UNIVERSITÀ DEGLI STUDI DI SASSARI

SCUOLA DI DOTTORATO IN

RIPRODUZIONE, PRODUZIONE, BENESSERE ANIMALE E SICUREZZA DEGLI ALIMENTI DI ORIGINE ANIMALE

Direttore Prof. Giovanni Garippa

INDIRIZZO IN: **Produzione e Sicurezza degli Alimenti di Origine Animale (XXIV CICLO)**

(coordinatore: prof. Basilio Remo Floris)

LONGITUDINAL STUDY ON *LISTERIA MONOCYTOGENES* IN SARDINIAN FERMENTED MEAT PRODUCTS PROCESSING PLANTS: CONTAMINATION ROUTES, PATHOGENIC PROFILE AND EVALUATION OF GENE EXPRESSION IN PERSISTENT AND NON-PERSISTENT ISOLATES

Docente Guida

Chiar.ma Prof.ssa R. Mazzette

Correlatore

Dott. Domenico Meloni

Direttore

Prof. Giovanni Garippa

Tesi di dottorato della

Dott.ssa ROBERTA MAZZA

ANNO ACCADEMICO 2010 - 2011

Abstract

Detection and enumeration of *L. monocytogenes* have been carried out in 385 samples of raw materials, fermented sausages and environments of different processing plants located in Sardinia (Italy). A subset of ninety - seven strains isolated during a period from 2004 to 2011 were characterized by multiplex PCR-based serogrouping, multiplex PCRs for the presence of virulence genes, PFGE, and quantitative assessment of the *in vitro* biofilm formation. In addition, Relative and Absolute Quantification of genes expression of *pocR*, *mdr*, *eutG* and *cbiD*, probably involved in the persistence of the pathogen in the environment have been evaluated, in persistent and non-persistent isolates by Real-Time PCR. The 22% of the samples were positive for the presence of *L. monocytogenes*. The 31.51% and 15% of the samples tested, food and environment, respectively, were positive for *Listeria monocytogenes*. Isolates were serotyped as 1/2a, 1/2c, 1/2c and 4b, and presented all the considered virulence genes. PFGE revealed a high heterogeneity of pulsotypes within the plants. PFGE results did not showed the presence of persistent strains among the selection included in this study. The 44% of the tested strains were able to attach to abiotic surfaces forming biofilm. Relative and Absolute qRTi-PCR showed that the genes selected were not involved in persistence.

INTRODUCTION

The microbiological and physicochemical characteristics of sausage allow contamination by *Listeria monocytogenes*, which was found at the surface level in 9% of sausages sold in Italy (Cantoni et al., 1998). Prevalences in sausages are between 25 and 32% (Barbuti et al. 1989; Cantoni et al., 1990, Comi et al. 1990; Cantoni, 1991).

Fermented sausages and semi-mature cheeses are "ready to eat" (RTE) products, for which the contamination by *Listeria monocytogenes* at consumption, represents a potential risk for human health (Levine et al. 2001; Moore, 2004).

Recently, a study aimed at the characterization of cured sausage, supported by the Ente Regionale di Sviluppo e Assistenza Tecnica in agricoltura (ERSAT), in collaboration with the Committee for the exploitation of Sardinian cured meats), which involved about a quarter of the meat processing plants in Sardinia, showed that over 40% of local sausages are contaminated by *Listeria monocytogenes* (Mazzette et al. 2005; Meloni et al., 2006).

The investigation of the sources of contamination of the pathogen at plant level, showed the presence of *Listeria monocytogenes* in raw materials and in surfaces with contact

and without contact with food. Phenotypic and molecular characterization of the strains allowed to highlight that the serotypes circulating in Sardinian sausages and their facilities, (1/2a, 1/2B, 1/2c and 4b) belong to clones (DUP-1038) which most frequently is involved in sporadic outbreaks of Listeriosis (Mazzette et al., 2006). At present, the information about the contamination levels of *Listeria monocytogenes* in RTE meat products marketed in Sardinia and their potential role in the spread and transmission of the pathogen to humans, are still limited. However, in the last years there has been an increase of sporadic cases of listeriosis, not only in patients considered at risk, but also in immunocompetent individuals (Walls et al., 2000).

Many cases of listeriosis are linked to secondary contamination by *Listeria monocytogenes* during or after food processing. *Listeria monocytogenes* is capable to survive to various stress conditions commonly found in food and food environment. It tends to maintain itself in niches in the processing environment, particularly in areas which are hard to clean, such as drains, rollers of conveyor belts, and worn or cracked rubber seals around doors (Tompkin et al. 2002). An individual strain, known as a persistent strain, can sometimes be repeatedly isolated from the same facility after a

period of months or even years, although sporadic contamination also occurs (Kathariou et al. 2002). The formation of biofilms involving *Listeria monocytogenes* is believed to be an important cause for such persistence. However, little is known, about the genetic mechanisms concerning the persistence of *Listeria monocytogenes* in the environment and the genes involved in these mechanisms.

Based on these assumptions, this work aims to deepen the knowledge of the prevalence, of the identification of sources of contamination and persistence of *Listeria monocytogenes*, and the genetic mechanisms that regulate it along the chain of Sardinian sausage.

LISTERIA MONOCYTOGENS: MICROORGANISM CHARACTERISTICS

Members of the genus *Listeria* are gram positive, nonsporeforming, facultatively anaerobic rod and they are 4 to 0.5 um in diameter, and 0.5 to 2 um in length.

They possess peritrichous flagella, which give them a characteristic tumbling motility, occurring only in a narrow temperature range. The genus *Listeria* is currently comprised of six species: *Listeria monocytogenes, Listeria ivanovii, Listeria innocua, Listeria seelingeri, Listeria welshimeri* and *Listeria grayi*, which were recognized decades ago.

Two novel species have been recently described, *Listeria marthii* and *Listeria recourti*.(graves et al., 2009; Leclercq et al., 2009)

Only two of them, *Listeria monocytogenes* and *L. ivanovii* are pathogenic for human and mammals. This two species are distinguishable by hemolysis and phospholipase activity and blood and chromogenic agars, respectively.

Listeria monocytogenes is catalase positive and oxidase negative and expresses a P-hemolysin which produces zones of clearing on blood agar. It can grow in presence of CO_2 at low temperatures, but at a concentration of 70% of CO_2 and a temperature <7 ° C becomes inert. Under the same conditions, the concentration of 5% of oxygen makes

Listeria monocytogenes able to grow (Wimpfheimer et al., 1990). *Listeria monocytogenes* is generally resistant to small changes of some environmental parameters. Significant changes or multiple complexes instead stimulate stress responses that are directed to survival rather than growth of the bacterium (Booth, 1998).

Listeria monocytogenes is widely present in plant, soil, and surface water samples (Weis and Seeliger, 1975) and has also been found in silage, sewage, slaughterhouse waste, milk of normal and mastitic cows, human and animal faeces (McCarthy, 1990.) *Listeria monocytogenes* has been isolated from cattle, sheep, goats, and poultry, but infrequently from wild animals (Gray and Killinger. 1966.)

TAXONOMY

The bacterium *Listeria monocytogenes* was first isolated by Murray et al. (1926) from cases of sudden death in rabbits, and named *Bacterium monocytogenes*. The name *Listeria* was first proposed by Pirie in 1940. *Listeria monocytogenes* remained the only member of the genus until 1948, when *L. denitrificans* was classified into this genus by Sohier et al.However, subsequent 16s rRNA studies reclassified *L. denitrificans* into

another genus, *Jonesia*. By 1985, the genus *Listeria* had 6 new members: *L. grayi, L. murrayi, L. innocua, L. welshimeri, L. seeligeri*, and *L.ivanovii*. There has been much debate over the taxonomy of *L. grayi and L. murrayi*. Recourt et al. (1992) carried out a study about the genomic relatedness of the two species using DNA-DNA hybridization, multilocus enzyme electrophoresis, and rRNA restriction length polymorphism analysis. The results suggested that both species should be reconsidered as members of a single species, *L.grayi*. Two recent species have been added to the genus *Listeria, L. rocourtiae* (Leclerq et al. 2010), and *Listeria marthii* (Graves et al. 2010). The taxonomic position of *L.grayi*, which is more distantly related to the other *Listeria* species, remains a subject of discussion.

Regarding the evolution of the genus *Listeria*, genomic analysis of the species within the genus has identified genetically stable genomes, with limited gene acquisition or less (den Bakker et al. 2010). A common ancestor of *Listeria* spp. has been proposed (den Bakker et al. 2010). The author divides the strains into two main clades (*L. recourtiae* was not included in the studies). One clade contains the pathogenic strain *Listeria monocytogenes, L. innocua* and *L.marthii*. The other one contains the pathogenic *L.ivanovii* and *L. seeligeri*. Placement of *L. welshimeri* varies. For example,

den Bakker et al. (2010) place it into the first clade, whereas Schmid et al. (2005) group *L. welshimeri* with the second clade. *L. grayi* did not cluster in either of these two clades.

Schmid et al. (2005) suggest that *L. grayi* represents the oldest branch of the genus, with the other species diverging more recently from a common ancestor

PHYSIOLOGY

A range of environmental conditions affects the growth, death or survival of *Listeria monocytogenes*

TEMPERATURE

Listeria monocytogenes grows between -0.4 and 50° C (Junttila et Al 1988; Walker et Al 1987), with optimal growth between 30 to 37 °C. It resists to thermal stress represented by the application of cooling temperatures, suffering minor damage and overcome this traditional barrier of the process of food preservation. One should not ignore the fact that at the household level, the temperature of the refrigerator is more often close to 9 ° C rather than 4 ° C (Sergelidis et al., 1997). However the pathogen is not able to grow and multiply at freezing temperatures. At temperatures above 50 ° C its growth is limited and the organism becomes inert. (Fabbi et al., 2005) Fleming et al. (1985) claim that the thermal tolerance of the microorganism is greater than that of other non-spore-forming pathogens in food. This property can increase after exposure to various stressful environmental conditions such as, for example, the heating temperatures or the presence of sub-lethal osmotic shock. The effect of thermal shock of

Listeria monocytogenes is highly dependent on temperature and duration of the treatment itself: high temperatures and long treatments will increase the resistance (Linton et al., 1992).

pH

Listeria monocytogenes can grow at pH ranges between 4.6 and 9.6 with an optimum at 7.1 (AFSSA, 2000). The meat pH (5.0-5.5), can induce in *Listeria monocytogenes* a response of tolerance to acidity, this reduces the sensitivity of the microorganism to further lower pH (Faleiro et al., 2003), especially those recorded during ripening of fermented sausages (Thevenot et al., 2005). Acidity adapting, can induce cross-protection against other potentially harmful factors, including osmotic stress (Vasseur et al., 2001).

In contrast, sub lethal stress, osmotic or thermal does not appear to affect the resistance of the organism to the acidity (Koutsoumanis et al., 2003). *Listeria monocytogenes* survives even at strongly acidic pH values, characteristic of the human stomach and macrophage phagosomes (Cotter and Hill, 2003).

WATER ACTIVITY

Listeria monocytogenes is able to multiply in substrates with aw ≥ 0.97, but it is also able to grow at aw values of 0.93. During the ripening of sausages, aw values tend to fall, as the pH, which is close to the isoelectric point (Tyopponen et al., 2003). Adding salt to sausages, change the a_w values, inhibiting the growth of both, bacteria of spoilage agents, and pathogenic bacteria, including *Listeria monocytogenes* (Lucke, 1985). At the end of the ripening, the a_w values are typically <0.90, values that inhibit the growth of the pathogen (Tyopponen et al., 2003). However, part of producers, show a growing tendency to reduce the ripening time, for economic reasons. Products not sufficiently mature, show a_w levels (0.92) which allow the growth of *Listeria monocytogenes* and must be classified as risk products (AFSSA, 2000).

SALT CONCENTRATION

Listeria monocytogenes is considered capable of growing in NaCl concentrations of 12%, even if it shows highest multiplication ability around values of approximately 6.5% (Fabbi et al., 2005). However, the tolerance to sodium chloride varies in relation to pH and temperature (AFSSA, 2000). *Listeria monocytogenes* usually encounters high

concentrations of NaCl in cured meat products, particularly in fermented sausages (Dabin and Jussiaux, 1994). In sausages, salt carries a major conservative action, linked to the ability to bind water (dehydration). It has also a selective function on some microbial species, through both indirect, as described above, and direct action, with a particular combination of protein-salt which is a bad way to the development of saprophytic germs of putrefaction. Halophilic microbial species (*micrococci* and *staphylococci*), which are of considerable importance in the success of the process of maturation, can found in an enriched environment of salt conditions optimal for development (Ghinelli, 1985). Glucose is the main energy source (Pine et al. 1989; Premaratne et al., 1991), but the organism is also able to hydrolyze esculin and sodium hypochlorite and produce acid from L-rhamnose, ametil-D manniside and mannitol.

Serology

Paterson described four serological types of *Listeria monocytogenes* based on somatic (O) and flagellar (H) antigens (Riser and Marth 1991).

Thirteen serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7) of *Listeria monocytogenes* are known. Most human disease is caused by serotypes 1/2a, 1/2b, and

4b; therefore, serotyping alone is not sufficiently discriminating for subtyping purposes. (Murray et Al, 1995)

Pathogenicity

Three of eight *Listeria spp*. can cause human and/or animal infections: *Listeria monocytogenes, L. ivanovii and L. seelingeri* (Hof and Hefner, 1988; Seeliger et al 1984.) Most of the human infections are caused by *Listeria monocytogenes*.

All the strains of *Listeria monocytogenes* are able to cause illness in humans. However several observations suggest that, the virulence of *Listeria monocytogenes* is considered to be rather heterogeneous, as there are differences in the expression of pathogenicity among different strains, which would allow classifying them in virulent, moderately virulent and avirulent. The variability in virulence of different subtypes of *Listeria monocytogenes* affects the amount of organisms required to cause infection and thereby influences the evolution and severity of clinical manifestations and the level of exposure of high-risk categories (Griffiths, 2002). Several epidemiological studies revealed differences in the virulence of strains isolated from food and the prevalence of some serotypes in cases of human disease (Norrung and Andersen, 1999). Many strains of

Listeria monocytogenes commonly isolated during outbreaks of listeriosis registered around the world belong to only 3 (1/2a, 1/2b and 4b) of the 13 known serotypes (Gasanov et al., 2005) and represent 95% of isolates from clinical cases of epidemic or sporadic listeriosis (Mc Lauchlin, 1990). Among these, serotype 4b is the most commonly associated with outbreaks of human listeriosis, while serotypes 1/2a and 1/2b are responsible of sporadic cases of infection (Ryser, 2003). The higher virulence of strains 1/2a, 1/2b and 4b may be related to some particular characteristics: it was observed that strains belonging to serotype 1/2a, survive to bacteriocins at 4 ° C, better than strains 4b, and also have greater ability to survive during the passage into the stomach and are able to replicate in the food with more speed and intensity than other serovars (Rocourt, 1996; Fabbi et al., 2005). This may explain the fact that the strains isolated from foods belong mostly to the antigen group 1/2 (1/2a, 1/2b, 1/2c) and are predominantly serotype 1/2a (Rocourt, 1996), while serotype 4b, are generally isolated in 10-12% of the contaminated food (Fabbi et al., 2005). In contrast, isolates of *Listeria monocytogenes* belonging to serotype 4b survive better at heat treatment after refrigeration compared to serotypes 1/2a. This feature may affect a greater ingestion of 4b strains. Furthermore, after refrigeration, strains 4b are able to replicate faster than

strains 1/2a at 37 ° C, sometimes showing a higher pathogenicity. These features justify the higher capacity of serotypes 4b to be pathogenic for humans (Fabbi et al., 2005).

Molecular typing methods (multilocus enzyme Electrophoresis - MEE), Pulsed Field Gel Electrophoresis - PFGE, Ribotyping, Amplified Fragment Lentgth Polymorphysm - Restriction Fragment Length Polymorphism AFLP and - RFLP, confirmed the existence of a different pathogenic potential of *Listeria monocytogenes* (Wiedmann et al., 1997), which has been divided into three distinct lineages (lineages I, II, III). In Lineage I serotype ranged 1/2b, 3b, 4b, 4d and 4e (Nadon et al., 2001, Norton et al., 2001), while Lineage II includes serotypes 1/2a, 1/2c, 3a and 3c. The Lineage III is considered a distinct taxonomic division and includes less commonly isolated serotypes, such as the fourth and 4c (Rasmussen et al. 1995; Wiedmann et al. 1997; Nadon et al., 2001).

VIRULENCE FACTORS

Listeria monocytogenes is a facultative intracellular pathogen that can invade and multiply in both professional phagocytic and in non-phagocytic cells (Cossart et al 2003; Farber et al 1991; Gaillard et al 1987; Severino et al 2007; Vazquez-Boland et al 2001). In order to achieve the invasion, *Listeria monocytogenes* tightly controls the

expression of a broad range of virulence genes to assure the production of the necessary virulence effectors at the specific sites along the process of invasion and infection of the host tissue.

Virulence factors can be classified into two categories:

1. **Virulence factors involved in the host cells adhesion and invasion**

- ***InlA*:** it is a protein that allows the invasion of epithelial cells and has its cellular receptor, the E-cadherin, a glycoprotein expressed involved in the process of adhesion (Cossart et al., 2000).

- ***InlB*:** it is a protein involved in the ignition of an invasion mechanism based on the signaling intracellular "pathway" mediated by growth factors. *InlB* is mainly involved in the hepatocytes invasion (Fabbi et al., 2005).

- ***IAP (invasion associated protein)*:** it seems to be involved particularly in the process of fibroblasts invasion (Swaminathan, 2001; Mc Lauchlin et al., 2004). The *IAP* protein also called *p60* has a very important function in the process of cell division, acting as a hydrolase constituent the cell wall (Schmid et al., 2005).

- ***Ami*:** it is a surface protein which has a lytic activity against the cell wall of *Listeria monocytogenes*, with an important role in the accession process.
- ***FbpA*:** it operates by modulating levels of *Listeriolisin O* and *InlB* and preventing the degradation of the major virulence factors.
- ***Listeriolisin O (LLO)*:** the essential role of *listeriolisin O* is the destruction of the phagosomes, but it is also involved in the host-parasite interaction. It could trigger various cellular responses such as interleukin-1 secretion by macrophages, apoptosis by dendritic cells, cell adhesion and adhesion molecules expression by the infected epithelial cells. Many of these functions are dependent on Ca^{+2}. Once secreted by the bacterium, they bind cholesterol on the membrane, and the oligomerization forming a pore. As a result, the internal pH of phagosomes rises, blocking the maturation of phospholipase C and allowing dissolving the entire phagosomes (Swaminathan, 2001).

  ***ActA*:** this protein, at this stage, is involved in the process of accession and entry into host cells.

2. Virulence factors involved in the intracellular life cycle

- ***Phospholipase C*:** the main function of *phospholipase C* (in association with the *listeriolisin O*) is to promote the release from the first phagocyte vacuole and subsequently mediate the dissolution of the double membrane after the diffusion from cell to cell (Vazquez-Boland et al. 2001a; Mc Lauchlin et al., 2004)
- ***ActA*:** its role is to induce the polymerization of actin filaments, promoting the motility of the bacterium in the cytoplasm of infected cells and the spread from cell to cell.
- ***Mpl*:** is a zinc-dependent protein that contributes to the activation of phospholipase and the maturation of cysteine of the host cell.

MECHANISMS OF VIRULENCE

Major virulence determinants, such as the *internalin A*, and *InlB*, the cholesterol dependent pore forming bacterial toxin *listeriolysin (LLO)*, phospholipases *PlcA* and *PlcB*, the actin-based motility surface protein *ActA*, or the master regulator of virulence *PrfA,* are conserved in virulent and in less virulent strains of *Listeria monocytogenes* (Balandytė L. et al 2010). Hence, the presence of these genes alone is not enough to explain the differences in virulence of any one particular strain

(Cossart et al 2003; Nelson et al 2004; Vazquez-Boland et al 2001). Several cell wall-associated proteins are involved in *Listeria monocytogenes* virulence, such as internalin-like proteins *InlE*, *InlF*, *InlG*, *InlH*, which are not involved in invasion process, but are important for the colonization of host tissues in vivo (Kirchner et al 2008; Vazquez-Boland et al 2001), as well as autolysin, which is necessary but not sufficient for the entry (Dussurget 2008; Dussurget et al 2004), and invasion-mediating protein *Vip*, which is required for invasion of several cell lines (Dussurget 2008). The recently discovered internalin-like protein *InlJ* is required for full virulence of *Listeria monocytogenes* in vivo (Dussurget 2008). Moreover, *InlC* and *InlC2* may play an important role in specific host-associated niche (Tsai et al, 2006). Particular to *Listeria monocytogenes* is that it can induce its entry into a broad range of non-phagocytic mammalian cells (Pizarro-Cerda et al, 2010). For this purpose, the bacterial surface-anchored protein *InlA* interacts with its cellular receptor *E-cadherin* to promote invasion into polarized intestinal epithelial cells, while *InlB* interacts with the hepatocyte growth factor (Met) to trigger entry into hepathocytes and non-polazized cells (Ireton and Cossart, 1997; Lecuit et al 2001; Mengaud et Al 1996; Shen et al 2000). Furthermore, interactions between host cell receptors and

InlA or *InlB* are species-specific (Kirchner and Higgins D, 2008). The concerted action between *InlA*, *InlB*, and *InlJ* (the latter being specifically expressed during infection in vivo) is required for the invasion of certain tissues under in vivo condition, such as placenta (Disson et al, 2008; Lecuit et al 2004). Interestingly, *Listeria monocytogenes* does not express the *inlJ* gene when infecting mammalian cell lines in vitro, but seems to play a role in colonization of liver and spleen, and particularly in causing bacteraemia in blood (Sabet et al, 2008).

The main genes encoding for pathogenicity factors are grouped into a cluster of 6 genes (9 kb), commonly identified as pVGC (Virulence gene clusters) or LIP 1 (Listeria pathogenicity island 1).

This cluster includes the genes *prfA*, *plcA*, *plcB*, *hly*, *mpl* and *actA* (Vazquez-Boland et al., 2001b, Ward et al., 2004, Schmid et al., 2005). Genes *inlB* and *inlA* instead form a separate operon (Batt, 1999; Renzoni et al., 1999). All genes encoding for virulence factors of *Listeria monocytogenes* are under the control of transcriptional regulatory factor *PrfA* (positive regulatory factor A), the only regulator of virulence identified in *Listeria monocytogenes* (Vazquez-Boland et al., 2001b). The virulence

expression, dependent on *PrfA*, can be affected by such various environmental factors. Among the activator signals, temperature values close to 37 ° C (Swaminathan, 2001) and the presence of stress conditions (Sokolovic et al., 1990) were detected. On the contrary, the high concentration of iron and temperatures below 20 °C are inactivating factors (Renzoni et al., 1999, Goebel et al., 2000, Kreft et al., 2001).

METHODS FOR DETECTION OF *LISTERIA MONOCYTOGENES*

There is a variety of conventional culture and rapid methods in use for the detection of *Listeria monocytogenes* in foods.

CULTURE METHODS

Food industry laboratories generally use conventional methods to detect and identify *Listeria spp*. and *Listeria monocytogenes*. Test for detection per 25 g are most commonly used for food. Most of the culture methods consist in five steps: 1) Pre-enrichment; 2) Selective enrichment; 3) Selective/differential plating; 4) Biochemical testing; 5) Serological testing. Detection methods involve selective enrichment in a broth, followed by the isolation of colonies in selective agar. These selective media contain various combinations of antibiotics and other selective/diagnostic chemicals. Colonies growing on the selective agar and exhibiting the typical morphology of *Listeria spp*. on that medium are considered presumptive *Listeria spp.* and are further identified to species level using biochemical and haemolytic characteristic as Gram stain, cell morphology, motility, catalase and oxidase, hydrolysis of aesculin, etc…

NON CULTURE METHODS

Immuno-separation: Immuno-separation makes use of antibody specific for a pathogen to concentrate that pathogen before other methods are used to amplify and identify the bacteria. Antibodies are attached to beads, and added to a homogenized sample. The beads are then separated from the slurry through either the use of a magnet (immunomagnetic separation with magnetic beads (Hudson et al., 2001), or through centrifugation (protein-A-linked sepharose beads) (Gray and Bhunia, 2005). Theoretically, the technique should concentrate the pathogens, thus making detection a feasible option without the long enrichment incubations required to amplify pathogen numbers to a detectable level. (Robin et al 2005.)

Automated methods for detection of *Listeria monocytogenes* (non-nucleic acid-based): a number of commercially available, semi-automated systems are available for the detection of various foodborne pathogens, including the MicroLog System (Biolog Inc., Hayward CA), the Microbial Identification System (MIS) (MIDI Inc., Newark DE), VITEK System (bioMerieux Vitek, Hazelwood, MO), and the Replianalyzer system (Oxoid Inc., Nepean, ON) (Odumeru et al., 1999).

Nucleic acid-based methods for detection of *Listeria monocytogenes*:

1. PCR-based detection of *Listeria monocytogenes*

With this method, total DNA is extracted from the food sample. Next, two oligonucleotide primers that bind a pathogen-specific target gene at the ends of opposing strands of DNA, are selected. A DNA polymerase is added along with the four types of deoxynucleotides (dNTPs) and appropriate buffers, and the mixture is inserted into a thermocycler. The resulting fragment is analyzed by agarose gel electrophoresis after staining with ethidium bromide and visualization under UV light. The presence of *Listeria monocytogenes*, both live and dead, can be detected by simply determining if a band, which represent the specific pathogen gene of interest, is present (Robin et al, 2005).

Some groups have eliminated the need for running agarose gels by using fluorescent resonance energy transfer (FRET)-based PCR (Koo and Jaykus, 2003). In this method, the DNA product is analyzed directly after PCR by measuring the fluorescence signal. This system works with two DNA probes for the gene of interest, one with a fluorescein label and the other one with a quencher label. The resulting fluorescence, due to free

fluorophore, is proportional to the number of pathogens in the original sample and obviates the need to run agarose gels for PCR product detection. (Robin et al, 2005). Another way to eliminate the need for agarose gel electrophoresis is to use real-time PCR in a 96-well PCR format. In this method a fluorescent dye, such as SYBR Green I, is used to follow the PCR amplification in real-time and can be used to detect the amplified products from a number of genes at the same time (Bhagwat, 2003). Multiplex PCR is a variation of the traditional PCR. This method makes use of multiple sets of primers to amplify a number of genes or gene fragments simultaneously (Robin et al, 2005). To address the need of detecting only living pathogens, one can detect the pathogen RNA rather than DNA. The presence of specific RNA sequences is an indication of live cells. When an organism dies, its RNA is quickly eliminated, whereas the DNA can last for years, depending on storage conditions. Reverse transcription-PCR (RT-PCR) makes use of a reverse transcriptase that is able, in the presence of a complementary primer, to create complementary DNA (cDNA) from an RNA strand corresponding to a transcribed gene. The cDNA is then amplified using oligonucleotide primers and DNA polymerase under normal PCR conditions. Analysis of the results is done in the same way as for PCR.

1-2. NASBA — nucleic acid sequence-based amplification

In nucleic acid sequence-based amplification (NASBA), total RNA in a sample is extracted. Messenger RNA is used as the target because it predicts viability better than rRNA or total RNA, then the PCR amplification step is not used and the thermocycler is not required (Robin et al, 2005).

1-3. DNA microarrays

Microarrays are composed of a number of discreetly located DNA probes fixed on a solid substrate such as glass. Each probe corresponds to an oligonucleotide specific to a target DNA sequence.

PATHOGEN SUBTYPING AND VERIFICATION METHODS

RFLP (restriction fragment length polymorphism), RAPD (randomly amplified polymorphic DNA), AFLP (amplified fragment length polymorphism), ribotyping and PFGE (pulsed-field gel electrophoresis) are molecular-based methods that are used for further verification after a food sample is suspected of harbouring a pathogen. (Robin et al, 2005).

These methods allow further characterization of the pathogen to determine which of the many possible subtypes within a serotype is causing the disease or is the contaminating factor.

RFLP — restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) analysis uses the restriction endonuclease patterns in DNA to determine differences in genetic profiles. (Robin et al, 2005).

Ribotyping

Ribotyping is similar to RFLP in that it uses restriction endonuclease digestion of DNA to create a pattern that can be analysed. This technique relies specifically on the ribosome encoding genes that are relatively conserved across the bacterial kingdom, and allows lineages to be traced through the appearance of mutations over time (Robin et al, 2005).

PFGE — pulsed-field gel electrophoresis

Pulsed Field Gel Electrophoresis is a method of sub-typing bacteria. PFGE is at present the most extensively used typing method applied for differentiation of *Listeria monocytogenes* strains. Differentiation of *L.monocytogenes* is achieved by cleaving the genomic DNA of the bacteria with a restriction enzyme, followed by separation using horizontal electrophoresis subjected to a pulsed- field current. The resulting bands can be compared to determine isolate relatedness.

AFLP — amplified fragment length polymorphism

Amplified fragment length polymorphism (AFLP) analysis is a DNA fingerprinting technique based on the selective amplification of genomic restriction fragments to generate a restriction pattern of the amplified bands (Aartset al, 1999). AFLP can be

used to differentiate strains of *Listeria monocytogenes* on a more discriminating basis than serotyping (Aarts et al., 1999; Vogel et al, 2004).

RAPD — randomly amplified polymorphic DNA

Randomly amplified polymorphic DNA (RAPD) analysis makes use of a short arbitrary primer (e.g. 10 bp) that anneals randomly along genomic DNA to amplify a number of fragments within the genome. As long as the same primer is used for all the test samples, the comparison of the number and sizes of fragments generated allows for discrimination between strains of a pathogen. It does require having a pure culture so that there are no contaminating bands from other organisms or from the DNA in the food sample (Lawrence and Gilmour, 1995). The technique can be used to trace the source of *Listeria monocytogenes* contamination in food processing plants.

FISH — fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) is often used to study, in a cultivation-independent way, the presence and distribution of specific strains in microbial communities. FISH can be used in phylogenetic studies, and in assessing the spatial

distribution of target microbes in communities such as biofilms (Wagner et al, 1998). The method has only recently been applied to detecting and analyzing microbes in food. (Robin et al, 2005).

Immunoassay-based methods for detection of *Listeria monocytogenes*

Immunoassays are based on the natural affinity of antibodies for their antigens. The antigen can be a hapten, a protein, or a carbohydrate on the surface of a cell. These assays are fast and relatively inexpensive. They allow accurate detection of antigens after very little sample purification (Hall et al, 1989b). Problems with immunoassay-based methods that may arise are the low sensitivity of the assays, low affinity of the antibody to the pathogen or other analyte being measured, and potential interference from contaminants. Improvements in these areas will likely expand the use of immunoassays in a variety of fields, including the food industry (Robin et al, 2005). A number of antibody types and formats are available for immunodetection. These include conventional and heavy chain antibodies, as well as polyclonal, monoclonal or recombinant antibodies (Robin et al, 2005).

ELISA — enzyme linked immunosorbent assay

ELISAs are the most common format used for immunodetection of pathogens. To achieve the detection limit often requires enrichment of the pathogens for at least 16–24 h before the concentration of the pathogen is adequate for detection by ELISA (De Boer and Beumer, 1999).

ELFA — enzyme-linked fluorescent assay

The ELISA assay can be made more sensitive by conjugating fluorescent labels to the antibodies.

Immunoprecipitation and agglutination assays

One simple assay that has been developed for the detection of food borne pathogens is the latex agglutination assay, which makes use of latex bead-bound antibodies specific to the antigen of choice. Antibodies on each bead bind to the antigen. Each antigen can bind to more than one antibody bead, causing the beads to agglutinate. The latex agglutination test still requires culture enrichment and then 48 h to give results; it does have one major advantage over most of the molecular methods available in that no specialized equipment is required. Only visual assessment is required to see the

agglutination result. Furthermore, there is no need for any expensive reagents, making the test suitable for use in the field (Matar et al, 1997).

PATHOGENICITY IN HUMANS

Listeria monocytogenes is a soil organism, which has evolved the ability to invade and mobilizes within eukaryotic cells, probably to some lower eukaryotic multi-cellular soil organism (McLauchlin, 1997). Ingestion of heavily contaminated foods is the principal route of infection (McLauchlin, 1996a). Contaminated food passes to the stomach where *Listeria monocytogenes* can be killed by the acid environment. (Fleming et al. 1995). The buffering capacity of some kinds of food may also be important in facilitating the survival of the organism, which may therefore achieve the invasion at sites along the gastrointestinal tract. Other routes of infection may occur. Schlech et al. (1983), report a food-borne outbreak in Canada in 1981 in which septicaemia and aspiration pneumonia developed after eating contaminated coleslaw salad, supporting the possibility of infection via the respiratory route. After surviving the gastric barrier, bacteria colonizes the small intestine, which is the primary site of invasion, although the exact anatomical site and the mechanism of infection are not well understood (Vazquez-Boland et al., 2001). Following this phase, invasion of the uterine contents or central nervous system (CNS, for patients with shorter incubation periods) may occur via the circulatory system. However, judging by the relatively mild course of *Listeria*

monocytogenes bacteraemia in pregnant women (McLauchlin et al., 1990), the majority of individuals will successfully resolve this initial invasion. The *Listeria* organisms that cross the intestinal barrier are carried by the lymph or blood to the mesenteric lymph nodes, the spleen, and the liver (Mielke et al, 1988; Pron et al, 1998). The principal site of bacterial multiplication in the liver is the hepatocyte (Vazquez-Boland et al., 2001). If the infection is not controlled by an adequate immune response in the liver, as may occur in immunocompromised individuals, unlimited proliferation of *Listeria monocytogenes* in the liver parenchyma may result in the release of bacteria into the circulation (Vazquez-Boland et al., 2001). *Listeria monocytogenes* is a multisystemic pathogen that can infect a wide range of host tissues. However, the principal clinical forms of listeriosis clearly show that *Listeria monocytogenes* has a pathogenic tropism towards the gravid uterus and the CNS.

Clinical outcome of *Listeria monocytogenes* infections depends on three major variables: the number of bacteria ingested with food, the pathogenic properties of the strain, and the immunological status of the host (Vazquez-Boland et al., 2001).

Listeriosis is usually a very severe disease, in fact, it is one of the most deadly bacterial infections currently known, with a mortality rate in systemic listeriosis estimated

between 20% and 40% (Farber and Peterkin, 1991). The clinical signs of *Listeria monocytogenes* infection are very similar in all susceptible hosts. *Listeria monocytogenes* causes two forms of listeriosis: non-invasive gastrointestinal listeriosis and invasive listeriosis. In immunocompetent individuals, non-invasive listeriosis develops as a typical febrile gastroenteritis (Allerberger and Wagner, 2010). For the invasive type, two basic forms of presentation can be distinguished: perinatal listeriosis and listeriosis in the adult patient. In both forms, the predominant clinical forms correspond to disseminated infection or to local infection in the CNS (Vazquez-Boland et al., 2001). In immunocompromised adults, such as the elderly and patients receiving immunosuppressive agents, listeriosis can manifest as septicaemia or meningoencephalitis.

Invasive listeriosis can also be acquired by the fetus via the placenta from its infected mother (Disson et Al, 2009). Perinatal listeriosis can lead to abortion, birth of a stillborn fetus or a baby with generalized infection (granulomatosis infantiseptica), and sepsis or meningitis in the neonate. Neonatal listeriosis is subdivided into two clinical forms: early-onset (usually defined as occurring within the first week of life) and late-on-set. The late-onset type may occur from one to several weeks after birth. Listeriosis during

pregnancy is a serious threat to the unborn child. One-third of culture-confirmed cases of maternal–fetal infections results in abortion or stillbirth. However, the prognosis for live-born babies is good, even in those severely ill (Smith et Al, 2009). Pregnancy-associated cases refer to listeriosis in pregnant women or in the neonates (up to 28 days of life), and the non-pregnancy-associated cases to older babies (>28 days) (Allerberger, 2007). Most maternal infections occur during the third trimester of pregnancy, when T-cell immunity is most impaired. Infected women typically develop non-specific flulike symptoms but may remain asymptomatic. Listeriosis has rarely been observed during the first trimester (Al-Tawfiq, 2008).

In non-pregnancy-associated cases, listeriosis mainly manifests as meningoencephalitis or septicaemia. The median incubation period is estimated to be 3 weeks. Outbreak cases have occurred 3–70 days following a single exposure to an implicated product. The onset of meningoencephalitis, which is rare in pregnant women, can be sudden, with fever, intense headache, nausea, vomiting and signs of meningeal irritation, or may be subacute, particularly in an immunocompromised or elderly host. *Listeria monocytogenes* can also produce a wide variety of focal infections: cases of conjunctivitis, skin infection, lymphadenitis, hepatic abscess, brain abscess,

cholecystitis, peritonitis, splenic abscess, pleuropulmonary infection, joint infection, osteomyelitis, pericarditis, myocarditis, arteritis, necrotizing fasciitis and endophthalmitis have been described (Allerberger, 2007; Pena-Sagredo et al. 2008; Cone et al. 2008). In Europe, approximately 10–20% of clinical cases are pregnancy-associated (including neonates within the first 4 weeks of birth), but the majority of cases occur in non-pregnant immunocompromised individuals, especially the elderly. Approximately 10% of patients have no known risk factor or underlying disease predisposing them to infection with *Listeria* (Kasper et al. 2009).

EPIDEMIOLOGY OF LISTERIA MONOCYTOGENES

The natural habitat of *Listeriae* is thought to be decaying plant matter, in which they live as saprophytes (Gray, 1960; Welshimer, 1968; Botzler et al, 1974; Weis and Seeliger, 1975). The maintenance of *Listeria spp.* can probably be traced back to a continous faecal-oral enrichment cycle by domesticated ruminants (Kampelmacher and Van Noorle Jansen, 1980; McCarthy, 1990; Fenlon, 1999). The organisms are ubiquitous in the rural environment and, consequently, contaminate raw materials used for the production of processed foods as well as production plants. As *Listeria spp.* tolerate high concentrations of salt of up to 4,5 %, relatively low pH values of about 4.3 to 4.5 (Buchanan et al, 1990) and are able to multiply at refrigeration temperatures, they appear to be well suited to survive food-processing technologies and thus cause problems in the food industry. Throughout the period from the first confirmed isolation of the organism by Murray et al. in 1924 (Murray et al, 1926) till the 1970's, clinical isolates of *Listeria spp*. were considered a mere laboratory rarity. From the start of the 1980's on, the number of reports on food-related *Listeria* isolations began to increase (Bille et al, 1988; Farber and Peterkin, 1991, McLauchlin et al, 2004). Foods at high risk of *Listeria monocytogenes* contamination are soft cheese, smoked salmon, salads,

raw vegetables and generally ready-to-eat foods that are not reheated prior to consumption. The first documented food-borne outbreaks of Listeriosis date back to the 1981 outbreak caused by contaminated coleslaw in Canada (Schlech et al, 1983), the 1983 outbreak caused by contaminated, pasteurized milk in Massachusetts / U.S. (Fleming et al, 1985) and to a four year period from 1983 - 1987 caused by contaminated "Vacherin" soft-cheese in Switzerland (Bille et al, 1988). A recent outbreak of human listeriosis amongst immunocompromised hospitalized patients in Finland caused by *Listeria monocytogenes* serovar 3a (a serotype rarely associated with human infection) was associated with butter consumption (Lyytika¨inen et al, 2000; Maijala et al, 2001). Data from Italy calculated that of 2189 individuals exposed to consumption of a contaminated corn salad containing 106 *Listeria monocytogenes*/g, 1566 (72%) developed gastroenteritis and fever, and 292 (19%) were hospitalized with more serious symptoms (Aureli et al., 2000). Although other routes than food-linked infections were reported (McLauchlin et al, 1986; McLauchlin and Low, 1994; O'Driscoll et al, 1999), the association of *Listeria monocytogenes* with several large food borne outbreaks suggests that contaminated food may be the primary source of the organism (Farber and Peterkin, 1991).

INCIDENCE OF *LISTERIA MONOCYTOGENES* IN HUMANS

Listeriosis is a serious human disease caused primarily (80-90% of cases) by the ingestion of food contaminated with *Listeria monocytogenes*. Other less common forms of transmission include vertical transmission (mother-son), zoonotic, nosocomial (Ryser, 1999) or through the dissemination, by aerosol, of the organism in laboratories (Farber et al., 1999).

Nevertheless, the demonstration of the origin in food is rare and is usually random (Fabbi et al., 2005). Up to 80 cases of listeriosis reported poor results, and then the organism has taken a leading role as an agent of serious food-borne infections, following some sensational outbreaks, occurring in the next two decades in America and Europe (Griffiths, 2002).

Symptoms of listeriosis are extremely variable, depending on the characteristics of the bacterium pathogenicity, the level of contamination of the food and health status of immune-competent host (McLauchlin, 2004). Asymptomatic infections are described, as gastrointestinal manifestations, rare skin lesions, flu-like symptoms and, in severe

cases, abortion, stillbirth, septicemia, meningitis and meningoencephalitis (Fabbi et al., 2005).

Although is not clearly defined the dose-response relationship in humans, a quantity of 100 cfu / g of *Listeria monocytogenes* in food is considered the threshold value for the consumer. Ingestion of foods contaminated by a large number of *Listeria monocytogenes* promotes the overcoming of local host defenses, initially in the gastrointestinal tract and later in the reticulo endothelial system in the liver and spleen. Then, by linfoematic way, the release occurs in different districts of the body, favored by the location of the microorganism within the monocytes which, after the release in the various districts, is engulfed by macrophages. The incubation period between exposure (consumption of contaminated foods) and onset of listeriosis varies between 1 day and 3 months (Linnan et al., 1988), and this, together with the long duration of contamination from a single manufacturing environment (McLauchlin, 1996a, b), means that related outbreaks can be very widely separated temporally and geographically. These two factors result in specific food items being rarely identified as associated with disease, although the majority of cases are assumed to be food-borne

(McLauchlin, 1996a). Data from naturally infected cases indicates that high doses are associated with infection (McLauchlin, 1996a). Listeriosis is defined by the WHO (World Health Organization) as a relatively rare disease, although infection by *Listeria monocytogenes* is less common than many other food borne diseases (e.g. those caused by *Escherichia coli* 0157:H7, *Campylobacter jejuni* or *Salmonella* spp.) (Manfreda et al., 2005). Currently in Europe and in the United States, listeriosis is considered a sporadic disease that affects mostly some segments of the population considered at risk (Wiedmann, 2002; Eurosurveillance, 2008). In the U.S., where the disease is notifiable, the CDC (Center for Disease Control) estimates that only half of all cases of listeriosis is notified (5 per million inhabitants). This finding is consistent with the low detection rates (3%) of most food-borne pathogens (Fabbi et al., 2005).

In Europe, in 2006, cases of listeriosis have been reported in all 23 states. Listeriosis is the fifth most common zoonoses in Europe, after the disease associated with *Campylobacter*, *Salmonella*, *Yersinia* and *E. coli* VTEC (EFSA, 2006). In Europe, listeriosis is fairly uncommon (0.3 cases per 100,000 inhabitants). It is characterized by an annual index of morbidity very low (2-11 per million inhabitants), but the severity of

the symptoms is that the individuals involved usually require hospitalization: in fact, is the food pathogen with the highest rate hospitalization, approximately 91% (Lukinmaa et al. 2003; Manfreda et al. 2005; Jemmi et al., 2006). The percentage of deaths related to complications of food poisoning is more than 30% (Goulet et al., 1998, De Valk et al., 2005).

In any case, host sensitivity plays a very important role in determining the clinical symptoms resulting from exposure to *Listeria monocytogenes*. The clinical manifestations are particularly severe in some categories of individuals with deficiencies of physiological or pathological state, interesting cell-mediated immunity (Vazquez-Boland et al., 2001a). The higher risk people categories for listeriosis are pregnant women, infants in the first 30 days of life, the elderly aged over 65 years, and immunosuppressed adults debilitated by previous disease such as cancer (leukemia, lymphoma or sarcoma) and antineoplastic chemotherapy, organ transplantation or corticosteroid use (immunosuppressive therapy), chronic liver disease (cirrhosis or alcoholism), kidney disease and diabetes (McLauchlin, 1990; Schuchat et al., 1991, Farber and Peterkin 1991; Rocourt, 1996, Koch and Stark, 2006). HIV infection (52

cases per 100,000 inhabitants) and the presence of AIDS are among the most important risk factors for listeriosis. It has been estimated that for patients with AIDS, the risk of contracting listeriosis is 300 to 1,000 times higher than the general population (Schlech, 2000).

In 2006, Europe has been recorded the highest number of cases (1,583 cases) during the last 8 years. In some states, such as Germany, France and Britain were recorded 64% of the total cases reported in 2006 in Europe. In some states (Denmark, Finland and Luxembourg) higher values of the incidence of disease have been found (≥ 0.9 cases per 100,000 inhabitants). From the statistical point of view, the most significant increases were registered in Germany, Ireland, Lithuania, Netherlands, Spain and Britain (Denny and McLauchlin, 2008). In 2006, the incidence of Listeriosis in patients aged > 65 years was 2.5 times higher than that recorded in any other age group. This trend was more significant (Denny and McLauchlin, 2008), in some states such as Belgium and Finland (64% of all cases) and especially Italy and Sweden (69% of all cases). In Italy listeriosis is subject to notification by doctors since 1991 (.. "significant diseases because of high

frequency and / or may control interventions ...") and is inserted into the SIMI, "Information System of infectious and contagious diseases "(D.Min.San.15.12.1990).

Cases reported in the last 8 years show that the disease is rare, but is rapidly increasing. In 1999, 17 cases were reported, while in 2005-2006 51 were registered (Gianfranceschi et al. 2002; Manfreda et al., 2005, Denny and McLauchlin, 2008). In 2005-2006, the incidence in our country was equal to 0.1 cases per 100,000 inhabitants (Denny and McLauchlin, 2008). However, these data are probably underestimated, considering that about 50% of meningitis is associated with an unknown causative agent, and often, episodes of non-invasive listeriosis, characterized by gastrointestinal symptoms are not notified and/or correctly diagnosed. Another limitation may be inherent to the notification device, which provides that the description of symptoms is associated with microbiological identification of the pathogen, usually performed only in cases of extreme gravity. Among other things, the extremely wide oscillation of the incubation period for listeriosis (1 to 90 days) does not facilitate the collection of reliable medical history and the retrieval and analysis of contaminated food (De Cesare et al., 2001).

INCIDENCE OF LISTERIA MONOCYTOGENES IN FOOD

Listeria monocytogenes is ubiquitous and almost all food categories can be contaminated with this pathogen, in some cases with a high frequency. The SCVPH opinion reported a range of contamination with *Listeria monocytogenes* (i.e. % positive findings) for various foods: 7-36% for minced meat, 0-52% for meat products; 9-85% for poultry meat, 4-60% for fish products, 1-12% for vegetable salads, 2-12% for raw milk. Quantitative data were also presented showing that most contaminated food samples contained less than 100 cfu/g but a minority (e.g. 0 to 1.8% according to the food type) contained more than 100 cfu/g. (The EFSA Journal 2007). In the Community Summary Reports on Trends and Sources of Zoonoses in the EU in 2004, 2005 and 2006 (EFSA, 2005; EFSA, 2006; EFSA, 2007a), the overall ranges of contamination of foods with *Listeria monocytogenes* were (presence in 25 g): 0-48% in meat products, 0-40% in poultry meat product, 0-30% in fish products, 0-100% in raw milk. In addition, the Community Summary Reports provided a range of contamination for other foods, not considered in SCVPH (1999): 0-38% for cheeses, 0-33% for fruit and vegetables, 0 to 33% for sandwiches.

In the Community Summary Reports, the highest frequencies of positive samples in ready-to-eat foods was found in meat and fish products: 2.7% for meat product and 7.5% for fish products in 2005, 3.5% for bovine meat product, 2.7% for pork meat products and 4.9% for fish products in 2006. The prevalence of food samples containing more than 100 *Listeria monocytogenes* per g was in accordance with those reported in the SCVPH opinion (0 – 1.8%), except for a few surveys on meat and fish products with around 5% (meat) or even up to 20% (fish) of samples containing more than 100 *Listeria monocytogenes* per g.

Data presented in the 2004, 2005 and 2006 Community Reports showed that ready-to-eat fish products were more frequently contaminated with *Listeria monocytogenes*, and contained a higher proportion of samples with more than 100 *Listeria monocytogenes* per g than other food categories (samples with > 100 cfu/g were found in 29% of surveys of fish products compiled in the 2005 Community Report). Ready-to-eat meat products then came next in decreasing order of contamination (The EFSA Journal, 2007). This is consistent with a survey of 2,217 samples of fish and meat products

imported to or exported from Switzerland which concluded that the highest prevalence of *Listeria monocytogenes* was in fish products (Jemmi et al., 2002).

Prevalence of *Listeria monocytogenes* in sausage at the end of the production process was 10% in France for dry fermented sausages (Thevenot et al., 2006), and from 3 to 4% for raw spreadable sausages in Germany (Hechelmann et al., 2002). In both cases, all contamination levels were below 100 cfu/g.

Tracing back foods implicated in sporadic listeriosis is rarely successful. For example, molecular typing of strains isolated from pork meat in France (Hong et al., 2007), cheeses in Portugal (Malak et al., 2001) and cheese in Belgium (Leite et al., 2006), failed to find any relationship with strains isolated from patients.

Parameters associated with the presence of the bacterium include: food packaging type, preparation practices (e.g. the use of slicing machines for meat products), storage temperatures, the stage of sampling with respect to shelf life, the lack of an effective HACCP system, and the lack of education and training of food handlers (Lewis et al., 2006; Lianou and Sofos, 2007; Little et al., 2007; Sagoo et al., 2007).

Regarding the sources of contamination, the SCVPH 1999 opinion reported an occurrence of *Listeria monocytogenes* in food animals between 1 to 10%.

The SCVPH 1999 described the establishment of *Listeria monocytogenes* in food processing facilities environments which becomes a source of contamination in foods during processing. This has been confirmed by several studies using molecular typing methods. In smoked fish, most strains isolated in finished ready-to-eat products were strains repeatedly isolated from processing plants (Lappi et al., 2004; Nakamura et al., 2004; Thimothe et al., 2004).

SURVEILLANCE OF ZOONOSES IN EUROPE

The Community system for monitoring and collection of information on Zoonoses is based on the Zoonoses Directive 2003/99/EC, which obligates the European Union Member States to collect relevant and comparable data of: 1) Zoonoses; 2) Zoonotic agents; 3) antimicrobial resistance; 4) foodborne outbreaks.

In addition, Member States shall assess trends and sources of these agents and outbreaks in their territory, and transmit to the European Commission, every year, a report covering the data collected. The European Food Safety Authority (EFSA) is assigned the tasks of examining the data collected and publishing the Community Summary Report.

The Decision 2119/98/EC on setting up a network for the epidemiological surveillance and control of communicable diseases in the Community, established the data collection on human communicable diseases from the Member States. The Decisions foresee that data from the networks shall be used in the Community Summary Report on Zoonoses. In such report the data related to the occurrence of Zoonotic agents in animals, foodstuffs and feed as well as to antimicrobial resistance in these agents are collected in the framework of Directive 2003/99/EC. This applies also to information of food borne outbreaks.

The Rapid Alert System for Food and Feed (RASFF) is a system which has been in place since 1979 and whose purpose is to provide the control authorities with an effective tool for exchange of information on measures taken to ensure food safety.

The legal basis of the RASFF is the Regulation EC/178/2002 that laid down the general principles and requirements of food law, established the European Food Safety Authority and lad down the procedures in matters of food safety.

The Annual Report on the RASFF provides useful data on the number of notifications received every year, as well as details on the origin of the notifications, the products and countries involved, and the identified risks. It also details the follow-up actions carried out in response to various food safety problems.

INTERNATIONAL REGULATORY FRAMEWORK IN RELATION TO THE PRESENCE OF *LISTERIA MONOCYTOGENES* IN FOOD

Canadian health authorities (Health Canada) have established the political control of *Listeria monocytogenes* in food on the application of HACCP principles. It is estimated that the risk of contamination by the organism may be reduced, but not always *Listeria monocytogenes* can be eliminated from food plants and food. The focus is on RTE products that are able to allow growth and development of *Listeria monocytogenes*.

More specifically, is given more attention to the foods whose consumption has been associated with the onset of outbreaks of listeriosis and that have a shelf life> 10 days.

In the European community, the Regulation (EC) n.2073/2005 on microbiological criteria for foodstuffs is the new and latest document on the criteria of food security (Chapter 1) for RTE products, which for the presence of *Listeria monocytogenes* are divided into three categories:

Ready to eat food for infants and for special medical purposes. Regular testing against the criterion is not useful in normal circumstances for the following RTE food:

- ✓ that have undergone heat treatment or other processing effective to eliminate *Listeria monocytogenes*, when recontamination is not possible after this treatment (eg. the products subjected to heat treatment at the time of the final package)
- ✓ fresh fruits and vegetables, not cut and processed, excluding sprouted seeds
- ✓ bread, biscuits and similar products
- ✓ water, soft drinks and alcoholic beverages bottled or packed

- ✓ sugar, honey and confectionery, including cocoa products and chocolate

- ✓ molluscs

For products placed on the market during their shelf-life, the sampling plan (n = 10, c = 0) provides the absence in 25 g. The reference analysis method is the EN / ISO 11290-1

Foods that support the growth of Listeria monocytogenes, other than those intended for infants and for special medical purposes. For products placed on the market during their shelf-life, the sampling plan (n = 5, c = 0) has a limit of 100 cfu / g. The method of analysis of reference is the EN / ISO 11290-2. This method can be applied if the manufacturer can demonstrate, with the satisfaction of the competent authority, that the product will not exceed the limit during the shelf-life. The operator may fix intermediate limits during the process, that are sufficiently low to ensure that the limit of 100 cfu / g is not exceeded at the end of the shelf-life. However, if the operator is not able to demonstrate that the product will not exceed the limit of 100 cfu / g during the shelf-life, the sampling plan that is applied (n = 5, c = 0) before the foods are no longer under the direct control of the operator, provides for the absence of the organism in 25 g of product. The method of analysis of reference is the EN / ISO 11290-1

Food that not support the growth of Listeria monocytogenes, other than those intended for infants and for special mediacal purposes. Regular testing against the criterion is not useful with normal circumstances for the following RTE foods:

- ✓ that have undergone heat treatment or other processing effective to eliminate *Listeria monocytogenes*, when recontamination is not possible after this treatment (eg. the products subjected to heat treatment at the time of the final package)
- ✓ fresh fruits and vegetables, not cut and processed, excluding sprouted seeds
- ✓ bread, biscuits and similar products
- ✓ water, soft drinks and alcoholic beverages bottled or packed
- ✓ sugar, honey and confectionery, including cocoa products and chocolate
- ✓ molluscs

Products with pH ≤ 4.4 or aw ≤ 0.92, or pH ≤ 5.0 and aw ≤ 0.94 and those with a shelf-life of less than 5 days are included in this category. Other types of products can also

belong to this category if there is scientific justification. For these products, placed on the market during their shelf-life is expected (n = 5, c = 0) a limit of 100 cfu / g. The method of analysis of reference is the EN / ISO 11290-2.

LISTERIA MONOCYTOGENES IN THE PORK MEAT PRODUCTS CHAIN

Slaughter

Listeria monocytogenes is commonly isolated in pigs' slaughterhouses (Sammarco et al. 1997; Korsak et al., 1998). The most contaminated areas are usually represented by the area of stun / hanging (Gobat 1991, Nesbakken et al., 1994, Saide-Albornoz 1995, Borch et al., 1996).

Carcasses of animals can be contaminated during the evisceration, because of breaking of the intestine (Norrung and Skovgaard, 1989; Adesiyun et al., 1995). However, some authors, (Kanuganti et al., 2002) have noted the presence of *Listeria monocytogenes* in only a small percentage (4%) of pig carcasses.

The pathogen was not isolated from the rectal contents of animals before slaughter, probably due to the emptying of the rectum before evisceration, an operation that

usually helps to reduce the extent of dissemination and the consequent faecal contamination of carcasses.

The authors believed that the presence of *Listeria monocytogenes* in the carcass was not to be charged exclusively with fecal origin.

Usually the prevalence of *Listeria monocytogenes* in pork faeces is between 0 and 4% (Felon et al., 1996). Several authors (Buncic et al. 1991; Ripamonti et al. 2002; Autio et al. 2003; Fabbi et al., 2005) have in fact shown that pigs carry the organism, preferably in tongues (14%) and tonsils (14-61%). This wide range is probably due to differences in sampling techniques and/or methods of farm management.

Kanuganti et al. (2002) showed that the detection of *Listeria monocytogenes* in meat or in the small intestines of freshly slaughtered pigs, varies from 0.8 to 2.4% to 8.3 to 9.3 % of the samples, respectively. Serotypes 1/2a, 1/2c were more frequently found in pork, while the serotype 1/2b was rare (Hof and Rocourt, 1992; Thevenot et al., 2005).

Raw materials, processing and transforming

Raw materials are an important source of contamination of working environments and equipment. In turn, due to the presence of favorable conditions for growth and

multiplication, represents the primary source of contamination of final products by *Listeria monocytogenes* (Giovannacci et al. 1999; Kathariou, 2002; Thevenot et al. 2005).

In such environments, *Listeria monocytogenes* appears to be almost ubiquitous, which makes difficult to control the contamination (Romanova et al., 2002). The most contaminated zones are the areas of receipt of raw materials, the cells of refrigeration and the processing rooms (Chasseignaux et al., 2002). During the processing stages of pork meat, the level of contamination can increase significantly (Nesbakken et al., 1996).

In minced meat intended to be processed, the prevalence of *Listeria monocytogenes* range between 16 and 50.2% of samples (Jay, 1996; Chasseignaux et al., 2002). The prevalence increases up to 71% during the grinding and bagging (Thevenot et al., 2005).

In a survey conducted among some French plants, levels of contamination of the surfaces in contact with food, were compared, before and during processing. 15% of the surfaces in contact with meat and 13.3% of those not in contact before the start of processing showed to be contaminated, and during processing these values increased, respectively, up to 50% and to 25.9 % (Thevenot et al., 2005).

In a study performed in 2003 in a pork meat processing plant, Pecci et al. reported the presence of *Listeria monocytogenes* both in raw meat (3.6 g-1 MPN) and in the mixture of minced meat, with spices and nitrite, ready for bagging (9.2 g-1 MPN).

Among the final products, some types of raw sausage (cotechino sausage and similar products) were found most frequently contaminated (3.6 to 23 g-1 MPN).

Among the equipment, *Listeria monocytogenes* was isolated from the meat grinder, and from the mixer (0.184 cm-2 MPN) while, samples taken from the bundler, the tables and other surfaces in contact with meat were negative.

Several studies have shown that strains of *Listeria monocytogenes* isolated from meat-processing environments belong to serotypes 1/2a, 1/2b, and 1/2c (Jay 1996; Chasseignaux et al., 2002).

Thevenot et al. (2005) have isolated the serotype 4b and 4e in some studies performed on French sausages. Recent studies have shown that the prevalence of strains 1/2b and 4b tends to increase after processing, in contrast, strains 1/2a and 1/2c decreases, confirming that the difference in distribution between the strains isolated from foods and from human may not necessarily reflect a difference in virulence, but a different

adaptation to different ecological "niches" (Farber and Peterkin, 1991; Rocourt et al. 1997; Thevenot et al., 2006).

Meat Products

Listeria monocytogenes can multiply in a huge variety of meat products (Farber and Peterkin, 1991) that may be contaminated at different times of the production cycle.

Among the predisposing factors, we consider the use of contaminated raw materials into products whose technological process does not provide effective barriers, cross-contamination of final products from raw materials and contaminated surfaces, or through incorrect manual procedures (Samelis et al. 1998; Chasseignaux et al. 2001; Aantrekker Reji et al, 2004).

Failure to follow good hygiene practices during processing is considereded one of the main ways of transmission of the pathogen (AFSSA, 2000). Cross-contamination can occur at any stage, even during the manipulation by the final consumer (Reji Aantrekker et al, 2004).

Contamination often occurs when domestic kitchen tools. Knives, for example, are used first on contaminated food and after on non-contaminated foods, or cooked foods or

prepared for immediate consumption. Informations concerning the presence of *Listeria monoytogenes* in pig meat products are sporadic.

In a study conducted in Veneto in 2003 levels of contamination in fresh and cured meats taken from retail supermarkets, butchers and delicatessens from this region were assessed, in order to establish the level of the consumer exposure to this emerging pathogen (AFSSA, 2000). Of 325 samples of fresh sausages (grilled sausages) and 250 fermented sausages (ready to eat) a positivity rate of 40.3% and 15% respectively was found. Levels of *Listeria monocytogenes* in fermented sausages were quite small (<10 cfu / g) (Mioni et al. 2004).

In a study performed in Emilia Romagna in 2003, on fresh and seasoned meat products taken from retail (Marzadori, 2004), in food preparation to be consumed after cooking, such as burgers and fresh sausages, high prevalence (58.88%), were reported. The pork meat products were the most contaminated (30.6%).

The results obtained as part of a national surveillance study in Italian salami like "cacciatori" in 2002-2003 (Fiore et al. 2004), showed that of 1020 samples taken at the end of the production cycle and before marketing, *Listeria monocytogenes* was present

in 22.7% of the samples (230 positive samples), even though always with restricted levels of contamination (<10 cfu / g).

In the U.S.A, 22.9% of products made from minced pork meat, mainly sausages, to be consumed after cooking, were contaminated (Duffy et al., 2001).

In Portugal, the prevalence of *Listeria monocytogenes* in raw fermented sausages was 60% (Ferreira et al, 2006).

In addition prevalences of 10.6% in Chile (Cordano and Rocourt, 2001), 10% in France (Thevenot et al., 2005), 22% in Italy (Gianfranceschi et al., 2006) and between 7 and 11% in Turkey were reported (Colak et al. 2005; Siriken et al., 2006).

During the fermentation process, in products with long maturation periods, levels of contamination by the organism tends to decrease (10%), due to the factor of the barriers (drop of the pH to 5, decrease of a_w up to 0.80, and the rise of concentrations of NaCl), the development of *Listeria monocytogenes* (Buncic et al. 1991; Mazzette al., 2005). However, levels of initial contamination of raw meat and especially the ability of the organism to adapt even to such conditions, can facilitate the detection of *Listeria monocytogenes* in the final product (Thevenot et al., 2005).

The contamination level during consumption is generally moderate (<10 to 100 cfu / g), due almost completely to the initial contamination of ingredients and raw meat (Thevenot et al., 2005, Ferreira et al. 2006; Gianfranceschi et al., 2006). Consequently, the risk level for number of meals is very low (2.1 × 10-12 cases per number of meals served). The amount of total annual human cases of listeriosis linked to consumption of this category product is estimated to be 0.0000055 per 100,000 inhabitants (Thevenot et al., 2005). As already noted for raw materials, also in the finished products made from pork meat, serotypes 1/2a, 1/2c and 1/2b are more often detected (Jay, 1996), while serotype 4b is more rarely seen (Greenwood et al., 1991, Hayes et al., 1991).

PERSISTENCE OF *LISTERIA MONOCYTOGENES* IN FERMENTED MEAT PRODUCT PLANTS

Several studies suggest that *Listeria monocytogenes* can become persistent in the workplace and survive for long periods. Giovannacci et al. (1999) and Lunden et al. (2002) reported the persistence of *Listeria monocytogenes* strains for more than a year in two cutting meat pork plants and for over three years in a meat grinder machine. The

finding of strains that have the same genetic profile in raw material, processing environments and final products, showed that some clones are adapted to survive in pork meat processing plants and in the finished products (Thevenot et al., 2006). Tompkin (2002) reported several studies that demonstrate the ability of *Listeria monocytogenes* to persist in processing environments. Some strains seem able to persist for even 12 years. Persistent strains belonged to different serotypes, as 1/2a, 1/2b, 4b, etc., usually implicated in cases of human disease. The Tompkin review (2002) demonstrates that many food production facilities, as meat, seafood, poultry and dairy, may have persistent resident *Listeria monocytogenes*.

The mechanisms and the factors governing the persistence of some of *Listeria monocytogenes* strains are not well known, and even studies showing the ability of several strains to persist are restricted. Several factors may be involved in its regulation, such as resistance to disinfectants, resistance to desiccation, internalization in protozoa, and the biofilms formation.

Role in persistence of disinfectant and desiccation resistance

Biocides, used as part of a thorough combined cleaning and disinfection program, are an essential weapon in the food industry hygiene armoury to control pathogenic and spoilage micro-organisms (Holah, 2000). Indeed, once disinfectants had been selected to combine effective biocidal action with minimal toxicity and taint issues, they have essentially remained the same for decades. The existence of populations of micro-organisms surviving thorough cleaning and disinfection has been demonstrated (Holah, 2000). It is not clear, however, whether such resistance is plasmid-mediated; Lemaıˆtre et al. (1998) found extrachromosoal DNA in all resistant *Listeria monocytogenes* strains tested, though Earnshaw and Lawrence (1998) and Mereghetti et al. (2000) found resistance in *Listeria monocytogenes* strains both with and without plasmids. None of these workers demonstrated *Listeria monocytogenes* strain resistance to disinfectant concentrations approaching disinfectant manufacturers in-use recommended concentrations (Holah, 2002).

Several studies have investigated the resistance of *Listeria monocytogenes* strains of disinfectants and desiccation, but the results are contradictor. In a study by Holah (2002), the author compares different strains growth at different concentrations of

disinfectants, but no difference in resistance was seen. In any case it seems that the resistance is supported by the ability of the strains to produce biofilms (Holah, 2002). Vogel et al. (2010) examined the role of resistance to desiccation in persistence, both the persistence and non-persistence strains shown similar resistance to desiccation.

Internalization and role in persistence

Another possible route interested in the mechanism of the persistence of some strains of *Listeria monocytogenes* could be the internalization in some protozoa that would allow *Listeria* to survive in hostile environments, offering a sort of protection.

Listeria monocytogenes cells are, in fact, ingested by protozoa such as *Acanthamoeba spp*. or *Tetrahyrnenapyrlformis.* However, they are not killed, but survive within the protozoa and may multiply intracellularly (Greub and Raoult, 2004).

Biofilm formation

Listeria monocytogenes is able to adhere to inert surfaces commonly used in the food industry (Marsh et al., 2003), although there are differences in the degree of adsorption and diffusion on the type of surface pretreatment, environmental conditions and bacterial serotypes. The multiplication of *Listeria monocytogenes* is favored by high

humidity, temperatures ≤ 10 ° C (Chasseignaux et al., 2002) and the presence of organic substances. This promotes its presence in biofilms of many different types of surfaces (stainless steel, glass, rubber) in contact with food, such as work tables, conveyors, storage tanks, refrigerators, freezers, haired and feather pecking machines, slicers, bagging and weighing equipment (Fonnesbech Vogel et al., 2001, Norton et al. 2001; Suihko et al., 2002) and not in contact with food, where there is condensed and stagnant water, such as sewage system or drainage, walls, doors, floors and equipment of rooms processing (Fonnesbech Vogel et al., 2001, Norton et al. 2001; Suihko et al., 2002).

The biofilm can be defined as a sessile community of bacteria adhering to a surface of biotic or abiotic, incorporated into a polysaccharide matrix (Roberts et al., 2003). The biofilms formation has basically two key points: the first is the process of adhesion of bacteria to the surface with which they come into contact with (modulated by physical-chemical phenomena). The second one is the production of polysaccharides by the bacteria, as a result of degradation of nutrients, organized into polymeric fibrils to give strength and adhesion to the surface of the structure (Blackman et al. 1996; Chavant et al., 2002).

Biofilm is resistant against environmental stress, antimicrobial agents and disinfection operations (Blackman et al., 1996). *Listeria monocytogenes* grows frequently in mixed biofilms. This occurrence results in a higher resistance of these biofilms, as the polysaccharides produced by different bacteria give greater solidity to the structure (Tompkin, 2002). For example, combining the protective effect of bacterial extracellular polymeric substances production, also increasing bacterial resistance to antimicrobial agents (Costerton et al., 1995).

A number of studies have shown that *Listeria monocytogenes* is able to increase its capacity of growth when associated with bacteria belonging to the genera *Flavobacterium* and *Pseudomonas* (Sasahara et al., 1993). Other studies have shown that biofilms made up of a single species consist of a concentration higher of bacteria than those mixed (Carpentier et al., 2004). Several studies have looked at the effectiveness of the products used for sanitizing and disinfecting surfaces against *Listeria monocytogenes*. In general, the pathogen is sensitive in vitro to acidic compounds, the iodophor, chlorine-based compounds and quaternary ammonium salts (Van de Weyer et al., 1993), while in vivo studies have shown different results. The quaternary ammonium compounds, are the most commonly used disinfectant in the

food industry. The massive use of these compounds is primarily due to their bactericidal properties (causing widespread damage to the cell membrane and inactivate cellular enzymes), combined with their non-aggressive, non-toxic and non corrosive (on human skin and hard surfaces), which makes them ideal products for use in the hand in the food industry. However, the minimum inhibitory concentration has increased over time, the resistance of *Listeria monocytogenes* to quaternary ammonium compounds (Mereghetti et al., 2000).

EXPERIMENTAL STUDY

The European legislation on food hygiene and microbiological criteria for foods safety gives to Food Service Operators (FSO) the responsibility for the safety of food products. This role requires a thorough knowledge by the FSO of the risk to the health of consumers resulting from their activities and, in particular, knowledge of the epidemiology and development related to the presence of pathogens, as well as how to manage the same during process and product. The verification methods of microbiological product safety and hygiene of the process are established by the EC Regulation 2073/2005, however, the guarantee of respect for the limits of the operators

implies a great responsibility. In full autonomy, through the application of HACCP and other hygiene measures, must demonstrate the proper management of process hazards to which they are responsible. With regard to *Listeria monocytogenes*, in order to properly implement procedures that enable management to meet the criteria, must be in possession of data on the level of risk involved in the process.

This step can be difficult when it comes to traditional products, which are often poor for the elements of knowledge. In this investigation attention has been given to Sardinian fermented sausage, the leading product among Sardinian salami, widely appreciated in the regional and extra-regional markets. The overall objective of this work was represented by the acquisition of epidemiological data concerning the risk level presented by the traditional meat-based products in the transmission of *Listeria monocytogenes* to humans. In particular, the specific aims were:

a) to evaluate *Listeria monocytogenes* prevalence in Sardinian fermented sausage

b) to evaluate the intrinsic (physical and chemical characteristics and composition) and extrinsic (production and processing technologies, sustained treatment, mode of distribution, intended use) risk factors for these products;

c) to identify the sources of entry of *Listeria monocytogenes* in fermented sausage production facilities located in the Sardinia region;

d) to study the mechanisms of persistent strains of *Listeria monocytogenes* in production facilities in order to acquire useful elements to define appropriate control strategies of the pathogen in the plant and to guarantee product safety.

The first part of the study was carried out in the laboratories of the Animal Biology Department, Food Hygiene division of the Faculty of Veterinary Medicine of Sassari. During the years 2010 and 2011 several meat product plants located in the Sardinia region were sampled in order to evaluate the occurrence of *Listeria monocytogenes.* The *Listeria monocytogenes* strains were further characterised by molecular methods. The contamination routes in the plants were traced by use of PFGE. In addition, a quantitative assessment of the in vitro biofilm formation was carried out in order to investigate the potential for persistency. The strains isolated during this period were compared with a selection of strains isolated from the same plants from 2004 to 2007. The following part of the study has been carried out in the period June-October 2011 next to the TEAGASC, Agriculture and Food Development Authority, laboratories,

placed at Moorepark, Fermoy, Co. Cork (Ireland). The specific aims of this part of the study were:

- Study of the genes responsible for the persistence of *Listeria monocytogenes* in food facilities;
- Evaluation of the expression of these genes in *Listeria monocytogenes* strains isolated from sausages and the processing environments

The purpose of this study was to evaluate the epidemiology of *Listeria monocytogenes,* the pathogenetic profile of the strains and the genetic mechanisms responsible for their persistence over time within the same facilities.

The working plan was divided in several following steps:

STEP 1 – Description of structural and technological requirements of the processing plants included in this study and types of fermented meat products produced

STEP 2 - Survey on the prevalence of *Listeria monocytogenes* during the processing of Sardinian sausages

STEP 3 - Phenotypic and molecular characterization of the *Listeria monocytogenes* isolates

STEP 4 - Study of the phenotypic and the genetic mechanisms of the *Listeria monocytognes* persistence in the processing environments.

MATERIALS AND METHODS

The six processing plants (A, B, C, D, E, F) included in our study were located in distinct areas of Sardinia, where different manufacture practices were used.

1 - Description of structural and technological requirements and types of fermented meat products produced in plants included in this study

In the pre-operational phase, a checklist for the acquisition of useful information and major structural and management issues in the plant has been prepared. The following informations were acquired during the first visit in each processing plant:

- Structural features, management of environments and equipment dedicated to the production of sausages

- Types of sausage produced

- Characteristics of the meat and the fat used: origin, storage conditions (refrigeration, freezing), meat cuts used and if known, weight of the pigs, characteristics and amount of fat used.

- Characteristics of the casings: species, section of origin, provenience, method of storage and use.

- Recipe: seasonings, flavorings, additives, use of starters, information on how to prepare the "tanning"

- Description of the process: identification and articulation of the stages, specification of process parameters (duration of the phases, temperature, relative humidity), technical aspects of interest for the product characteristics, such as the diameter of the holes of the drow-plate, the time of work and rest of the cells, the possible introduction of periodic outside air.

- Presentation, characteristics and the weight of the sausages

- Packaging and labeling.

The acquisition of these information involved the owners of the information and / or staff responsible for producing and was complemented by the inspection of premises and, where possible, assisting the production stages, or by checking the product characteristics and process parameters during the different phases.

2 – Survey on the prevalence of *Listeria monocytogenes* during the processing of Sardinian sausages (raw material, semifinished and finished products) and in the processing environments.

Samples collection

A total of n. 341 samples taken from 2 different production lots in the 5 sausage processing plants included in the survey were examined. In one processing plants (A), were collected samples from one production lot only. The samples were divided as follows:

- Ground meat and fermented sausages:

-No.55 samples of raw material (ground pork fat and meat)

-No.55 samples of Sardinian sausages at the end of acidification

-No. 55 samples of Sardinian fermented Sausages at the end of ripening

Environmental samples

-No. 88 samples from swabbed surfaces without contact with meat (walls and gutters of the cells for the storage of raw materials, drying, curing and processing rooms)

- No. 132 samples from surfaces in contact with meat (counter tops, trolleys, hooks, meat grinder machines, mixer and bagging).

Sampling locations were chosen in order to represent the most likely to present *Listeria monocytogenes* contamination

Environmental samples were collected during the production stages by swabbing with 10 cm by 10 cm sterile gauze pads rehydrated with 10 ml of neutralizing buffer (Solar-cult sampling kit, Biogenetics, Italy) using a sterile template to delineate the swabbed area of 100cm2.Each sponge was diluted with 90 ml of Fraser broth base (Biolife, Italy) and used for the qualitative research of *Listeria monocytogenes*, according to the method described below.

Analysis performed

All the samples were submitted to the following chemical-physical and microbiological analysis, unless otherwise indicated:

Chemical and physical parameters

1. Free acidity (pH): the potentiometric measurement of pH was carried out diluting 10 g of sample in 1:1 ratio with sterile distilled water using a pH meter Forlab 710 (Carlo Erba, Italy). Two measurements were performed and averaged.

2. Water activity (aw) was determined using an Aqualab CX3 (Decagon, Pullman, USA).

Microbiological parameters

Detection and enumeration of Listeria monocytogenes

Detection and enumeration of *Listeria monocytogenes* were carried out by using the ISO 11290-1:1996 and 11290-2:1998 protocols respectively. Samples of pork ground meat, sausages at the end of acidification, ripened sausages and swabbed samples were homogenized 1/10 with Fraser broth base (Biolife, Italy) in a stomacher Lab-Blender 400 (Seward Medical, London, UK) for 2 min. The homogenates were incubated at 20°C for 1h, in order to resuscitate stressed microorganisms. For the enumeration of *Listeria monocytogenes*, 1 ml of each inoculum was distributed over the

surface of three Aloa (Biolife) 90mm plates (0.3, 0.3, 0.4 ml) using a sterile spreader. The plates were incubated at 37°C for 48h and subsequently examined for the presence of suspect colonies of *Listeria spp*. and *Listeria monocytogenes.* The Aloa media allowed differentiation of colonies of *Listeria monocytogenes* from other colonies of the genus *Listeria spp*.: After 48 hours of incubation, colonies of *Listeria monocytogenes* are colored blue or bluish-green surrounded by an opaque halo. From plates containing up to 150 suspect colonies, 5 (or fewer if the number of suspect colonies was less than 5) were selected and isolated on Tryptone Soya extract agar plates (TSYEA, Biolife, Italy). After incubation at 37 ° C for 48 hours, colonies of typical appearance were subjected to confirmatory tests for *Listeria spp*. and *Listeria monocytogenes* described further below. The number of colonies of *Listeria monocytogenes* was determined applying the following formula: a = b / A x C, where b was the number of colonies corresponding to the identification criteria, A was the number of suspect colonies subjected to identification, while C was the number of colonies suspected for *Listeria spp*. For detection of *Listeria monocytogenes*, the homogenates were supplemented by Fraser half selective supplement (Biolife) and incubated at 30°C for 24 hours. Afterwards, 0.1 ml of the primary enrichment was inoculated in 10 ml of Fraser broth

supplemented (Biolife) by Fraser selective supplement (Biolife) and incubated at 37°C for 48 hours. Cultures were streaked onto Oxford (Oxoid, Milan, Italy) and Aloa (Biolife) plates and incubated at 30°C and 37°C for 48 hours respectively. The plates were examined for the presence of suspect colonies of *Listeria spp*. and *Listeria monocytogenes*. On the Oxford media, after 24 hours of incubation, colonies showed small (1 mm in diameter) grayish in color and surrounded by a black halo. After 48 hours of incubation, the colonies became darker in color (sometimes with a greenish reflection), with a diameter of 2 mm, with a central depression, surrounded by a black halo. On Aloa media, colonies of *Listeria monocytogenes* are colored blue or bluish-green surrounded by an opaque halo.

From each plate of the primary and secondary enrichment, 5 colonies presumed to be *Listeria spp* were streaked on TSYEA plates (Biolife) and incubated at 37°C for 24 hours. Colonies were selected for typical appearance on TSYEA (convex, colorless and opaque, with a diameter between 1 and 2 mm) and submitted to to the following confirmatory tests for *Listeria spp*. and *Listeria monocytogenes* (gram staining, catalase test) Hemolytic activity and CAMP tests on sheep blood agar were performed for

Listeria monocytogenes confirmation. Biochemical characterization of all the isolates was performed using the API Listeria identification system (bioMérieux, Marcy l'Etoile, France).

1. Confirmatory test for Listeria spp.:

- *Gram staining:* one isolated colony was taken and then a microscope slide set up and stained. At the observation by light microscopy, the suspect colonies of *Listeria spp*. appeared as short bacilli, Gram +

- *Catalase reaction*: an isolated colony was suspended in an hydrogen peroxide drop (3%) on a microscope slide. The formation of gas bubbles indicated a positive reaction.

2. *Confirmatory test for Listeria monocytogenes:*

- *Hemolysis test*: an isolated colony was sown in 5% Sheep blood agar media (Oxoid, Italy). After incubation at 35-37 ° C for 24 hours, the plates were examined in transparency: *Listeria monocytogenes* showed a clear and restricted β-hemolysis area.

- *Camp Test:* 5% Sheep blood agar media (Oxoid, Italy) was inoculated in parallel with two reference cultures of *Staphylococcus aureus* (DSM 12463) and *Rhodococcus equi* (DSM 20307). Strains to be tested, were sown perpendicular to the two control strains, avoiding touching and being spaced at least 1-2 mm. The plates were incubated at +35-37 ° C for 18-24 hours. The test was considered positive in the presence of an enhanced β-hemolysis zone at the intersection of the strain analyzed with *Staphylococcus aureus* (zone of 3-4 mm of increased hemolysis within a weakly hemolytic zone caused by the growth of *Staphylococcus aureus*) and *Rhodococcus equi* (hemolysis dictates to a large "arrowhead").

- *Biochemical profile determination:* The test consisted of ten micro tubes containing dehydrated substrates for the detection of enzymatic activities and sugars fermentation. The reactions produced during the incubation were translated into color change, spontaneous or revealed after the addition of auxiliary reagents. Colonies taken from “fresh” (18-24h) TSYEA plates, were suspended in a vial of API suspension medium

(2ml), until reaching an opacity equal to that of McFarland standard 1. Subsequently, the bacterial suspension was distributed in tubes, dispensing 100µl in the DIM test tube, and 50µl in the remaining tubes (tests from ESC to TAG). The galleries containing tubes were aerobically incubated for 18-24 hours at +36 ° C. For the reading, 1 drop of ZYM B reagent was added to the test DIM, and within 3 minutes, all the tests were evaluated, giving a + / - on the appropriate results registration form. The identification was achieved by creating a profile number: the tests were separated into 3 groups, assigning to each a value of 1, 2, 4. Within each triplet, only the values corresponding to positive reactions were added together, resulting in a 4-digit numerical profile. DIM test (presence / absence of arilamidase) allows to distinguish between *Listeria monocytogenes* (negative reaction, color from gray to pale orange-beige) and *Listeria innocua* (positive reaction, orange color). With regard to the other tests, *Listeria monocytogenes* showed a positive reaction to the tests for aesculin hydrolysis (production of iron citrate), fermentation of αD mannopiranoside, acidification of D-arabitolo, L-

Rhamnose of Methyl-αD-glucopyranoside. The tests for acidification of D-Xylose, D-Ribose, Glucose-1-phosphate, D-tagatose. showed negative reactions

All isolates with typical morphological and growth characteristics of *Listeria spp*. were frozen at -80 ° C, to be subjected to further confirmatory tests. All strains having the characteristics of *Listeria monocytogenes* have been submitted to biomolecular confirmation and characterization.

3 - Molecular identification, characterization and typing of Listeria monocytogenes

All the isolates having the characteristics of *Listeria monocytogenes* have been identified by molecular test, considering a strain for each positive sample. Among these, a subset of strains was also submitted to characterization and typing, using biomolecular investigations. These strains were selected in relation to the prevalence of the pathogen within the plants, being higher the number from those more contaminated, except for

the plant F. Then the results of this study were compared with those obtained by the analysis of a selection of 36 Listeria monocytogenes strains isolated, during a period from 2004 to 2007, from food and environmental samples, in the same plants included in this survey,

DNA extraction

Listeria monocytogenes strains were harvested from BHI cultures incubated overnight at 37°C. The bacterial cells were lysed with lysozyme (1 mg/ml) for 1 h at 37°C, and genomic DNA was isolated with the Wizard Genomic Purification kit (Promega Corp., Madison, WI). Briefly, after cell lysate 600 µl of Nuclei Lysis Solution are added and incubated for 5 minutes at 80 C. Subsequently, RNase Solution was added and incubated at 37 C for 45 minutes. After that, the Protein Precipitation Solution is added. The solution was centrifuged and the supernatant transferred to a new tube containing isopropanol. The mixture was centrifuged again and the pellet obtained was washed and resuspended in 100 µl of the Rehydration Solution and stored at -20 C for further analysis.

Genotypic and serotype identification

A multiplex PCR assay was set up using primer sets and conditions as described by Doumith et al. (2004). This assay allowed to separate the four major *Listeria monocytogenes* serovars isolated from food and patients (1/2a, 1/2b, 1/2c, and 4b) into distinct groups.The marker genes selected for the multiplex PCR assay were *lmo0737, lmo1118*, ORF2819 and ORF2110.The *prs* gene, specific for strains of the genus *Listeria*, was targeted for an internal amplification control. Each PCR product amplifies distinct fragment sizes between 370 and 906 bp. GoTaq® Hot Start Master Mixes (Promega, Italy) was used for amplification of the target DNA fragments, as per manufacturer's instructions. PCR products were visualized by running 6 µl of the completed reaction mix on a 2,5% agarose gel in 1X TAE and stained with ethidium bromide (0.1 mg/ml) for 20 minutes. The gel images were visualized by Quantity-One software (Bio-Rad, USA) and captured using the Gel-Doc UV trans-illuminator (Bio-Rad, Hercules, CA, USA). Primers sequences (Roche diagnostics, Milan, Italy) used in this protocol are set in the table below:

Primer	Sequence (5'-3')	Concentration of each primer	Product size (bp)
lmo 0737	F AGGGCTTCAAGGACTTACCC	1 µM	691
	R ACGATTCTGCTTGCCATTC		
lmo 1118	F AGGGGTCTTAAATCCTGGAA	1.5 µM	906
	R CGGCTTGTTCGGCATACTTA		
ORF2819	F AGCAAAATGCCAAAACTCGT	1 µM	471
	R CATCACTAAAGCCTCCCATTG		
ORF 2110	F AGTGGACAATTGATTGGTGAA	1 µM	597
	R CATCCATCCCTTACTTTGGAC		
prs	F GCTGAAGAGATTGCGAAAGAAG	0.2 µM	370
	R CAAAGAAACCTTGGATTTGCGG		

The program used in the thermal cycler GeneAmp PCR System 9700 (Applied Biosystems, USA) was as follows:

Steps	no. of cycles		Temperature	Time
Initial denaturation	1		94 °C	3 min
Amplification	35	*denaturation*	94 °C	40 sec
		annealing	54 °C	75 sec
		elongation	72 °C	75 min
Final extension	1		72 °C	7 min

PCR analysis of virulence genes

Three different multiplex PCRs were standardized in order to detect the following 10 virulence-associated genes: multiplex PCR 1): rrn, hlyA, actA and prfA; multiplex PCR 2): inlA, inlB and iap; multiplex PCR 3): plcA, plcB and mpl by modifying the protocols of Border et al. (1990) and Jaradat et al. (2002).

GoTaq® Hot Start Master Mixes (Promega, Italy) was used for amplification of the target DNA fragments, as per manufacturer's instructions.

Primers sequences used in this protocol are set in the table below:

PCR	Primers		Sequence (5'-3')	Concentration	Size (bp)
1	*rrn*	F	CAG CAG CCG CGG TAA TAC	*0.9 µM*	938
		R	CTC CAT AAA GGT GAC CCT		
	hlyA	F	CCT AAG ACG CCA ATC GAA		702
		R	AAG CGC TTG CAA CTG CTC		
	actA	F	GAC GAA AAT CCC GAA GTG AA		268 o 385
		R	CTA GCG AAG GTG CTG TTT CC		
	prfA	F	CTG TTG GAG CTC TTC TTG GTG AAG CAA TCG		1060
		R	AGC AAC CTC GGA ACC ATA TAC TAA CTC		
2	*inlA*	F	CCT AGC AGG TCT AAC CGC AC	*0.9 µM*	255
		R	TCG CTA ATT TGG TTA TGC CC		
	inlB	F	AAA GCA CGA TTT CAT GGG AG		146
		R	ACA TAG CCT TGT TTG GTC GG		
	iap	F	ACA AGC TGC ACC TGT TGC AG		131
		R	TGA CAG CGT GTG TAG TAG CA		
3	*plcA*	F	CGA GCA AAA CAG CAA CGA TA	*0.9 µM*	129
		R	CCG CGG ACA TCT TTT AAT GT		
	plcB	F	GGG AAA TTT GAC ACA GCG TT		261
		R	ATT TTC GGG TAG TCC GCT TT		
	mpl	F	TTG TTC TGG AAT TGA GGA TG		502
		R	TTA AAA AGG AGC GGT GAA AT		

- Multiplex PCR 1: aimed to amplify the following genes: rrn, hlyA, actA e prfA and included the primers: U1, LI1, LM1, LM2, actAF, actAR, prfA0 and prfA1. The program used in the thermal cycler GeneAmp PCR System 9700 (Applied Biosystems, USA) was as follows:

steps	**n. cycles**		**temperature**	**time**
Amplification	24	*denaturation*	94 °C	80 sec
		annealing	55 °C	90 sec
		elongation	72 °C	120 sec
Final extension	1		72 °C	10 min

- Multiplex PCR 2: aimed to amplify the following genes: inlA, inlB and iap and included the primers: inlAF, inlAR, inlBF, inlBR, iap1, iap2. The program used in the thermal cycler GeneAmp PCR System 9700 (Applied Biosystems, USA) was as follows:

steps	n. cycles		temperature	time
Denaturation	1		94 °C	180 sec
Amplification	35	*denaturation*	94 °C	60 sec
		annealing	60 °C	120sec
		elongation	72 °C	1 min
Final extension	1		72 °C	5 min

- Multiplex PCR 3: aimed to amplify the following genes: plcA, plcB and mpl and included the primers: plcAF, plcAR, plcBF, plcBR, mpl0 and mpl1. The program used in the thermal cycler GeneAmp PCR System 9700 (Applied Biosystems, USA) was as follows:

steps	n. cycles		temperature	time
Denaturation	1		94 °C	180 sec
Amplification	35	*denaturation*	94 °C	60 sec
		annealing	60 °C	120 sec
		elongation	72 °C	1 min
Final extension	1		72 °C	5 min

PCR products were visualized by running 5 µl of the completed reaction mix on a 2,8% agarose gel in 1X TAE and stained with ethidium bromide (0.1 mg/ml) for 20 minutes.

The gel images were visualized by Quantity-One software (Bio-Rad, USA) and captured using the Gel-Doc UV trans-illuminator (Bio-Rad, Hercules, CA, USA).

PFGE

A selection of *Listeria monocytogenes* isolates were submitted to Pulsed Field Gel Electrophoresis (PFGE) according to PulseNet standardized protocol (Graves and Swaminathan, 2001). Isolates were inoculated onto TSYEA plates (Oxoid) and incubated overnight at 37°C to evaluate the purity. A reference strain of *Salmonella* Braenderup H9812 to serve as a molecular weight standard was also inoculated onto TSYEA plates (Oxoid). A few isolates colonies of *Listeria monocytogenes* isolates were inoculated onto 10 ml TSYEA slants (Oxoid) and incubated overnight at 37°C. 3 ml of TE Buffer 0,01 M was added to resuspend the colonies. 1 ml of culture was transferred into a sterile 1.5 Eppendorf tube and subsequently, the optical density of bacterial cell suspension was adjusted to 1.3 (range of 1.25 to 1.35) using a PharmaSpec spectrophotometer (Shimadzu, Kyoto, Japan) at OD 610 nm. 240 µl of cell suspension were transferred to a new sterile 1.5 Eppendorf tube and mixed with 60 µl of 2 mg/ml lysozyme solution (Sigma, St. Louis, USA). The suspension was incubated in a

waterbath at 37 °C for 10 minutes. Agarose plugs were prepared as follows: 1.2% of seakem gold agarose (Pulsed Field Certified Agarose, Biorad) was dissolved in milliQ H_2O by gentle heating in a microwave (0,5 ml per sample) and transferred to a heated waterbath (56°C) to equilibrate. SDS buffer plus Proteinase K (0,2mg/ml, Sigma Sigma, St. Louis, USA) were added. Plugs moulds were prepared by sealing the underside with tape to prevent leakage. 300 µl of agarose were added to the resuspended cells, mixed thoroughly and immediately dispensed into the wells of the plug moulds. Wells were slightly overfilled to allow for shrinkage of cooled agarose (about 100 µl per well). Moulds were placed at 4°C for 15-20 minutes to facilitate setting of the agarose. For the cell lysis, 1% N-lauroylsarcosline (Sarkosyl NL-30) dissolved in TE buffer pH 8 milliQ water, and Proteinase K (0,15mg/ml, Sigma P-0390) were added were dispensed into labeled 5 ml Falcon tubes. The plugs were gently pushed into the lisys buffer and incubated in a heated waterbath (54°C) for 2 hours to allow cell lysis. Following lysis the plugs were transferred into a labelled 5ml Falcon tube containing 3 ml of sterile milliQ water preheated to 50°C and incubated in shaking waterbath at 54 °C for 10 minutes. This stage was repeated 2 times. Afterwards, sterile milliQ water was decanted and the plugs were washed with 3 ml of TE buffer (pH 7.5) (10mM Tris methylamine,

10mM EDTA) and incubated in shaking waterbath at 54 °C for 10-15 minutes. This stage was repeated at least 4 times to thoroughly wash the plugs. After the last TE wash was completed, TE buffer was decanted and 5 ml of fresh TE buffer (pH 7.5, room temperature) were added. Plugs slices were stored at 4-6°C for the following restriction step. Sample plugs were digested with 25 U of *Asc*I (New England Biolab, Beverly, MA, USA) at 37 °C for 3 h or 40 U of *Apa*I (New England Biolab) at 30 °C for 6 h. For the restriction endonuclease digestion of DNA, 100 µl per sample of 1x reaction buffer (supplied with enzyme by manufacturer) were dispensed into labelled Eppendorf tubes. A thin slice (about 1mm) was cut from across the width of the agarose plug with a clean scalpel blade onto the surface of a clean Petri plate, placed in the reaction buffer and incubated at at 37 °C for 3 h for *Asc*I and 30 °C for 6 h for *Apa*I. An agarose gel was prepared as follows: 1% Seakem Gold agarose gel (BioRad Pulsed Field Certified) was dissolved by heating in 0.5X TBE buffer consisting in 50mM Tris, 45 mM Boric Acid and 0.5mM EDTA, allowed to cool to 50°C, poured on a tray and allow to set for at least 30 minutes. Once set, the gel was cooled to 4°C. Approximately 2 ml of gel were retained to seal the wells. The plugs were inserted into the wells, ensuring that they touched the front wall of the well and that there were not air bubbles present. Size

markers were loaded in the same manner. The plugs were sealed into the wells by using the reserved agarose and allowed to set before placing the gel into the electrophoresis tank. The separation of the restriction fragments was carried out by PFGE in a CHEF Mapper XA system (Bio-Rad) using the PFGE-PulseNet protocol (Graves and Swaminathan, 2001). The tank was filled with 2.25 litres of 0.5X TBE buffer and chilled to 14°C before loading, using the following parameters: initial switch time, 4 s; final switch time, 40 s; run time, 2 h; angle, 120°; gradient, 6 V/cm; temperature, 14 °C; ramping factor, linear. Gels were stained with ethidium bromide (0.1 mg/ml) for 30 minutes and destained in water for 60 minutes. The gel images were visualized by Quantity-One software (Bio-Rad, USA) and captured using the Gel-Doc UV trans-illuminator (Bio-Rad, Hercules, CA, USA). The banding patterns for each enzyme were assigned through visual analysis of the restriction profiles. Isolates were designated genetically indistinguishable (same pulsotype) when their restriction pattern had the same number of bands and the corresponding bands were the same apparent size (Tenover et al., 1995; Graves et al., 2005). *Asc*I and *Apa*I macrorestriction patterns were analysed using GelCompar software (Applied Maths, Sint-Martens-Platen, Belgium). The similarity between restriction patterns, based on bands position, was

expressed as a Dice coefficient correlation. The position tolerance was optimal when set at 1.0% and 2.0% for *Asc*I and *Apa*I respectively. Clustering and construction of dendrogram were performed by the Unweighted Pair Group Method using arithmetic Averages (UPGMA) combining both *Asc*I and *Apa*I macrorestriction patterns into one unique PFGE profile.

4 - Study of the phenotypic and the genetic mechanisms of the *Listeria monocytognes* persistence.

Quantitative assessment of in vitro biofilm formation

A subset of 97 of *Listeria monocytogenes* strains isolated from 2004 to 2011 and representative of the meat plants included in the study were tested for their capabilities to attach to abiotic surfaces forming biofilm. The quantitative assessment of the in vitro biofilm formation was carried out on sterile 96- well polystyrene microtiter plates using the method described by STEPANOVIC et al., (2004) with some modifications. All the strains were sown on TSYEA plates (Biogenetics) and incubated at +37 ° C for 24h. Some typical colonies were suspended in 2 ml of Brain Heart Infusion Broth (BHIb) and incubated overnight at +37°C.

All the wells of a polystyrene microtiter plate were filled up with 230 µl of BHIb. Afterwards, 21 wells per strain were filled up with 20 µl of culture. Each plate included 12 wells of BHIb without inoculum, as negative control. Each strain was inoculated into two different microplates in order to assess biofilm formation after incubation at +30 ° C for 20 and 40 hours. At the end of the incubation the content of the wells was removed and the microtiter plate washed three times with 300 µl of sterile distilled water for each well in order to remove loosely attached bacteria. The remaining attached bacteria were fixed with 250 µl of methanol per well, and after 15 min the wells were emptied and air dried for 45 minutes. Each well was stained with 250 µl of Crystal violet for 5 min. After staining, the microtiter plates were washed under running tap water, then air dried and the dye bound to the adherent cells was resolubilized with 250 µl of 33% (v/v) glacial acetic acid per well. The microtiter plates were read spectrophotometrically (OD620) using a Sunrise RC absorbance reader (Tecan, Maennedorf, Switzerland). In relation to the OD values produced by bacterial films adherent to microtiter plates, the different strains of *Listeria monocytogenes* have been classified under the following categories: non-producers, weak, moderate and strong biofilm producers. The cut-off (ODC) was obtained by adding the average value

of optical density (OD), calculated on the negative control, three times the standard deviation of the same. In relation to the attitude to produce biofilms, the strains were then classified into 4 categories: a) non-producers (NP), if OD ≤ ODC b) weak (WP), if ODc<OD≤2ODc c) moderate (MP) 2ODc<OD≤4ODc d) strong (SP) 4ODc<OD.

The part below has been conducted in the period June-October 2011 next to the TEAGASC, Agriculture and Food Development Authority, laboratories, placed at Moorepark, Fermoy, Co. Cork (Ireland).

Differential gene expression by persistent and non persistent strains of Listeria monocytogenes.

Selection of genes, strains and primers design

Using transcriptomic analysis, Fox et al 2011 studied the differential expression of genes in a persistent strain of *Listeria monocytogenes*. For this study, some of these genes were carefully chosen basing on their function and the fact that in the presence of BZT, a quaternary ammonium compound commonly used in the food industry, were up-regulated in a strain considered to be persistent and down-regulated in a strain considered to be non-persistent, the strain LM 6179 and strain LM 272, respectively.

The purpose was to determine if these genes were upregulated in all persistent strains, and therefore if these genes could be used as a marker for persistence. Based on their functionality as likely candidates for marker genes, the genes selected for this work were *pocR*, *eutJ*, *cbiD* and *MDR*. *PocR* is a metabolism regulatory gene and controls the expression of other genes in different genomes (Bobik, T.A. et Al. 1992; Chen, P. et Al, 1995). These are involved in the metabolism of ethanolamine and 1,2-propanediol in only 3 pathogenic species: *Listeria monocytogenes*, *Salmonella*, and *Clostridium perfrigens*, using these compounds as a sole carbon source (Srikumar, S., and Fuchs, T.M. 2011). Authors suggest that these genes may confer an advantage in proliferation to *S. Typhimurium*, not only within the host but also in the external environment. These pathways could have implications in the persistence of *Listeria monocytogenes.* In many genomes the *eut* and *cbi* genes are responsible for cobalamin-dependent propanediol degradation, and both are regulated by the common regulatory gene *pocR* when *Salmonella* grows in conditions of poor carbon sources (Bobik, T. A. et Al. 1992; Bobik, T. A. et Al. 1997; Chen, P. et Al. 1995.; Rondon, M. R. et Al**.** 1996; Walter, D. et Al. 1997).These genes could also have implications in the persistence of *Listeria monocytogenes.*

As regards the gene MDR, this is a multidrug efflux pump that is involved in protecting the cell against several antibiotics, heavy metals, Ethidium bromide and quaternary ammonium compounds like Benzethonium chloride (BZT), allowing the cell to be persistent, and this may be involved in the persistence mechanism.

Primers were designed using DNASTART LaserGene 8 software. The primers used are shown in Table 13. The specificity of the primer sequences was further determined using the NCBI BLASTN database, and analyses revealed that the primer sequences showed 100% homology to *Listeria monocytogenes* strain EGD-e (GenBank accession number NC_003210).

Potential housekeeping genes to be used as internal controls were identified from Taurai and Stephan (2007). Of the three housekeeping genes *16S rRNA*, *rpoB*, and *gap*, *rpoB* displayed the best characteristics for our purpose and was chosen as the internal control gene for relative quantification. The primers for this gene were: rpoB F: 5'-AATCGGGGACAATGACT-'3, rpoB R: 5'-GTGTGCGGAAACCTAC-'3. Primers were synthesized and purchased from SIGMA.

Five known persistent strains (6179, 4423, R479A, CZ48 and 4898) and 5 presumed non persistence strains (N22-2, CZ70, 2566, 251 and 272) were tested for relative expression of selected genes. In addition, 34 *Listeria monocytogenes* strains, isolated from different sources in different meat processing plants (raw materials, final products and environmental sources) between 2004 to 2011 were selected, having assessed their PFGE profiles (of 97 strains) for comparative purposes. Strains belonging to different PFGE profiles were analysed.

Growth conditions

The different *Listeria monocytogenes* strains were available as frozen (70°C) stock cultures. They were revived by plating onto TSA agar plates (Biogenetics), and single colonies were obtained after incubation for 18–24 hours at 37°C. Single colonies from each strain were inoculated into 50 ml TSB. The cultures were grown for 42 hours at 14°C to reach the stationary phase. The 10 strains of known phenotype (persistent and non persistent) were grown in either the presence (4.0 ppm or 0.5 ppm) or absence of the quaternary ammonium compound benzylthonium chloride (BZT) at 14°C for 72 hours (the temperature of the ripening room at the processing facility where strains in this study were isolated). Thereafter, the samples were processed for total RNA isolation.

Total RNA isolation

Total RNA was isolated from each sample using the RNeasy Midi kit (Qiagen), and following the guidelines of the kit protocol. Briefly, *Listeria monocytogenes* cells, prepared under the conditions of the stress adaptation described above were harvested by centrifugation of 50 ml cultures (4500 rpm for 5 minutes at 4°C). The pellets were

resuspended in 500 µl of lysis buffer and transferred onto an RNeasy midi column. Thereafter, total RNA was isolated from the bacterial cell lysates following the RNeasy Midi kit protocol. This included two steps to remove or minimize genomic DNA contamination. The lysates were first passed over a DNA-binding column provided in the kit, followed by an on-column DNAse I digestion of the samples bound to the RNA-binding column. The RNA templates were eluted in 150 µl of RNAse free water. The RNA concentration was measured by absorbance at 260 nm, while purity was monitored by inspection of the 260/280nm and 230/260nm ratios to assess protein and organic substances contamination, respectively, using the Nanodrop instrument (Nanodrop Technologies, Delaware). The quality and integrity of the total RNA templates were assessed by electrophoresis of aliquots of selected samples from each run on a 1,5% (w/v) denaturing formaldehyde agarose gel electrophoresis.

cDNA preparation

The reverse transcription step, to convert mRNA to cDNA, was performed using the SuperScript III First-Strand Synthesis SuperMix Kit (Invitrogen). Eight-hundred nanograms of the RNA template from each sample were converted into cDNA in a 20

µl reaction. The cDNA template pool from each sample was subsequently used to assess the various target gene transcript levels in gene-specific real-time PCR assays.

Real time PCR

The real-time PCR reactions were performed in the Light Cycler 480 instrument (Roche Molecular Diagnostics, Rotkreuz, Switzerland) in a total reaction volume of 20 µl. This reaction contained 2 µl of cDNA template, 0.5 mM (1 µl)of each primer and 2 µl of LightCyclerR 480 SYBR Green I master mix (Roche Molecular Diagnostics, Penzburg, Germany) made up to the required volume with purified water. The real-time PCR run protocol consisted of (1) preincubation (10 minutes at 95°C); (2) amplification (10 seconds at 95°C; 10 seconds at 53-54°C; 12 seconds at 72°C); (3) melting curve (65–95°C at continuous fluorescent measurement); and (4) cooling. Amplification was monitored in the appropriate LC 480 channel and specific amplification confirmed by single peaks in melting curve analysis. For the purposes of quantifying the results obtained by real-time RT-PCR both the Absolute Quantification (based on the standard curve method) and the Relative Quantification method were used. The method of relative quantification allows evaluation of the differences in the levels of expression of

a gene between strains. A housekeeping gene, assumed to have equal expression in all strains, was used to normalise expression. Relative expression of the target gene, normalised on the housekeeping gene, between two strains can then be assessed. However, as this process can lead to errors, due for example to differential expression of the housekeeping gene, absolute quantification of the copy number of the target gene is more definitive. For absolute quantification a standard curve is first constructed from a DNA of known concentration. Spectrophotometric measurements at 260 nm can be used to assess the concentration of this DNA, which can then be converted to a copy number value based on the molecular weight of the sample used. This curve is then used as a reference standard for calculating quantitative information for cDNA targets of unknown concentrations. cDNA plasmids are the preferred standards for standard curve quantification.

Cloning in the vector pCRII-TOPO (Invitrogen)

For absolute quantification the two genes pocR and MDR were selected as targets. Both genes, one a regulator and one a multi-drug resistant pump, would be a likely candidate gene, over-expression of which could result in improved persistence. To prepare

standard curves for absolute quantification, the two genes were obtained through the PCR technique and purified by running on agarose gel. They were then cloned into commercial plasmid vector (pCRI-TOPO). The plasmid vector was then used to transform competent *E. coli* bacterial cells that were grown in liquid culture before extracting the plasmid DNA which was tested to evaluate the effective transfer of the gene of interest. The cloning was performed using the TOPO ® TA-Cloning Kit (Invitrogen), following the manufacturer's instructions.

Ligation was carried out by simply incubating the following ligation mix, prepared according to the instructions of manufacturer, for 5-30 minutes at room temperature:

4µl of PCR product

1 µl of salt Solution

1 µl TOPO ® vector

The plasmid vector which was ligated was used to transform chemically competent cells of *E. coli* TOP10 (Invitrogen) by heat shock, which allows the transfer of the plasmid inside the cell. The cells were thawed on ice and then 2 µl of the product of ligation was

added. The tubes were incubated 30 minutes on ice, prior to thermal shock at 42°C for 30 seconds and transfer immediately to ice for 2 minutes, after which 250 µl of SOC medium was added at room temperature. The cells thus transformed, were incubated with shaking at 37°C for one hour while keeping the tube horizontal. At this point the cells were streaked onto LB media + Ampicillin (50 µg / ml) for selection of transformed clones. Ten and 50 µl were plated and the plates were incubated overnight at 37°C. The day after, the extraction of plasmid DNA was performed. The plasmid DNA thus obtained was used to amplify the gene of interest to confirm its presence into the competent cells, and visualized on agarose gel. Two bands were displayed. One belonging to the plasmid and one to the fragment of the gene of interest.

RESULTS AND DISCUSSION

1 - Description of structural and technological requirements and types of fermented meat products produced in plants included in this study

Main Features of the facilities

The six plants included in the survey, located in the province of Sassari (A), Carbonia - Iglesias (C), Medio Campidano (D), and Nuoro (B, E, F), were a representative sample of the regional reality. All the facilities were authorized under the Legislative Decree 537/92 and following amendments and provided with EU recognition (stamp L). Of these, three had an industrial capacity (plants A, B, and D) and the other a non industrial capacity (C, E, F).

General features of productions

All the facilities produced different types of meat products (ham, salami, sausages, cups, loin, bacon). The "Salsiccia Sarda" represented the main product in all the facilities. All plants produced the classic and the smoked Sardinian sausage, while the thin and the cylindrical kind were processed only by the facility D. The supply of

raw materials (meat) for the production of sausages was predominantly national (Northern Italy) and a small part cames from our region (facility F). The meat purchased from national slaughterhouses belongs to the heavy type pigs. Mainly fresh refrigerated meat pork was used. It cames entire in the plants to be cut in half (usually the main product in this case is the ham; the sausage is obtained from the trimmings) and /or in anatomical and commercial cuts. Only one facility has declared to use frozen meat. The main cuts used were rump steak, cut from bench, lorgnette, cut throat, cutting ham, minced bacon, slices of bacon, pack-saddle.

Definition the diagram process of ripened sausage

Recipe: The fat is usually represented by muscle cuts thoroughly infiltrated or fat cover. When use lean cuts the fat is added in form of bacon, rarely, lard is used. The mix ingredients are common to all plants, in addition to meat and fat are used: salt, nitrite and nitrate, ascorbic acid, pepper. Each facility characterizes its product with additional ingredients and additives, including whole or ground garlic, nutmeg, vinegar, white wine, Vernaccia wine, cinnamon, cloves, fennel seeds, myrtle,

anise, chili pepper. Sometimes milk powder or skimmed, milk proteins, sugars (lactose, dextrose), etc were used. All ingredients and additives are usually from Italy, but sometimes foreign products are used. Some facilities declare to use microbial starter, consisting of lactic bacteria or staphylococci coagulase negative (MI100, Briostarter CAP, dried SH101 Texel,SA301 Milan). Instead yeast and molds were not used. All plants used natural tort bowel beef.

Received meat: the meat is stored in cool room at 0-4 ° C until processing

Preparation of the mix: half-carcasses, quarters and cuts are first subjected to boning and / or peeling. The meat is then minced using a mincer with different number of blades. The size of the holes of drawing machines varies from a minimum of 8 mm (rare) to a maximum of 22 mm. More widely, drawing machines of 16-18 mm are used. For the mix composition, to the ground beef, salt is added, eventually wine, and then the "tanning", previously weighed in relation to the amount of meat. The mixture is processed with automatic mixers. Generally, the mixture is subjected to a rest period of time ranging from 12 to 24 hours under refrigeration (0-4°C). Rest allows the absorption and amalgamation of the various ingredients, and also the acquisition of

a more compactness that facilitates bagging. In addition, low temperatures are able to limit the development of bacteria indicators of hygiene and spoilage present in raw materials or from environmental origin.

Bowel preparation: bowels are generally purchased in salt, packed in drums refrigerated. The origin maybe various and the health measures provided from Europe to control the spread of TSE, have encouraged the importation of bowels from third countries such as Argentina. Preparation includes the removal of salt and washing in hot and cold water, accompanied by a thorough massage that promotes the recover by the intestinal walls of the necessary elasticity.

Sacking: is performed by asausage filler with an automatic dosing device, which acts with vacuum by entering a constant pre-set amount of mixture. The closure of the ends is made by clipping, usually automatic. From the sausage filler the product is shaped manually by operators that give the characteristic form of a "rock" and carry out the binding of the ends with string.

Wiping and drying process: after sausage-making, sausages are placed in a controlled environment where they are subjected to drying and curing stages. Sometimes, the real

seasoning is done in distinct environments. The Drip-drying stage is carried out at temperatures ranging from initial 20 to 28 ° C and relative humidity of 60-70%, for a period ranging from 6 to 36 hours. Rooms work alternating between periods of work with periods of rest, progressively more extended. In this phase the product exudes visibly and looks swollen with stretched bowel, the mixture coloris lighter red, while the presence of humidity under the bowel, gives it a greyish exterior color. During drying, the sausages are kept in the same room, where the temperature undergoes a gradual reduction, of 1 or 2 degrees per day, while the relative humidity increases gradually up to values equal to 75-80%. The process ends with the achievement of room temperature values equal to 15-17 ° C, after a period ranging from 3 to 5 days. During the wiping and drying process, we are witnessing a massive loss of humidity, accompanied by the heating of the product, which promotes the development of the lactic micro flora and the resulting acidification.

Seasoning: the duration and the management of this stage are strictly dependent on the previous phase of drying. Generally, the temporal limits between the two phases are difficult to distinguish. In most plants these are in planned rooms , which are often the

same of the previous phase. The temperature is approximately 14-17 °C, with relative humidity of 75-80%. The duration of this phase is varying, from 7 to 25 days.

Grooming: All plants perform a final wash with a drying in cell (20-22 ° C), or a brushing. Sometimes two washes, before and after drying, are performed.

Presentation features of sausages: sausages appear in a horseshoe bend, the so-called rock, size and weight varies from a minimum of 300 to a maximum of 600 g. Regarding the way of packaging and labeling, several facilities package the entire product under vacuum, with heat-shrink film. Some after portioning or slicing, using the vacuum packaging of portions. All plants will label the product with typical seals, but many have developed innovative solutions for commercial packaging and labeling (brochures and adhesive labels).

2 - Survey on the prevalence of *Listeria monocytogenes* during the processing of Sardinian sausages (raw material, semifinished and finished products) and in their environmental plant.

Chemical and physical parameters

a) Ground meat (*GM*)

The results for the pH are shown in table 1. The values (mean±s.d.) were equal to 5.81±0.18 in ground meat. These values are similar of those found in pork stored at refrigeration temperature.

b) Sausages at the end of curing (*AS*)

The average value of pH (table 1) was equal to 5.58 ± 0.33, but it has been shown a wide variability in a range between 4.8 and 5.9, probably dependent by the use of starter cultures. The mean value of aw was equal to 0.95 ± 0.01. The range of aw values ranged from a minimum of 0.98 to a maximum of 0.93

c) Sausage at the end of ripening (*RS*)

The average value of pH (table 1) was equal to 5.38 ± 0.29, but it has been shown a wide variability in a range between 4.82 and 6.05, dependent by the use of starter cultures. The values fall between those typical of sausages with short and very short maturity. The mean value of aw was equal to 0.90 ± 0.03 and generally highlights a dehydration process not very intense. The range of aw values ranged from a minimum of 0.84 to a maximum of 0.97 and was influenced by the length of seasoning and the use of hygroscopic substances. In general, chemical and physical parameters found at the end of ripening are index of stability, but the temperature of refrigeration during storage is the most reliable criterion of safety for a short sausage seasoning.

Detection and enumeration of Listeria spp and Listeria monocytogenes

- Prevalence in food samples

The overall prevalence (Table 2) of *Listeria spp* in food samples (*GM*, *AS* and *RS*) was 63.03 % (n.104/165). In particular the higher prevalence was found in *AS* (74.5%), while decreased in *RS* (50.9%). With regard to the overall prevalence of *Listeria*

monocytogenes in food samples, it was 31.51% (n.52). The prevalence was higher in *GM* samples (36.3%), decreased in *AS* (29.1%), and remained constant in *RS* (29.1%). These results highlighting the ability of *Listeria monocytogenes* to survive during sausages fermentation, and overcome the current hurdles of the manufacturing process.

All the samples did show detectable levels always below 100 CFU/g, complying with the food safety criteria provided for the RTE foods able to support the growth of *Listeria monocytogenes* (EC Reg. 2073/2005).

The prevalence of *Listeria spp*. and *Listeria monocytogenes* differed between plants and, within plants, between production batches (Table 4→9).

The higher prevalence of *Listeria* spp was observed in the plant A (86.66%), even if was only sampled once, while the lower prevalence was in the plant C (40%). The higher prevalence of *Listeria monocytogenes* was found in the plant F (46.66%), while the lower was in plant D (20%). Plant C was the only plant where the pathogen was not detectable. Among the plants, the following prevalence was showed:

- in plant A, the prevalence for *Listeria spp.* was 60% in *GM*, and all the samples of *AS* and *RS* were positive. Concerning the prevalence of *Listeria monocytogenes*, it was 40% in *GM*, increased in *AS* (60%), and then decreased in *RS* (20%).

- in plant B, the prevalence of *Listeria spp.* was constant between the batches: the overall prevalence in food samples was 100% in *GM* and in *AS*, while was 50% in *RS*. Concerning the contamination rate of *Listeria monocytogenes*, a higher prevalence was found in the second batch (*GM* 80%, *AS* 40%, *RS* 60%), while in the first one was lower (not detected in *GM*, 20% in *AS* and in *RS*).

- in plant C, differences between the batches in prevalence of *Listeria spp* were found: in the first batch prevalence was 40% in *GM*, 50% in *AS* and 30% in *RS*. During the second sampling day any *Listeria spp.* was isolated. Plant C was the only one where *Listeria monocytogenes* was not detectable.

- in plant D, during the first sampling, *Listeria spp.* was not detectable in *GM*, while its prevalence was 80% in *AS* and 40% in *RS*. In the second batch the prevalence of *Listeria spp.* was 80% in *GM* and in *RS*, while was not detectable in *AS*. Regarding the prevalence of *Listeria monocytogenes,* differences within the two batches were

observed. The first batch was not contaminated, while prevalence in the second batch was 40% in *GM*, not detectable in *AS*, and 80% in *RS*.

- in plant E, the prevalence of *Listeria spp*. was 40% in *GM* of both batches, while in *AS* it was 60% and 100%, and in *RS* 20% and 80%, respectively in the first and in the second batch. Prevalence of *Listeria monocytogenes* was higher in the second batch, more specifically in *AS* (80%), and in *RS* (60%).

- in plant F, no difference in prevalence of *Listeria spp* was observed between the batches for *GM* contamination (100%), while prevalence in *AS* was higher during the second sampling (100%). In *RS* the prevalence was 80% in the first batch, but *Listeria* spp was not detected in the second batch. Likewise the prevalence of *Listeria monocytogenes* was higher in samples from the second batch: 60 → 100% in *GM*, 20 → 40% in *AS*, while in *RS* the decrease of the pathogen in the second batch was confirmed.

- Prevalence in surface samples by swabbing

Listeria spp. could be isolated from 49 of 220 environmental samples. In general the prevalence observed in samples from surfaces in contact with meat was slightly higher (23.5%), than in surfaces not in contact with meat (20.5%).

The results concerning *Listeria monocytogenes* confirmed the higher prevalence (17.42%) in surface in contact with meat (*Scm*), than in those not in contacts (*Swcm*, 11.4%). Results are showed in table 3.

Contamination of SCM

Samples positive for *Listeria spp*. were recovered in all plants. The higher contamination was found in plant A (58.33.5%), followed by plant E (37.5%), and plant B (33.33%). A very low prevalence (< 16%) was observed in the other plants.

Listeria monocytogenes were recovered from 5 of the six plants (Table 4→9); 10/30 (33.33%) samples were from the ground meat trolleys, and 5/33 (15.15%) from the working tables of the processing room. The remaining positive samples were from different *Scm* (9.09%).

Plant A was the most contaminated (41.66%), followed by plant E (37.5%), plant B (25%), plant F (8.33%), and plant D (4.16%). Large plants (A and B) seemed to have relatively high contamination rate by *Listeria monocytogenes*, except for the plant E, which was a small one. Within the plant, differences between the batches were found. In plant D *Listeria monocytogenes* was detected only during the second sampling day, while the first one resulted negative. In plant E, prevalence was twice (50%) in the first batch compared to the second (25%). However in plant B and F no differences between batches were found.

Contamination of Swcm

Listeria spp. was not detected in plant E, while positive samples were detected in all the other plants. The higher prevalence was observed in plant F (43.75%), followed by D and E (25%), A and B (12.5%).

Positive samples for *Listeria monocytogenes* were recovered in 3 plants (B, E, F) from floor drains, while the pathogen was not detected in *Swcm* taken in plants A, C, and D. In plant E, the pathogen was recovered also from the walls of the seasoning and drying rooms (n. 3). The higher level of contamination was observed in plants F and E (37.5%

and 18.75% respectively). Even for this kind of samples, differences between batches were observed. In particular in plant B, positive samples were detected only in the second batch (12.5%), while the pathogen was not detectable in the first one. In plant E, prevalence of *Listeria monocytogenes* was twice in the second batch (25%), than the first (12.5%). Finally in plant F prevalence of *Listeria monocytogenes* was much higher in the second (62.5%), than in the first (12.5%).

Results analysis and discussion

These results seem to confirm the considerable diffusion of *Listeria monocytogenes* in meat processing plants and showed interesting epidemiological aspects. Over 31 % of food samples were positive for *Listeria monocytogenes*. Furthermore the pathogen was isolated from 17.42% of *Scm*, and 11.4% from *Swcm*.

In plant A, *Listeria monocytogenes* was detected in a significant number of ground meat samples (40%), which were a probable source of entry of the pathogen into the plant. During the process, prevalence increased until the end of the curing stage (60%), then decreased in drying period (20% in finished products). As further source of *Listeria monocytogenes*, in addition of the primary contamination by raw meat, cross

contamination by surfaces in contact with meat should be considered (prevalence 41.66%). These results confirm that the hurdles of microbial growth, as low pH and a_w and salt concentration, should properly to take place during the ripening of the product, to prevent the *Listeria monocytogenes* multiplication.

In plant B, significant differences in *Listeria monocytogenes* contamination between and within the batches were showed, in particular for the ground meat (80% in B2 *vs* absence in B1). In the second batch, the high contamination rate decrease in *AS*, but increase again in *RS*. Instead in the first batch, the pathogen was not detectable in ground meat, but it was detected in *AS* and *RS* (contamination rate of 20%). On the basis of these results, we could suppose a contamination route similar to that described above for the plant A. In fact, *Listeria monocytogenes* was present also in environmental samples, both in surface in contact and not in contact with meat.

In plant D, as in the previous plant, we have found an unhomogeneous contamination of *Listeria monocytogenes*, which was not detectable in all samples of the first batch, while in the second one was found in ground meat (40%) and in the finished products (80%).

The remarkable increase, in the last case, seems to depend on cross-contamination by *Scm* (12.5%).

In plant E, the pathogen was present in all ground meat samples; both in the first and second sampling day, increased in contamination rate during the curing stage, and then decreased in an unhomogeneous way in the finished products. In fact, in the second batch, prevalence in sausages at the end of ripening remained high (60%). Surfaces were always positive too, with prevalence until 50% (*Scm*). On the base of these results, the contamination source could be due to raw meat, but the presence of the pathogen in all the products and in environmental surfaces sampled, highlights its constant persistence, and lack of measures of hygienic control. Further information could be acquired by PFGE analysis.

In plant F, in both batches, a high prevalence was found in ground meat (60% and 100%), probably due to a poor hygienic quality of raw meat. Prevalence decreased during the curing stage, but increase again in the finished product (60%) of the first batch, while the pathogen was not detectable in *RS* of the second batch. In this plant,

Swcm resulted the most contaminated (overall prevalence 37.5%). The same remarks reported for the plant E, could be made.

3 - Molecular identification, characterization and typing of Listeria monocytogenes

A total of 292 strains of *Listeria spp* were isolated, of which 173 were identified as *Listeria monocytogenes*. N. 102 strains were isolated from food samples, while 71 were from environmental samples. A subset of 97 strains was characterized by Multiplex PCR-based Serogrouping, multiplex PCR analysis of virulence factors, PFGE and in vitro biofilm formation, as previous described. Among these, 61 strains were isolated in 2011, and 36 strains from the same facilities during a period from 2004 to 2007.

Multiplex PCR-based Serogrouping

The prevalent serotype (n. 97 strains) was 1/2a (41.24%), followed by 1/2c (30.93%), 1/2b (19.59%), and 4b (8.25%). Results are shown in table n. 10, divided in relation of the origin of the samples. The serotype distribution was various, as reported in tables 11 and 12, where the results are showed per plants and per year of isolation, in food and environmental samples, respectively. As described by other authors (Thevenot et al.. 2005), no specific serotype was recovered from food and environmental samples, or in

relation to the plant. Table 13 shows the details of *Listeria monocytogenes* serotypes isolated in 2011, from the specific matrices, and in relation to the plants and the batches (I and II).

Concerning serotype distribution within the facilities, differences were observed.

In plant A, during the survey carried out in 2011, the prevalent serotype was 1/2c, isolated in food, except *GM*, and in all the environmental samples. Serotype 1/2b was also isolated, except in *RS* and *Swcm*. The former was not detected in the previous study carried out in 2004-07; on the contrary, the latter was the only one serotype isolated, in both food and environmental samples.

Similarly, in plant B, two serotypes, 1/2a and 1/2c, were recovered, in 2011, both in food and environmental samples, and during the two sampling days. In the previous survey, only serotype 1/2a was found, but only in *RS* and in *Swcm*.

In plant D, where the prevalence was low, serotypes 1/2a (*GM*) and 1/2c (*GM*, *Scm*) were isolated. Both serotypes were found occasionally in *RS* in 2004 and in *Scm* in 2007. It is interesting to highlight that in the previous survey, serotype 4b was isolated

sporadically in food and environmental samples, while this serotype was not detected in the latter sampling.

In plant E, that was one of the most contaminated, different serotypes were isolated in relation to the year of sampling. The prevalent serotype, in 2011, was 1/2a in all the samples of both batches, followed by 4b, isolated only in food samples, and, occasionally, 1/2c. On the contrary, the serotype 1/2a was rarely isolated in 2004, while in 2007, 1/2b serotype was the only found.

In plant F, two serotype, 1/2a and 1/2c (prevalent), were recovered from both food and environmental samples. Only the former was occasionally isolated from food samples in 2004.

PCR analysis of virulence factors

The amplification products of the analyzed virulence genes (results are shown in table n. 14) were found in all 97 isolates of *Listeria monocytogenes*, with the following prevalence: 79.38% *hlyA;* 97.94% *rrn*; 99% *prfA*; 100% *iap*; 96.9% *inlB*; 68% *inlA*; 62.9% *plcA*; 100% *plcB* and 90.7% *mpl*. Primers used for the detection of *actA* allowed the amplification of two different products (268 bp and 385 bp), showing a polymorphism for this gene. Only 26.80% of isolates showed the 268 bp amplification band (Fig. 1), while the 61.85% of isolates showed that of 385 bp.

Some authors (Wiedmann *et al.,* 1997; Jiang *et al.*, 2006) hypothesized that a deletion of nucleotides in this gene, causing the polymorphism described, might be related to different virulence properties among the isolates. Recent studies have reported no statistical correlation between the presence of the 268 bp *actA* band and the ability to invade HeLa cells, multiplying intracellularly. For this reason, the role of *actA* as marker of unambiguous identification of foodborne isolates, which are attenuated or non-virulent, should be revised (Conter et al*.,* 2009).

With regard to the low prevalence of the virulence factor *inlA* (68%), strains that didn't show the expected product (255 bp) were from *GM* (n.2), *AS* (n.4), *RS* (n.1), and *Scm* (n.1). Polymorphism had been reported for *inlA* and *inlB* (Ericsson et al., 1995, 2000) gene sequences. Recent studies support the idea that a significant proportion (approximately 45%) of *Listeria monocytogenes* isolates from RTE foods carry unique virulence - attenuating mutations, leading to a "premature stop codon" (PMSC) in *inlA* (Nightingale et al. 2008; Van Stelten et al. 2010; Ward et Al. 2010). However, we did not observe any polymorphism in the PCR products of these genes. It is important to highlight that the lack of this gene, although present in some strains, is probably not demonstrable whith this protocol. This is due to the fact that the primers used in our study are able to amplify only a portion of the *inlA* gene, that could be located outside the areas of polymorphism, or the target regions may have been too small to detect any variations.

DNA macrorestriction and Pulsed-field gel electrophoresis (PFGE)

A high heterogeneity of pulsotypes occurred within the plants, 34 with *Apa*I and 40 with *Asc* I, respectively. Among the *Listeria monocytogenes* strains presenting the same pulsotype, a selection of them was selected for further numerical analysis (Figure n. 2). The distribution of the *Apa* I and *Asc* I pulsotypes (tables 15 →19), within the plants was as follows.

Plant A: with the *Apa* I restriction enzyme, during the sampling in 2011, 4 pulsotypes were found: P4 (*GM* and *Scm)*, P5 (*AS*, *RS* and *Scm)*, P10 (*AS*), and P20 (*AS* and *Scm)*. Strains isolated during the period 2004/2007 belonged to 4 different pulsotypes: P2 (*GM* and *RS)*, P13, P18, and P19 (*Swcm)*. With the *Asc* I restriction enzyme, during the sampling in 2011, 3 pulsotypes were found: P11 (*AS)*, P23 (*GM*, *AS*, *RS* and *Scm)*, P25 (*AS)* and P26 (*Scm)*. Strains isolated during the period 2004/2007 belonged to 3 different pulsotypes: P9 (*GM* and *RS)*, P31 and P35 (*Swcm*). *Plant B*: with the *Apa* I restriction enzyme, during the sampling in 2011, 4 pulsotypes were found during the first sampling session: P15 (*Scm)*, P23 (*RS* and *Scm)*, P24 (*Scm)* and P26 (*AS)*; during the second sampling session, P15 was recovered again (*GM* and *Scm)*, while other 2

additional pulsotypes were found: P16 (*Scm)*, and P25 (*GM)*. Strains isolated during the period 2004/2007 belonged to 4 different pulsotypes: P7 (*RS)*, P12 (*Scm)*, P22 (*RS),*and P34 (*Swcm*). With the *Asc* I restriction enzyme, in 2011, during the first sampling day 6 different pulsotypes were found: P9 and P15 (*RS)*, P12, P13, and P19 (*Scm)*, P21 (*AS)*. During the second sampling session the isolated strains belonged to 3 different pulsotypes: P16 (*GM)*, P18 (*GM* and *Scm)*, and P20 (*Scm)*. Strains isolated during the period 2004/2007 belonged to 3 different pulsotypes: P32 (*Scm)*, P34 (*Swcm)*, and P36 (*RS)*. *Plant D*: with the *Apa* I restriction enzyme, during the sampling in 2011, 2 pulsotypes were found: P6 (*GM* and *Scm)*, and P11 (*GM)*. Strains isolated during the period 2004/2007 belonged to 3 different pulsotypes: P12 (*GM)*, P21 (*Swcm)*, and P29 (*RS)*. With the *Asc* I restriction enzyme, in 2011, during the first sampling day 3 different pulsotypes were found: P14 and P24 (*GM*), P22 (*Scm)*. Strains isolated during the period 2004/2007 belonged to 3 different pulsotypes: P33 (*GM)*, P37 (*Swcm)*, and P38 (*RS)*. *Plant E*: with the *Apa* I restriction enzyme, during the sampling in 2011, only pulsotype P8 was recovered during the first sampling session. During the second sampling session, 5 different pulsotypes were found: P1 and P14 (*AS)*, P9 (*GM)*, P17

(*Scm)*, and P28 (*Swcm)*. Strains isolated during the period 2004/2007 belonged to the pulsotype P3 (*RS)*. With the *Asc* I restriction enzyme, in 2011, during the first sampling day 4 different pulsotypes were found: P1 (*GS* and *Scm)*, P2 (*AS* and *RS)*, P5 (*Scm* and *Swcm)*, and P10 (*GM*, *AS* and *Scm*). During the second sampling session strains belonged to 6 different pulsotypes: P2 (recovered again, *Scm)*, P3 (*Scm* and *Swcm)*, P4 (*Scm*), P6 and P7 (*GM)*, P17 (*AS)*. Strains isolated during the period 2004/2007 belonged to the pulsotype P40 (*RS)*. *Plant F*: with the *Apa* I restriction enzyme, during the sampling in 2011, 4 pulsotypes were found: P27 (*Swcm)*, P30 (*GM* and *Scm)*, P31 (*GM)*, and P33 (*GM* and *RS)*. The strain isolated during the period 2004/2007 belonged to pulsotype P32 (*RS)*. With the *Asc* I restriction enzyme, during the sampling in 2011, 4 pulsotypes were found: P27 and P29 (*GM)*, P28 (*RS)*, P30 (*Scm)*, and P8 (*Swcm)*. One strain isolated from *RS* during the period 2004/2007 belonged to pulsotype P39

The *Apa* I and *Asc* I restriction patterns of these strains were combined in 65 different PFGE profiles, indicating a great level of diversity among the strains collected from the Sardinian processing plants and their products. The high number of PFGE profiles and their heterogeneous distribution within the plants agreed with the results of previous

surveys carried out in meat processing plants (Thevenot et al., 2006) and such subtypes appearing unique to each processing plant, can be considered as plant-specific (Fugett et al., 2007). These results may be due to the great diversity of the *Listeria monocytogenes* strains collected from ground meat, but also to the production capacity of each plant. The large plants (A, B, and E) exhibited the highest PFGE profiles heterogeneity, since these plants also used the greatest amount of raw meat from different geographical origin (National and European). Numerical analysis of the PFGE profiles showed that the majoriy of strains could be allotted into 3 main PFGE clusters ($\geq$ 5 strains, similarity $\geq$ 60%) labelled as A, B, and C. These three genomic divisions were not linked with the flagellar antigen type, in spite of what was described by several authors (Chasseignaux et al., 2001; Autio et al., 2003; Thevenot et al., 2006). Cluster A included isolates (n.18) of serotype 1/2a and 4b, from 3 different processing plants. Cluster B included isolates belonging to serotypes 1/2b, and 1/2c, from one processing plants. Cluster C (n.20) included isolates of serotype 1/2a, 1/2c and 4b from 3 different processing plants. Several isolates of different serotypes (1/2a, 1/2b, 1/2c and 4b) were outliers, showing low similarity ($\leq$ 60%) with the other clusters. *Listeria monocytogenes*

strains were thought to be persistent when the same PFGE profile occurred in samples collected from the same environmental niche of the same plant after an interval of months or years. Numerical analysis of the PFGE results did not showed the presence of such strains among the subset included in this study.

4 - Study of the phenotypic and the genetic mechanisms of the Listeria monocytognes persistence.

Quantitative assessment of in vitro biofilm formation

After 40 h of incubation, 44% of the strains were able to attach to abiotic surfaces forming biofilm (table 20). Among these, the 41.2% showed a weak production, while 3.09% a moderate production. The 19.6% of strains able to produce biofilms belonged to serotype 1/2a, the 12.3% to 1/2b, and the remaining strains to serotypes 1/2c (9.28%) and 4b (3.09%).

The microtiter plate assay confirmed its utility as an indirect way of assessing the ability of *Listeria monocytogenes* strains to attach to abiotic surfaces, enabling researchers to rapidly analyze adhesion of multiple bacterial strains within each experiment (Stepanovic et al.. 2004).

Differential gene expression by persistent and non persistent strains of Listeria monocytogenes.

Primer design

The first designed primers were made not on the gene sequence, but rather on the protein sequence (degenerate primers), in order to have a better chance of alignment in case of gene mutations. After having tested them, the primers did not show the correct amplified band on agarose gel, and several nonspecific bands were seen. Primers were then re-designed on the gene sequence.

The primers were optimised at different temperatures and different concentrations of magnesium, until the expected band was obtained on the agarose gel. The primer sequences used are shown in Table 21. The genes amplified by PCR end-points were then checked by agarose gel electrophoresis, to ensure the reaction occurred and the accuracy of the size of the amplicon obtained.

Total RNA extraction

Total RNA was isolated from each sample using the RNeasy Midi Kit (Qiagen), and following the guidelines of the Kit protocol. The RNA concentration was measured by absorbance at 260nm, while purity was monitored by inspection of the 260/280 nm and 230/260 nm ratios, to assess protein and organic substances contamination, respectively, using the Nanodrop instrument. Data are shown in Tables 22, 23 and 24 for the different strains of *Listeria monocytogenes*, in relation to the growth conditions. As demostrated by the low ratios of 260/280 nm and 230/260 nm, the quality of the RNA was good in all cases. The quality and the integrity of the total RNA templates were also assessed by a 1.5% denaturing formaldehyde agarose gel electrophoresis. The integrity of the nucleic acid can be estimated by comparative observation of the ribosomal bands 28 S and 18 S. In a good preparation, the upper band (28 S) should be twice as reflective of the lower band (18 S); the 5 S band (small RNA) should be little evident and there should be no effects of *smear*. Representative data are shown in Figure 3.

Real time PCR

The optimization of the Real Time PCR assay included determination of amplification specificity, appropriate primer concentration and amplification conditions. Single product bands in agarose gels and single peaks in melting curve analysis confirmed the amplification specificity of the selected primer set. Amplification with Real Time PCR of the 4 target genes are shown in figures 4, 5, 6 and 7.

Relative quantification

Analysis of expression profiles of target genes (*pocR*, *mdr*, *eutG* and *cbiD*) in *Listeria monocytogenes* strains was conducted (in duplicate) by amplifying cDNA of all samples for each target gene and the HK gene. The relative quantification values were then calculated using the 2-ΔΔCT method, by expressing the results as fold change, as normalized on the average of the selected group as a control, to which it is given a value of 1. The results for the persistent and non-persistent strains are shown in Table 25 (*pocR*), 26 (*mdr*) and 27 (*eutG*). Results of *cbiD* gene are not shown because it was not possible to use a persistent phenotypic marker as a reference.

The relative quantification was optimized first on the two strains LM 6179 and LM 272, respectively known as persistent and non-persistent, compared to the expression of a housekeeping gene. For this work, the *rpoB* gene was chosen, as a reference gene, taken from the work of T. Tauraa and Roger S. (2007). The primers designed on this gene were chosen because they had characteristics (annealing temperature and magnesium concentrations), which best corresponds to primers used in this work. The Relative Quantification by RT-PCR was done for all 4 genes selected and the strains isolated

from a meat processing environment. To obtain a confirmation of the data obtained the Relative Quantification of ten strains of *Listeria monocytogenes*, 5 known to be persistent and 5 known as non-persistent, was also done. For the 10 strains of known phenotype the CT values were evaluated, but the predicted phenotype based on Relative Quantification did not correspond with the observed phenotype, because the genes studied were not always over expressed in strains persistent and down-regulated in non-persistent. In fact, the Relative Quantification is based on the assumption that the housekeeping genes are always equally expressed in different strains. This is not always true, because even if the expression is normalized there can always be mistakes. For this reason the Absolute Quantification that allows knowing more accurately the number of copies of the gene expressed was calculated.

Cloning of pocR and MDR in vector pCRII-TOPO (Invitrogen)

The presence of the correct insert in the clones, grown in medium containing ampicillin, was determined by extraction of the plasmid DNA and subsequent amplification of the gene of interest (data shown in Figures 8 and 9). The correct sequence was verified by sequencing the plasmid by an external laboratory (Source BioScience, Dublin, Ireland).

Absolute quantification

Standard curves were constructed for the two genes studied, using serial dilutions, at known concentrations, of the plasmid DNA, obtained from the competent cells after cloning the PCR product of interest in plasmid vectors. Dilutions were set up by a factor of 10. Each dilution, for a total of 10, was amplified in triplicate, to have greater reproducibility. The standard curve was constructed plotting the ordered values of Ct (or rather, the average Ct for each triplicate) and in abscissa the logarithm base 10 of dilutions: it was possible to determine the reaction efficiency considering the slope of the line. Standard curves for both gene *pocR* and *MDR* are shown in Figures 10 and 11. The absolute quantification for expression of the target genes was determined in the presence and absence of BZT for the set of known persistent and non-persistent strains (Table 28 and Figure 12 and 13). The results show no consistent high or low expression of either gene by persistent and non-persistent strains in the presence or absence of BZT. Regarding the gene *pocR*, first tested in strains grown in absence of BZT, this was over expressed in all strains but the degree of overexpression varied between all strains. In the presence of BZT, only 3 Persistent strains showed an increase in gene expression

of that gene (compared to the degree of overexpression in the absence of BZT), and the same increase was also observed for the strain LM CZ70, considered Non Persistent.

With regard to *MDR* gene expression, in strains grown in absence of BZT, within the persistent strains, only 2 (LM 6179 and LM 4423) showed a higher gene expression, for non-persistent strains two of them (LM N22-2 and LM 2566) had a high gene expression too. In the presence of BZT, within the persistent strains, only LM 6179 shown an increase in gene expression, while, within non-persistent strains, LM N22-2 and LM 2566 shown an increase in expression of that gene.

Therefore neither of these genes can be used as a marker to indicate persistence.

CONCLUSIONS

The present survey provides a contribution to the knowledge of prevalence, enumeration, virulence potential and traceability of *Listeria monocytogenes* in Sardinian fermented sausages and their production facilities. Furthermore, our study gives an interesting contribution for understanding the genetic mechanisms of persistence of the pathogen in the processing plant environments.

Our results underlined the overall presence of *Listeria monocytogenes* in environmental niches, ground meat, semi-finished and finished products. *Listeria monocytogenes* was isolated from 31.51% of food samples, where the main contaminated matrix was the *GM* (36.3%). In agreement with Thevenot et al. (2004), an unhomogeneous decrease of the pathogen prevalence was observed in *RS* (29.1%) highlighting the presence of *Listeria monocytogenes* strains able to survive during sausages fermentation, overcoming the current hurdles of the Sardinian sausages manufacturing process. All the positive food samples presented a level of contamination <100 CFU/g (EC Reg.2073/2005). All the processing plants except one (C) presented *Listeria monocytogenes* contamination, with different prevalences, varying from 4.16% (D) to 41.66% (A).

Listeria monocytogenes was isolated from 15% of the environmental samples, most of which were floor drains. Contamination of the plants appears to be due to strains already present in the processing environments (LOPEZ et al., 2005), with all the facilities serving as source of meat contamination (CHASSEIGNAUX et al., 2002). Environmental niches as the floor drains can be a site critical to the control of contamination of the processing plant environment and food products (TOMPKIN, 2002). Decontaminating floor drains is challenging because especially when entrapped in a biofilm, *Listeria monocytogenes* is afforded unusual protection against available disinfectants and treatments (ZHAO et al., 2004). *Listeria monocytogenes* strains can become well established in the floor drains and persist as resident microbial flora for up to several years.

The *Listeria monocytogenes* serotype 1/2a was prevalent (41%), followed by 1/2c (31%), 1/2b (20%), and 4b (8%). Previous studies have reported the presence of these in meat processing environments (Garrido et al. 2009; Thevenot et al., 2004). The serotype distribution within the plants was various and unhomogeneous. As described by other

authors in similar surveys (Thevenot et al. 2005), no specific serotypes were recovered from food and environmental samples, or in relation to the plant.

The main serotypes associated with human cases of listeriosis are 4b, 1/2a, and 1/2b. Then the recovery of these serotypes frequently associated with epidemic or sporadic cases of listeriosis (Farber and Peterkin, 1991, Recourt and Cossart, 1997; Kiss et al, 2006), is an important result in terms of importance for public health.

In particular the presence of serotype 4b is important for the food industry, and could be a source of listeriosis, as reported by other authors (Thevenot et al, 2004). Rocourt and Bille (1997) showed that most of cases of listeriosis associated with the consumption of meat products were caused by strains of serotype 4b. It must be emphasized that the presence of such strains in the processing plants and as a consequence in fermented sausages, may represent a hazard if the pathogen is able to multiply during the ripening of the product and reach levels higher than 1000 ufc/g (ROSS et al., 2002; THEVENOT et al., 2006).

The molecular findings revealed the presence of all the considered virulence genes, suggesting that all the isolates could be potentially dangerous for public health.

Nevertheless, from a risk analysis perspective, it is important to remark that 10-20% of strains isolated from the food industry are weakly or non-pathogenic both *in vivo* and *in vitro* tests (Tabouret et al*.,* 1991; Roche *et al.,* 2009). In this study low prevalence of the *inlA* (68%) was observed. This could be due to the presence of virulence - attenuating mutations (PMSC) in *inlA*, as recent studies reported in relation to *Listeria monocytogenes* strains isolated from RTE foods (Nightingale et al. 2008; Van Stelten et al. 2010; Ward et Al. 2010). Polymorphism had been reported for inlA and inlB (Ericsson et al., 1995, 2000) gene sequences. However, we did not observe any polymorphism in the PCR products of these genes, probably because it is not demonstrable with the protocol used in our study.

PFGE results showed a great level of diversity among the isolates collected from the Sardinian processing plants and their products. As previously said, the high number of PFGE profiles, and their heterogeneous distribution within the plants, agreed with the results of previous surveys carried out in meat processing plants (Thevenot et al., 2006). These results may be positively linked to the amount and the variability of geographical

origin of raw meat processed by the plants (Thevenot, 2006). Furthermore, this multiple contamination could be due to the ineffective cleaning-disinfecting procedures.

Some subtypes appeared to be unique for a processing plant and should be considered as plant-specific (Fugett et al., 2007).

Listeria monocytogenes strains were thought to be persistent when the same PFGE profile occurred in samples collected from the same environmental niche of the same plant after an interval of months or years (THEVENOT et al., 2006). Our results did not show the presence of such strains among the selection included in this study. These results were partially confirmed by those concerning the *in vitro* biofilm production: strains able to produce biofilm were mostly weak producers (41.2%) and only n. 3 were considered as moderate producers.

Regarding the investigation of expression of the genes probably interested in persistence of *Listeria monocytogenes*, the main purpose of this part of the study was to clarify the genetic mechanisms related to the persistence of this pathogen in the environment, and to attempt to identify marker genes for persistence. According to the studies of Fox et

al. (2011), strains known as Persistent and Non-Persistent have been studied for the expression of *pocR* and *MDR* genes, grown both in the presence and absence of BZT.

This hypothesis was not supported by the results of the Absolute quantification, suggesting that other genes are involved in the persistence of the strains in the processing environments. The complex mechanism of the persistence of *Listeria monocytogenes* is not due probably, to a single gene but a group of genes. Further studies will be useful on other genes in order to better assess how the genes we studied are involved in this mechanism and if other genes are involved in it. Although a clear role of the genes included in our study in the complex mechanism of the persistence of *Listeria monocytogenes* in the meat processing plants was not shown, however the results of the PFGE and the evaluation of *in vitro* biofilm production confirmed that the *Listeria monocytogenes* strains included in the study did not showed phenotypic and genotypic features correlated with a persistent contamination of the processing plant environments.

To decrease the presence of *Listeria monocytogenes* in the traditional fermented sausages at the end of ripening, food business operators should adhere to accurate

application of the hurdles technology. Although fermented pork meat RTE products as dry and semi-dry sausages have been rarely implicated in food poisoning, more risks should be linked to the consumption of these products: mainly in the manufacturing of traditional products, an empirical application of the hurdles technology often occurs. Products can also become contaminated in contact with work surfaces and equipment even after routine cleaning and disinfecting operations (THEVENOT et al., 2006): more attention should be focused on the respect of the good manufacturing practices and the application of the HACCP principles.

TABLES AND FIGURES

Table n.1 – Mean values of pH in relation to the type of samples tested: ground meat (GM), sausages at the end of acidification (AS) and sausages at the end of ripening (RS). (mean ± sd)

	GM	***AS***	***RS***
n.	55	55	55
pH	5.81±0.18	5.58±0.33	5.38±0.29
aW	0.97 ± 0.01	0.95 ± 0.01	0.90 ± 0.03

Table n.2 – Prevalence of *Listeria spp* and *Listeria monocytogenes* in food samples: ground meat (*GM*), sausages at the end of acidification (*AS*) and sausages at the end of ripening (*RS*).

	GM (n.55)		***AS (n.55)***		***RS (n.55)***		***total(n.165)***	
	n°*	%	n°*	%	n°*	%	n°*	%
Listeria spp.	35	63.6	41	74.5	28	50.9	104	63.03
L.monocytogenes	20	36.3	16	29.1	16	29.1	52	31.51

*= positive samples

Table n.3 – Prevalence of *Listeria spp* and *Listeria monocytogenes* in environmental samples: surfaces without contact (Swcm) and in contact with meat (Scm)

	Scm (n.132)		***Swcm (n.88)***		***Total (n.220)***	
	n°*	%	n°*	%	n°*	%
Listeria spp	31	23.5	18	20.5	49	22.27
L.monocytogenes	23	17.42	10	11.4	33	15

*= positive samples

Table n.4 – Prevalence of *Listeria spp* and *Listeria* monocytogenes in the facility A

Samples	***n°***	***Listeria spp*** % (n.)	***L. monocytogenes*** % (n.)
GM	5	60 (3)	40 (2)
AS	5	100 (5)	60 (3)
RS	5	100 (5)	20 (1)
Scm	12	58.3 (2)	41.66 (5)
Swcm	8	12.5 (1)	-

Table n.5 – Prevalence (%) of *Listeria spp* and List*eria monocytogenes* in samples from facility B, in relation to the batch: ground meat (GM), sausages at the end of acidification (AS), sausages at the end of ripening (RS), Surface in contact (Scm) and without contact (Swcm) with meat. Between bracket are reported the number of positive samples when different of 100%.

samples	*n°*	*Listeria spp*			*L. monocytogenes*		
		B 1	*B 2*	*Tot*	*B 1*	*B 2*	*Tot*
GM	10	100	100	100	-	80	40 (4)
AS	10	100	100	100	20	40	30 (3)
RS	10	40	60	50 (5)	20	60	40 (4)
Scm	24	41.6	33.3	37.5 (9)	25	25	25 (6)
Swcm	16	12.5	12.5	12.5 (2)	-	12.5	6.25 (1)

Table n.6 – Prevalence (%) of *Listeria spp* and *Listeria monocytogenes* in samples from facility C, in relation to the batch: ground meat (GM), sausages at the end of acidification (AS), sausages at the end of ripening (RS), Surface in contact (Scm) and without contact (Swcm) with meat. Between bracket are reported the number of positive samples when different of 100%.

Samples	*n°*	*Listeria spp*			*L. monocytogenes*		
		B 1	*B 2*	*Tot*	*B 1*	*B 2*	*Tot*
GM	10	80	-	40 (4)	-	-	-
AS	10	100	-	50 (5)	-	-	-
RS	10	60	-	30 (3)	-	-	-
Scm	24	8.33	-	4.16 (1)	-	-	-
Swcm	16	-	-	-	-	-	-

Table n.7 – Prevalence (%) of *Listeria spp* and *Listeria monocytogenes* in samples from facility D, in relation to the batch: ground meat (GM), sausages at the end of acidification (AS), sausages at the end of ripening (RS), Surface in contact (Scm) and without contact (Swcm) with meat. Between bracket are reported the number of positive samples when different of 100%.

samples	*n°*	*Listeria spp*			*L. monocytogenes*		
		B 1	*B 2*	*Tot*	*B 1*	*B 2*	*Tot*
GM	10	-	80	40 (4)	-	40	20 (2)
AS	10	80	-	40 (4)	-	-	-
RS	10	40	80	60 (6)	-	80	40 (4)
Scm	24	-	16.66	8.33 (2)	-	12.5	4.16 (1)
Swcm	16	25	25	25 (4)	-	-	-

Table n.8 – Prevalence (%) of *Listeria spp* and *Listeria monocytogenes* in samples from facility E, in relation to the batch: ground meat (GM), sausages at the end of acidification (AS), sausages at the end of ripening (RS), Surface in contact (Scm) and without contact (Swcm) with meat. Between bracket are reported the number of positive samples when different of 100%.

samples	*n°*	*Listeria spp*			*L. monocytogenes*		
		B 1	*B 2*	*Tot*	*B 1*	*B 2*	*Tot*
GM	10	40	40	40 (4)	40	40	40 (4)
AS	10	60	100	80 (8)	60	80	70 (7)
RS	10	20	80	50 (5)	20	60	40 (4)
Scm	24	41.66	25	37.5 (9)	50	25	37.5 (9)
Swcm	16	25	25	25 (4)	12.5	25	18.75 (3)

Table n.9 – Prevalence (%) of *Listeria spp* and *Listeria monocytogenes* in samples from facility F, in relation to the batch: ground meat (GM), sausages at the end of acidification (AS), sausages at the end of ripening (RS), Surface in contact (Scm) and without contact (Swcm) with meat. Between bracket are reported the number of positive samples when different of 100%.

samples	*n°*	*Listeria spp*			*L. monocytogenes*		
		B 1	*B 2*	*Tot*	*B 1*	*B 2*	*Tot*
GM	10	100	100	100	60	100	80 (8)
AS	10	80	100	90 (9)	20	40	30 (3)
RS	10	80	-	40 (4)	60	-	30 (3)
Scm	24	16.66	16.66	16.66 (4)	8.33	8.33	8.33 (2)
Swcm	16	25	62.5	43.75 (7)	12.5	62.5	37.5 (6)

Table n.10– Prevalence of *Listeria monocytogenes* serotypes (n.97 strains)

Serotype	Environmental samples (n.43)		products (n.54)		Total (n.97)	
	n°	%	n°	%	n°	%
1/2a	19	44	21	39	40	41
1/2b	9	21	10	19	19	20
1/2c	13	30	17	31	30	31
4b	2	5	6	11	8	8

Table n.11–*Listeria monocytogenes* serotypes distribution per plants in food samples.

Serotype	***Tot***	***Plant A***		***Plant B***		***Plant C***		***Plant D***		***Plant E***		***Plant F***	
		10/11	***04/07***	***10/11***	***04/07***	***10/11***	***04/07***	***10/11***	***04/07***	***10/11***	***04/07***	***10/11***	***04/07***
1/2a	21	-	-	4	6	-	-	1	1	6	1	1	1
1/2b	12	3	3	-	-	-	-	-	-	-	6	-	-
1/2c	15	3	-	4	-	-	-	1	-	1	-	6	-
4b	6	-	-	-	-	-	-	-	1	5	-	-	-

Table n.12–*Listeria monocytogenes* serotypes distribution per plants in environmental samples.

Serotype	*Tot*	*Plant A*		*Plant B*		*Plant C*		*Plant D*		*Plant E*		*Plant F*	
		10/11	*04/07*	*10/11*	*04/07*	*10/11*	*04/07*	*10/11*	*04/07*	*10/11*	*04/07*	*10/11*	*04/07*
1/2a	19	-	-	3	3	-	-	-	-	12	-	1	-
1/2b	9	1	8	-	-	-	-	-	-	-	-	-	-
1/2c	13	4	-	3	-	-	-	1	4	-	-	1	-
4b	2	-	-	-	-	-	-	-	2	-	-	-	-

Table n.13 – Distribution of *Listeria monocytogenes* serotypes isolated in 2011, in food and environmental samples per plants (A→ F) and sampling day (I →II).

	Plant A				***Plant B***								***Plant D***								***Plant E***								***Plant F***							
	I				***I***				***II***				***I***				***II***				***I***				***II***				***I***				***II***			
origin	*1/2a*	*1/2b*	*1/2c*	*4b*	*1/2a*	*1/2b*	*1/2c*	*4b*	*1/2a*	*1/2b*	*1/2c*	*4b*	*1/2a*	*1/2b*	*1/2c*	*4b*	*1/2a*	*1/2b*	*1/2c*	*4b*	*1/2a*	*1/2b*	*1/2c*	*4b*	*1/2a*	*1/2b*	*1/2c*	*4b*	*1/2a*	*1/2b*	*1/2c*	*4b*	*1/2a*	*1/2b*	*1/2c*	*4b*
GM		**1**							**2**		**2**						**1**		**1**		**2**						**2**				**3**						
AS		**2**	**2**		**1**						**2**										**2**		**1**		**1**		**3**				**1**						
RS			**1**		**1**																**1**								**1**		**2**					
SCM		**1**	**3**		**2**		**1**		**1**		**2**								**1**		**6**				**3**						**1**					
SWCM			**1**																		**1**				**2**				**1**							

Table n. 14– prevalence (%) of virulence factors among *Listeria monocytogenes* strains analyzed.

Strains (%)	VIRULENCE FACTORS										
	1st PCR Multiplex					2nd PCR Multiplex			3rd PCR Multiplex		
	actA (268pb)	actA (385bp)	hlyA (702)	rrn (938 bp)	prfA (1060 bp)	Iap (131bp)	inlB (146bp)	inlA (255bp)	plcA (129bp)	plcB (261pb)	Mpl (502bp)
	26.80	61.85	79.38	97.94	99	100	96.9	68	62.9	100	90

Table n. 15 – Distribution of pulsotypes, with APA I and ASC I, across sampling day (B1, B2), year of isolation and sources (*GM*, *AS*, *RS*, *SCM* and *SWCM*) in plant A

PLANT	BATCH AND YEAR	ORIGIN	APA I	ASC I
PLANT A	B1 2011	GM	P4	P23
		AS	P5, P10, P20	P11,P23,P25
		RS	P5	P23
		SCM	P4,P5,P20	P23,P26
		SWCM		
	2004/2007	GM	P2**	P9**
		AS		
		RS	P2**	P9**
		SCM		
		SWCM	P13**,P18**,P19**	P31**,P35**

* = Strains isolated in 2004

**= Strains isolated in 2005

***= Strains isolated in 2007

Table n. 16 – Distribution of pulsotypes, with APA I and ASC I, across sampling day (B1, B2), year of isolation and sources (*GM*, *AS*, *RS*, *SCM* and *SWCM*) in plant B

PLANT	BATCH AND YEAR	ORIGIN	APA I	ASC I
PLANT B	B1 2011	GM		
		AS	P26	P21
		RS	P23	P9,P15
		SCM	P15,P23,P24	P12,P13,P19
		SWCM		
	B2 2011	GM	P15,P25	P16,P18
		AS		
		RS		
		SCM	P15,P16	P18,P20
		SWCM		
	2004/2007	GM		
		AS		
		RS	P7**,P22**	P36**
		SCM	P12**	P32**
		SWCM	P34**	P34**

* = Strains isolated in 2004

**= Strains isolated in 2005

***= Strains isolated in 2007

Table n. 17 – Distribution of pulsotypes, with APA I and ASC I, across sampling day (B1, B2), year of isolation and sources (*GM*, *AS*, *RS*, *SCM* and *SWCM*) in plant D

PLANT	BATCH AND YEAR	ORIGIN	APA I	ASC I
PLANT D	B1 2011	GM	P6,P11	P14,P24
		AS		
		RS		
		SCM	P6	P22
		SWCM		
	B2 2011	GM		
		AS		
		RS		
		SCM		
		SWCM		
	2004/2007	GM	P12**	P33**
		AS		
		RS	P29*	P38*
		SCM		
		SWCM	P21**	P37**

* = Strains isolated in 2004

**= Strains isolated in 2005

***= Strains isolated in 2007

Table n. 18 – Distribution of pulsotypes, with APA I and ASC I, across sampling day (B1, B2), year of isolation and sources (*GM*, *AS*, *RS*, *SCM* and *SWCM*) in plant E

PLANT	BATCH AND YEAR	ORIGIN	APA I	ASC I
PLANT E	B1 2011	GM	P8	P1,P10
		AS	P8	P2,P10
		RS	P8	P2
		SCM	P8	P1,P5,P10
		SWCM	P8	P5
	B2 2011	GM	P9	P6,P7
		AS	P1,P14	P17
		RS		
		SCM	P17	P2,P3,P4
		SWCM	P28	P3
	2004/2007	GM		
		AS		
		RS	P3*	P40*
		SCM		
		SWCM		

* = Strains isolated in 2004

**= Strains isolated in 2005

***= Strains isolated in 2007

Table n. 19 – Distribution of pulsotypes, with APA I and ASC I, across sampling day (B1, B2), year of isolation and sources (*GM*, *AS*, *RS*, *SCM* and *SWCM*) in plant F

PLANT	BATCH AND YEAR	ORIGIN	APA I	ASC I
PLANT F	B1 2011	GM	P30,P31,P33	P27,P29
		AS		
		RS	P33	P28
		SCM	P30	P30
		SWCM	P27	P8
	2004/2007	GM		
		AS		
		RS	P32*	P39*
		SCM		
		SWCM		

* = Strains isolated in 2004

**= Strains isolated in 2005

***= Strains isolated in 2007

Table n. 20 – Biofilm production in 97 Listeria monocytogenes strains tested

Origin	*Biofilm production (no. of positive out of 97 strains)*							
	NP		**WP**		**MP**		**SP**	
	10/11	**04/07**	**10/11**	**04/07**	**10/11**	**04/07**	**10/11**	**04/07**
GM	**7**	**-**	**7**	**2**	**-**	**-**	**-**	**-**
AS	**12**	**-**	**3**	**4**	**-**	**1**	**-**	**-**
RS	**4**	**1**	**2**	**11**	**-**	**-**	**-**	**-**
Scm	**16**	**4**	**5**	**1**	**-**	**-**	**-**	**-**
Swcm	**4**	**6**	**1**	**4**	**-**	**2**	**-**	**-**

Table n. 21 – Details of primers and amplicons for each of the four evaluated genes for *Listeria monocytogenes*

Primers	**Sequence**	**Size (bp)**	**Tm**
pocR_F	TTTCTTCATGATGCGCTACTTTTG	**322**	54°C
pocR_R	CCACGCCGGATTAACTGATTTT		
eutG_F	CCGCGAGTCTGTAGAAGTTGTGA	**313**	54°C
eutG _R	CATATGCGTTCCTCCTGTTGGTTC		
cbiD_F	AAATCCCCGTGGCGTATCAAAA	**315**	54°C
cbiD_R	CGCGCTCTTCTCCTTCTGGTG		
MDR_F	GTGGGGGAAATTATTGGCTCTT	**315**	53°C
MDR_R	ACTTGCTTTATGATCAGGTTCTC		

Table n. 22 –Total RNA isolated from persistent and non-persistent strains of *Listeria monocytogenes* grown at 14°C for 48 h. Analysis was done in independent triplicate.

Strain	RNA concentration (ng/µl)	Ratio 260:280 nm	Ratio 260:230 nm
Persistent strains			
LM 6179	37	1.96	2.02
LM 6179	38.1	2.11	2.20
LM 6179	60.09	1.98	1.97
LM 4423	712.7	2.12	2.42
LM 4423	158.2	1.97	2.31
LM 4423	152.1	2.05	2.33
LM R479A	353.2	2.00	2.59
LM R479A	733.6	2.09	2.43
LM R479A	304.5	2.09	2.29
LM CZ48	90	2.00	1.90
LM CZ48	96.5	1.92	2.00
LM CZ48	150.3	1.98	1.86
LM 4898	332.5	1.89	2.18
LM 4898	523.1	2.10	2.34
LM 4898	441.3	1.99	2.42
Non-persistent strains			
LM 2566	50.09	1.98	2.13
LM 2566	41.1	1.89	1.98
LM 2566	50.6	1.98	2.02
LM N22-2	319.6	2.05	2.40
LM N22-2	266.2	2.04	2.31
LM N22-2	216.4	2.06	2.31
LM CZ70	234.3	2.04	2.31
LM CZ70	300.1	2.01	2.38
LM CZ70	331	1.98	2.21
LM 251	806.3	2.02	2.39
LM 251	779.5	2.10	2.39
LM 251	823.9	2.09	2.42
LM 272	135	1.91	2.00
LM 272	130.5	2.04	2.20
LM 272	172.6	2.01	2.24

Table n. 23 –Total RNA isolated from persistent and non-persistent strains of *Listeria monocytogenes* grown at 14°C for 72 h in the presence of benzylthonium chloride (BZT).
Analysis was done in independent triplicate.

Strain	BZT(ppm) concentration	RNA concentration (ng/μl)	Ratio 260:280 nm	Ratio 260:230 nm
Persistent strains				
LM 6179	4	83.6	2.00	1.97
LM 6179	4	97.9	2.08	2.23
LM 6179	4	65.4	2.13	2.09
LM 4423	0.5	283.1	2.08	2.4
LM 4423	0.5	590.9	2.10	2.42
LM 4423	0.5	251.7	1.92	1.98
LM R479A	0.5	297.7	1.97	2.2
LM R479A	0.5	160.7	1.94	2.29
LM R479A	0.5	403	1.86	1.93
LM CZ48	4	2533	2.05	2.42
LM CZ48	4	944.3	2.17	2.44
LM CZ48	4	1809.9	2.13	2.43
LM 4898	0.5	366	1.88	1.91
LM 4898	0.5	269.4	1.89	1.97
LM 4898	0.5	331.3	1.92	1.97
Non-persistent strains				
LM 2566	4	48.8	1.98	1.98
LM 2566	4	90.9	2.18	2.16
LM 2566	4	96.1	2.19	2.25
LM N22-2	4	68.8	2.21	2.13
LM N22-2	4	122	2.19	2.33
LM N22-2	4	66.8	2.18	2.15
LM CZ70	0.5	378.3	1.91	2.01
LM CZ70	0.5	652.7	2.14	2.41
LM CZ70	0.5	1119.7	2.12	2.44
LM 251	0.5	1339.9	2.11	2.48
LM 251	0.5	1573.5	2.06	2.27
LM 251	0.5	1315.5	2.11	2.40
LM 272	0.5	125.9	1.91	1.98
LM 272	0.5	88.3	2.02	2.36
LM 272	0.5	143.2	1.85	2.04

Table n. 24 –Total RNA isolated from strains of *Listeria monocytogenes* of unknown phenotype, grown at 14°C for 48 h.

Strain	RNA concentration (ng/μl)	Ratio 260:280 nm	Ratio 260:230 nm
LC1003	61.4	1.97	2.04
LC1007	76.3	2.21	2.20
LC1011	253.3	2.05	1.95
LISA 3	207.8	2.08	2.14
LISA 4	342.3	1.94	2.20
LISA 13	334	1.96	2.08
LISA 15	286.9	1.98	2.02
LISA 17	1135.4	2.08	2.40
LISA 24	329.9	1.92	2.21
LISA 25	257.6	2.04	2.04
LISA 26	263.4	1.91	2.07
LISA 27	132.2	2.05	1.96
LISA 29	155.1	1.98	2.03
LISA 39	53.5	2.08	2.22
LISA 40	193.7	1.89	2.03
LISA 42	53	2.02	2.09
LISA 46	101.1	1.85	1.98
A2I	157.4	2.05	2.42
A5I	127.4	2.64	2.11
A8	223.1	2.00	2.20
E1	224.5	1.89	1.99
E7	124.5	2.02	2.20
F2S	641.3	1.89	2.88
F3S	99.7	1.87	2.28
F5	105.5	1.89	2.13
F17	194	1.96	2.67
G1T	266.7	1.81	1.94
L3I	45.6	2.03	1.98
L3T	379.9	1.87	1.98
L4	268.2	1.84	1.94
L9	237.5	1.84	1.81
M2I	54	1.85	2.05
M4T	112	1.86	2.20
M13	164.9	1.94	2.34

Table n. 25 –Relative expression of *pocR* gene in both Persistent and Non-Persistent strains grown at 14 C° for 42h in absence of BZT. The results are shown as the number of fold of Differential Expression (DE) of strain 272 used as a reference of non-persistent strain.

Relative quantification analysis using the $2^{-\Delta\Delta CT}$ method											Normalised Expression Relative to 272	(2-fold DE) Apparent Phenotype	Environmental Phenotype
STRAIN	**C_T pocR**	**Mean C_T**	**SD on C_T**	**CV on C_T**	**C_T HKG**	**Mean C_T**	**SD on C_T**	**CV on C_T**	**ΔCT**	**ΔΔCT**			
LM 6179	20,34	20,33	0,02	0,10	22,29	22,29	0,00	0,00	-1,97	-1,97	3,92	**Persister**	**Persister**
	20,31				22,29								
LM 4423	19,32	19,30	0,03	0,15	17,52	17,34	0,25	1,47	1,96	1,96	0,26	**Non-Persister**	**Persister**
	19,28				17,16								
LM R479A	18,74	18,74	0,01	0,04	18,61	18,64	0,04	0,23	0,09	0,09	0,94	**Non-Persister**	**Persister**
	18,73				18,67								
LM CZ48	21,19	21,16	0,05	0,23	21,17	21,16	0,01	0,07	0,00	-0,01	1,01	**Non-Persister**	**Persister**
	21,12				21,15								
LM 4898	19,76	19,77	0,01	0,04	19,73	19,58	0,22	1,12	0,19	0,18	0,88	**Non-Persister**	**Persister**
	19,77				19,42								
LM 2566	17,49	17,50	0,01	0,08	17,86	17,83	0,05	0,28	-0,32	-0,33	1,26	**Non-Persister**	**Non-Persister**
	17,51				17,79								
LM N22-2	21,19	21,22	0,04	0,17	20,63	20,66	0,04	0,21	0,56	0,55	0,68	**Non-Persister**	**Non-Persister**
	21,24				20,69								
LM CZ70	22,56	22,54	0,03	0,13	22,63	22,57	0,09	0,41	-0,02	-0,03	1,02	**Non-Persister**	**Non-Persister**
	22,52				22,5								
LM 251	27,3	27,31	0,01	0,05	20,28	20,27	0,01	0,07	7,04	7,04	0,01	**Non-Persister**	**Non-Persister**
	27,32				20,26								
LM 272	16,48	16,48	0,01	0,04	16,52	16,47	0,07	0,43	0,01	0,00	1,00	**Non-Persister**	**Non-Persister**
	16,47				16,42								

Table n. 26 – Relative expression of *MDR* gene in both Persistent and Non-Persistent strains grown at 14 C° for 42h in absence of BZT The results are shown as the number of fold of Differential Expression (DE) of strain 272 used as a reference of non-persistent strain.

Relative quantification analysis using the $2^{-\Delta\Delta CT}$ method											Normalised Expression Relative to 272	(100-fold DE) Apparent Phenotype	Environmental Phenotype
STRAIN	**C_T mdr**	**Mean C_T**	**SD on C_T**	**CV on C_T**	**C_T HKG**	**Mean C_T**	**SD on C_T**	**CV on C_T**	**ΔCT**	**ΔΔCT**			
LM 6179	19,74	19,51	0,33	1,70	22,87	22,45	0,59	2,65	-2,95	-15,91	61572,56	**Persister**	**Persister**
	19,27				22,03								
LM 4423	17,27	17,30	0,04	0,20	17,18	17,21	0,04	0,25	0,09	-12,88	7538,18	**Persister**	**Persister**
	17,32				17,24								
LM R479A	30,05	30,03	0,04	0,12	18,56	18,66	0,13	0,72	11,37	-1,60	3,02	**Non-Persister**	**Persister**
	30				18,75								
LM CZ48	32,13	32,10	0,05	0,15	21,74	21,59	0,22	1,02	10,51	-2,46	5,48	**Non-Persister**	**Persister**
	32,06				21,43								
LM 4898	31,4	31,37	0,04	0,14	18,52	18,58	0,08	0,46	12,79	-0,18	1,13	**Non-Persister**	**Persister**
	31,34				18,64								
LM 2566	20,56	20,57	0,01	0,03	21,98	22,00	0,02	0,10	-1,43	-14,40	21544,02	**Persister**	**Non-Persister**
	20,57				22,01								
LM N22-2	19,26	19,26	0,00	0,00	19,14	19,09	0,07	0,37	0,17	-12,80	7106,88	**Persister**	**Non-Persister**
	19,26				19,04								
LM CZ70	30,02	30,05	0,04	0,14	16,42	16,28	0,21	1,26	13,78	0,81	0,57	**Non-Persister**	**Non-Persister**
	30,08				16,13								
LM 251	27,82	28,37	0,77	2,72	19,88	19,93	0,07	0,35	8,44	-4,53	23,10	**Non Persister**	**Non-Persister**
	28,91				19,98								
LM 272	29,32	29,38	0,08	0,26	16,5	16,41	0,13	0,78	12,97	0,00	1,00	**Non-Persister**	**Non-Persister**
	29,43				16,32								

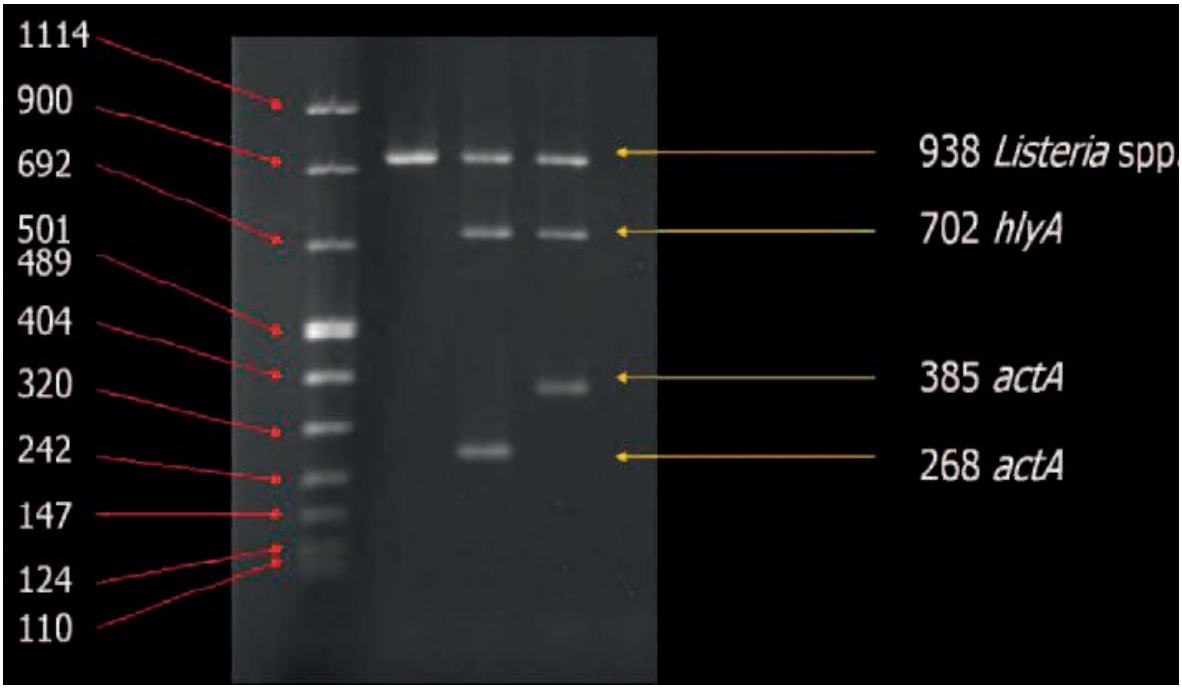
Table n. 27 – Relative expression of *eutG* gene in both Persistent and Non-Persistent strains grown at 14 C° for 42h in absence of BZT. The results are shown as the number of fold of Differential Expression (DE) of strain 272 used as a reference of non-persistent strain.

Relative quantification analysis using the $2^{-\Delta\Delta CT}$ method											Normalised Expression Relative to 272	(5-fold DE) Apparent Phenotype	Environmental Phenotype
STRAIN	**C_T mdr**	**Mean C_T**	**SD on C_T**	**CV on C_T**	**C_T HKG**	**Mean C_T**	**SD on C_T**	**CV on C_T**	**ΔCT**	**ΔΔCT**			
LM 6179	18,58	18,14	0,63	3,47	22,31	22,32	0,01	0,03	-4,18	-4,45	21,86	**Persister**	**Persister**
	17,69				22,32								
LM 4423	19,71	19,64	0,11	0,54	19,9	19,83	0,10	0,50	-0,20	-0,46	1,38	**Non-Persister**	**Persister**
	19,56				19,76								
LM R479A	19,05	19,19	0,19	1,00	18,98	18,99	0,01	0,07	0,20	-0,07	1,05	**Non-Persister**	**Persister**
	19,32				19								
LM CZ48	16,48	16,51	0,04	0,21	16,5	16,41	0,13	0,78	0,10	-0,17	1,13	**Non-Persister**	**Persister**
	16,53				16,32								
LM 4898	19,17	19,20	0,04	0,22	19,16	19,21	0,06	0,33	0,00	-0,27	1,21	**Non-Persister**	**Persister**
	19,23				19,25								
LM 2566	20,34	20,40	0,08	0,42	21,9	21,84	0,08	0,39	-1,44	-1,71	3,27	**Non-Persister**	**Non-Persister**
	20,46				21,78								
LM N22-2	19,14	19,12	0,03	0,15	18,9	18,90	0,00	0,00	0,22	-0,05	1,04	**Non-Persister**	**Non-Persister**
	19,1				18,9								
LM CZ70	20,6	20,61	0,01	0,03	21,43	22,00	0,80	3,63	-1,39	-1,66	3,16	**Non-Persister**	**Non-Persister**
	20,61				22,56								
LM 251	17,82	17,83	0,01	0,04	18,01	18,04	0,04	0,20	-0,21	-0,48	1,39	**Non-Persister**	**Non-Persister**
	17,83				18,06								
LM 272	21,62	21,58	0,06	0,26	21,19	21,31	0,17	0,80	0,27	0,00	1,00	**Non-Persister**	**Non-Persister**
	21,54				21,43								

Table n. 28 – Expression of *pocR* and *MDR* in Persistent and in Non-Persistent strains, grown either in presence and absence of BZT, in terms of Number of Copies.

Strains	Expression of pocR in absence of BZT Number of copies	Standard Deviation	Expression of pocR in presence of BZT Number of copies	Standard Deviation	Expression of MDR in absence of BZT Number of copies	Standard Deviation	Expression of MDR in presence of BZT Number of copies	Standard Deviation
Persistent strains								
LM 6179	98420	93066	198267	272828	147834	13155	1058678	98993
LM 4423	172426	141232	38930	26783	273975	243220	46920	31347
LM CZ48	972134	1407581	323646	1846	18	6	180	1
LM R479A	84971	32146	134552	72967	192	112	6	2
LM 4898	39783	11041	59486	3591	164	63	1	1
Non-persistent strains								
LM N22-22	28946	18589	8543	5471	44692	23170	600738	33753
LM 272	374244	14075	79691	26522	46	39	116	5
LM 251	1354	702	500	138	114	52	8	8
LM CZ70	100953	3842	59547	60580	30	11	2	3
LM 2566	74124	2538	31400	2288	83235	109	367701	209414

Figure n. 1– Multiplex PCR showing the polymorphism of *actA* gene. The lanes from left to right are: marker; *L. innocua*; *Listeria monocytogenes* (268 bp *actA*); *Listeria monocytogenes* (385 bp *actA*).



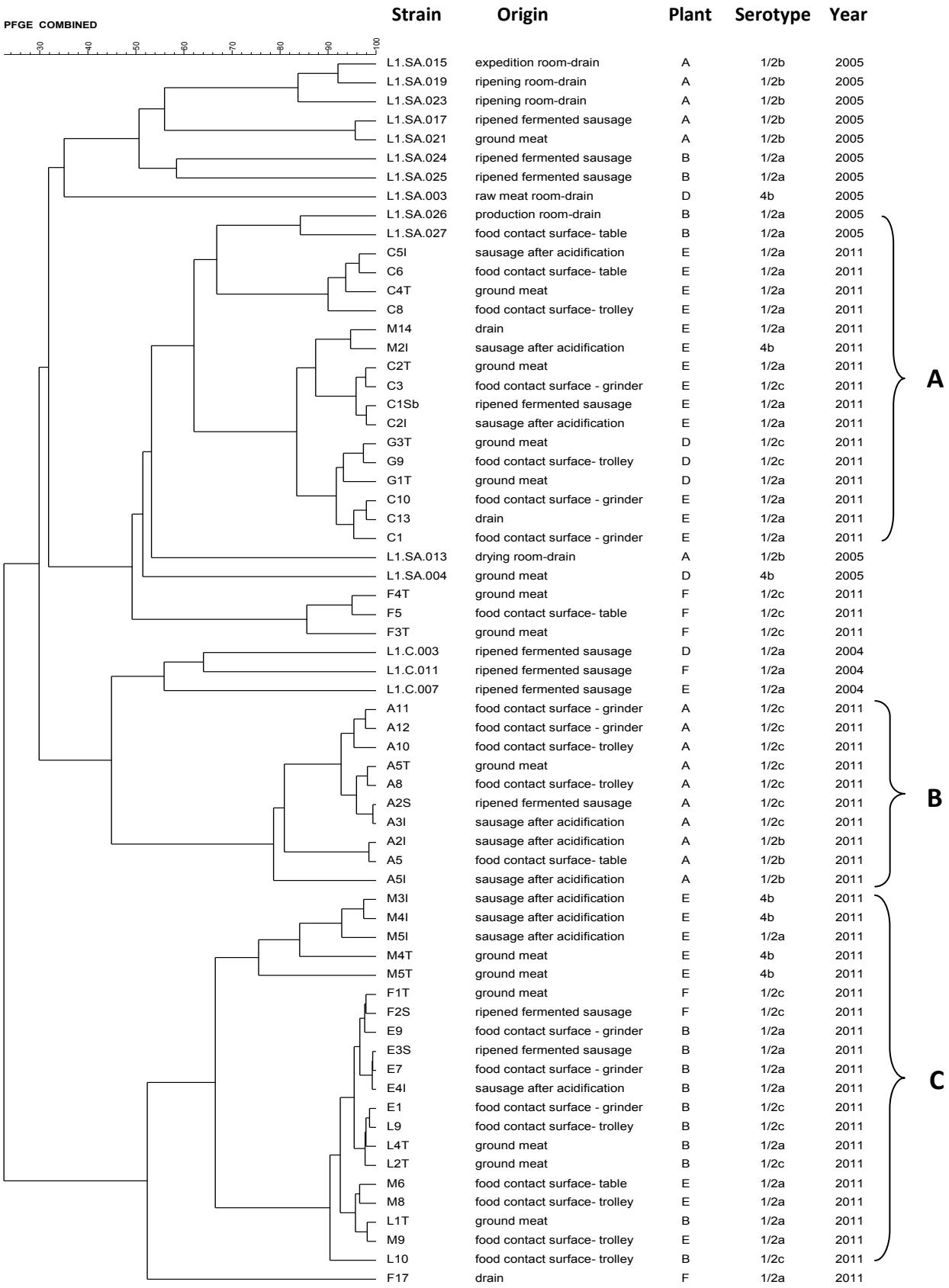


Figure n.2 – PFGE combined profiles of Listeria monocytogenes isolates, in relation to the origin, to the plant, to the serotype and to the year of isolation.

Figure n. 3 – Electrophoresis in denaturing formaldehyde agarose gel to check the integrity of the RNA. Four representative samples are shown. All other samples gave similar results. All the samples are intact, according this analysis, as observed by the distinct ribosomal RNA bands.

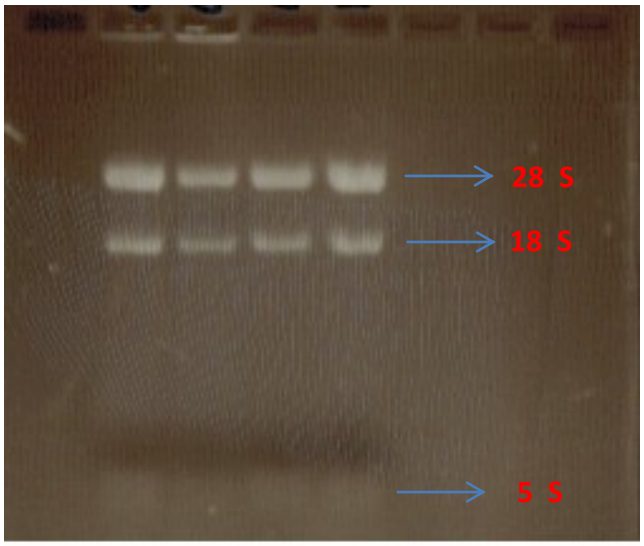


Figure n. 4 – Amplification of the gene pocR. The blue line represents the negative control. Seven representative samples are shown. All other samples gave similar results. As you can see the peaks of fluorescence starting from different numbers of cycles. This indicates that the initial number of copies of the gene is higher (low number of cycles) or less (high number of cycles)

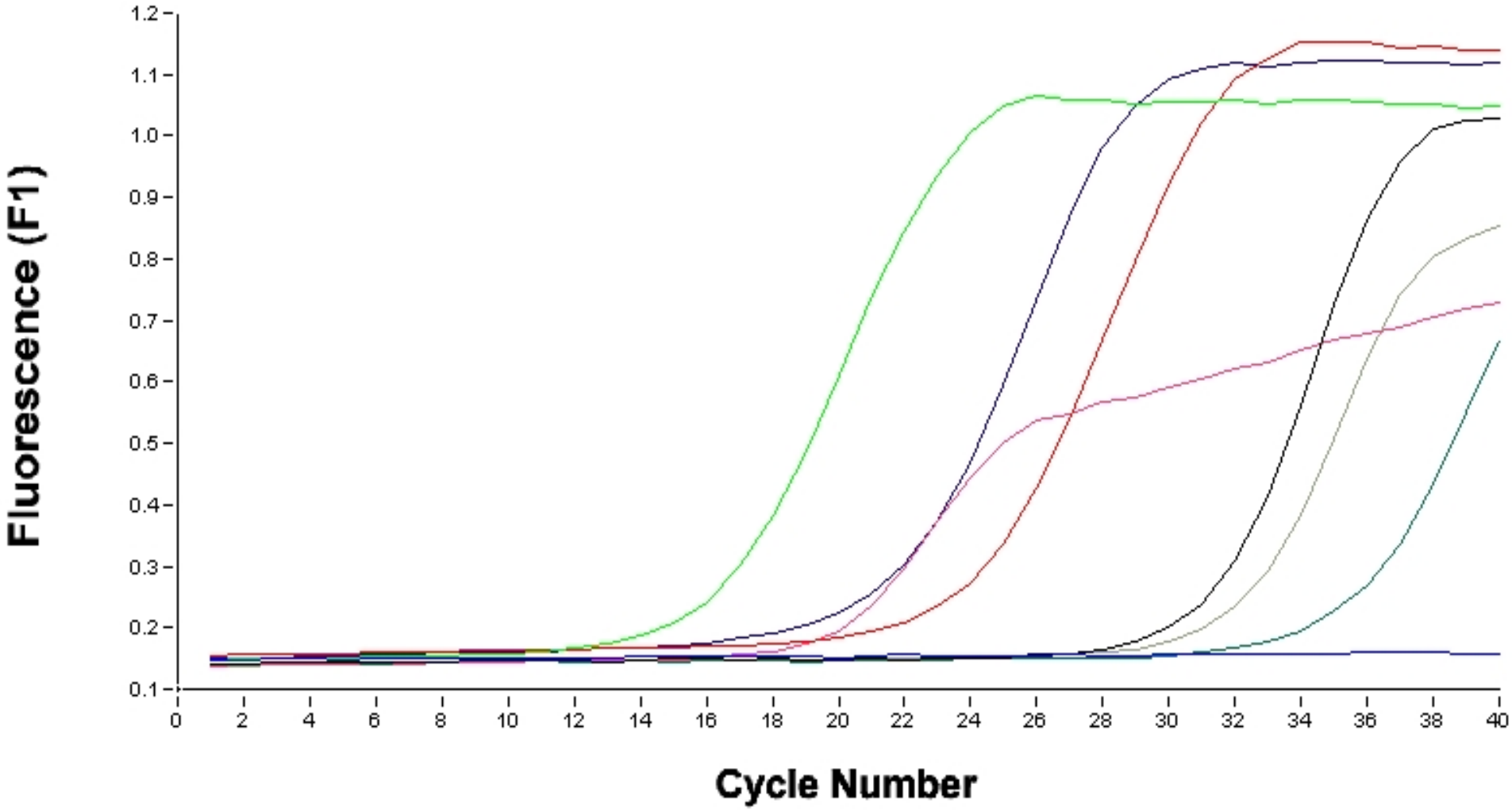


Figure n. 5 – Amplification of the gene MDR. The blue line represents the negative control. Four representative samples are shown. All other samples gave similar results. As you can see the peaks of fluorescence starting from different numbers of cycles. This indicates that the initial number of copies of the gene is higher (low number of cycles) or less (high number of cycles).

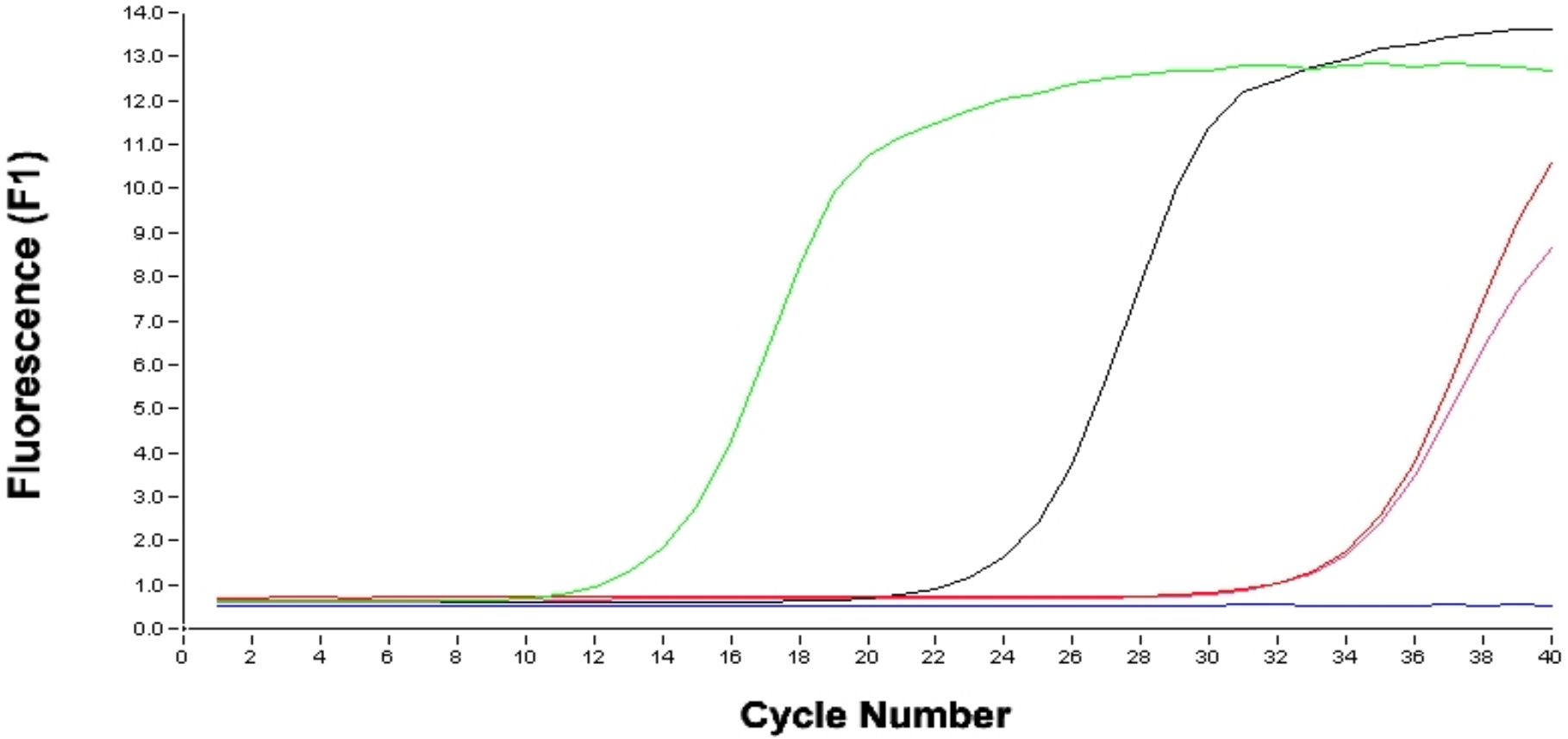


Figure n. 6 – Amplification of the gene eutG. The blue line represents the negative control. Four representative samples are shown. All other samples gave similar results. As you can see the peaks of fluorescence starting from different numbers of cycles. This indicates that the initial number of copies of the gene is higher (low number of cycles) or less (high number of cycles).

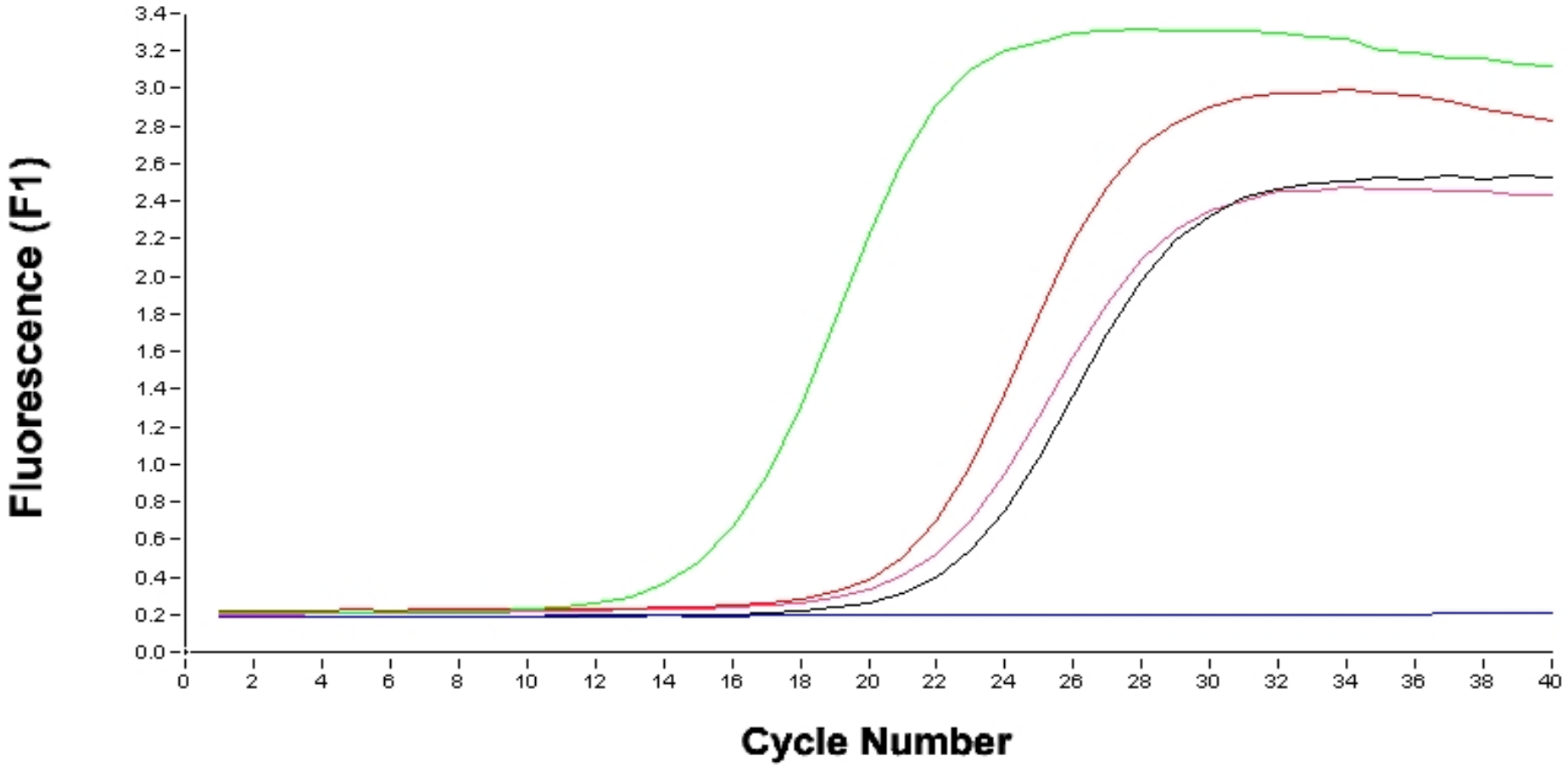


Figure n. 7 – Amplification of the gene cbiD. The blue line represents the negative control. Four representative samples are shown. All other samples gave similar results. As you can see the peaks of fluorescence starting from different numbers of cycles. This indicates that the initial number of copies of the gene is higher (low number of cycles) or less (high number of cycles).

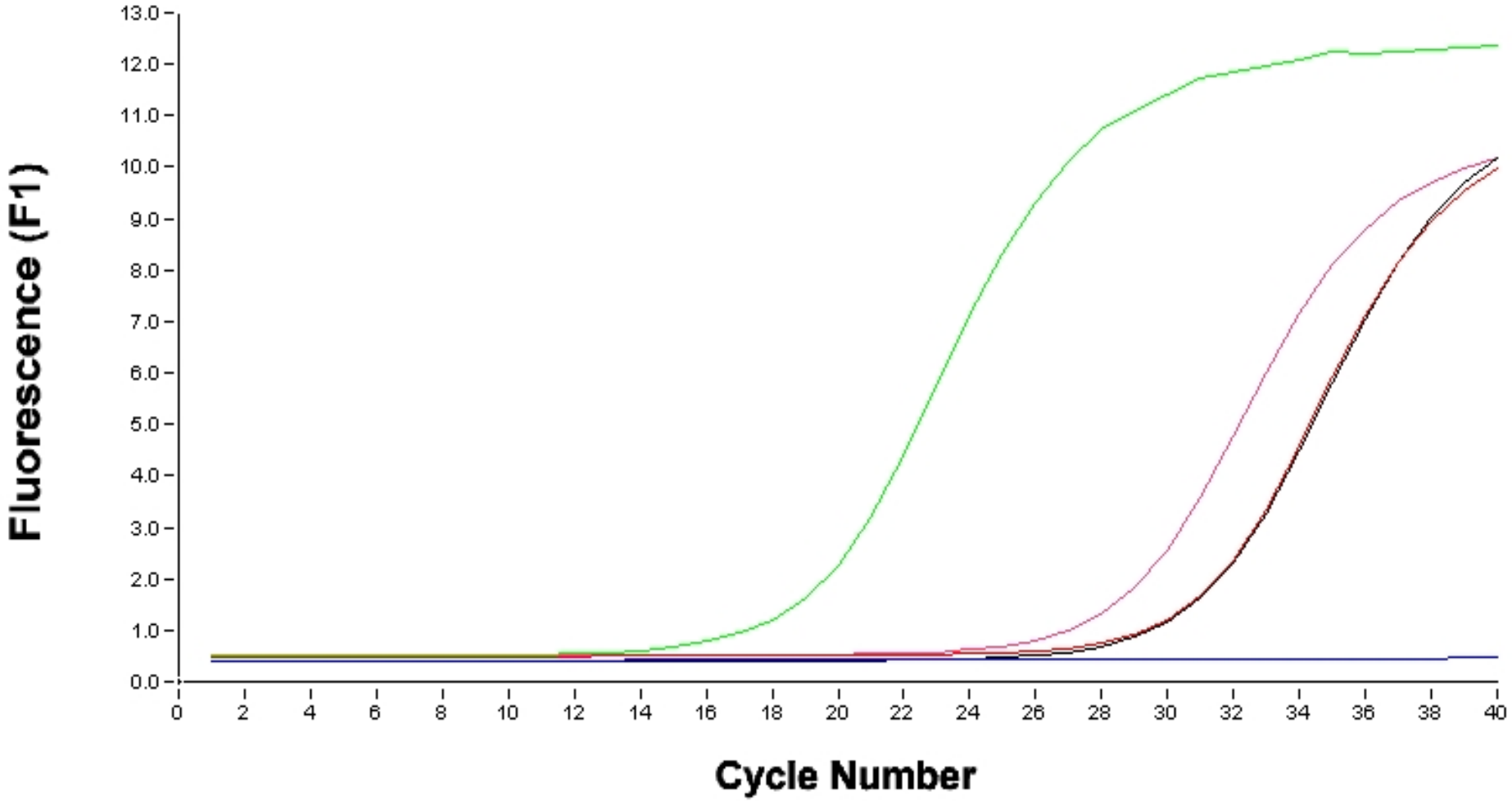
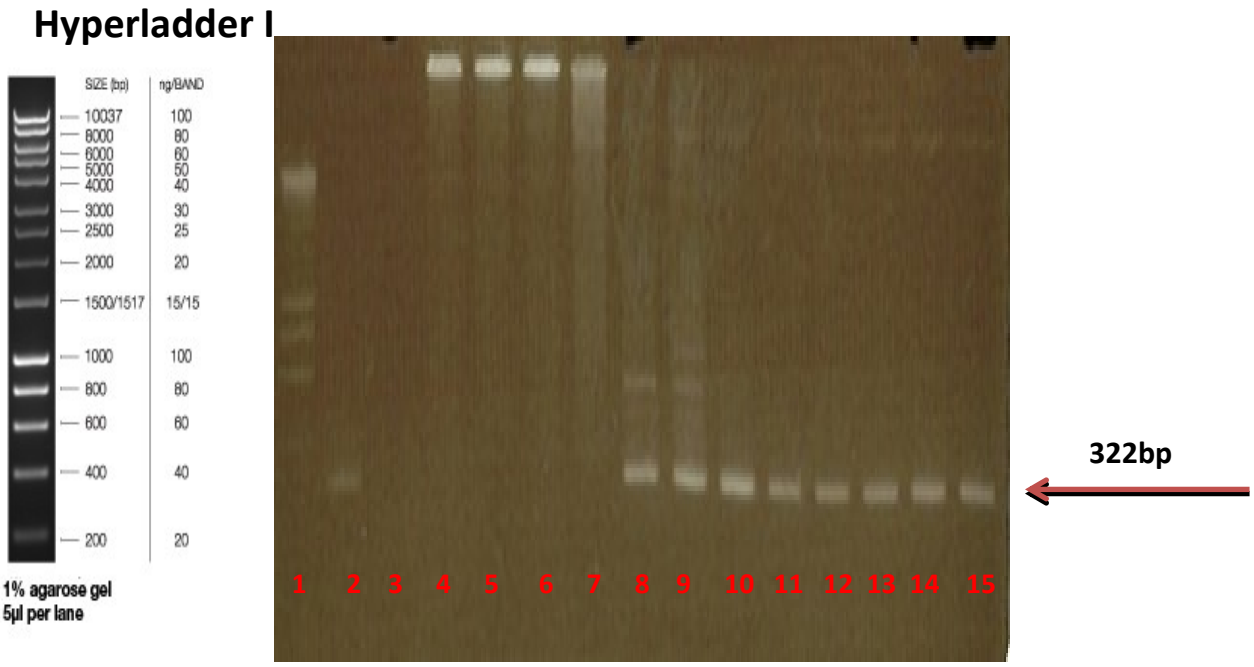
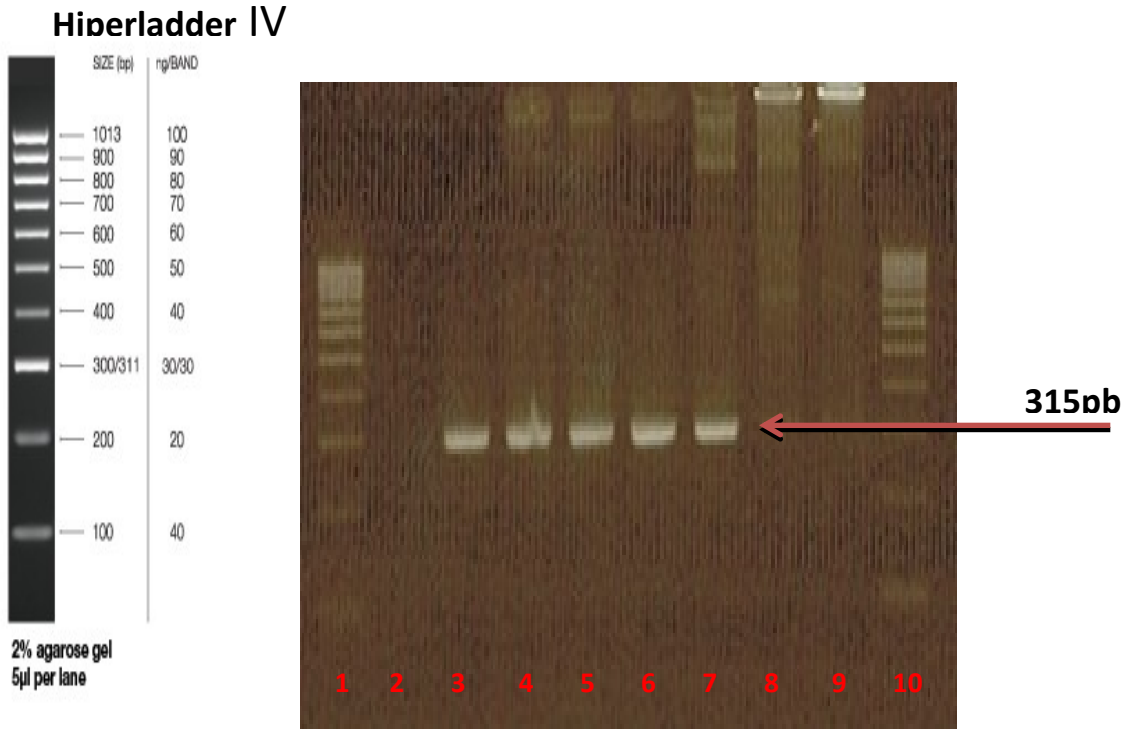


Figure n. 8 – Amplification product of pocR gene in plasmid DNA (322 bp)



Lane 1: Marker; lane 2: positive control; lane 3: negative control; lane 4- 15: different plasmid DNA samples.

Figure n. 9 – Amplification product of MDR in plasmid DNA (315 bp)



Lane 1: Marker; lane 2: negative control; lane 3- 15: different plasmid DNA samples.

Figure n. 10 – Standard curve for the target gene *pocR*. Triplicate analysis was undertaken and average values are shown with error bars as the standard deviation.

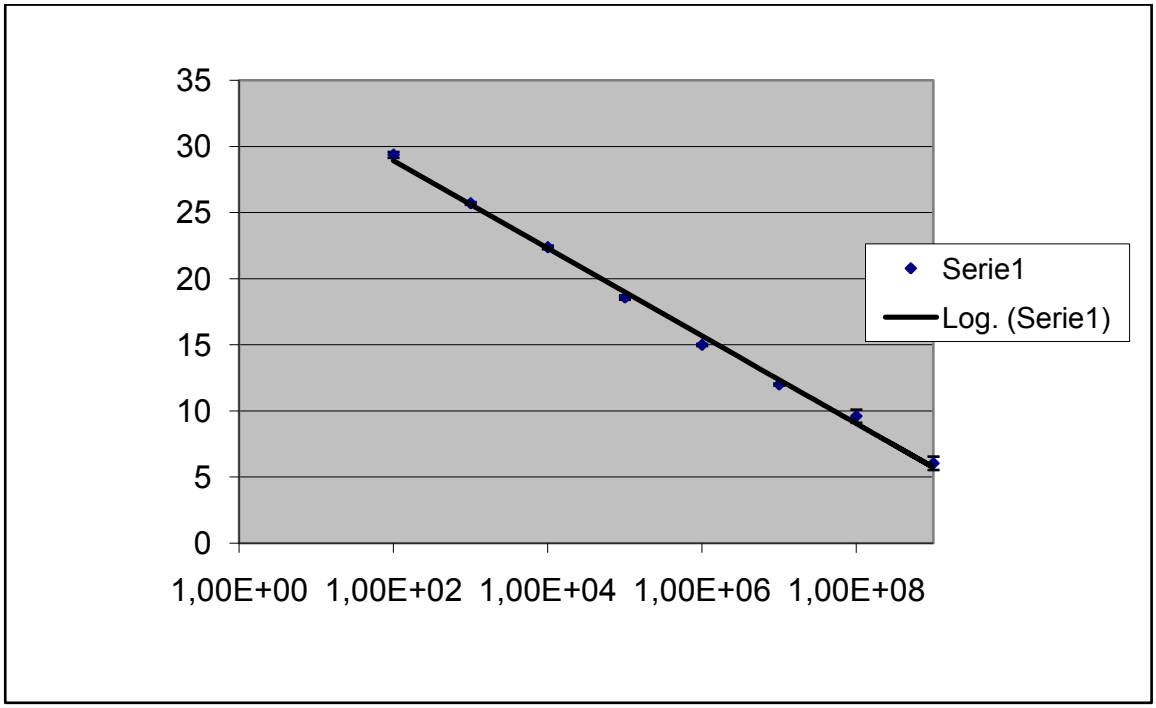


Figure n. 11 – Standard curve for the target gene *MDR.* Triplicate analysis was undertaken and average values are shown with error bars as the standard deviation.

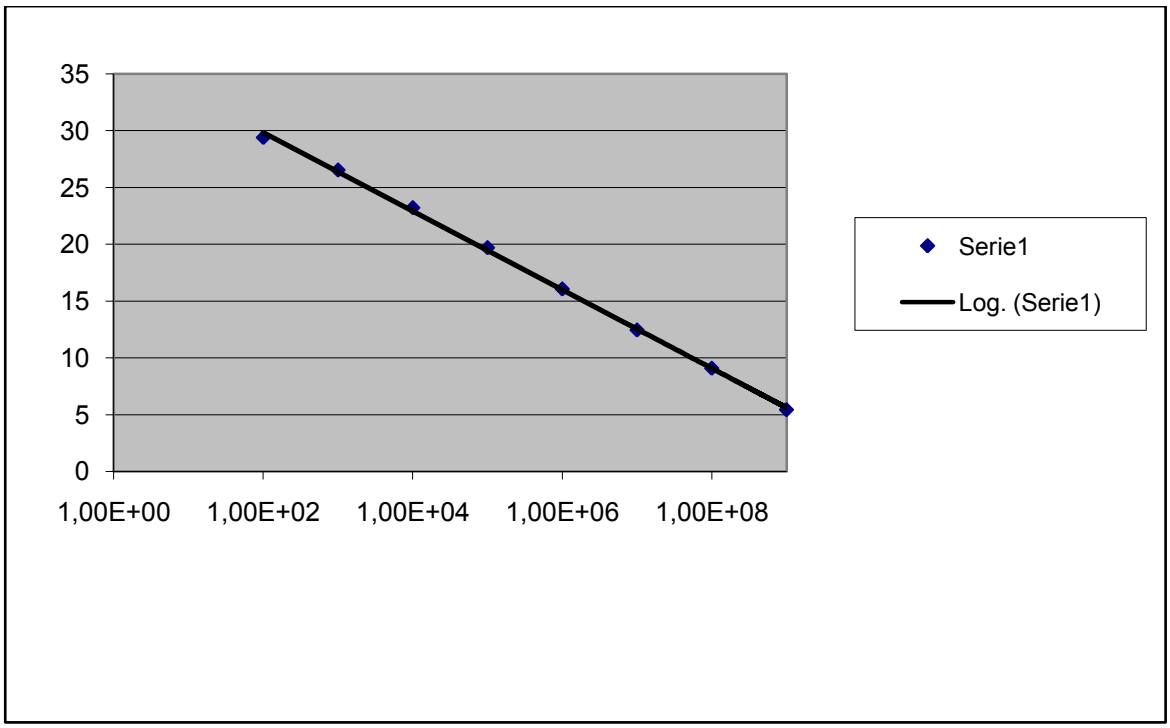


Figure n. 12 – Differences in expression of *pocR* gene in Persistent and Non-Persistent strains grown in presence and absence of BZT

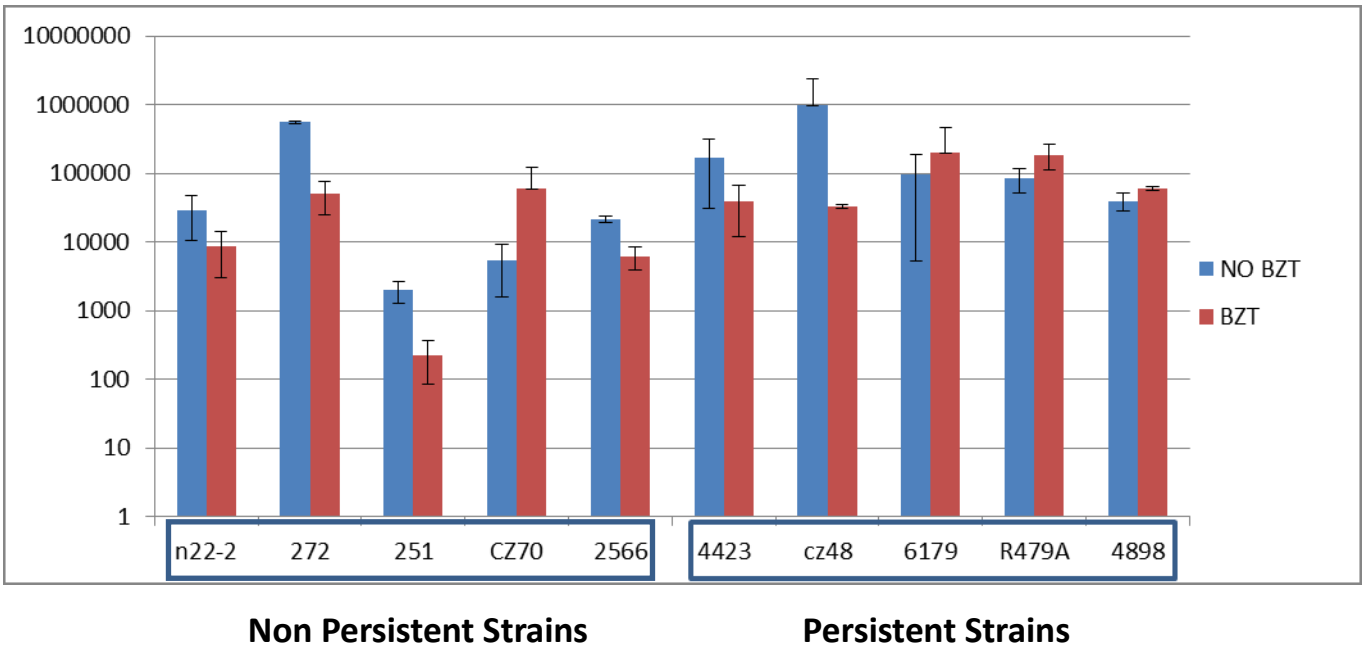
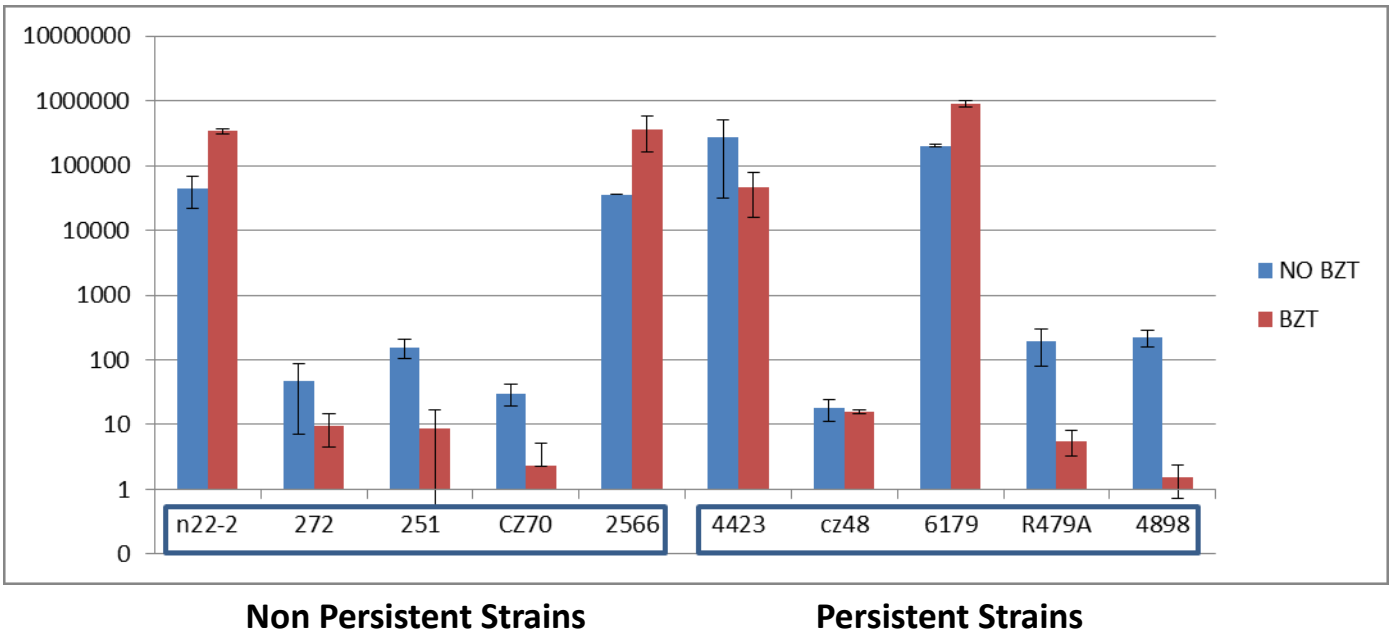


Figure n. 13 – Differences in expression of *MDR* gene in Persistence and in Non-Persistence strains grown in presence and absence of BZT



BIBLIOGRAPHY

1. ***Aarts. H.J.M., Hakemulder. L.E., Van Hoef. A.M.A.,(1999)*** "Genomic typing of *Listeria monocytogenes* strains by automated laser fluorescence analysis of amplified fragment length polymorphism fingerprint patterns". Int. J. Food Microbiol. 49. 95– 102.
2. ***Adesyiun A.A., Krishnan C. (1995)*** "Occurrence of Yersinia enterocolitica O:3. *Listeria monocytogenes* O:4. and thermophilic *Campylobacter spp*. in slaughter èpigs and carcasses in Trinidad". Food Microbiol., 12:99-107.
3. ***AFSSA (2000)*** « Rapport de la Commission D'e´tude des Risques lie`s a` *Listeria monocytogenes »*. Available at: http://www. afssa.fr
4. ***Allerberger F (2007)*** "*Listeria*. In: Simjee S (ed) Foodborne Diseases". ISBN 1-58829-046-8. Humana Press. Totowa. New Jersey. pp 27–3
5. ***Allerberger F. Wagner M. (2010)*** "Listeriosis: a resurgent foodborne infection". *Clin Microbiol Infect* ;**16**:16–23.
6. ***Al-Tawfiq JA. (2008)*** "*Listeria monocytogenes* bacteremia in a twin pregnancy with differential outcome: fetus papyraceus and a full-term delivery." J Microbiol Immunol Infect; 41: 433–436.
7. ***Anne Schuchat, Bala Swaminathan., and Claire V. Broom (1991).*** "Epidemiology of Human Listeriosi" Clinical Microbiology Reviews. Apr.. p. 169-183.
8. ***Aureli P., Fiorucci G.C., Caroli D., Marchiaro G., Novara O., Leone L., Salmaso S. (2000).*** "An outbreak of febrile gastroenteritis associated with corn contaminated by *Listeria monocytogenes*". New England J. Med.. 27. 1236-1241
9. ***Autio T., Timonen-Keto R., Lunden J., Bjorkroth J., Korkeala H. (2003)*** "Characterizzation of persistent and sporadie *Listeria monocytogenes* strains by pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP)" System. Appl. Mierobiol. 26. 539-545.

10. ***Balandytė L., Oevermann A., Brodard I., Frey J., Abril C. (2010)***. "MLVA typing of *Listeria monocytogenes* strains isolated from brain samples of sheep, goat and cattle, and comparison with strains of human, food and environmental origin".
11. ***Batt C. (1999)*** "*Listeria*" in "Encyclopedia of food microbiology" edited by: Robinson R., Batt C., Patel P.D. 1195-1251.vol. two. Academic press.
12. ***Bhagwat AA. (2003).*** "Simultaneous detection of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* strains by real-time PCR". Int J Food Microbiol 84:217–24
13. ***Bille. J. and Glauser. M-P. (1988)*** *Listeriose* - Situation in der Schweiz (Listeriosis in Switzerland). Bulletin des Bundesamtes fiir Gesundheitswesen/Bulletin de I'Office f6dCral de la sant6 publique. no. 3. 28-29.
14. ***Blackman I.C., Frank J. (1996)*** "Growth of *Listeria monocytogenes* as biofilm on various food processing surfaces". J. Food Prot. 59. 827-831.
15. ***Bobik. T. A., Y. Xu. R. M. Jeter. K. E. Otto, and J. R. Roth.(1997).*** Propanediol utilization genes (pdu) of *Salmonella typhimurium*: three genes for the propanediol dehydratase. J. Bacteriol. 179:6633-6639
16. ***Bobik. T.A., M.Ailion. and J.R.Roth. (1992).*** A single regulatory gene integrates control of vitamin B12 synthesis and propanediol degradation. J. Bacteriol. 174:2253-2266;
17. ***Booth. I. (1998)*** "The bacteria strike back". Biochemistria 5.8–11.
18. ***Borch. E., Nesbakken. T., Christensen. H. (1996)*** "Hazard identification in swine slaughter with respect to foodborne bacteria". Int. J. Food Microbiol. 30: 9–25.
19. ***Border P.M., Howard J.J., Plastow G.S,. Siggens K.W.; (1990)*** "Detection of *Listeria* species and *Listeria monocytogenes* using polymerase chain reaction" Letters in Applied Microbiology Volume 11. Issue 3. pages 158–162.
20. ***Botzler RG. Cowan AB. Wetzler TF (1974).*** "Survival of *Listeria monocytogenes* in soil and water". S. wildl Dis 10:204-212.
21. ***Buchanan. R. L, and L. A. Klawitter. (1990).*** "Effects of temperature and oxygen on the growth of *Listeria monocytogenes* at pH 4.5". J. Food Sci. 55:1754–1756.
22. ***Buncic. S. (1991).*** "The incidence of *Listeria monocytogenes* in slaughtered animals, in meat, and in meat products in Yugoslavia". Int. J. Food Microbiol. 12: 173–180.

23. ***Carpentier B., Chassaing D. (2004)*** "Interactions in biofilms between *Listeria monocytogenes* and resident microorganisms from food industry premises" International J. Of Food Microbiology 97 111-122.

24. ***Chakraborty T., Leimeister-Wachter M., Domann M., Hartl E., Goebel W., Niechterlein T., Notermans S. (1992)*** "Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the prfA gene" J.Bacteriol.174.568-574.

25. ***Chasseignaux E., Toquin M.T., Ragimbeau C., Salvat G., Colin P., Ermel G. (2001)*** "Molecular epidemiology of *Listeria monocytogenes* isolates collected from the environment, raw meat and raw products in two poultry and pork processing plants" Journal of Applied Microbiology. 91. 888-899.

26. ***Chasseignaux. E., Gérauld. P., Toquin. M.-T., Salvat. G., Colin. P., Ermel. G. (2002).*** "Ecology of *Listeria monocytogenes* in the environment of raw poultry meat and raw pork meat processing plants". FEMS Microbiol. Lett. 210: 271-275.

27. ***Chavant. P., Martinie. B., Meylheuc. T., Bellon-Fontaine. M.N., Hebraud. D. (2002)*** "*Listeria monocytogenes* LO28: Surface Physicochemical Properties and Ability To Form Biofilms at Different Temperatures and Growth Phases" Applied and Environmental Microbiology. 728-737. 68.2.

28. ***Chen. P. M. Ailion. T. Bobik. G. Stormo and J. Roth. (1995).*** "Five promoters integrate control of the cob/pdu regulon in *Salmonella typhimurium*.) J. Bacteriol. 177:5401-5410.

29. ***Colak H., Hampikyan H., Ulusoy B., Baris Bingol E. (2005)*** "Presence of *Listeria monocytogenes* in Turkish style fermented sausage (sucuk)" Food Control. 45. 78-96

30. ***Commission Regulation (EC) No 1441/2007*** of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs. Official Journal of the European Union 7.12.2007. L 322/12

31. ***Cone LA., Somero MS., Qureshi FJ, et al. (2008)*** "Unusual infections due to *Listeria monocytogenes* in the Southern California Desert." Int J Infect Dis; 12: 578–581

32. ***Conter M., Vergara A., Di Ciccio P., Zanardi E., Ghidini S., Ianieri A. (2009)."*** Polyporphism of actA gene is not related to in vitro virulence of Listeria monocytogenes". Int. J. Food Microbiol. 137:100.

33. ***Cordano A.M. Rocourt J. (2001)*** "Occurrence of *Listeria monocytogenes* in food in Chile". Int. J. Food Microbiol., 70. 1-2. 175-178.

34. ***Cossart P., Pizarro-Cerda J., Lecuit M., (2003)*** "Invasion of mammalian cells by *Listeria monocytogenes*: functional mimicry to subvert cellular functions". Trends Cell Biol. 13:23-31.

35. ***Cossart P., Portnoy D.A.,(2000)*** "The cell biology of invasion and intracellular growth by *Listeria monocytogenes* in: Gram-positive pathogens". American society for microbiology. Washington DC. 2000. pp.509-515.

36. ***Costerton. J.W., Lewandowski. Z., Caldwell. D.E., Korber. D.R. and Lappin-Scott. H.M. (1995)*** "Microbial biofilms". Annu Rev Microbiol 49. 711–745.

37. ***Cotter. P.D. and Hill. C. (2003)*** "Surviving the acid test: responses of gram positive bacteria to low pH". Microbiol Mol Biol Rev 67. 429–453.

38. ***Dabin. E. and Jussiaux. R. (1994)*** "Modifications des saucissons au cours de l'e´tuvage et du se`chage". In Le Saucisson Sec ed. Frentz. J.C. pp. 136–138. Paris: Erti.

39. ***de Boer. E., Beumer. R.R. (1999)*** "Methodology for detection and typing of foodborne microorganisms". Int. J. Food Microbiol. 50. 119– 130.

40. ***De Cesare A. Bruce JL. Dambaugh TR. Guerzoni ME. Wiedmann M. (2001)*** "Automated Ribotyping using different enzymes to improve discrimination of *Listeria monocytogenes* isolates with a particular focus on serotype 4b strains". Journal of Clinical Microbiology. 39:3002-3005.

41. ***De Valk H., Jacquet C., Goulet V., Vaillant V., Perra A., Simon F., (2005)*** "Surveillance of *Listeria* infections in Europe" Euro Surveill 2005;10(10):251-5. Available from: http://www.eurosurveillance.org/em/v10n10/1010-225.asp

42. ***Denny J. McLauchlin J. (2008)*** "Human *Listeria monocytogenes* infections in Europe - an opportunity for improved European surveillance". EuroSurveill. 2008;13(13):pii=8082. Available online: http://www.eurosurveillance.org. ViewArticle.aspx?ArticleId=8082

43. ***Disson O., Grayo S., Huillet E., Nikitas G., Langa-Vives F., Dussurget O., Ragon M., Le Monnier A., Babinet C., Cossart P., Lecuit M., (2008)*** "Conjugated action of two species specific invasion proteins for fetoplacental listeriosis". Nature; 455: 1114–1118.

44. ***Doumith M., Buchrieser C., Glaser P., Jacquet C., Martin P. (2004).*** "Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR". J. Clin. Microbiol.. 42 (8). 3819-3822.

45. ***Duffy. E.A., Belk. K.E., Sofos. J.N., Bellinger. G.R., Pape. A. and Smith. G.C. (2001)*** "Extent of microbial contamination in United States pork retail products". J Food Prot 64. 172– 178.

46. ***Dussurget O., (2008)*** "New insights into determinants of *Listeria monocytogenes* virulence". Int Rev. Cell Mol. Biol. 270:1-38.

47. ***Dussurget O., Pizarro-Cerda J., Cossart P., (2004)*** "Molecular determinants of *Listeria monocytogenes* virulence". Annu. Rev. Microbiol. 58:587-610.

48. ***Earnshaw AM. Lawrence LM. (1998).*** "Sensitivity to commercial disinfectants, and the occurrence of plasmids within various *Listeria monocytogenes* genotypes isolated from poultry products and the poultry processing environment". *J Appl Microbiol* **84**:642–8.

49. ***Edward M. Fox, Nola Leonard, Kieran Jordan(2011)*** **"**Physiological and Transcriptional Characterization of Persistent and Nonpersistent Listeria monocytogenes Isolates**".** Appl. Environ. Microbiol. September 2011 77:6559-6569

50. ***Ericsson, H., Stalhandske, P., Danielsson-Tham, M.L., Bannerman, E., Bille, J., Jacquet, C., Rocourt, J., Tham, W.,(1995).*** "Division of Listeria monocytogenes serovar 4b strains into two groups by PCR and restriction enzyme analysis". Appl. Environ. Microbiol. 61, 3872 – 3874.

51. ***Ericsson, H., Unnerstad, H., Mattsson, J.G., Danielsson-Tham, M.L., Tham, W., (2000).*** "Molecular grouping of Listeria monocytogenes based on the sequence of the inlB gene". J. Med. Microbiol. 49, 73 – 80.

52. ***European Food Safety Authority (EFSA) 2007***. The Community Summary Report on Trends and Sources of Zoonoses. Zoonotic Agents. Antimicrobial Resistance and Foodborne Outbreaks in the European Union in 2006. The EFSA Journal. 130: 3-352

53. ***European Food Safety Authority (EFSA) 2007***. Updated SCVPH opinion on *Listeria monocytogenes* risk related to ready-to-eat foods and scientific advice on different levels of *Listeria monocytogenes* in ready-to-eat foods and the related risk for human illness. The EFSA Journal 599: 1-42.

54. ***European Food Safety Authority. (EFSA) 2006.*** "The community summary report on trends and sources of zoonoses, zoonotic agents, antimicrobial resistance and foodborne oubreaks in the European Union in 2005". The EFSA Journal 130. 1–310.

55. ***European Food Safety Authority. (EFSA) 2005.*** The Community Summary Report on Trends and Sources of Zoonoses. Zoonotic Agents. Antimicrobial Resistance and Foodborne Outbreaks in the European Union in 2004. The EFSA Journal 310: 1-97.

56. ***Fabbi M., Andreoli G., De Giuli L., Carretto E. (2005)*** "*Listeria monocytogenes*" in "Trattato sulle tossinfezioni alimentari". pp.357-389, edited by Rondanelli E.G.. Fabbi M.. Marone P.. ed Selecta Medica.

57. ***Faleiro. M.L., Andrew. P.W. and Power. D. (2003)*** "Stress response of *Listeria monocytogenes* isolated from cheese and other foods". Int J Food Microbiol 84. 207–216.

58. ***Farber J.M., Peterkin P.I. (1991)*** "*Listeria monocytogenes*. a food-borne pathogen". Microbiol. Rev., 55. 476-511.

59. ***Farber J.M., Peterkin P.I. (1999)*** "Incidence and behavior of *Listeria monocytogenes* in meat products" p.505-564 in "*Listeria*. listeriosis and food safety". 2nd edition, edited by E.T. Ryser and E.H. Marth. Marcel Dekker. New York.

60. ***Fenlon D.R. (1999)*** "*Listeria monocytogenes* in the natural environment. In: Ryser ET. Marth EH (eds) Listeria.Listeriosis, and food safety. 2nd edn. Mrcel Decker. New York. pp 21-37.

61. ***Fenlon. D.R., Wilson. J. and Donachie. W. (1996)*** "The incidence and level of *Listeria monocytogenes* contamination of food sources at primary production and initial processing" J Appl Microbiol 81. 641–650.

62. ***Ferreira V., Barbosa J., Silva J., Felício M.T., Mena C., Hogg T., Gibbs P., Teixeira P. (2006)*** "Characterisation of alheiras. traditional sausages produced in the North of Portugal. with respect to their microbiological safety" Food Control. 56. 78-89

63. ***Fiore A. et al., (2004)*** "Valutazione del rischio di Listeriosi in gravidanza con particolare attenzione alla situazione in Piemonte". AIVEMP newsletter. 3. 10

64. ***Fleming. D.W., Cochi. S.L., McDonald. K.L., Brondum. J., Hayes. P.S., Plikaytis. B.D., Holmes. M.B., Audurier. A. et al. (1985)*** "Pasteurized milk as a vehicle of infection in an outbreak of listeriosis". New English J Med 3120. 404–407.

65. ***Fonnesbech Vogel. B., Huss. H. H., Ojeniyi. B., Ahrens. P., Gram. L. (2001)*** "Elucidation of *Listeria monocytogenes* contamination routes in cold-smoked salmon processing plants detected by DNA-based typing methods". Appl. Environ. Microbiol. 67: 2586–2595.

66. ***Fugett E.B., Schoonmaker-Bopp D., Dumas N.B., Corby J. and Wiedmann M.(2007).*** "Pulsed-Field Gel Electrophoresis (PFGE) analysis of temporally matched Listeria monocytogenes isolates from human clinical cases, foods, ruminant farms, and urban and natural environments reveals source-associated as well as widely distributed PFGE types". J. of Clin. Microbiol. 865:873

67. ***Gaillard J.L., Berche P., Mounier J., Richard S., Sansonetti P. (1987)*** " In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2". Infect. Immun. 55:2822-2829.

68. ***Gasanov U. Hughes D. Hansbro PM. (2005)*** "Methods for the isolation and identification of *Listeria spp*. and *Listeria monocytogenes*" FEMS Microbiology Reviews (29). 851-875.

69. ***Ghinelli I. (1985)*** "Le carni conservate: laboratori di carne" - vol.2. Piccin Ed.. Padova.

70. ***Gianfranceschi M., Gattuso A., Fiore A., D'Ottavio M.C., Casale M., Palombo A. Aureli P. (2006)*** "Survival of *Listeria monocytogenes* in Uncooked Italian Dry Sausage (Salami)" J. of Food Prot.. 69.7. 1533-1538.

71. ***Gianfranceschi M., Pourshaban M., Gattuso A., Wedell-Needegaard C., Aureli P.(2002)*** " Characterization of *Listeria monocytogenes* strains isolated from food and humans in Italy by pulsed-field gel electrophoresis" Food Microbiology. 2002.19.47-55.

72. ***Giovannacci. I., Ragimbeau. C., Queguiner. S., Salvat. G., Vendeuvre. J. L., Carlier. V. and Ermel. G. (1999)*** "Listeria monocytogenes in pork slaughtering and cutting plants. Use of RAPD. PFGE. and PCR-REA for tracing and molecular epidemiology". Int J Food Microbiol 53. 127–140.

73. ***Gobat. P.F., Jemmi. T. (1991).*** "Epidemiological studies on *Listeria spp*. in slaughterhouses". Fleischwirtsch. 1: 44–49.

74. ***Goebel W. Kreft J., Bockmann R. (2000)*** "Regulation of virulence genes in pathogenic *Listeria spp*." in: Gram-positive pathogens. American society for microbiology. Washington DC. 2000. pp.499-506.

75. ***Goulet. C., Rocourt. J., Rebiere. I., Jacquet. C., Moyse. C., Dehaumont. P., Salvat. G. and Veit. P. (1998)*** "Listeriosis outbreak associated with the consumption of rillettes in France in 1983. J Infect Dis 177. 155–160.

76. ***Graves L. M., Helsel L. O., Steigerwalt A. G., More. R. E., Daneshvar M. I., Roof S. E., Orsi R. H., Fortes E. D., Milillo S. R., den Bakker H. C., Wiedmann M., Swaminathan B., and Sauder. B. D. (2009).*** "*Listeria marthii* sp. nov., isolated from the natural environment". Finger Lakes National Forest. Int. J. Syst. Evol. Microbiol. doi:10.1099/ijs.0.014118-0

77. ***Graves LM. Swaminathan B. (2001)***"PulseNet standardized protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis". International Journal Food Microbiology; 65:55-62.

78. ***Gray Kristen M., Bhunia Arun K. (2005)*** "Specific detection of cytopathogenic *Listeria monocytogenes* using a two-step method of immunoseparation and cytotoxicity analysis" Journal of Microbiological Methods Volume 60. Issue 2. Pages 259-268.

79. ***Gray. M. L., and A. H. Killinger. (1966).*** "*Listeria monocytogenes* and listeric infections." Bacteriol. Rev. 30:309-382.

80. ***Gray. M.L. (1960)*** "Isolation of Listeria monocytogenesfrom oat silage". Science 132:1767:1768.

81. ***Greenwood. M., Roberts. H.D. and Burden. P. (1991)*** "The occurrence of *Listeria* species in milk and dairy products: a national survey in England and Wales" Int J Food Microbiol 12. 197–206.

82. ***Greub G. Raoult D. (2004)*** "Microorganisms resistant to free-living amoebae". Clin Microbiol Rev. 17:413-33.

83. ***Griffiths M.W. (2002)*** "*Listeria*, properties and occurrence" in Encyclopedia of food sciences and nutrition, edited by Caballero B. Trugo LC. Finglas PM. 3562-3581 2nd edition, vol.6, Academic press.

84. ***Hall. J.C., Deschamps. R.J.A., McDermott. M.R., (1989b).*** "Immunoassays for detecting pesticides in the environment". Highlights 12. 19–23.

85. ***Hayes. P.S., Graves. L.M., Ajello. G.W., Swaminathan. B., Weaver. R.E., Wenger. J.D., Schuchat. A. and Broome. C.V. (1991)*** "Comparison of cold-enrichment and U.S. Department

of Agriculture methods for isolating *Listeria spp*. from naturally contaminated foods". Appl Environ Microbiol 57. 2109–2113.

86. ***Hechelmann. H., Albert. T., Gareis. M., (2002).*** "*Listeria monocytogenes* in streichfähiger Rohwurst und Rohwurstbrät - Vorkommen und quantitative Nachweis". Fleischwirtschaft 82 (8). 92–94.
87. ***Hof. H. and Rocourt. J. (1992)*** "Is any strain isolated detected in food a health risk?" Int J Food Microbiol 16. 683–692.
88. ***Hof. H., and P. Hefner. (1988)*** "Pathogenicity of *Listeria monocytogenes* in comparison to other *Listeria spp*." infection 16 (suppl. 2):141-144.
89. ***Holah J.T., Taylor J.H., Dawson D.J. and Hal K.E. (2002)*** "Biocide use in the food industry and the disinfectant resistance of persistent strains of *Listeria monocytogenes* and *Escherichia coli*". Journal of Applied Microbiology Symposium Supplement. 92. 111S–120
90. ***Holah. J.T. (2000)*** "Cleaning and disinfection. In Chilled Foods: a Comprehensive Guide" ed. Dennis. C. and Stringer M.F. pp. 397–428. Cambridge. UK: Woodhead Publishing.
91. ***Honga E., Doumitha M., Duperrierc S., Giovannaccib I., Morvana A., Glaserc P., Buchrieserc C., Jacqueta C., Martina P. (2007)*** "Genetic diversity of *Listeria monocytogenes* recovered from infected persons and pork. seafood and dairy products on retail sale in France during 2000 and 2001". International Journal of Food Microbiology 114 - 187 – 19
92. ***Hudson. J.A., R.J. Lake. P. Savill. P. Scholes and R.E. Mc-Connick. (2001)***. "Rapid detection of L. monceytogenes in harn sample using irnmunornagnetic seperation followed by PCR". J. Applied Microbiol., 90: 614-621.
93. ***International Organization for Standardization, 1996***. Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of Listeria monocytogenes. Part 1: Detection method". ISO method 11290-1, 1st ed. International Organization for Standardization. Genève, Switzerland.
94. ***International Organization for Standardization, 1998***. Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of Listeria monocytogenes.

Part 2: Enumeration method". ISO method 11290-2, 1st ed. International Organization for Standardization. Genève, Switzerland.

95. ***Ireton K., Cossart P., (1997)*** "Host-pathogen interactions during entry and actin-based movement of *Listeria monocytogenes*" Annu. Rev. Genet31:113-138.
96. ***Jaradat. Z. W., Schutze. G. E. & Bhunia. A. K. (2002).*** "Genetic homogeneity among *Listeria monocytogenes* strains from infected patients and meat products from two geographic locations determined by phenotyping, ribotyping and PCR analysis of virulence genes". Int J Food Microbiol 76. 1–10.
97. ***Jay J. (1996)*** "Prevalence of *Listeria spp*. in meat and poultry products" Food Control 7. 209-214.
98. ***Jemmi. T., Pak. S.I. and Salman. M.D. (2002)*** "Prevalence and risk factors for contamination with *Listeria monocytogenes* of imported and exported meat and fish products in Switzerland. 1992–2000". Prev Vet Med 54. 25–36
99. ***Jemmy T., Stephan R. (2006)*** "*Listeria monocytogenes*: foodborne pathogen and hygiene indicator" Rev. Sci.Tech.. 25 (2):571-80.
100. ***Jiang L.L., Xu J.J., Chen N., Shuai J.B. and Fang W.H. (2006).*** "Virulence phenotyping and molecular characterization of a low-pathogenicity isolate of Listeria monocytogenes from cow's milk". Acta Biochim. Biophys. Sin. 38:262.
101. ***Juntilla. J. R., Niemela. S. I., Hirn. J. (1988).*** "Minimum growth temperatures of *Listeria monocytogenes* and non-haemolytic listeria". J. Appl. Bacteriol. 65: 321-327.
102. ***Kampelmacher E. H. and Van Noorle Jansen. (1980)*** "Listeriosis in Humans and Animals in the Netherlands (1958-1977)". Zentralblatt fur Bakteriologie. Mikrobiologie und Hygiene. I. Abteilung Oroginale A 256:211-27
103. ***Kanuganti. S.R., Wesley. I.V., Reddy. P.G., McKean. J. and Hurd. H.S. (2002)*** "Detection of *Listeria monocytogenes* in pigs and pork". J Food Prot 65. 1470–1474.
104. ***Kasper S. Huhulescu S. Auer B et al. (2009)*** "Epidemiology of listeriosis in Austria". Wien Klin Wochenschr; 121: 45–51.
105. ***Kathariou S. (2002)*** "*Listeria monocytogenes* virulence and pathogenicity. a food safety perspective". J Food Prot. 65(11):1811 – 1829.

106. ***Kirchner M., Higgins D.E., (2008)*** "Inhibition of ROCK activity allows InlF-mediated invasion and increased virulence of *Listeria monocytogenes*". Mol. Microbiol. 68:749-767.

107. ***Koch J. Stark K. (2006)*** "Significant increase of listeriosis in Germany – Epidemiological patterns 2001-2005". Euro Surveill 2006;11(6):85-8. Available from: http://www. eurosurveillance.org/em/v11n06/1106-224.asp

108. ***Koo. K., Jaykus. L.A., (2003).*** "Detection of Listeria monocytogenes from a model food by fluorescence resonance energy transfer-based PCR with an asymmetric fluorogenic probe set". Appl. Environ. Microbiol. 69. 1082–1088.

109. ***Korsak. N., Daube. G., Ghafir. Y., Chahed. A., Jolly. S., Vindevogel. H. (1998).*** "An efficient sampling technique used to detect four foodborne pathogens on pork and beef carcasses in nine Belgian abattoirs". J. Food Prot. 61: 535–541.

110. ***Koutsoumanis. K.P., Kendall. P.A. and Sofos. J.N. (2003)*** "Effect of food processing-related stresses on acid tolerance of *Listeria monocytogenes*". Appl Environ Microbiol 69. 7514– 7516.

111. ***Kreft J., Vazquez-Boland J.A. (2001)*** "Regulation of virulence genes in Listeria" Int.J. Med.Microbiol. 291. 145-157.

112. ***Lappi. V.R., Thimothe. J., Nightingale. K.K., Gall. K., Scott. V.N., Wiedmann. M., (2004).*** "Longitudinal studies on *Listeria* in smoked fish plants: impact on intervention strategies on contamination patterns". Journal of Food Protection 67 (11). 2500–2514.

113. ***Lawrence. L.M., Gilmour. A., (1995)*** "Characterization of *Listeria monocytogenes* isolated from poultry products and from the poultry- processing environment by random amplification of polymorphic DNA and multilocus enzyme electgrophoresis". Appl. Environ. Microbiol. 61. 2139–2144.

114. ***Leclercq A. Clermont D. Bizet C. Grimont PAD. Le Fleche-Mateos A. Roche SM. Buchrieser C. Cadet-Daniel V. Le Monnier A. Lecuit M. Allerberger F (2009).*** "Listeria rocourtiae sp." nov. Int. J. Syst. Evol. Microbiol.. 60: 2210-2214.

115. ***Lecuit M., Nelson D.M., Smith S.D., Khun H., Huerre M., Vacher-Lavenu M.C., Gordon J.I., Cossart P., (2004)*** "Targeting and crossing of the human maternofetal barrier by *Listeria monocytogenes*: role of internalin interaction with trophoblast E-cadherin". Proc. Natl. Acad. Sci. U. S. A 101:6152-6157.

116. ***Lecuit M., Vandormael-Pournin S., Lefort J., Huerre M., Gounon P., Dupuy C., Babinet C., Cossart P., A (2001)*** "Transgenic model for listeriosis: role of internalin in crossing the intestinal barrier". Science 292:1722-1725.

117. ***Leite P. Rodriguez R. Ferreira M. Ribeiro G. Jacquet C. Martin P. Brito L (2006):*** "Comparative characterization of *Listeria monocytogenes* isolated from Portuguese farmhouse ewe's cheese and from humans". International Journal of Food Microbiology 106. 111–121

118. ***Lemaitre J.P., Echchannaoui H., Michaut G., Divies C., Rousset A. (1998):*** "Plasmid-mediated resistance to antimicrobial agents among *listeriae*". J. Food Prot, 61. 1459–1464.

119. ***Lewis. H. C., Little. C. L., Elson. R., Greenwood. M., Grant. K. A. and McLauchlin. J. (2006).*** "Prevalence of *Listeria monocytogenes* and other *Listeria* species in butter from United Kingdom production, retail, and catering premises". J. Food Prot. 69 (7): 1518-1526Lianou and Sofos. 2007;

120. ***Linnan. M.J., Mascola. L., Dong Lou. X., Goulet. V., May. S., Salminen. C., Hird. D.W., Yonekura. L.M., Hayes. P., Weaver. R., Audurier. A., Plikaytis. B.D., Fannin. S.L., Kleks. A., Broome. C.V., (1988).*** "Epidemic listeriosis associated with Mexican-style cheese". N. Engl. J. Med. 319. 823-828.

121. ***Linton. R.H., Wesber. J.B., Pierson. M.D., Bishop. J.R. and Hackney. C.R. (1992)*** "The effect of sublethal heat shock and growth atmosphere on the heat resistance of *Listeria monocytogenes*" J Food Prot 55. 84–87.

122. ***Little CL. Taylor FC. Sagoo SK. Gillespie LA. Grant K. McLauchlin J (2007).*** "Prevalence and level of *Listeria monocytogenes* and other *Listeria* species in retail pre-packaged mixed vegetable salads in the UK". Food Microbiol. 24: 711-717.

123. ***Lucke. F. (1985)*** "Fermented sausages". In Microbiology of Fermented Foods. Vol. 2 ed. Wood. B. pp. 41–84. London: Elsevier.

124. ***Lukinmaa. S., M. Miettinen. U. M. Nakari. H. Korkeala. Siitonen A. (2003).*** "*Listeria monocytogenes* isolates from invasive infections: variation of sero- and genotypes during an 11-year period in Finland". J. Clin. Microbiol. 41. 1694-1700.

125. ***Lunden. J.M., Autio. T.J. and Korkeala. H.J. (2002)*** "Transfer of persistent *Listeria monocytogenes* contamination between food-processing plants associated with a dicing machine". J Food Prot 65. 1129–1133.

126. ***Lyytika¨ inen. O., T. Autio. R. Maijala. P. Ruutu. T. Honkanen-Buzalski. M. Miettinen. M. Hatakka. J. Mikkola. V.-J. Anttila. T. Johansson. L. Rantala. T. Aalto. H. Korkeala and A. Siitonen. (2000)***. "An outbreak of *Listeria monocytogenes* serotype 3a from butter in Finland". J. Infect. Dis. 181:1838–1841

127. ***Maijala R., O. Lyytika¨ inen, T. Johansson, T. Autio, T. Aalto, L. Haavisto, and T. Honkanen-Buzalski. (2001)***. "Exposure of *Listeria monocytogenes* within an epidemic caused by butter in Finland". Int. J. Food Microbiol. 70:97–109.

128. ***Malak. M., Vivier. A., Andre. P., Decallonne. J. and Gilot. P. (2001).*** "RAPD analysis, serotyping, and esterase typing indicate that the population *Listeria monocytogenes* strains recovered from cheese and from patients with listeriosis in Belgium are different". Can. J. Microbiol. 47 (9): 883-887.

129. ***Manfreda G. De Cesare A. Stella S. Cozzi M. Cantoni C. (2005)*** "Occurrence and ribotypes of *Listeria monocytogenes* in Gorgonzola cheeses" International Journal of Food Microbiology. (34). 45-49.

130. ***Marsh E. Luo L. Wang H. (2003)*** "A three-tiered approach to differentiate *Listeria monocytogenes* biofilm-forming abilities". FEMS Microbiol Lett. 228. 203-10.

131. ***Marzadori F.. 2004*** "Valutazione del rischio di Listeriosi in gravidanza con particolare attenzione alla situazione in Piemonte". AIVEMP newslettre 3. 9

132. ***Matar. G.M., Hayes. P.S., Ribb. W.F., Swaminthan. B., (1997).*** "Listeriolysin O-based latex agglutination test for the rapid detection of *Listeria monocytogenes* in foods". J. Food Prot. 60.1038–1040.

133. ***Mazzette R., Meloni D., Mureddu A., Lepori L., Cosseddu A.M. (2006)*** "Prevalence and molecular typing of *Listeria monocytogenes* in Ready to eat Meat Products and in the Processing Plants" Proceedings of Food Micro 2006 "Food safety and food biotechnology:diversity and global impact. Bologna. Italy. August 29-September 2. 2006. p.184.

134. ***McCarthy. S. A. (1990).*** "Listeria in the environment". p. 25-29.

135. ***McLauchlin J. (1990)*** "Distribution of serovars of *Listeria monocytogenes* isolated from different categories of patients with listeriosis" Eur. J. Clin. Microbiol.Infect. Dis., 9.210-213.

136. ***McLauchlin J., Mitchell R.T., Smerdon W.J., Jewell K. (2004)*** "*Listeria monocytogenes* and listeriosis: a review of hazard characterisation for use in microbiological risk assessment of foods" Int. J. of Food Microbiology 92. 15-33.

137. ***McLauchlin. J., (1996a)***. "The relationship between *Listeria* and listeriosis". Food Control 7. 187 – 193.

138. ***McLauchlin. J., (1996b)***. "The role of the PHLS in the investigation of listeriosis during the 1980s and 1990s". Food Control 7. 235 – 239.

139. ***McLauchlin. J., (1997)***. "The pathogenicity of *Listeria monocytogenes*: a public health perspective". Rev. Med. Microbiol. 8. 1 – 14.

140. ***McLauchlin. J., Audurier. A., Taylor. A.G., (1986)***. "Aspects of the epidemiology of human *Listeria monocytogenes* infections in Britain 1967 – 1984. The use of serotyping and phage-typing". J. Med. Microbiol. 22. 367 – 377.

141. ***McLauchlin. J., Low. C., (1994)***. "Primary cutaneous listeriosis in adults: an occupational disease of veterinarians and farmers". Vet. Rec. 135. 615 – 617.

142. ***Mengaud J., Ohayon H., Gounon P., Mege R.-M., Cossart P., (1996)*** "E-cadherin is the receptor for internalin. a surface protein required for entry of *Listeria monocytogenes* into epithelial cells". Cell 84:923-932.

143. ***Mereghetti L., Quentin R., Marquet-Van Der Mee N., Audirier A. (2000)*** "Low sensitivity of *Listeria monocytogenes* to quaternary ammonium compounds" Appl. Environm Microbiol. 66:5083-5086.

144. ***Mielke. M. E., S. Ehlers, and H. Hahn. (1988).*** "T-cell subsets in delayed-type hypersensitivity, protection, and granuloma formation in primary and secondary *Listeria* infection in mice: superior role of Lyt-21 in acquired resistance". Infect. Immun. 56:1920–1925.

145. ***Miller A. J., Smith J. L., and Somkuti G. A.*** (ed.)." Foodborne listeriosis". Society for Industrial Microbiology. Elsevier Science Publishing. Inc.. New York.

146. ***Ministerial Decree of the Italian Health Minister 15.12.1990*** "Information System of infectious and contagious diseases" (G.U. Serie Generale n. 6 del 8 gennaio 1991).

147. ***Mioni et al., (2004)*** "Valutazione del rischio Listeriosi in gravidanza con particolare attenzione alla situazione in Piemonte" AIVEMP newsletter. 3. 8

148. ***Murray EGD. Webb RE. Swann MBR. (1926)*** "A disease of rabbits characterized by a large mononuclear leucocytosis, caused by a hitherto undescribed bacillus *Bacterium monocytogenes* (n. sp.)". J Pathol Bacteriol ;29:407–439

149. ***Nadon CA. Woodward DL. Young C. Rodgers FG. Wiedmann M. (2001)*** "Correlations between molecular subtyping and serotyping of *Listeria monocytogenes*" Journal of Clinical Microbiology. 39:2704-2707.

150. ***Nakamura. H., Hatanaka. M., Ochi. K., Nagao. M., Ogasawara. J., Hase. A., Kitase. T., Haruki. K., Nishikawa. Y., (2004).*** "*Listeria monocytogenes* isolated from cold-smoked fish products in Osaka City. Japan". Int. J. Food Microbiol. 94. 323–328

151. ***Nelson K.E., Fouts D.E., Mongodin E.F., Ravel J., DeBoy R.T., Kolonay J.F., Rasko D.A., Angiuoli S.V., Gill S.R., Paulsen I.T., Peterson J., White O., Nelson W.C., Nierman W., Beanan M.J., Brinkac L.M., Daugherty S.C., Dodson R.J., Durkin A.S., 21 Madupu R., Haft D.H., Selengut J., Van Aken S., Khouri H., Fedorova N., Forberger H., Tran B.. Kathariou S., Wonderling L.D., Uhlich G.A., Bayles D.O., Luchansky J.B., Fraser C.M., (2004)*** "Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species". Nucl. Acids Res. 32:2386-2395.

152. ***Nesbakken. T., Kapperud. G., Caugant. D.A. (1996)*** "Pathways of *Listeria monocytogenes* contamination in the meat processing industry". Int. J. Food Microbiol. 31: 161–171.

153. ***Nesbakken. T., Nerbrink. E., Rotterud. O.-J., Borch. E. (1994).*** "Reduction of *Yersinia enterocolitica* and *Listeria spp*. on pig carcasses by enclosure of the rectum during slaughter". Int. J. Food Microbiol. 23: 197–208.

154. ***Nightingale, K. K., et al. (2008)***. "inlA premature stop codons are common among Listeria monocytogenes isolated from foods and yield virulence-attenuated strains that confer protection against fully virulent strains". Appl. Environ. Microbiol. 74:6570–6583.

155. ***Nørrung. B., Andersen. J. K., and Schlundt. J. (1999)*** "Incidence and control of *Listeria monocytogenes* in foods in Denmark". Int. J. Food Microbiol. 53: 195–203.

156. ***Norton D.M., Scarlett JM. Horton K. Sue D. Thimothe J. Boor KJ. Wiedmann M. (2001)*** "Characterization and pathogenic potential of *Listeria monocytogenes* isolates from the smoked fish industry" Applied and Environmental Microbiology 67: 646–653.

157. ***O'Driscoll. J., Nnadi. C., McLauchlin. J., (1999)***. *Listeria monocytogenes* septic arthritis in an immunocompetent adult. Clin. Microbiol. Infect. 5. 234 – 235.

158. ***Odumeru. J.A., Steele. M., Fruhner. L., Larkin. C., Jiang. J., Mann. E., McNab. W.B., (1999)***. "Evaluation of the accuracy and repeatability of identification of food-borne pathogens by automated bacterial identification systems". J. Clin. Microbiol. 37. 944 – 949.

159. ***Patrick R. Murray. Ellen Jo Baron. Michael A.Pfaller. Fred C. Tenover. Robert H. Yolken (1995).*** Manual of clinical microbiology.

160. ***Pena-Sagredo JL. Hernandez MV. Fernandez-Llanio N et al (2008).*** "*Listeria monocytogenes* infection in patients with rheumatic disease on TNFalpha antagonist therapy: the Spanish Study Group experience". Clin Exp Rheumatol; 26: 854–859.

161. ***Pine. L., Malcolm. G. B., Brooks. J. B. & Danshevar. M. I. 1989.*** "Physiological studies on the growth and utilization of sugars by *Listeria* species" Can J Microbiol 35. 245-254.

162. ***Pizarro-Cerda J., Bonazzi M.. Cossart P., (2010)*** "Clathrin-mediated endocytosis: what works for small, also works for big". Bioessays 32:496-504.

163. ***Premaratne. R. J., Lin. W. J. & Johnson. E. A. (1991)*** "Development of an improved chemically defined minimal medium for *Listeria monocytogenes*" Appl Environ Microbiol 57. 3046–3048.

164. ***Pron. B., C. Boumaila. F. Jaubert. S. Sarnacki. J.-P. Monnet. P. Berche. and J.-L. Gaillard. (1998)*** "Comprehensive study of the intestinal stage of listeriosis in a rat ligated ileal loop system". Infect. Immun. 66:747–755.

165. ***Rasmussen O.F. Skouboe P. Dons L. Rossen L. Olsen JE. (1995)*** "*Listeria monocytogenes* exists in at least three evolutionaty lines: evidence from flagellin, invasive associated protein and lysteriolisin O genes". Microbiology 141:2053-2061.

166. ***Reij. M. and De Aantrekker. E.D. (2004)*** "Recontamination as a source of pathogens in processed foods". Int J Food Microbiol 91. 1–11.

167. ***Renzoni A., Cossart P., Dramsi S. (1999)*** "PrfA, the transcriptional activator of virulence genes is upregulated during interaction of *Listeria monocytogenes* with mammalian cells and in eukaryotic cell extracts" Molec. Microbiology. 34 (3).552-561.

168. ***Ripamonti B., Zecchini A., Colombo F., Stella S., Cantoni C. (2002)*** "Localizzazione di *Listeria monocytogenes* e *Salmonella spp*. nei macelli" Ingegneria alimentare 3/2002.

169. ***Riser E. T., Marth E. H. (1991).*** "*Listeria*, Listeriosis, and food safety".

170. ***Roberts A.J., Wiedmann M. (2003)*** "Pathogen, host and environmental factors contributing to the pathogenesis of listeriosis". Cell. Mol. Life Sci. 60. 904-918.

171. ***Robin L.T. Churchill. Hung Lee. J. Christopher Hall. (2005).*** "Detection of *Listeria monocytogenes* and the toxin listeriolysin O in food".

172. ***Roche S.M., Gracieux P., Velge P. (2009)***." Poor detection of low-virulence field strains of L. monocytogenes is related to selective agents in selective media and is unrelated to PrfA". Food Microbiol. 26:21.

173. ***Rocourt J. (1996)*** "Risk factors for listeriosis" Food Control. 7. 195-202.

174. ***Rocourt J., Bille J. (1997)*** "Foodborne listeriosis" World Health Statistics Quartely 50. 67-73.

175. ***Rocourt. J., Hogue. A., Toyofuku. H., Jacquet. C., Schlundt. J., (2001).*** "*Listeria* and listeriosis: risk assessment as a new tool to unravel a multifaceted problem". Am. J. Infect. Control 29. 225–227.

176. ***Romanova. N., Favrin. S., Griffiths. M. W. (2002).*** "Sensitivity of *Listeria monocytogenes* to sanitizers used in the meat processing industry". Appl. Environ. Microbiol. 68: 6405–6409.

177. ***Rondon. M. R.. and J. C. Escalante-Semerena. 1996.*** In vitro analysis of the interactions between the PocR regulatory protein and the promoter region of the cobalamin biosynthetic (cob) operon of *Salmonella typhimurium* LT2. J. Bacteriol. 178:2196-2203

178. ***Ryser E.T., (1999)*** "Foodborne listriosis" p.299-358 in "*Listeria*, listeriosis and food safety". 2nd edition, edited by E.T. Ryser and E.H. Marth. Marcel Dekker. New York.

179. ***Ryser E.T., (2003)*** "*Listeria monocytogenes*" in "Encyclopedia od dairy sciences" vol. Three, edited by H.Roginski. J.W. Fuquay. P.F. Fox. Academic Press.

180. ***Sabet C., Toledo-Arana A., Personnic N., Lecuit M., Dubrac S., Poupel O., Gouin E., Nahori M.-A., Cossart P., Bierne H., (2008)*** "The *Listeria monocytogenes* virulence factor InlJ is specifically expressed in vivo and behaves as an adhesion". Infect. Immun. 76:1368-1378.

181. ***Sagoo. S.K., Little. C.L., Allen. G., Williamson. K., & Grant. K.A. (2007).*** "Microbiological safety of retail vacuum-packed and modified-atmosphere-packed cooked meats and end of shelf life". Journal of Food Protection 70(4). 943-951

182. ***Saide-Albornoz. J. J., Knipe. C. L., Murano. E. A., Beran. G. W. (1995).*** "Contamination of pork carcasses during slaughter, fabrication, and chilled storage". J. Food Prot. 58: 993–997.

183. ***Samelis. J., Metaxopoulos. J., Vlassi. M. and Pappa. A. (1998)*** "Stability and safety of traditional Greek salami – a microbiological ecology study". Int J Food Microbiol 44. 69–82.

184. ***Sammarco. M. L., Ripabelli. C., Ruberto. A., Iannitto. G., Grasso G. M. (1997).*** "Prevalence of *Salmonellae*. *Listeriae*. and *Yersinae* in the slaughterhouse environment and on work surfaces, equipment, and workers". J. Food Prot. 60: 367–371.

185. ***Sasahara. K.C., Zottola. E.A., (1993)*** "Biofilm formation by *Listeria monocytogenes* utilizes a primary colonizing microorganism in flowing system" J. Food Prot. 56. 1022-1028.

186. ***Schlech W.F., (2000)*** "Epidemiology and Clinical manifestations of *Listeria monocytogenes* infection" in "Gram-positive pathogens". edited by Fischetti C. Novick R. Ferretti J. Portnoy DA. Rood J.473-498.ASM press. Washington.

187. ***Schlech W.F., Lavigne P.M., Bortolussi R.A., Allen C.A., Haldane E.V., Won A.J., Hightower A.W., Johnson S.E., King S.H., Nicholls E.S., Broome C.V. (1983)*** "Epidemic listeriosis-evidence for transmission by food" N.Engl. J. Med. 308:203-206.

188. ***Schmidt MW. Eva Y.W., Lampidis R. Emmerth M., Walcher M. Kreft J.Goebel W. Wagner M., Schleifer W. (2005)*** "Evolutionary history of the genus *Listeria* and its virulence genes" Systematic and Applied Microbiology 28:1–18.

189. ***Seeliger H. P. R. .J. Rocourt. A. Schrettenbrunner. P. A. D. Grimont. D.Joners. (1984)*** "*Listeria ivanovii* spp." Nov. int. j. syst. Bacyeriol. 34:336-337.

190. ***Sergelidis. D., Abrahim. A., Sarimvei. A., Panoulis. C., Karaioannoglou. P. and Genigeorgis. C. (1997)*** "Temperature distribution and prevalence of *Listeria spp*. in domestic, retail and industrial refrigerators in Greece". Int J Food Microbiol 34. 171–177.

191. ***Severino P., Dussurget O., Vencio R.Z., Dumas E., Garrido P., Padilla G., Piveteau P., Lemaitre J.P., Kunst F., Glaser P., Buchrieser C.. (2007)*** "Comparative transcriptome analysis of *Listeria monocytogenes* strains of the two major lineages reveals differences in virulence, cell wall, and stress response". Appl. Environ. Microbiol. 73:6078-6088..

192. ***Shen Y., Naujokas M., Park M., Ireton K., (2000)*** "InIB-dependent internalization of *Listeria* is mediated by the Met receptor tyrosine kinase". Cell 103:501-510.

193. ***Sırıken B., Pamuk S., Cuneyt Z., Gedikoglu S., Eyigo M. (2006)*** "A note on the incidences of *Salmonella spp., Listeria spp*. and *Escherichia coli O157:H7* serotypes in Turkish sausage (Soudjouck)" Meat Science 72. 177–181

194. ***Skovgaard. N., Nørrung. B., (1989).*** "The incidence of *Listeria spp*. In faeces of Danish pigs and in minced pork meat". Int. J. Food Microbiol. 8. 59 – 63

195. ***Smith B. Kemp M. Ethelberg S et al. (2005)*** "*Listeria monocytogenes*: maternal–foetal infections in Denmark 1994". Scand J Infect Dis 2009; 41: 21–25.

196. ***Sokolovic Z., Fuchs A., Wuenscher M., Goebel W. (1990)*** "Synthesis of species-specific stress proteins by virulent strains of *Listeria monocytogenes*". Infect. Immun. 58. 3582-3587.

197. ***Srikumar. S., and Fuchs. T.M. 2011.*** Ethanolamine utilization contributes to proliferation of *Salmonella enterica Sevorar Typhimurium* in food and in nematodes. Appl. Environ. Microbiol. 77:281-290.

198. ***Stepanovic. S., Cirkovic. I., Ranin. L., Svabic-Vlahovic. M. (2004)*** "Biofilm formation by *Salmonella spp*. and *Listeria monocytogenes* on plastic surfaces" Letters in applied microbiology. 38. 428-432.

199. ***Suihko. M. L., Salo. S., Niclasen. O., Gudbjörnsdóttir. B., Torkelsson. G., Bredholt. S., Sjöberg. A.-M., Gustavsson. P. (2002).*** "Characterization of *Listeria monocytogenes* isolates from the meat. poultry and seafood industries by automated ribotyping". Int. J. Food Microbiol. 72: 137–146.

200. ***Swaminathan B. (2001)*** "*Listeria monocytogenes*" in: Food Microbiology: fundamentals and frontiers. 2nd edition, edited by M.P.Doyle. ASM press. Washington. D.C.

201. ***Tabouret M., Rycke J., Audurier A., Poutrel B. (1991).*** **"**Pathogenicity of Listeria monocytogenes isolates in immunocompromised mice in relation to listeriolysin production". J. Med. Microbiol. 34:13.

202. ***Taurai T. and Roger S. 2007.*** Evaluation of housekeeping genes in *Listeria monocytogenes* as potential internal control references for normalizing mRNA expression levels in stress adaptation models using real-time PCR. FEMS Microbiol Lett. 269:265–272

203. ***Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., B.E., Persing, D.H., Swaminathan, B., (1995)***. "Interpreting chromosomal DNA restriction patterns produced by pulsedfield gel electrophoresis: criteria for bacterial strain typing". J. Clin. Microbiol. 33, 2233–2239.

204. ***Thèvenot D., Delignette-Muller M.L., Christieans S., Leroy S., Kodjo A. Vernozy-Rozand C. (2006)***"Serological and molecular ecology of *Listeria monocytogenes* isolates collected from 13 French pork meat salting-curing plants and their products".International Journal of Food Microbiology. 112 (2006)153-161.

205. ***Thèvenot D., Delignette-Muller M.L., Christieans S., Vernozy-Rozand C. (2005)*** "Prevalence of *Listeria monocytogenes* in 13 dried sausage processing plants and their products" International Journal of Food Microbiology. 102. 85-94.

206. ***Thimothe J. Nightingale KK. Gall K. Scott VN. Wiedmann M. (2004)***. "Tracking of *Listeria monocytogenes* in smoked fish processing plants". J Food Protect 67(2):328 – 341.

207. ***Tompkin R.B., (2002)*** "Control of *Listeria monocytogenes* in the food-processing environment". J. Food Prot. 65. 709- 725.

208. ***Tsai Y.H., Orsi R.H., Nightingale K.K., Wiedmann M., (2006)*** "*Listeria monocytogenes* internalins are highly diverse and evolved by recombination and positive selection". Infect. Genet. Evol. 6:378-389.

209. ***Tyopponen S., Markkula A., Petaja E., Suihko M.L., Mattila-Sandholm T.(2003)*** "Survival of *Listeria monocytogenes* in North European type dry sausges fermented by bioprotective meat starter cultures" Food Control 14. 181-185.

210. ***V. Garrido, A.I. Vitas, I. García-Jaló (2009)*** "Survey of Listeria monocytogenes in ready-to-eat products: Prevalence by brands and retail establishments for exposure assessment of listeriosis in Northern Spain" Food Control 20 (2009) 986–99

211. ***van de Weyer. A., Devleeschouwer. M.-J. Dony. J. (1993)*** "Bactericidal activity of disinfectants on *Listeria*". J. Appl. Microbiol. 74: 480-483.

212. ***Van Stelten, A., J. M. Simpson, T. J. Ward, and K. K. Nightingale (2010)***. "Revelation by single-nucleotide polymorphism genotyping that mutations leading to a premature stop codon in inlA are common among Listeria monocytogenes isolates from ready-to-eat foods but not human listeriosis cases". Appl. Environ. Microbiol. 76:2783–2790.

213. ***Vasseur C., Rigaud. N., Hebraud. M. and Labadie. J. (2001)*** "Combined effects of NaCl. NaOH and biocides (Monolairin or Lauric acid) on inactivation of *Listeria monocytogenes* and *Pseudomonads spp*." J Food Prot 64. 1442–1445.

214. ***Vazquez-Boland J. A., Kuhn M., Berche P., Chakraborty T., Domi´Nguez-Bernal G. . Goebel W., Gonza´Lez-Zorn B., Wehland J.. And Kreft J., (2001)*** "*Listeria* Pathogenesis and Molecular Virulence Determinants". Jouve. J.L., 2000. ILSI Europe session on microbial risk assessment. Int. J. Food Microbiol. 58. 141– 142.

215. ***Vazquez-Boland J.A., Dominguez-Bernal G., Gonzalez-Zorn B., Kreft J., Goebel W. (2001b)*** "Pathogenicity island and virulence evolution in *Listeria*" Microbes and infection. 3. 2001. 571-584.

216. ***Vazquez-Boland J.A., Kuhn M., Berche P., Chakraborty T., Domingues-Bernal G., Goebel W., Gonzalez-Zorn B., Wehlamd J., Kreft J. (2001a)*** "*Listeria* pathogenesis and molecular virulence determinants" Clinical Microbiology Reviews. 2001. 584-640.

217. ***Vogel B. F., Hansen L. T., Mordhorst H., Gram L. (2010).*** "The survival of *Listeria monocytogenes* during long term desiccation is facilitated by sodium chloride and organic material". International Journal of Food Microbiology 140 (2010) 192–20.

218. ***Vogel B.F., Fussing. V., Ojeniyi. B., Gram. L., Ahrens. P., (2004).*** "High-resolution genotyping of *Listeria monocytogenes* by fluorescent amplified fragment length polymorphism analysis compared to pulsed-field gel electrophoresis. random amplified polymorphic DNA

analysis, ribotyping, and PCR-restriction fragment length polymorphism analysis". J. Food Prot. 67. 1656–1665.

219. ***Wagner M., Schmid. M., Juretschko. S., Trebesius. K.-H., Bubert. A., Goebel. W., Schleifer. K.-H., (1998).*** "In situ detection of a virulence factor mRNA and 16S rRNA in *Listeria monocytogenes*". FEMS Microbiol. 160. 159– 168.

220. ***Walker S. J., and M. F. Stringer. (1987).*** "Growth of *Listeria monocytogenes* and *Aeromonas hydrophila* at chill temperatures". J. Appl. Bacteriol. 63:R20.

221. ***Walter. D., M. Ailion. and J. Roth. 1997.*** Genetic characterization of the pdu operon: use of 1.2-propanediol in Salmonella typhimurium. J. Bacteriol. 179:1013-1022.

222. ***Ward T.J., Gorski L., Borucki M.K., Mandrell R.E., Hutchins J., Pupedis K. (2004)*** "Intraspecific phylogeny and lineage group identification based on the prfA virulence gene cluster of *Listeria monocytogenes*" Journal of Bacteriology. Aug.2004. 4994-5002.

223. ***Ward, T. J., et al (2010)***. "Molecular and phenotypic characterization of Listeria monocytogenes from U.S. Department of Agriculture Food Safety and Inspection Service surveillance of ready-to-eat foods and processing facilities". J. Food Prot. 73:861–869

224. ***Weis J., and H. P. R. Seeliger. (1975).*** "Incidence of *Listeria monocytogenes* in nature". Appl. Microbiol. 30:29-32.

225. ***Welshimer. H.J. (1968)*** "Isolation of *Listeria monocytogenes* from vegetation". .I. Bacterial. 95. 300-303

226. ***Wiedmann M. (2002)*** "Molecular subtyping methods for *Listeria monocytogenes*" JAOAC Int. 85:524-531.

227. ***Wiedmann M. Bruce JL. Keating C., Johnson AE. Mc Donough PL. Batt CA (1997)*** "Ribotypes and virulence gene polymorphism suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential". Infection and Immunity. 65:2707-2716.

228. ***Wiedmann M., Bruce J.L., Keating C., Johnson A.E., Mc- Donough P.L. and Batt C.A. (1997)***. "Ribotypes and virulence gene polymorphisms suggest three distinct Listeria monocytogenes lineages with differences in pathogenic potential". Infect. Immun. 65:2707.

229. ***Wimpfheimer L., Altman NS.S. Hptchkiss J.H. (1990)*** "Growth of *Listeria monocytogenes* Scott A. serotype 4 and competitive spoilage organisms in raw chicken packaged under modified atmospheres and in air". Int J Food Microbiol. Dec; 11(3-4):205–214.

INDEX