytogenes* environmental contamination was twice as much in dairy A as compared to dairy B. However, great part of the difference in *L. monocytogenes* prevalence was accountable to contamination observed along the Pecorino Romano production line. Little differences between cheese-making plants were observed for ricotta salata and common production areas. Environmental conditions, pre-operational sanitation and the adoption of good manufacturing practice reduce the risk of colonization in cheese curdling and whey floating areas (Pilo *et al*., 2007). Therefore, contamination of the products in these areas should be considered as a sporadic event. Instead, *L. monocytogenes* prevalence observed in washing, drying and packaging rooms was higher, resulting in an increased risk for products contamination (FSIS, 2010). The higher prevalence could be explained by specific condition observed in these areas, such as low temperatures, high salt concentration, presence of environmental surfaces difficult to clean and sanitize (i.e. wood). Cheese cleaning and ripening rooms are areas shared between Pecorino Romano and ricotta salata, therefore they may represent a potential source of cross-contamination between the two production lines. With regard to the production phases the probability of contamination in ripening rooms was about 6 times lower during the resuming of production as compared to other phases. This could be explained by the effectiveness of cleaning and sanitizing procedures conducted during the break production period. Differences in *Listeria monocytogenes* prevalence were observed between the two cheese-making plants based

on the sampling period. In dairy B a significant decrease in the contamination prevalence was observed in the break and resuming production phases, while in dairy A the reduction was limited. Moreover, in diary A the contamination of food contact surfaces was higher than in dairy B. Dairy A was also characterized by a greater strains variability, with isolates belonging to *L. monocytogenes* lineage I (serotypes 1/2b and 4b) and II (serotype 1/2a), while in dairy B all strains belonged to lineage II. Persistent contamination was observed in sites with favourable conditions for microorganism survival. In cheese washing machine the increased risk was represented by the presence of organic residues and the poor hygienic design of the equipment which limit the cleaning and sanitization effectiveness. Other sites, such as floors, drains, mainly in salting ripening and storage area, served as niches for *L. monocytogenes*. Persistent contamination seems to be related to the inability to remove and inactivate strains from niche sites rather than specific strain properties (Carpentier and Cerf, 2011). The regular implementation of environmental sampling programs is strongly recommended in order to monitor the level of *L. monocytogenes* contamination and the presence of persistent harbourage sites (trend analysis). The application of strict cleaning and sanitizing procedures is essential in order to prevent on one hand the re-introduction of *L. monocytogenes* into the plant environment and on the other hand the establishment of niches which serve as a potential source of product contamination.

Tables

Table 3.1 *Listeria monocytogenes (Lm)* prevalence on surfaces in relation to the period of dairies activity (A, B).

Dairy	Cheese-making period								
	peak [a]			break [b]			resuming [c]		
	n	*Lm*+	%	n	*Lm*+	%	n	*Lm*+	%
A	79	29	36.7	76	21	27.6	83	16	19.3
B	94	30	31.9	94	8	8.5	111	5	4.5
total	173	38	34.3	170	29	17.1	194	21	10.8

[a]: January, March, June; [b]: September and October; [c]: November e December

Table 3.2 *Listeria monocytogenes* (*Lm*) prevalence in different areas of the two dairies.

	dairy A			dairy B				
Line / Room	n	*Lm*+	%	n	*Lm*+	%	*P* value	*Odds ratio*
Pecorino Romano								
cheese-making	28	0	0.0	33	0	0.0	-	-
ripening	102	20	19.6	99	8	8.1	<0.05	2.8
Ricotta salata								
ricotta processing	42	1	2.4	48	2	4.2	NS	0.6
ricotta storage	17	8	47.1	26	3	11.5	<0.05	6.8
Common areas								
product washing	68	30	44.1	42	19	45.2	NS	0.9
packaging	7	4	57.1	12	3	25.0	NS	4.0
salting	-	-	-	21	7	33.3	-	-
other	21	3	14.3	47	1	2.1	<0.05	7.7
total	285	66	23.2	328	43	13.1	<0.01	2.0

Table 3.3 Comparison of *Listeria monocytogenes (Lm)* prevalence in different areas in the three production periods.

Areas	n	*Lm+*	%	*P value*	*Odds ratio*
Cheese-making					
Peak of production	25	0	0.0	.	.
Break of production	18	0	0.0	.	.
Resuming of production	18	0	0.0	.	.
Packaging					
Peak of production	7	3	42.9	NS	1.5
Break of production	6	2	33.3	.	1
Resuming of production	6	2	33.3	.	1
Washing products					
Peak of production	46	25	54.4	NS	2.2
Break of production	30	12	40.0	NS	1.2
Resuming of production	34	12	35.3	.	1
Ricotta processing					
Peak of production	37	2	5.4	NS	1.5
Break of production	26	0	0.0	.	.
Resuming of production	27	1	3.7	.	1
Salting					
Peak of production	9	4	44.4	.	1
Break of production	6	3	50.0	NS	1.2
Resuming of production	6	0	0.0	.	.
Ripening					
Peak of production	90	16	17.8	0.01	6.1
Break of production	53	10	18.9	0.01	6.5
Resuming of production	58	2	3.5	.	1
Storage salted ricotta					
Peak of production	20	7	35.0	NS	1.1
Break of production	11	0	0.0	.	.
Resuming of production	12	4	33.3	.	1
Other					
Peak of production	25	2	8.0	.	1
Break of production	20	2	10.0	NS	1.3
Resuming of production	23	0	0.0	.	.

Lm, *Listeria monocytogenes*; NS, not significant. *Note that peak of production occurred in January, March, and June, break of production in September and October, and resuming of production in November and December.

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CHAPTER 4

Occurrence and traceability of *Listeria monocytogenes* strains isolated from sheep's milk cheese-making plants environment.

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The contribution of the doctoral candidate to the paper presented in this chapter concerned several aspects of the experiment, from samples collection at the cheese-making plants and to the execution of the microbiological and molecular analysis. In particular the candidate was primary responsible for conducting strains serotyping and pulsed field gel electrophoresis. The candidate contributed also in the interpretation of their output traceability and population study. The contribution of the candidate was also in the writing and editing of the publication.

Occurrence and traceability of *Listeria monocytogenes* strains isolated from sheep's milk cheese-making plants environment

4.1 Abstract

The aim of the study was to conduct an extensive survey on *Listeria monocytogenes* and *Listeria spp.* environmental contamination in 13 cheese-making plants. A total of 409 environmental and food samples were collected during years 2011-2013. Listeria spp. contamination was observed in all the facilities, while *L. monocytogenes* was recovered from 12 facilities with a prevalence ranging between 3.0% and 22.6%. Floor drains were the most contaminated sampling sites (48.8% of positive samples), serving as harbourage site for subsequent contamination. Out of 616 isolates, 277 (45.0%) were *Listeria innocua*, 274 (44.5%) *Listeria monocytogenes*, 41 (6.6%) *Listeria ivanovii,* 14 (2.3%) *Listeria welshimeri* and 10 (1.6%) *Listeria gravyi*. Serotyping carried out by PCR and agglutination method for *L. monocytogenes* revealed that 169 strains (61.7%) were serotype 1/2a, 65 (23.7%) 4b, 20 (7.3%) 1/2b, 10 (3.6%) 3a, 7 (2.5%) 1/2c and 3 (1.1%) 3b. PFGE conducted on *L. monocytogenes* isolates using *Asc*I and *Apa*I restriction enzymes, yielded 6 clusters. Two predominant PFGE clusters were observed including respectively 36 and 32 strains. Within cheese-making plants, *L. monocytogenes* showed wide variability with strains distributed up to 4 different clusters. Pulsotypes isolated from raw milk filter were never detected in the processing environment, indicating that the contamination originated from sources other than raw milk. The isolation of strains with similar profile from different sampling sites, within and among cheese-making plants, indicated the possible transfer of *L. monocytogenes* contamination along production lines and from one facility to another. Strains recovered from food were confirmed as originating from the processing environment.

4.2. Introduction

Listeria monocytogenes is an important pathogen that can be transmitted to human through the consumption of contaminated foods (Farber & Peterkin, 1991). According to the EU summary report on zoonosis, zoonotic agents and foodborne outbreaks, *L. monocytogenes* is the main leading cause of hospitalization and death in Europe (EFSA, 2014). Listeriosis is frequently associated with ready-to eat (RTE) foods (Lianou & Sofos, 2007; Liu, 2006). Of particular risk are considered RTE foods that are able to support the growth of *L. monocytogenes.* In addition to product testing, food business operator should monitor also processing areas and equipment, through the adoption of effective environmental sampling schemes in the processing plant (Regulation EC 2073/2005). Recontamination from processing environment has been demonstrated as being the principal route of Listeria contamination of RTE foods (Tompkin, 2002). Cheese-making plants are characterized by areas with conditions favourable to *L. monocytogenes* growth, such as refrigeration temperature, moisture and high saline concentration (Tompkin, 2002; Unnerstad *et al.,* 1996).

Furthermore, *L. monocytogenes* is frequently recovered from floors, drains and equipment and is able to colonize for many years different niches in the processing environment (Unnerstad *et al.,* 1996), despite them being routinely cleaned and disinfected, thus leading to the consideration that this is a contamination of ―clean premises‖ (Carpentier & Cerf, 2011). In order to reveal niches in the environment it is advisable to conduct environmental sampling during or at the end of production runs since microorganisms can be dislodged from harborage sites during process operations (FDA, 2008; Tompkin, 2004). To prevent post process contamination it is essential to determine the source of such contamination and to implement corrective action to avoid

the transfer of *L. monocytogenes* from the environment to foods. Previous studies reported the isolation of *L. monocytogenes* from different environmental sites within sheep's cheese-making plants (Ibba *et al.,* 2013; Almeida *et al.,* 2013). Since *Listeria spp*. and *L. monocytogenes* share similar ecology, contamination sources and spreading pathways in the processing environment, an effective environmental monitoring program should include also testing for *Listeria spp*. Their presence is a good indication for the potential presence of *L. monocytogenes* (Tompkin *et al.*, 1999). During epidemiological investigation it is essential to subtype *L. monocytogenes* isolates. The traditional agglutination serotyping method allows to classify *L. monocytogenes* into 13 serotypes (Seeliger & Jones, 1986), while the use of a multiplex PCR assay discriminate the major serovars 1/2a, 1/2b, 1/2c and 4b (Doumith *et al.*, 2004). Since most of cases of human listeriosis is associated with serotypes 1/2a, 1/2b and 4b (Borucki & Call, 2003; Jacquet *et al.*, 2002; Liu, 2006; Rocourt, 1994; Wiedmann *et al.,* 1996), the utility of the conventional serological test is questioned during epidemiological investigations (McLauchlin *et al.*, 2004). However, the use of classical serological procedure makes it possible the identification of the two main phylogenetic lineages: lineage I including serotypes 1/2b, 3b, 3c, 4b and lineage II including serotype 1/2a, 1/2c and 3a (Nadon *et al.*, 2001; Piffaretti *et al.,* 1989). Other molecular subtyping techniques such as Pulsed-field gel electrophoresis (PFGE), Multi-locus variable number tandem repeat analysis (MLVA) or fluorescent amplified length polymorphism (fAFLP) should be used in association with serotyping to trace the source of *Listeria* in food processing plants (Almeida *et al.,* 2013; Doijad *et al.,* 2011; Fox *et al.* 2011; Lomonaco *et al.*, 2011; Lunestad *et al.*, 2012). PFGE is the ―gold standard‖ typing method used during epidemiological investigations and it can also be appropriately used for tracing contamination with *L. monocytogenes* in processing plants.

Sardinia (Italy) has a long tradition in the production of various types of cheese using sheep milk. Among these, Pecorino Romano PDO (protected designation of origin) and Ricotta salata are widely exported in many European and third countries, mainly in North America, thus representing a great economic value for regional economy.

The aim of the present study was to identify the niches and environmental contamination pathways of *Listeria spp*. and *L. monocytogenes* in cheese-making plants using sheep milk. Another objective was to subtype *L. monocytogenes* isolates circulating in sheep's milk cheese-making plants, to investigate their genetic diversity and the possible routes of contamination between and within the analyzed premises.

4.3. Materials and Methods

4.3.1 Samples collection

Samples were collect from 13 industrial sheep's milk cheese-making plants operating in Sardinia (Italy) over a two-year period from 2011 to 2013 during the period of maximum production (from January to March). The cheese-making plants selected for the study were representative of great part of the total regional sheep cheese production. The maximum distance between the plants was about 200 km with no personnel exchange from one to another. Each facility was visited once for samples collection, which was performed after at least two hours of production or at the end of production runs (Tompkin, 2004). Environmental sampling was conducted from different areas along the processing stream of Pecorino Romano PDO and Ricotta salata cheese, such as curd production, whey heating, moulding, salting, washing, drying, ripening and packaging areas. Overall, were collected 326 environmental samples: 143

from food contact surfaces (raw milk filters, drainage tables, ripening shelves, moulds, trolleys, cheese and ricotta washing machines) and 183 from non-food contact surfaces (external surfaces of equipment, walls, floors, floor drains). Environmental samples were collected using a commercial environmental sponge sampling kit (3M, St. Paul, Minnesota, USA) containing each a sterile sponge moistened with 10 mL of buffer peptone water, sterile gloves and sterile bag to transport the sponge. Sponges were dragged back and forth on the selected site in order to cover, when possible, an area of about 0.3 m^2 (Carpentier & Barre, 2012). Sponges were also used to sample the rind of Pecorino Romano and the surface of Ricotta salata wheels. Each sponge was used to obtain comingled samples scrubbing the surface of five different wheels of the same batch, for a total area of 0.5 m^2. After collection each sponge was placed back into its sterile bag and kept refrigerated until analysis, performed within 24 hours.

4.3.2 Microbiological examination

The presence/absence of *L. monocytogenes* was investigated using conventional qualitative culturing methods according to ISO 11290-1:1996/Amd 1:2004. Briefly, sponges were submitted to primary and secondary enrichment. After incubation pre-enrichment and enrichment broths were streaked onto Oxford and ALOA (Biolife, Milan, Italy) agar plates. From each positive sample up to 5 presumptive *Listeria spp*. colonies were subcultured onto Triptic Soy Yeast Extract Agar (TSYA, Biolife) and incubated at 37°C for 24 h. Species confirmation was obtained with the following test: gram staining, catalase and oxidase test, haemolytic activity and CAMP tests on sheep blood agar and the biochemical test API *Listeria*® (BioMérieux, Marcy l'Etoile, France). All the strains were stored at -80°C in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, UK) with glycerol (15% v/v) for further analysis.

4.3.3 *Listeria monocytogenes* serotyping

Multiplex PCR was performed in order to differentiate *L. monocytogenes* major serovars (1/2a, 1/2b, 1/2c and 4b) according to the protocol proposed by Doumith *et al.* (2004). After PCR based screening, the strains were submitted to serological serotyping (Denka Seiken Co., Tokyo, Japan) following manufacturer's instructions. A specific subset of somatic (O) and flagellar (H) antigens were selected according to the cost-saving flow-chart proposed by Tamburro *et al.* (2009). Concordance between the two methods was also evaluated.

4.3.4 *Listeria monocytogenes* lineages

In the present study lineage classification was conducted according to the designation described by Piffaretti *et al.* (1989) where serotypes 1/2b, 3b, 3c and 4b were assigned to lineage I and serotypes 1/2a, 3a and 1/2c to lineage II. The serotypes most frequently recovered from human cases are included in this classification.

4.3.5 Pulsed-field gel electrophoresis

All *L. monocytogenes* isolates were analyzed using PFGE according to the protocol proposed by Graves & Swaminathan (2001). After DNA extraction, agarose plugs were digested with *Asc*I (New England Biolabs, Massachusetts, USA) and *Apa*I (Promega, Madison,WI, USA) restriction enzymes. Electrophoretic run was performed in Tris Borate EDTA 0.5x using CHEF MAPPER XA System (Bio-Rad). Lambda DNA Ladder (New England Biolabs, MA, USA) marker was loaded on each gel and used as a reference standard. Gels were stained with ethidium bromide and visualized by a UV trans-illuminator (Bio-Rad). A preliminary screening of the combined profiles obtained

with the two restriction enzymes was conducted by visual examination. In order to avoid overrepresentation of potential clones, one strain for each positive sample was selected. In case that more than one PFGE profile was obtained from isolates of the same sample, all different profile were included in the population study. The restriction profiles of the selected strains were analysed with GelCompar II software version 6.5 (Applied Math, Sint-Martens-Latem, Belgium) using the Dice correlation coefficient with 1.0% optimization setting and represented by Unweighted Pair Group Method using Arithmetic averages (UPGMA). A cut off of 70% was used to obtain PFGE clusters. Restriction profiles were used to determine the genetic variability of the isolates and to trace the contamination along the processing plant. Variability was evaluated by the recovery, from the same independent sample, of strains with different PFGE pattern, while traceability along the processing environment, was assessed by the comparison of similar PFGE profiles from different samples.

4.4. Results

4.4.1 Prevalence of *Listeria spp*. and *Listeria monocytogenes*

In the present study *Listeria* spp. was isolated from all the 13 cheese-making plants, *L.monocytogenes* from 12 out of 13 cheese-making plants (92.3%), while the presence of *Listeria* spp. in combination with *Listeria monocytogenes* occurred in 11 out of 13 (84.6%) premises (Table 4.1). Among *Listeria* other than *L .monocytogenes*, *L. innocua* was recovered from all the plants investigated *L. ivanovii* and *L. gravyi* from 6 plants (46.2%), while *L. welshimeri* was detected in 5 (38.5%) plants. The prevalence of *L. monocytogenes* and other *Listeria* spp. from different sampling sites is reported in Table 4.2. A total of 616 *Listeria* spp. were isolated and speciated. *Listeria*

monocytogenes was the most prevalent species with 274 (44.5%) isolates recovered, while 342 (55.5%) belonged to other *Listeria* spp. The prevalence for each of the 5 identified species is reported in Table 4.3.

4.4.2 *Listeria monocytogenes* serotyping

Multiplex PCR allowed to differentiate *L. monocytogenes* isolates into four major serovars: 1/2a with 179 strains (65.3%), 1/2c with 7 strains (2.5%), 1/2b with 23 strains (8.4%), and 4b with 65 strains (23.7%). The use of traditional serotyping confirmed molecular serotyping in 261 out of 274 strains (95.3%) showing a high concordance between PCR and the traditional agglutination method (Table 4.4). Strains belonged to two evolutionary lineages: lineage I and lineage II represented respectively by 88 (32.1%) and 186 (67.9%) strains (Table 4.4).

4.4.3 Strains variability and traceability

Overall 274 *L. monocytogenes* strains were typed by PFGE and 104 were retained for the population study (figure 4.1). The combined *Asc*I and *Apa*I restriction profile yielded 6 CLs (CLI-CLVI) and one unique pulsotype. Two predominant CLs were observed (CLII and CLIII), including respectively 36 and 32 strains. The remaining CLs included 15 strains (IV), 11 strains (I), 6 strains (V) and 3 strains (VII). Strain isolated from 9 cheese-making plants (A, B, C, D, E, F, G, H and L) showed a wide variability and belonged from two up to four different CLs. A lower variability was observed in three cheese-making plants (I, M and N) with strains included in a single CL for each plant. Strains isolated from the same samples showing different restriction profiles were observed in 6 out of 12 facilities (A, C, D, G, H, I). In 5 out of 12 cheese-making plants (B, D, F, L, M) strains isolated from different samples showed

the same PFGE profile. This allowed tracing the contamination along the processing facilities. The presence of *L. monocytogenes* was observed in filters at milk reception in 2 cheese-making plants (F and H). The isolates were characterized by PFGE pattern never detected in samples collected from other working areas. In general the comparison of the PFGE profiles showed similar pattern in strains isolated from food samples with strains isolated from food contact and non-food contact surfaces. Exception was represented by the cheese-making plant B, where strains isolated from Pecorino Romano showed PFGE pattern which differed from strains isolated from the environment and by cheese-making plant N where *L. monocytogenes* was isolated only from Ricotta salata samples.

4.5 Discussion

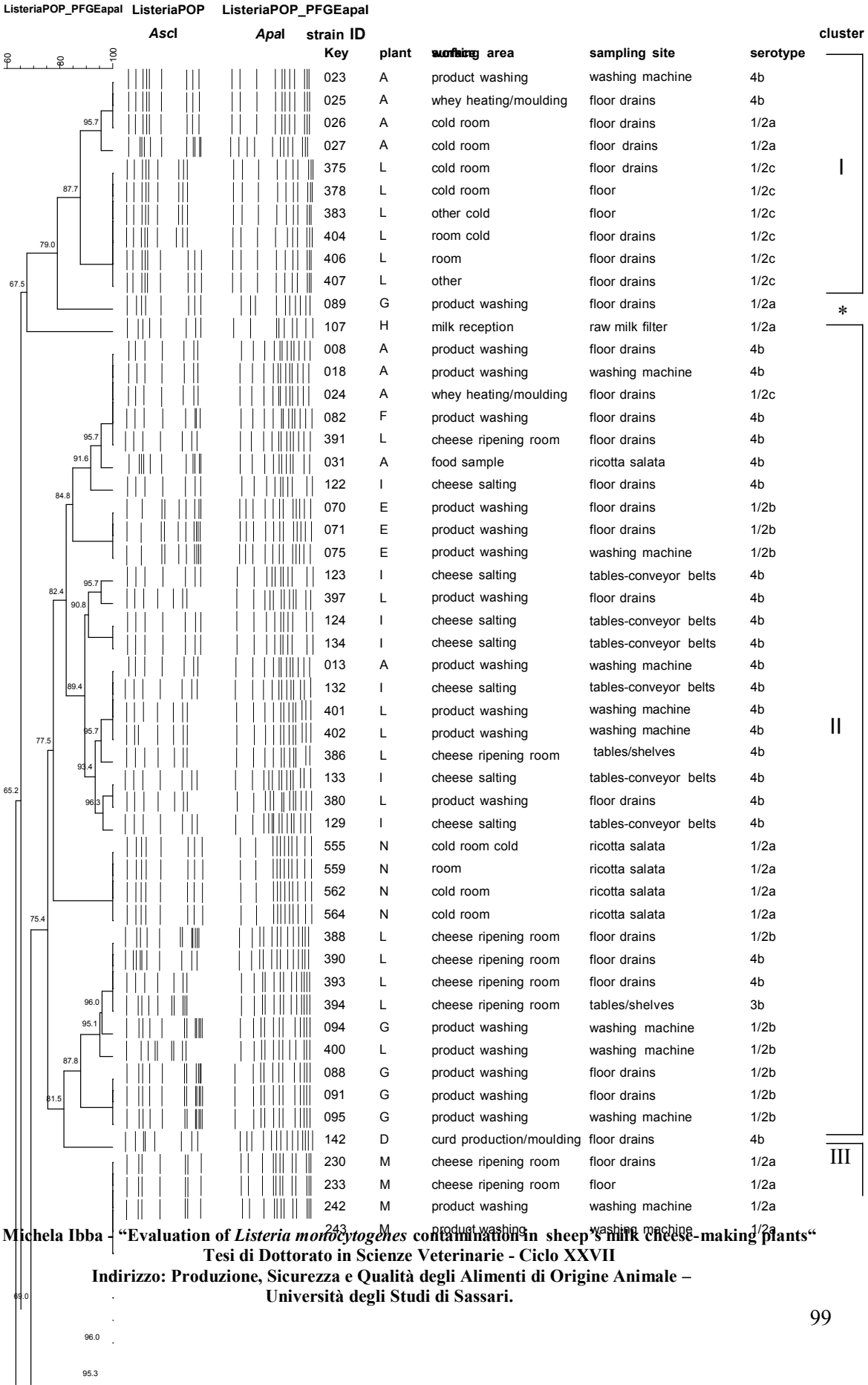
Our results showed that *L. monocytogenes* and other *Listeria* spp. were frequently isolated either alone or in association from cheese-making plants environment represented 44.5% of the isolates, while among *Listeria* other than *L. monocytogenes, L. innocua* was the most represented with 45% of the isolates, followed by L. *ivanovii, L. welshimeri* and *L. gravy*. Although *Listeria* spp. does not represent a threat for human health, they share the same habitat with *L. monocytogenes* and their presence is a good indication of the potential presence of this important foodborne pathogen (CAC, 2007). Therefore, testing for *Listeria* spp. represents an essential part of an effective environmental monitoring program. In the present study contamination in the processing environment was greater in areas where the product is submitted to manipulations such as product washing, salting areas and cheese ripening rooms. It is well known that the presence of *Listeria* niches in processing areas downstream a listericidal treatment represents a potential source of product post-process

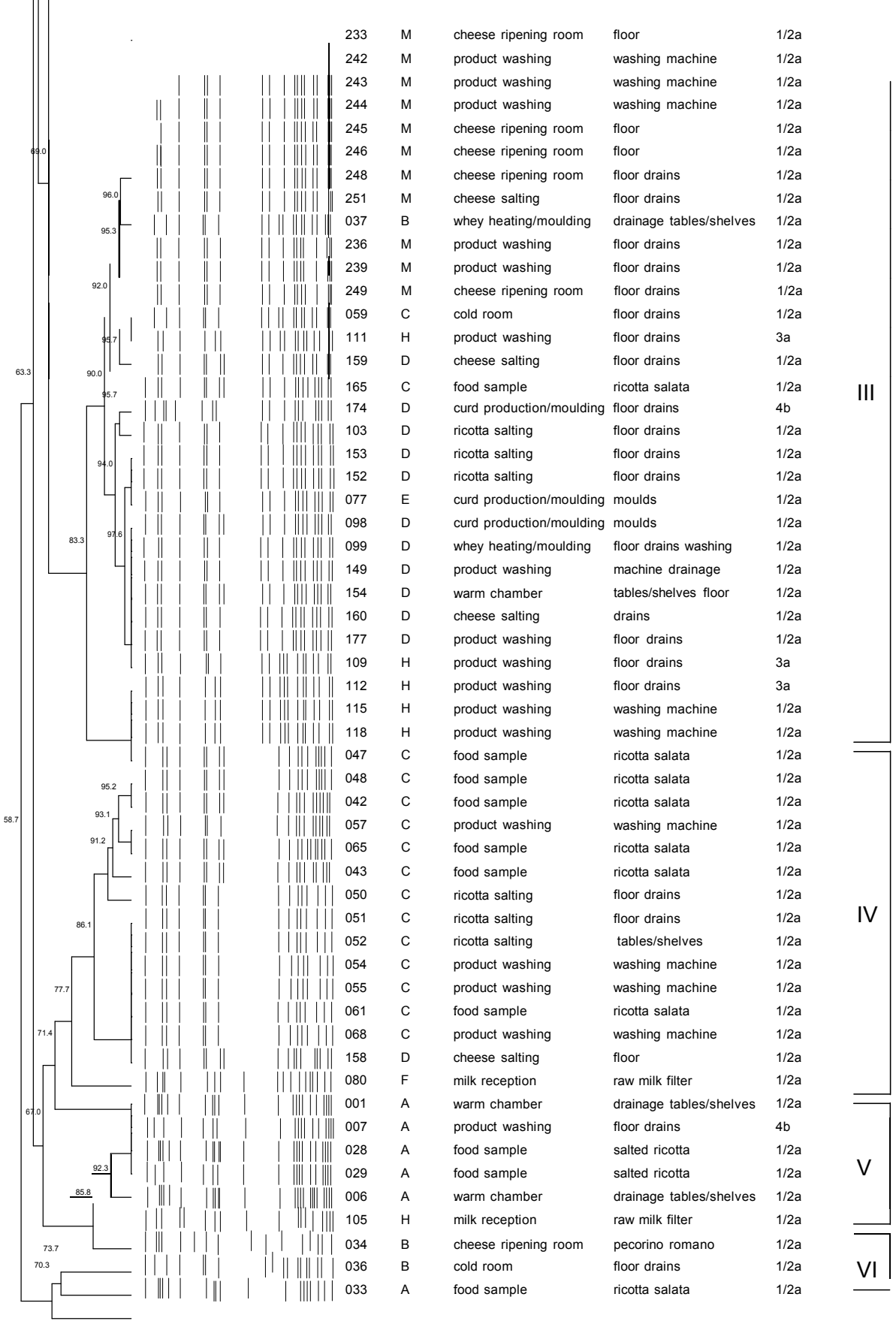
contamination (Tompkin *et al.*, 1999). With regard to sampling sites, the floor drains were the sites with the highest contamination (over 40%). This highlights the importance of monitoring floor drains as sentinel sites to detect Listeria contamination in the environment. The genetic diversity of *L. monocytogenes* isolates was investigated by serotyping and PFGE. All the four major serovars were identified using PCR technique. Molecular typing does not allow differentiation between serotypes within the same serogroup, which can be performed by the agglutination method. However, only in few cases the latter discriminated serotypes other than the major serovars (De Santis *et al.,* 2007). Serotypes 1/2a, 1/2b and 4b observed in the present study are those most frequently associated with foodborne listeriosis in human (Rocourt, 1994). The greater prevalence of lineage II is consistent with the existing literature regarding *Listeria monocytogenes* isolated from food and food plant environments (Chen *et al.*, 2009; Lomonaco *et al.,* 2009; Manfreda *et al.*, 2005; Zhou & Jiao, 2006). Overall, *L. monocytogenes* environmental contamination showed a wide variability of strains within and among cheese-making plants. *Listeria monocytogenes* genetic variability was demonstrated by PFGE patterns of strains isolated from the same facility which were distributed up to four different clusters. A possible explanation is that in plants characterized by the greater variability, the introduction of products originating from other plants is a common commercial practice. The circulation of strains from one plant to another and the transmission of contamination among facilities were demonstrated by the presence within the two main clusters of *L. monocytogenes* recovered from all the cheese-making plants. Three plants represented an exception with strains belonging to a single CL for each plant, indicating the presence of strains variability also among cheese-making plants. As expected the sites showing the greater variability were product washing machines and floor drains. Data obtained by PFGE also allowed

tracing the sources of contamination. Contamination by raw milk was excluded since all the strains isolated from milk filter were never detected in the processing environment. This could be related with the separation of raw milk equipment (reception, storage and heat treatment) from open processing areas and the listericidal effect applied during heat treatment of raw milk. Isolates from various sampling sites (i.e. floor drains, tables and shelves, product washing machine) of different processing areas showed similar PFGE profiles thus indicating that the contamination is spread downstream along the processing line. *Listeria monocytogenes* isolated from food samples clustered together with environmental strains, indicating the processing environment as contamination source. In particular strains recovered from Ricotta salata showed similar profile observed in strains isolated from handling and storing areas. Product washing represented an important route of cross-contamination, since products coming from different processing lines (Pecorino Romano and other cheeses) merge at this particular point. This was demonstrated by the presence of the same pulsotypes in Ricotta salata and Pecorino Romano rind. However, differences exist between the two products in the prevalence of contamination (30% and 10%, respectively) and in their associated risk for human health. In fact, Ricotta salata is a food that supports the growth of *L. monocytogenes* (Spanu *et al.,* 2012; Spanu *et al.,* 2013) while the microorganism can only survive on Pecorino Romano rind. The present study shows that *L. monocytogenes* and *Listeria* spp. are widely distributed among processing environment in cheese-making plants. The adoption of monitoring programs for this microorganism, in supporting continuous control measures to reduce environmental contamination, plays a key role in preventing food contamination and the associated risk for human health. The identification of environmental sites which serve as niches of contamination is essential in order to prevent spreading in the processing plant.

Figures and Tables

Figure 4.1 PFGE profile comparison conducted on a selection of 104 strains isolated from different cheese-making plants.





*= unique pulsotype;

Table 4.1 Prevalence of *Listeria monocytogenes* and other *Listeria* spp. alone and in combination isolated from 13 sheep's cheese-making plants.

Cheese-making plant	n. samples	*Listeria monocytogenes*		Other *Listeria**		*L. monocytogenes* + other *Listeria* **		Total *Listeria* spp.	
		n	%	n	%	n	%	n	%
A	26	4	15.4	3	11.5	4	15.4	11	42.3
B	33	1	3.0	12	36.4	3	9.1	16	48.5
C	31	4	12.9%	6	19.4	5	16.1	15	48.4
D	31	7	22.6	5	16.1	2	6.5	14	45.2
E	28	1	3.6	2	7.1	3	10.7	6	23.4
F	27	1	3.7	2	7.4	1	3.7	4	14.8
G	26	-	-	3	11.5	3	11.5	6	23.1
H	27	3	11.1	1	3.7	1	3.7	5	18.5
I	25	3	12.0	2	8.0	1	4.0	6	24.0
L	54	14	25.9	3	5.6	2	3.7	19	35.2
M	49	11	22.4	7	14.3	-	-	18	36.7
N	30	3	10.0	2	6.7	1	3.3	6	20.0
O	22	-	-	7	31.8	-	-	7	31.8
Total	409	52	12.7	55	13.4	26	6.4	133	32.5

**Listeria* other than *monocytogenes*; ** simultaneous presence of *Listeria monocytogenes* and *Listeria* other than *L. monocytogenes*.

Table 4.2 Prevalence of *Listeria* spp. isolated from different samples.

Working area	Sampling site	n	*Listeria monocytogenes*		Other *Listeria*		*Listeria* spp. + *L.monocytogenes*		total *Listeria* spp.	
			n	%	n	%	n	%	n	%
Milk reception	raw milk filters	10	-	-	4	40.0	2	20.0	6	60.0
Curd production and moulding	moulds	15	3	20.0	1	6.7	-	-	4	26.7
	Floor/ Floor drains	36	1	2.8	7	19.4			8	22.2
Warm chamber	drainage tables/shelves	15	2	13.3	1	6.7	-	-	3	20.0
	Floor drains	14	-	-	2	14.3	-	-	2	14.3
Cheese salting	Tables/conveyor belts	16	2	12.5	2	12.5	-	-	4	25.0
	Floor drains	23	2	8.7	8	34.8	1	4.3	11	47.8
Product washing	Washing machine	32	9	28.1	8	25.0	6	18.8	23	71.9
	Floor drains	19	8	42.1	4	21.1	4	21.1	16	84.2
Whey heating and moulding	drainage tables	17	1	5.9	1	5.9	-	-	2	11.8
	Floor drains	29	2	6.9	5	17.2	3	10.3	10	34.5
Cold rooms	Tables/shelves	13	-	-	2	15.4	-	-	2	15.4
	Floor drains	23	2	8.7	1	4.3	4	17.4	7	30.4
Ricotta salting	Tables/shelves	6	1	16.7	-	-	-	-	1	16.7
	Floor drains	8	-	-	4	50.0	-	-	4	50.0
Food samples	Pecorino Romano	50	3	6.0	1	2.0	1	2.0	5	10.0
	Ricotta salata	33	7	21.2	2	6.1	1	3.0	10	30.3
Cheese ripening room	Tables/shelves	11	2	18.2	-	-	-	-	2	18.2
	Floor drains	20	5	25.0	2	10.0	-	-	7	35.0
Other[1]	miscellaneous[2]	8	-	-	-	-	-	-	-	-
	miscellaneous[3]	11	2	18.2	4	36.4	-	-	6	54.5
Total		409							133	

[1]packing, moulds washing, ice production; [2]Cheese cutter, cheese grating machine, moulds and shelves washing machine, conveyor belt; [3]floor/floor drains, forklift wheels, boot washer.

Table 4.3 Prevalence of *Listeria monocytogenes* and other *Listeria* spp. by sampling sites in 13 sheep's milk cheese-making plants.

Sampling site	Number of isolates	*L. monocytogenes*		*L. innocua*		*L. ivanovii*		*L. welshimeri*		*L. gravyi*	
Food contact surfaces											
Raw milk filters	23	6	26.1%	4	17.4%	13	56.5%	-	-	-	-
Cheese moulds	17	11	64.7%	-	-	6	35.3%	-	-	-	-
Drainage tables/shelves	27	17	63.0%	4	14.8%	6	22.2%	-	-	-	-
Ripening wooden shelves	23	11	47.8%	11	47.8%	-	-	1	4.3%	-	-
Product washing machine	104	47	45.2%	54	51.9%	-	-	3	2.9%	-	-
Tables/conveyor belts	18	12	66.7%	6	33.3%	-	-	-	-	-	-
Non-food contact surfaces											
Floor	16	9	56.3%	6	37.5%	-	-	-	-	1	6.3%
Floor drains	302	109	36.1%	158	52.3%	16	5.3%	10	3.3%	9	3.0%
Other[1]	9	-	-	9	100%	-	-	-	-	-	-
Food samples											
Pecorino Romano	21	11	53.4%	10	47.6%	-	-	-	-	-	-
Ricotta salata	56	41	73.2%	15	26.8%	-	-	-	-	-	-
Total	616	274	44.5%	277	45.0%	41	6.6%	14	2.3%	10	1.6%

[1]forklift wheels, boot washer.

Table 4.4 Correlation between serotyping by multiplex PCR and agglutination methods on *Listeria monocytogenes* strains isolated from food and environmental samples in 13 sheep's cheese-making plant.

Samples	Number of isolates	Serotyping method		Concordance	Lineage
		Serogroups	Serotypes		
		PCR	Agglutination		
Food	41	1/2a	41 (1/2a)	100 %	II
	3	1/2b	3 (1/2b)	100 %	I
	8	4b	8 (4b)	100 %	I
Food contact surfaces	67	1/2a	63 (1/2a), 4 (3a)	94%	II
	9	1/2b	6 (1/2b), 3 (3b)	66.7%	I
	28	4b	28 (4b)	100 %	I
Non-food contact surfaces	71	1/2a	65 (1/2a), 6 (3a)	91.5%	II
	7	1/2c	1/2c	100 %	II
	11	1/2b	11 (1/2b)	100 %	I
	29	4b	29 (4b)	100 %	I

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CHAPTER 5

Genotypic characterization and virulence genes analysis of *Listeria monocytogenes* isolated from sheep cheese-making plants

Most of the laboratory work presented in this chapter was carried out by the candidate during a 6 months period of internship at the Foodborne Pathogens Reference Services of Public Health of England (PHE, London, UK). The candidate contributed to define the selection criteria of the strains to be processed by fAFLP and genome sequencing, performed the molecular experiments and collected the data. The candidate participated to the analysis of the data and to the drafting of the manuscript. The results presented in this chapter are part of a more extensive work that is intended to be completed and submitted for possible publication in a scientific journal.

Genotypic characterization and virulence genes analysis of *Listeria monocytogenes* isolated from sheep cheese-making plants

5.1. Introduction

Listeria monocytogenes is an important foodborne pathogen responsible for a threatening illness known as listeriosis that affects elderly, pregnant women, newborns and immunocompromised individuals (Jadhav *et al.*, 2012). In Europe, the invasive form of listeriosis, despite a lower incidence (0.3 cases/100,000 inhabitants/year) as compared to other food-borne diseases, has a strong impact on human health due to its high hospitalization (>90 %) and high lethality (20-40%) rate (European Food Safety Authority, 2014).

The foods that are more exposed to *L. monocytogenes* contamination are ready-to-eat (RTE) foods, products that are not subjected to any treatment able to reduce *Listeria monocytogenes* loads before their consumption (Prencipe *et al.*, 2012). RTE foods include a wide array of products such as dairy products, fishery products, meat products and deli-type salads (EFSA, 2007). In Europe the incidence of listeriosis has increased since 2000 (Allenberg and Wagner, 2010). In 2011 the Rapid Alert System for Food and Feed (RASFF) reported 117 notifications for the presence of *L. monocytogenes*. The notifications interested fishery products (smoked salmon in 42 cases) and different types of cheese; in 23 circumstances the cheeses were produced by France and Italy (RASFF Annual Report, 2011). In the United States it is estimated that every year occur about 2,500 cases of human listeriosis (Mead *et al.*, 1999). In Europe, half of the reported cases of listeriosis outbreaks are caused by the ingestion of dairy products. Outbreaks associated with dairy products occurred in 1983 and 1987 in Switzerland (Farber and Peterkin, 1991) from unpasteurized soft cheeses, in 1989 and

1990 in Denmark (Jensen *et al.*, 1994) following the consumption of unpasteurized milk, and in 1995 in France, where a Brie style cheese, made from unpasteurized milk, was responsible for an epidemic case of listeriosis (Lundén *et al.*, 2004). However, even pasteurized products were linked to cases of listeriosis, as reported in Finland in 1998 to 1999 as result of the consumption of contaminated butter (Maijala *et al.*, 2001). In 2012 Ricotta salata, a traditional ripened and salted whey cheese, produced in Sardinia (Italy) was responsible for an outbreak occurred in U.S.A that caused 20 hospitalization and 4 deaths (CDC, 2012).

Almeida *et al*. (2010) highlighted how listeriosis has long been underestimated not being a notifiable disease. Since listeriosis became a notifiable disease, in 2001, an increase in the number of reported cases was observed in many European countries (Eurosurveillance, 2008). Mild cases of listeriosis often go undiagnosed or underreported, making it difficult to estimate the real incidence. In addition, considering the long period of incubation of listeriosis (from 3 to 60 days), in many cases it can be difficult link the symptoms with the contaminated meal (Yde *et al.*, 2010).

For this reason it became necessary to develop in Europe an effective surveillance system based on the obligatory notification. Collection of epidemiological data and the serological and molecular subtyping of isolates by reference laboratories improve the possibility to detect the listeriosis cases and to trace the sources of infection (Allerberger and Wagner, 2010).

Since 2010 listeriosis is part of the new surveillance network, the European Surveillance System (TESSy), coordinated by European Centre for Disease Prevention and Control (ECDC) (ISS, 2013). Following the example of the American PulseNet, the European Centre for Disease Prevention and Control (ECDC) has developed a surveillance network shared within the community National Reference Laboratories

(NRLs) networks (Van Walle, 2013). The network consists in a database in which are shared epidemiological data, the serotyping and the typing results of the strains that are isolated from food, environments, animal and human clinical cases (Felix *et al.*, 2014). The reference molecular typing method chosen is the Pulsed-Field Gel Electrophoresis (PFGE).

Due its high discriminatory power and reproducibility PFGE is the most frequently used method during epidemiological investigation of many foodborne bacteria (Heir *et al*., 2000). The use of PFGE typing allows controlling the routes of contamination throughout the food system and in the products helping to trace the contamination source during outbreaks (Felix *et al.*, 2014). This method is easily standardized and has high sensitivity in detecting changes in the DNA sequence as mutations, insertions and deletions which differentiate the genetic profile of the different strains (Jadhav *et al*., 2012). PFGE is considered the ―gold standard‖ method during listeriosis epidemiological surveillance (Graves and Swaminathan, 2001) and it has been used to subtype several isolates worldwide. An international database is available containing environmental, food and human isolates that are of fundamental relevance in epidemiological investigations (Lomonaco *et al.*, 2011). However, PFGE it is an expensive method that requires qualified personnel, long processing times and the interpretation of banding patterns may be subjective (Murphy *et al*., 2007).

Several studies have showed that the typing method fluorescent amplified fragment length polymorphism (fAFLP) have equal or sometimes a higher discriminatory power than the PFGE (Rousell *et al.*, 2013; Lomonaco *et al.*, 2011; Akiba *et al.*, 2003). In addition fAFLP is faster (48 h), cheaper and less labour intensive as compared to PFGE (Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail- ANSES, 2011; Rousell *et al.*, 2013). This method

combines whole genome digestion using one or two restriction enzymes with the use of fluorescent primers PCR that amplify and detect the obtained fragments. The use of automatic sequencer to size the amplified fragments and the use of specific software for the visualization of the fingerprint profiles allow a more rapid and accurate analysis respect PFGE (Lomonaco *et al.,*2011; Parisi *et al.*, 2010). Authors suggest that the use of fAFLP in the routine surveillance in food processing plants could be useful in revealing the source and the routes of *Listeria* contamination (Jadhav *et al*., 2012; Lomonaco *et al.*, 2011).

In recent years the development of sequencing molecular methods has led to nucleic acid sequencing for determining the exact order of nucleotides present in the DNA (Grada and Weinbrecht, 2013).

The second-generation sequencing methods, or next-generation sequencing (NGS), are becoming the future epidemiological investigations systems and will allow an accurate identification and characterization of bacterial isolates. NGS includes a group of methods in which several sequencing reactions take place simultaneously producing enormous amounts of sequencing data (Abel and Duncavage, 2013) that permit a rapid and complete analysis of the genome (Dheilly *et al.*, 2014). Comparison of the genome sequences allows a more accurate discriminatory power in subtyping microorganism and moreover, allows to identify and to analyze genes or proteins that could contribute to persistence in the environment or that are responsible of the virulence (Hoch *et al.*, 2013).

The large amount of data supplied by NGS are still far from being rapidly interpreted and analysed and therefore those methods are not yet suitable for routine surveillance (Sabat *et al*., 2013). Despite the widespread use of NGS, limiting factors to the implementation of this technology are the high cost for samples processing, the need

of bioinformatics training for the staff and high capacity electronic devices for data processing (Daber *et al,* 2013; Hui, 2014).

An ideal molecular diagnostic test should be a rapid and reliable method able not only to find and to type the microorganisms, but also able to predict the potential pathogenicity of the isolates (Jacquet *et al.*, 2004).

It is accepted that *L. monocytogenes* includes strains with different virulence and pathogenicity and for this reason it would be necessary the development of laboratory procedures able to recognize virulent from no-virulent strains present in foods and environment (Liu *et al.*, 2007).

The pathogenesis of *L. monocytogenes* is mediated by a number of virulence factors that play an important role in the onset of human listeriosis. Several virulence testing protocols have been developed to enhance the laboratory evaluation of *L. monocytogenes* virulence, as *in vivo* bioassays and *in vitro* cell assays that are efficient but not useful as routine techniques due to their cost, to the long time needed for the assay, the necessity of skilled personnel and to the little reproducibility of the analysis (Liu *et al.*, 2003). PCR assays for the detection of the presence of virulence-associated proteins and their corresponding genes, as *inlA, inlB, inlC, inlF, inlJ , hly, acta*, were described (Liu *et al.*, 2007; Lomonaco *et al.*, 2012: Balandyte´ *et al.*, 2011). Many authors agree that the *inlJ*, the *inlF*, *inlC* and *hly* genes could be used as markers for the determination of *Listeria monocytogenes* virulence (Shen *et al.*, 2013; Chen *et al.*, 2008; Dussurget, 2008; Liu *et al*., 2003, 2006, 2007; Sabet *et al.*, 2005). *In vivo* experiments showed that the presence of *inlJ* is essential for full virulence of *L. monocytogenes* (Sabet *et al.*, 2005) and Liu *et al.* (2006) showed that the *inlJ* is absent in non-pathogenic serotypes, such as serotype 4a. Sabet *et al.* (2005) in a work *in vivo* showed that the absence of *inlJ* and deletions in *inlC* reduced significantly the virulence of the

strains, data confirmed later by Dusserget (2008). The authors suggest that is not the detection of the gene but rather the analysis of differences in the nucleotides sequences that can allow differentiation of virulent from avirulent strains (Liu *et al.,* 2007; Shen *et al.,* 2013; Bierne *et al.,* 2007). Nucleotides mutations are more frequent in *hly* genes, leading to non-pathogenic strain, while mutations in *inlF*, *inlJ*, *inlC* and *actA,* would lead to a reduction of virulence (Liu *et al*., 2007). The presence of full-length internalins is highly associated with virulence, while the presence of truncated internalins is more frequent in non-clinical strains rather than clinical strains. This suggests that the risk associated with foods contaminated with *L. monocytogenes* could be assessed not only on the basis of the level of contamination but also on the basis of the functionality of the internalins (Balandyte *et al.,* 2011; Jacquet *et al.,* 2004). Balandyte *et al.* (2011) analyzed possible allelic differences in 9 internalins, in *pfrA* and *actA* in strains from clinical (human and animal) cases and from non-clinical (foods and environment) cases, highlighting significant differences in *actA* and *inlJ* genes. The authors concluded that the presence of particular alleles of the genes in clinical strains is correlated to their virulence.

Sequencing of the virulence factors genes by NGS provides useful information during epidemiological investigation and in the future may be the election tool for the evaluation of *L. monocytogenes* strains pathogenicity.

Previous studies showed a widespread *L. monocytogenes* contamination in cheese making plants transforming ewe's milk and that some areas in processing facilities (salting and washing rooms) play a critical role as reservoir of the microorganism (Ibba *et al.,* 2013; Spanu *et al.,* 2015). However, no investigation has been performed to explore the correlation between strains collected from Sardinian cheese making plants environment and their potential pathogenicity.

With this aim a selection of *L. monocytogenes* strains isolated from Sardinian cheese making plants environment and food products was compared using fAFLP with strains isolated from clinical cases of human listeriosis. NGS was used to characterize differences in the strains virulence. The present study was carried out in collaboration with the Foodborne Pathogens Reference Services of Public Health of England (PHE, London, UK) that provides national and international service for *L. monocytogenes*.

5.2. Materials and methods

5.2.1 Strains selection

Listeria monocytogenes strains were obtained from a collection of the Veterinary Medicine Department, Food Hygiene Sector, University of Sassari, Italy. The collection included strains isolated over a two year period from environmental and cheese samples collected in 13 cheese making plants using sheep milk operating in Sardinia (Italy). An extensive description of the sampling plan, methods and results has been previously described in Chapter 4 (Spanu *et al.*, 2015).

After screening by Pulsed-field gel electrophoresis (PFGE) (Graves & Swaminathan, 2001), 100 *L. monocytogenes* strains were selected for fAFLP analysis. The selection criteria was aimed to avoid overrepresentation of potential clones, therefore one strain for each positive sample was selected; in case that more than one PFGE profile was obtained from isolates of the same sample, all different profiles were included in the selection.

5.2.2 DNA extraction

DNA extraction was performed using Microlysis Plus DNA release reagent (Microzone Ltd, UK). Briefly, from each plate a single colony was picked with a 1 µl disposable loop and added to 19 µl of Microlysis reagent in a PCR tube. After centrifugation at 3,000 rpm for 10 seconds, samples were placed in a thermocycler to complete the DNA extraction. Six different time-temperature parameters were used: 1) 5 min at 65 °C; 2) 2 min at 96 °C; 3) 4 min at 65 °C; 4) 1 min at 96 °C; 5) 1 min at 65°C; 6) 30 sec at 96 °C. DNA was stored at 4 °C and used within 24 hours after the extraction.

*5.2.*3 Molecular Serogrouping

A Real Time PCR was carried out to classify *L. monocytogenes* isolates into serogroups (I-IV) and to confirm the results previously obtained using the multiplex PCR technique (Doumith *et al*, 2004). Gene detection for serogrouping was carried out using primers *lmo0737*, *lmo1118*, *ORF2819* and *ORF2110*. In addition the *prs* gene, specific for *Listeria* spp., and *plcA* gene, specific for *L. monocytogenes* were used as internal amplification controls. Samples were processed using a Real Time PCR platform Prism 7500, Fast Sequence Detector Taqman (Applied Biosystems Inc, CA, USA). The protocol consisted in two TaqMan-based Real Time PCRs: one triplex-PCR combined *ORF2110*, *ORF2819*, and *lmo1118* target genes, while second triplex-PCR targeted *lmo0737*, *plcA*, and *prs*. Combining the results of both triplex-PCRs, each isolate could be clustered into one of the four serogroups (Table 5.1).

5.2.4 Fluorescent amplified fragment length polymorphism (fAFLP)

Fluorescent amplified fragment length polymorphism (fAFLP) of the selected strains was performed at PHE laboratories, using the protocol described by Desai et al. (2001) with minor modification.

15-50 ng of DNA was digested with 6U of two restriction enzymes *HindIII* and *HhaI* (New England Biolabs Ltd, UK) with addition of RNase A and bovine serum albumine. The digested fragments were ligated with two adapters for *HindIII* and *HhaI* (New England Biolabs). The digestion/ligation was carried out by a single step rather than 3 different steps as proposed by Desai *et al.* (2001). The digest was then amplified by PCR using adapters-specific primers: *Hind-A*, labeled with the blue fluorescent dye 5-carboxyfluorescein (FAM), and *Hha-A*, non-labeled (Eurogentec, Seraing, Belgium). The amplified digested fragments were separated using an ABI 3730XL 96 capillary DNA Analyzer (Applied Biosystems) alongside a GeneScan - 600 LIZW Size standard. The fAFLP fragments were displayed using Peak Scanner v.1.0 software (Applied Biosystems) and visualized as fluorescent peaks. A peak profile differing by the presence or absence of at least one peak would be considered a different profile. The percentage of similarity between each fAFLP types selected was 100%.

5.2.5 Next Generation Sequencing

Total genomic DNA was extracted and purified using the Qiagen Biorobot Automated Extractors after a pre-extraction procedure with Lysozime (100mg/ml) and Proteinase K (20 mg/mL). After extraction the yield was evaluated using the Life Technologies® Quant-iT™ Broad Range dsDNA assay and the fluorometer GloMax® Multi+ Detection System with Instinct Software (Promega corp., Madison, WI, USA). Purity was also evaluated using LabChip® DS (PerkinElmer). DNA concentration was

also evaluated and samples with a concentration ranging between 10-30 ng/µl and with a DNA OD ratio (260/280) between 1.8 and 2 were submitted to NGS analysis.

The sequencing process consisted in two main phases. The first phase included the preparation and amplification of the DNA by Illumina® Nextera® XT Sample Preparation Kit. DNA was simultaneously fragmented and tagged using transposomes preparing a library of sequencing- fragments. Sequencing libraries contained 1ng of DNA. In the second phase the sequencing was carried out: ultra-high-throughput sequencing was runned by Illumina® HiSeq 2500 System sequencer.

5.2.6 Virulence factors analysis

In order to find possible *L. monocytogenes* virulent strains among the environmental and food isolates, the fAFLP profiles obtained were compared with the fAFLP profiles of strains isolated from human clinical cases available from the PHE collection. Environmental and food isolates showing the same fAFLP profile of the human strains were retained for further analysis, i.e. high-throughput sequencing with next generation sequencing (NGS).

Investigation of the potential pathogenicity of the selected strains was carried out by the analysis of the genes coding for internalin A (*inlA*), internalin J (*inlJ*) and hemolysin (*hly*). Genes were selected among the *L. monocytogenes* virulence factors showing a greater genetic variability.

Multiple alignments of the nucleotide sequences for *hly*, *inlA* and *inlJ* genes were performed using the ‒ClustalW‖ multiple alignment tool in BioEdit software version 7.2.5 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) to investigate the potential presence of different haplotypes. For this purpose haplotype

was defined as a group of strains sharing the same nucleotide sequences for a given gene. Each haplotype

sequence was then compared with reference sequences available in GeneBank international database (http://www.ncbi.nlm.nih.gov/genbank/). By aligning query sequence against all sequences in database, similarity of the selected genes was evaluated considering the percentage of identity and the coverage.

The reference strains for lineage I were *L. monocytogenes* Clip81459 serotype 4b (accession number: NC_012488) for the analysis of the *inlA* and *hly* genes and *L. monocytogenes* ATCC19117 serotype 4b (accession number: FR733643.1) for the analysis of the *inlJ* gene. As representative of Lineage II *L. monocytogenes* EGDe, serotype 1/2a (accession number: NC_003210.1) was used to investigate *inlA* and *hly* genes, while *L. monocytogenes* LSCC5850, serotype 1/2a (accession number: NC_018592.1) was selected for *inlJ* gene analysis.

Pairwise alignment (optimal GLOBAL alignment) was performed using BioEdit software. To assess variation in the protein sequence and the possible presence of Premature Stop Codons (PMSC), nucleotide sequences were translated in amino acid sequence using the BioEdit. The reference protein sequence was used as backbone for the investigated strain sequence frame.

Differences in the gene sequences were evaluated either at nucleotide and protein level. The presence of substitution, deletion and insertion of bases in the nucleotide sequence was used to classify the type of mutation occurred.

On the other hand, the effect on the protein sequence was assessed by the evaluation of the number of silent, missense and non-sense mutation. A silent mutation was observed when the modification in DNA codon produced no change of the encoded amino acid, a missense mutation when the mutation in the DNA codon leaded to a different amino acid while a non-sense mutation when the change in the genetic code resulted in a stop codon.

5.3. Results

5.3.1 Molecular Serogrouping

Molecular serogrouping conducted on the 100 selected strains by Real-Time PCR resulted in 57 strains belonging to serogroup I, 8 strains to serougroup II, 10 to serogroup III and 25 strains to serougroup IV (Figure 5.1).

5.3.2 fAFLP

Two main fAFLP profiles were obtained: the profile ‒VIIa.139‖ (including 20 strains isolated from three different cheese-making plants and the ‒IX.52‖ profile with 14 strains isolated from a single cheese-making plant. Twenty-five out of 29 fAFLP patterns (86.2%) were exclusively found in one premises, indicating a little variability of strains within cheese-making plant, while 4 (16.80%) fAFLP types (I.5, I.8b, VIIa.139 and VIIc.37a) were observed in two or more facilities.

Figure 5.1 shows the fAFLP profiles investigated that clustered according to PFGE analysis. For each strain is reported also the origin, the serogroups and the serotypes.

Comparison of fAFLP profiles of University of Sassari *L. monocytogenes* collection with strains belonging to the PHE collection allowed finding 20 strains of food processing environment origin showing a similarity level of 100% with strains originating from human clinical cases. This represented a selection criterion of strains, for further investigation on the potential pathogenicity by NGS analysis.

5.3.3 Virulence genes analysis

Out of 100 *L. monocytogenes* strains isolated from sheep's cheese making-plants environment, 20 showed the same fAFLP profile of strains isolated from human clinical cases. The strains collected either from food, food contact and non-contact surfaces were clustered into 6 different fAFLP profiles: I.8b (n.7, 35%), XII.17 (n.5, 25%), II.34 (n.3, 15%), I.5 (n.2, 10%), I.52 (n.2, 10%) and VIIa.104 (n.1, 5%).

The comparison of the nucleotide and amino acid sequences of *hly*, *inlA* and *intJ* genes between wild-type strains and reference strains showed nucleotide mutations in 14 (70%), 19 (95%) and 9 (45%) strains, respectively.

- *Haplotypes*

Multiple gene alignment of the target sequences with the reference sequences allowed identifying 4 *hly* haplotypes, 7 *inlA* haplotypes and 5 haplotypes for *intJ*.

- *BLAST analysis* (*hly*, *inlA* and *intJ*)

The BLASTN analysis revealed at least 80% of identity with reference sequences available in GeneBank. In particular for *hly* genes an identity ranging from 83% to 100% and query coverage of 99-100% was detected; for *inlA* the identity found was 99-100% and, with the exception of one haplotype, the coverage was 98-100%. A greater variability was observed for *inlJ*, with an identity ranging from 88% to 100% and a coverage from 23% to 100%. Detailed information on each gene for each haplotype is reported in table 5.2.

- *Nucleotide and protein mutations*

The number of nucleotide mutations occurring in *hly* and *inlA* genes ranged respectively from 3 to 4 and from 10 to 13. Mutations were exclusively for substitution of a single nucleotide (single base or point mutations). These mutations resulted in no change of the amino acid (silent mutation) or in the substitution with a different amino

acid (missense mutation) as reported in Table 5.3. A greater number of nucleotide mutations were observed for *inlJ* gene. *Listeria monocytogenes* strains belonging to lineage II were characterized by a greater variability as compared with lineage I strains. Unlike *hly* and *inlA* genes, in *inlJ* sequence more than one mutation occurred in a single triplet.

Most of the mutations were for transitions with substitution between A and G (purines) or between C and T (pyrimidines), while more rare were transversion mutations with substitutions between purine and pyrimidine. Transition accounted respectively for: ca. 65% for *hly* and 60-70% for *inlA* genes. For the gene encoding *inlJ* differences were observed between strains belonging to serotype 1/2a (ca. 50% transition) and serotype 1/2b (> 90%).

Differences were observed in the number of protein mutations between lineage I and lineage II, being silent mutations more frequent in lineage I and missense mutations more frequent in strains of lineage II (Table 5.3).

Most of the mutations were for transitions with substitution between A and G (purines) or between C and T (pyrimidines), while more rare were transversion mutations with substitutions between purine and pyrimidine. These mutations resulted in no change of the amino acid (silent mutation) or in the substitution of a different amino acid (missense mutation). The number of substitution for each strain and the mutation type is reported in table 5.3.

5.4 Discussion

Listeria monocytogenes is a food borne pathogen that includes a wide variety of strains characterised by differences in their virulence (Liu *et al*., 2003). Among all the 13 known serotypes of *L. monocytogenes* great part of human infections are caused by

serotypes 1/2a, 1/2b and 4b. This may imply differences in the virulence of the strains with some strains being somehow unable to cause infection in the host, despite they are frequently recovered from animal, from food and from various types of environment (Wiedmann *et al*., 1997). Therefore, it is important during surveillance and epidemiological studies investigate the potential pathogenicity of the strains. The recovery of avirulent strains from food could reduce the impact of sanitary measures taken during product recalls. A number of methods have been proposed to determine the virulence of *L. monocytogenes* strains: *in vivo* assessment, *in vitro* cell assays, determination of specific virulence-associated proteins (Liu *et al.*, 2003). However, a deeper characterisation of the potential virulence of strains should include DNA sequencing analysis. In fact, the potential pathogenicity of a strain could be related to the specific nucleotide composition rather than the presence of a virulence gene.

In the present study the potential virulence of *L. monocytogenes* strains isolated from sheep cheese-making plants and cheeses were investigated looking at the presence of mutations in nucleotide and protein sequences of specific virulence genes. Comparison of fAFLP profiles with strains obtained from human clinical cases was used to screen strains with a potential virulence attitude. After analysis by high-throughput sequencing with next generation sequencing (NGS) technique, sequences of the genes *hly*, *inlA* and *intJ* were investigated.

The *hly* gene encodes listeriolysin O (LLO) which is involved in the lysis of the host cell vacuole membrane. Nucleotide differences were observed in 19 out of 20 strains. Mutations occurred exclusively for nucleotides substitution which leaded to silent or missense mutations. The *inlA* gene encodes for a surface protein which plays a key role in the invasion of the host cells, mediating the entry of *L. monocytogenes* in the bloodstream through the intestinal barrier. The *inlJ* of the internalins family is a

virulence factor which mediates the adherence of *L. monocytogenes* with epithelial cells of the intestinal mucosa.

Overall the investigated virulence genes of wild-type strains were almost identical to the reference strains with regard to potential pathogenicity.

Although mutations occurred, they were exclusively point mutation with little (missense) if no change (silent) on the corresponding protein, as demonstrated by protein sequence.

5.5 Conclusion

Out of 20 wild-types strains isolated from cheese and cheese-making plants environment only one showed a sequence identical to the reference strains for all the three virulence genes investigated. The mutations observed in *hly*, *inlA* and *inlJ* genes were in most of the cases for substitution of nucleotides suggesting little change in the potential pathogenicity. However, in order to evaluate the relation between nucleotide polymorphism, protein structure and the definition of *L. monocytogenes* strain virulence further investigation should be conducted.

Tables

Tab.5.1 Target genes amplification in each triplex-PCR for serogroup classification of *Listeria* strains.

	Target	I (1/2a, 3a)	II (1/2c, 3c)	III (1/2b, 3b)	IV (4b, 4c, 4e)	Rare 4b strains	*Listeria* other than *L.monocytogenes*
Triplex 1	Orf2819	-	-	+	+	+	-
	Orf2110	-	-	-	+	+	-
	lmo 118	-	+	-	-	-	-
Triplex 2	*lmo737*	+	+	-	-	+	-
	Plc	+	+	+	+	+	-
	Prs	+	+	+	+	+	+

Tab. 5.2 Sequence identity and query cover percentages exhibit by the haplotypes.

Gene	Haplotype	Sequence identity*	Query cover*
inlA	$A_{(A)}$	99 - 100%	100%
	$B_{(A)}$	99 -100%	99 -100%
	$C_{(A)}$	99 -100%	98 - 100%
	$D_{(A)}$	89 - 100%	34 - 100%
	$E_{(A)}$	99 - 100%	98 - 100%
	$F_{(A)}$	99%	98 - 100%
	$G_{(A)}$	99%	98 - 100%
inlJ	$A_{(J)}$	88 - 100%	31 - 100%
	$B_{(J)}$	88 - 100%	83 – 100%
	$C_{(J)}$	99 %	33 – 100 %
	$D_{(J)}$	79 – 100 %	10 – 100 %
	$E_{(J)}$	89 – 100 %	34 – 100 %
hly	$A_{(hly)}$	83 - 100%	100 %
	$B_{(hly)}$	99 - 100%	100%
	$C_{(hly)}$	99 - 100%	99 - 100%
	$D_{(hly)}$	99%	99 - 100%

*Sequence identity: percentage of identity between two aligned sequences.

*Query cover: percentage of the query sequence that overlaps the subject sequence.

Table 5.3 Alignment results of *inlA, inlJ* and *hly* virulence genes in 20 *Listeria monocytogenes* wild-type strains (isolated from sheep cheese-making plants environment) and the reference strains EGDe1, Clip814592, SLCC58503 and ATCC191174.

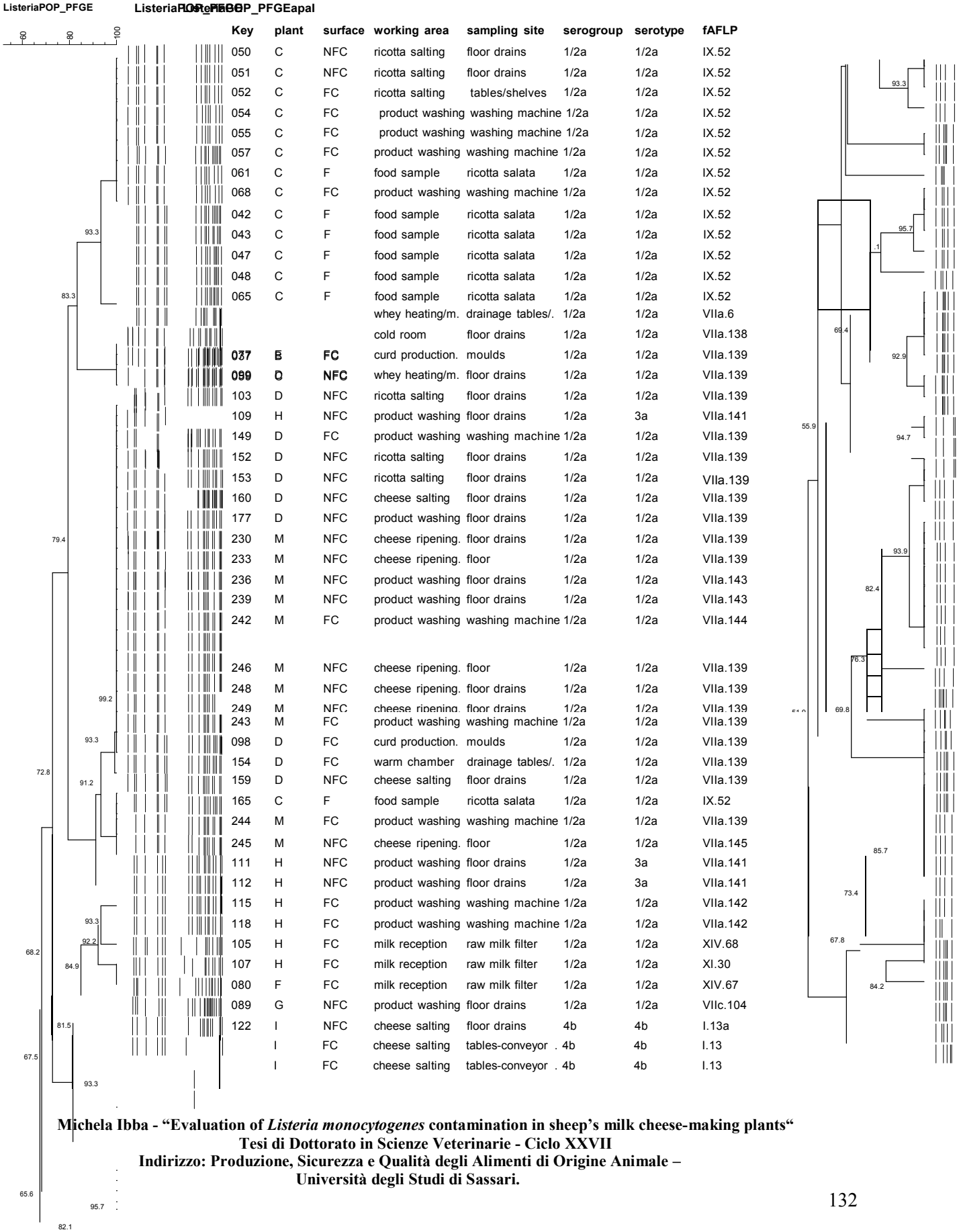
Strain ID	Virulence genes					
	inlA		*inlJ*		*hly*	
	type of nucleotide mutation (n)	**type of aa mutation (n)**	**type of nucleotide mutation (n)**	**type of aa mutation (n)**	**type of nucleotide mutation (n)**	**type of aa mutation (n)**
LM001	Substitution (10)	Silent (7) Missense (3)	Substitution (61)	Silent (19) Missense (33)	0	Silent (0) Missense (0)
LM006	Substitution (10)	Silent (7) Missense (3)	Substitution (61)	Silent (19) Missense (33)	0	Silent (0) Missense (0)
LM007	Substitution (11)	Silent (6) Missense (5)	-	-	3	Silent (2) Missense (1)
LM008	Substitution (10)	Silent (6) Missense (4)	-	-	3	Silent (2) Missense (1)
LM013	Substitution (10)	Silent (6) Missense (4)	-	-	3	Silent (2) Missense (1)
LM018	Substitution (10)	Silent (6) Missense (4)	-	-	3	Silent (2) Missense (1)
LM028	Substitution (10)	Silent (7) Missense (3)	Substitution (61)	Silent (19) Missense (33)	0	Silent (0) Missense (0)
LM029	Substitution (10)	Silent (7) Missense (3)	Substitution (61)	Silent (19) Missense (33)	0	Silent (0) Missense (0)
LM033	Substitution (10)	Silent (7) Missense (3)	Substitution (61)	Silent (19) Missense (33)	0	Silent (0) Missense (0)

LM070	Substitution (11)	Silent (8) Missense (3)	Substitution (15)	Silent (10) Missense (5)	3	Silent (2) Missense (1)
LM071	Substitution (11)	Silent (8) Missense (3)	Substitution (15)	Silent (10) Missense (5)	3	Silent (2) Missense (1)
LM075	Substitution (11)	Silent (8) Missense (3)	Substitution (16)	Silent (11) Missense (5)	3	Silent (2) Missense (1)
LM082	Substitution (12)	Silent (7) Missense (5)	-	-	3	Silent (2) Missense (1)
LM089	(0)	Silent (0) Missense (0)	Substitution (5)	Silent (3) Missense (2)	0	Silent (0) Missense (0)
LM134	Substitution (10)	Silent (6) Missense (4)	-	-	4	Silent (2) Missense (2)
LM380	Substitution (10)	Silent (6) Missense (4)	-	-	3	Silent (2) Missense (1)
LM390	Substitution (13)	Silent (9) Missense (4)	-	-	4	Silent (2) Missense (2)
LM391	Substitution (10)	Silent (6) Missense (4)	-	-	3	Silent (2) Missense (1)
LM393	Substitution (10)	Silent (6) Missense (4)	-	-	3	Silent (2) Missense (1)
LM397	Substitution (10)	Silent (6) Missense (4)	-	-	4	Silent (2) Missense (2)

Reference strains were selected according to the strains lineage: [1]for *inlA* and *hly* in lineage II strains (serotypes 1/2a, 3a and 1/2c); [2] for *inlA* and *hly* in lineage I strains (serotypes 1/2b, 3c, 4b and 4d); [3] for *inlJ* in lineage II strains (serotypes 1/2a, 3a and 1/2c); [4] for *inlJ* in lineage I strains (serotypes 1/2b, 3c, 4b and 4d).

Figures

Fig.5.1 Dendrogram showing the PFGE clustering of strains with the corresponding fAFLP profiles.



Key	plant	surface	working area	sampling site	serogroup	serotype	fAFLP
050	C	NFC	ricotta salting	floor drains	1/2a	1/2a	IX.52
051	C	NFC	ricotta salting	floor drains	1/2a	1/2a	IX.52
052	C	FC	ricotta salting	tables/shelves	1/2a	1/2a	IX.52
054	C	FC	product washing	washing machine	1/2a	1/2a	IX.52
055	C	FC	product washing	washing machine	1/2a	1/2a	IX.52
057	C	FC	product washing	washing machine	1/2a	1/2a	IX.52
061	C	F	food sample	ricotta salata	1/2a	1/2a	IX.52
068	C	FC	product washing	washing machine	1/2a	1/2a	IX.52
042	C	F	food sample	ricotta salata	1/2a	1/2a	IX.52
043	C	F	food sample	ricotta salata	1/2a	1/2a	IX.52
047	C	F	food sample	ricotta salata	1/2a	1/2a	IX.52
048	C	F	food sample	ricotta salata	1/2a	1/2a	IX.52
065	C	F	food sample	ricotta salata	1/2a	1/2a	IX.52
			whey heating/m.	drainage tables/.	1/2a	1/2a	VIIa.6
			cold room	floor drains	1/2a	1/2a	VIIa.138
037	E	FC	curd production.	moulds	1/2a	1/2a	VIIa.139
099	D	NFC	whey heating/m.	floor drains	1/2a	1/2a	VIIa.139
103	D	NFC	ricotta salting	floor drains	1/2a	1/2a	VIIa.139
109	H	NFC	product washing	floor drains	1/2a	3a	VIIa.141
149	D	FC	product washing	washing machine	1/2a	1/2a	VIIa.139
152	D	NFC	ricotta salting	floor drains	1/2a	1/2a	VIIa.139
153	D	NFC	ricotta salting	floor drains	1/2a	1/2a	VIIa.139
160	D	NFC	cheese salting	floor drains	1/2a	1/2a	VIIa.139
177	D	NFC	product washing	floor drains	1/2a	1/2a	VIIa.139
230	M	NFC	cheese ripening.	floor drains	1/2a	1/2a	VIIa.139
233	M	NFC	cheese ripening.	floor	1/2a	1/2a	VIIa.139
236	M	NFC	product washing	floor drains	1/2a	1/2a	VIIa.143
239	M	NFC	product washing	floor drains	1/2a	1/2a	VIIa.143
242	M	FC	product washing	washing machine	1/2a	1/2a	VIIa.144
246	M	NFC	cheese ripening.	floor	1/2a	1/2a	VIIa.139
248	M	NFC	cheese ripening.	floor drains	1/2a	1/2a	VIIa.139
249	M	NFC	cheese ripening.	floor drains	1/2a	1/2a	VIIa.139
243	M	FC	product washing	washing machine	1/2a	1/2a	VIIa.139
098	D	FC	curd production.	moulds	1/2a	1/2a	VIIa.139
154	D	FC	warm chamber	drainage tables/.	1/2a	1/2a	VIIa.139
159	D	NFC	cheese salting	floor drains	1/2a	1/2a	VIIa.139
165	C	F	food sample	ricotta salata	1/2a	1/2a	IX.52
244	M	FC	product washing	washing machine	1/2a	1/2a	VIIa.139
245	M	NFC	cheese ripening.	floor	1/2a	1/2a	VIIa.145
111	H	NFC	product washing	floor drains	1/2a	3a	VIIa.141
112	H	NFC	product washing	floor drains	1/2a	3a	VIIa.141
115	H	FC	product washing	washing machine	1/2a	1/2a	VIIa.142
118	H	FC	product washing	washing machine	1/2a	1/2a	VIIa.142
105	H	FC	milk reception	raw milk filter	1/2a	1/2a	XIV.68
107	H	FC	milk reception	raw milk filter	1/2a	1/2a	XI.30
080	F	FC	milk reception	raw milk filter	1/2a	1/2a	XIV.67
089	G	NFC	product washing	floor drains	1/2a	1/2a	VIIc.104
122	I	NFC	cheese salting	floor drains	4b	4b	I.13a
	I	FC	cheese salting	tables-conveyor .	4b	4b	I.13
	I	FC	cheese salting	tables-conveyor .	4b	4b	I.13

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CHAPTER 6

General conclusion

The present survey provides a contribution to the knowledge of prevalence, traceability and potential pathogenicity of *L. monocytogenes* strains isolated from industrial sheep's milk cheese-making plants operating in the regional territory of Sardinian (Italy).

According to previous studies (Pritchard *et al*., 1995; Pilo *et al*., 2008; Parisi *et al.*, 2010) our results showed a high prevalence of *L. monocytogenes* in the processing environment and all facilities analysed were found to be positive for the presence of the microorganism. The prevalence of contamination in the plants ranged from 3% to 22.6%. Contamination by *L. monocytogenes* was observed, although with different prevalence, in all cheese making steps.

The study showed the greater contamination in the areas where the products are subjected tointensive handling such as product washing, salting areas, cheese ripening areas and packaging areas. According with the data reported in literature (Tompkin, 2002), the sites with highest contamination are the floor drains (over 40%). Contamination of the plants appears to be due to strains already present in the processing environments, with all the facilities serving as source of products contamination. Persistent contamination was observed in sites know to be niches of persistence of *L. monocytogenes* and presenting favourable conditions for microorganism survival: floors, floor drains and washing machine. The washing machine represents an important source of contamination of the finished products: the presence of organic residues within brushes and the design of the machine don't allow a correct application of cleaning and sanitizing procedures and the use facilitate the cross contamination between products. Environmental niches as the floor drains are critical sites for the control of contamination in the dairies processing environments and food products (Tompkin, 2002). For this reason it is important to monitor the contamination in this sites which serve as sentinel to detect *Listeria* contamination in the premises. Since all *Listeria* spp share the same ecological niches, testing for *Listeria* spp. can be used as a useful indicator of a decline of process hygiene conditions during food production (McLauchlin, 1997; Tompkin, 1999). Our results showed that *Listeria* spp. other than *L. monocytogenes* were isolated in all the plants analysed. Well-

designed control and sanitation procedures, as sampling and cleaning programs, are important to decrease the incidence of *L. monocytogenes,* to avoid the diffusion of the contamination in the environment and in the products (Ryser *et al.*, 2011) and to avoid the instauration of niches of persistent strains. Regular environmental sampling programs are essential to monitor the presence of the persistent harbourage sites of contamination (trend analysis). The identification of niches is essential to prevent spreading of the contamination in the processing plants. Sites that should be tested are those that are expected to harbour the microorganism and where niches may occur, generally they are sites hard to reach and clean (e.g. hollows part, conveyor belts, machines) with the routine cleaning and sanitizing procedures.

The *L. monocytogenes* serotypes most isolated in the study were 1/2a, 1/2b and 4b, that are the main serotypes associated with human cases of epidemic (4b) and or sporadic cases of listeriosis (1/2a, and 1/2b) (Rocourt, 1994). The recovery of these serotypes from food processing areas have plays an important role in terms of impact on public health.

With the exception of three plants, PFGE results showed a great level of diversity among the isolates collected from cheese making plants and their products, in contrast with those reported for other dairy products (Lomonaco *et al.,* 2007; Carminati *et al.,* 2004). The strains variably can be attributed to the custom of introducing products originating from other plants that favour the cross-contamination between different plants. The study of *L. monocytogenes* throughout the whole processing flow was useful to identify the most likely sources of contamination of the final product. Isolates from food samples clustered together with environmental strains, confirming that the processing environment is a source of *L. monocytogenes* contamination in Sardinian cheese-making plants. In particular the strains recovered from Ricotta Salata samples showed a similar profile with strains recovered from washing machine, confirming the important role played in product contamination. Strains isolated from filter used to filter raw milk were never found in the final product neither in the processing areas, allowing to exclude the raw materials as source of contamination.

L. monocytogenes includes strains with different virulence and pathogenicity and for this reason it would be necessary the development of laboratory procedures able to recognize virulent from not virulent strains present in foods and food processing environment (Liu et al., 2007). The analysis of differences in the nucleotides sequences could be used to differentiate virulent from avirulent strains (Liu et al., 2007; Shen et al., 2013; Bierne et al., 2007). In the study NGS was used for the evaluation of *L. monocytogenes* strains pathogenicity. The presence of mutations in nucleotide and protein sequences of the *hly, inlA* and *inlJ* genes were investigated. In all the strains analysed the mutation observed were exclusively point mutation with little (missense) if no change (silent) on the corresponding protein, as demonstrated by protein sequence. The findings suggest that all the isolates analysed could be potentially dangerous for public health. In order to clarify the relationship between nucleotide polymorphism, protein structure and the definition of *L. monocytogenes* strain virulence further analyses should be conducted on more virulence genes. However, according with other authors our study suggests that to assess the risk associated with consumption of food products contaminated with *L. monocytogenes* it is possible to evaluated the functionality of the genes encoding for the virulence factors. This approach should allow for more-rational risk assessment and, thus, more-efficient and cost-effective control measures for foodborne listeriosis.

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