UNIVERSITA' DEGLI STUDI DI PARMA

Dottorato di ricerca in Scienze Medico-Veterinarie

Ciclo XXVII

DETECTION OF *LISTERIA* SPP. IN TYPICAL COOKED AND CURED MEAT PRODUCTS: ANALYSIS OF THE HYGIENE PROCESS AND FOOD SAFETY CRITERIA IN "READY-TO-EAT FOOD" IN COMPLIANCE WITH EUROPEAN LAW

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Alla mia splendida famiglia

Summary

1. Introduction

1.1 LISTERIA MONOCYTOGENES OUTBREAKS

In the European Union (EU), listeriosis is a relatively rare but serious food-borne illness in humans, with high morbidity, hospitalization and mortality in vulnerable populations. The bacterial genus *Listeria* currently comprises 10 species, but human cases of listeriosis are almost exclusively caused by the species *Listeria monocytogenes* (*L. monocytogenes*). *Listeria* species are ubiquitous organisms that are widely distributed in the environment, especially in plant matter and soil. The principal reservoirs of *Listeria* are soil, forage and surface water. The main route of transmission to humans is believed to be through consumption of contaminated food. The bacterium can be found in raw foods and in processed foods that are contaminated during and/or after processing. The fact that *L. monocytogenes* is able to multiply in various foods at temperatures as low as 2 to 4 °C makes the occurrence of *L. monocytogenes* in ready-to-eat (RTE) foods with a relatively long shelf-life, such as fishery products, heat-treated meat products and RTE cheese, of particular concern.

In order to estimate at the EU level the prevalence and level of *L. monocytogenes* in packaged hot or cold smoked or gravad fish, packaged heat-treated meat products and soft and semi-soft cheeses (excluding fresh cheeses), an EU wide *L. monocytogenes* baseline survey was conducted at retail. The foods to be sampled were randomly selected from the customer display in the outlet and each sample weighed at least 100 g. The survey was designed to yield estimates at the EU level only and not at the Member State level.

Sampling took place between January 2010 and January 2012. A total of 3 053 batches of packaged hot or cold smoked or gravad fish, 3 530 packaged heat-treated meat products and 3 452 soft or semi-soft cheeses were sampled from 3 632 retail outlets in 26 EU Member States, plus Norway. For fish, two samples were collected from each sampled batch and one was analyzed on arrival at the laboratory (at the time of sampling) and the other one was analyzed at the end of shelf-life. For the meat products and cheese samples one sample was taken from the selected batch and was analyzed at

the end of shelf-life. All 13 088 food samples were examined for the presence of *L. monocytogenes*, in addition to the determination of the *L. monocytogenes* counts.

The EU prevalence of *L. monocytogenes*-contaminated fish samples at time of sampling was 10.4 % while at the end of shelf-life it was 10.3 %. The EU level proportion of samples exceeding the food safety limit of 100 colony forming units (cfu)/g at sampling was 1.0 % while for fish at the end of shelf-life it was 1.7 %. Among meat products, the EU prevalence of *L. monocytogenes*-contaminated samples at the end of shelf-life was 2.07 % while the EU level proportion of samples exceeding the level of 100 cfu/g was 0.43 %.

The EU prevalence of *L. monocytogenes*-contaminated cheese samples at the end of shelf-life was 0.47 % while the EU level proportion of samples exceeding the level of 100 cfu/g was 0.06 %.

Considering only the enumeration test, the proportion of fish samples considered positive, defined as a *L. monocytogenes* count of 10 cfu/g or more, was 2.2 % and 3.2 % at the time of sampling and at the end of shelf-life, respectively. Of the 66 fish samples at time of sampling having a count of 10 cfu/g or more, 29 samples contained *L. monocytogenes* exceeding the level of 100 cfu/g. At the end of shelf-life of the 99 fish samples with a count of 10 cfu/g or more, 52 samples contained *L. monocytogenes* exceeding the level of 100 cfu/g. The proportion of packaged heat-treated meat products samples considered negative by the enumeration test was 99.1 % at the end of shelf-life whereas 0.9 % had a positive enumeration result. Of the 32 meat products samples at the end of shelf-life having a count of 10 cfu/g or more, 15 samples contained *L. monocytogenes* exceeding the level of 100 cfu/g. Enumeration showed that only four soft or semi-soft cheese products were positive, and in only two of these products the *L. monocytogenes* count exceed 100 cfu/g at the end of shelf-life.

RTE foods with a relatively long shelf-life, such as fishery and heat-treated meat products, and ready-to-eat cheese are considered an important food-borne source of human *L. monocytogenes* infections in the EU. The risk for human health arises from exposure to *L. monocytogenes* in such foods and in particular foods containing *L. monocytogenes* exceeding the level of 100 cfu/g. In the European survey a low proportion of fish samples contained *L. monocytogenes* at levels exceeding the food safety limit of 100 cfu/g at the end of shelf-life. This is of concern to public health as the risk of

human listeriosis increases with increasing numbers of ingested cells. The proportion of cooked meat samples exceeding the level of 100 cfu/g was very low and soft and semi-soft cheeses samples exceeding this level were rare. However, even a very low proportion of samples exceeding the level of 100 cfu/g may raise concern for public health.

Good manufacturing practices, appropriate cleaning, sanitation and hygiene programs and effective temperature control throughout the food production, distribution and storage chain are required for prevention of contamination or inhibition of growth of *L. monocytogenes* to levels exceeding 100 cfu/g in foods that may pose a *L. monocytogenes* risk. The selected foods were RTE and therefore intended to be consumed without any further heat treatment. The findings indicate the ongoing presence of *L. monocytogenes* in such foods. All food business operators and consumers should keep the temperatures of their refrigerators low, in order to limit potential growth of *L. monocytogenes* if this is present in RTE products (EFSA, 2013).

1.1.1 Prevalence of L. monocytogenes-Contaminated Meat Product Samples

The EU prevalence of *L. monocytogenes*-contaminated meat products was 2.07 % (72 positive samples out of 3 470), at the end of shelf-life. The proportion (and number) of meat products samples with a *L. monocytogenes* count exceeding the level of 100 cfu/g was 0.43 % (15 samples) at the end of shelf-life (EFSA, 2013).

1.1.2 Description of Meat Product Samples with a Count of L. monocytogenes Exceeding the Level of 100 cfu/g

These 15 samples originated from nine MSs, and the distribution of the animal species of the origin of the meat product for those samples was the following: eight pork, one beef, two broiler, two poultry, one turkey, and one mixed. Twelve were reported as ‘cold, cooked meat product’, two as ‘pate’ and one as ‘sausage’. All, except one, were sliced meat products. Seven samples were packaged in modified atmosphere, two in normal atmosphere, five in vacuum one and one an in ‘other’ one. Concerning their suitability for human consumption at

the end of shelf-life on the basis of visual and smell (olfactory) evaluation, 11 samples were reported as suitable for human consumption, while this information was missing for the remaining four samples (EFSA, 2013).

1.1.3 L. monocytogenes Enumeration Results in Packaged Heat-Treated Meat Products

At EU level, the percentages of meat product samples, with enumeration results (cfu/g of food) below 10, between 10-39, between 40-100, above 100-1 000, above 1 000-10 000, above 10 000-100 000 and above 100 000 were 99.1 %, 0.3 %, 0.2 %, 0.3 %, 0.03 %, 0.1 % and 0 %, respectively. Norway's data are included in these results.

Thirty-two samples (0.9 %) had a *L. monocytogenes* count of at least 10 cfu/g, out of the 3 530 samples that were examined. Approximately half of these products (17 out of the 32) contained *L. monocytogenes* at levels ranging from 10 to 100 cfu/g. The levels of the pathogen exceeded 100 cfu/g in 15 meat products (0.42%, i.e. 15 out of 3 530). In three of these meat products *L. monocytogenes* counts were in excess of 1 000 cfu/g (EFSA, 2013).

1.1.4 Relevance of the Findings to Human Health

Among the recognized species of the genus *Listeria, L. monocytogenes* is essentially the only pathogenic species for humans. Human cases of listeriosis are usually sporadic, but outbreaks of various magnitudes also occur. The disease usually manifests itself as a febrile gastroenteritis in otherwise healthy human hosts, but also as an invasive disease in high-risk individuals. Although the incidence of invasive listeriosis in developed countries is rather low, the disease is severe, with a high (20-30 %) mortality rate. The risk of invasive listeriosis is higher among certain population groups such as the elderly, pregnant women, neonates and patients under iatrogenic immune-suppression, as well as patients with underlying immune-suppressive conditions (Painter and Slutsker, 2007). According to the EU summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2011 (EFSA and

ECDC, 2013), 26 EU MSs reported 1 476 confirmed cases of human listeriosis, with an incidence of 0.32 cases per 100 000 individuals. Sixteen MSs provided information on hospitalisation for listeriosis for all or the majority of their cases and on average 93.6 % of the cases were hospitalised, in 2011. In ten MSs this proportion was 100 %. This is the highest hospitalisation of all zoonoses under EU surveillance. A total of 134 deaths due to listeriosis were reported by 19 MSs in 2011 resulting in an EU case fatality rate of 12.7 %.

Listeriosis acquired from food is mostly due to the consumption of RTE foods which support the growth of *L. monocytogenes* and develop a high concentration of *L. monocytogenes* along the food chain. The ability of *L. monocytogenes* to proliferate under refrigeration temperatures is probably the most salient feature of the pathogen, given that refrigeration is the most commonly used method of food preservation in developed countries.

Since RTE foods do not require any bactericidal treatment on behalf of the consumer prior to consumption, contamination of RTE foods that can support the growth of *L. monocytogenes* can pose public health risks.

Recent risk assessment concluded that most listeriosis cases are due to foods having a *L. monocytogenes* count markedly above the level of 100 cfu/g. The impact on public health would depend on whether levels greatly exceeding 100 cfu/g are reached (EFSA, 2007). To protect public health a count exceeding the level of 100 cfu/g at the end of the product's shelf-life is considered unsafe in EU legislation and products containing such levels must be withdrawn or recalled from the market.

The results of the baseline European survey show that, at the end of shelf-life, a low proportion of smoked and gravad fish samples contained counts exceeding the food safety limit of 100 cfu/g. This is of concern for public health as the risk for human listeriosis increases with increasing numbers of ingested cells. For meat products and cheeses respectively, a very low and rare proportion was observed. However, taking into account the

popularity of these meat and cheese products, these results may be still a concern for public health. It is noteworthy that the ‘time of sampling‘ concept in the survey was a random and arbitrary point in the shelf-life of the RTE food products and can be regarded as a typical time at which these products are available for retail purchase. However, products purchased at this point followed by home storage might be expected to produce higher counts at end of shelf-life as a result of temperature abuse. In addition and of cause for concern, work by the UK Advisory Committee on the Microbiological Safety of Food (ACMSF) has shown a disregard for dates of minimum durability (i.e. use-by-dates) of RTE products in some sectors of the population at risk for listeriosis (ACMSF, 2009). On the other hand, it seems that, in general, the surveyed food samples were stored at the laboratory under satisfactory temperature conditions; therefore, in this sense providing scenario of the presence of *L. monocytogenes* in the surveyed foods at the end of shelf-life that was not the worst case.

Good manufacturing practices, appropriate cleaning, sanitation and hygiene programs and effective temperature control throughout the food production, distribution and storage chain are required for prevention of contamination or inhibition of growth of the pathogen to levels exceeding 100 cfu/g in foods that may pose a *L. monocytogenes* risk. An effective food safety management system implemented by trained staff is important to control the prevalence and numbers of *L. monocytogenes* in these at risk food products. Consumers can protect themselves by following storage instructions and respecting use-by-dates as *L. monocytogenes* can grow at refrigeration temperatures. The consumers, particularly the vulnerable groups such as pregnant women and the elderly and chronically ill, who are more susceptible to invasive listeriosis, are also advised to follow the guidelines given by the national authorities regarding the consumption of foodstuffs related to higher risk of *L. monocytogenes* contamination.

The data provided by the EFSA survey, gathered in all EU countries using a similar and representative nationwide sampling plan, will be useful in assessing the exposure of EU consumers to *L. monocytogenes* via the three specific RTE food categories (EFSA, 2013).

1.2 CHARACTERISTICS OF L. MONOCYTOGENES

1.2.1 Microbiology

L. monocytogenes is a gram-positive, nonsporeforming, facultatively anaerobic rod which grows between -0.4 and 50°C (Juntilla et al., 1988; Walker and Stringer, 1987). It is catalase positive and oxidase negative and expresses a P-hemolysin which produces zones of clearing on blood agar. The hemolysin acts synergistically with the 3-hemolysin of *Staphylococcus aureus* on sheep erythrocytes; the substance mediating this effect is known as the CAMP factor after Christie, Atkins, and Munch-Petersen (Christie et al., 1944), the workers who first described the phenomenon in group B streptococci. The organism possesses peritrichous flagella, which give it a characteristic tumbling, motility, occurring only in a narrow temperature range. When the organism is grown between 20 and 25°C, flagellin is both produced and assembled at the cell surface, but at 37°C flagellin production is markedly reduced (Peel et al., 1988). The colonies demonstrate a characteristic blue-green sheen by obliquely transmitted light (Henry, 1993). *L. 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, and human and animal feces (Mc Carthy, 1960). *L. monocytogenes* has been isolated from cattle, sheep, goats, and poultry, but infrequently from wild animals (Gray and Killinger, 1966). In tryptic soy broth supplemented with 0.6% yeast extract, incubated at 30°C, *L. monocytogenes* F5027, F5069, S4b, and Scott A grew at pH values from 4.5 to 7.0, with no growth at pH 4.0 and lower (Parish and Higgins, 1989). Of several acids (acetic, lactic, citric, and hydrochloric acids)

used to lower the pH of brain heart infusion broth before using it as the growth medium for four *L. monocytogenes* strains, acetic acid was the most effective growth inhibitor (Aharam and Marth, 1989; Farber et al., 1989). The authors found that the minimum pH required for initiation of growth ranged from 5.0 to 5.7 at 4°C and from 4.3 to 5.2 at 30°C. Buchanan and Phillips (1990) developed a mathematical model describing the effects of temperature (5 to 37°C), pH (4.5 to 7.5), NaCl (5 to 45 g/liter), $NaNO_2$ (0 to 1,000 p.g/ml), and atmosphere (aerobic or anaerobic) on the growth kinetics of *L. monocytogenes* Scott A in tryptone phosphate broth. Studies on carbohydrate fermentations by *Listeria* spp. were reported by Pine et al. (1989). Under anaerobic conditions only hexoses and pentoses supported growth; aerobically, maltose and lactose, but not sucrose, also supported growth. *L. monocytogenes* and *L. innocua* utilize glucose, lactose, and rhamnose under aerobic conditions; *L. grayi* and *L. murrayi* also utilize galactose. *L. ivanovii* and *L. seeligeri* are the only *Listeria* spp. to ferment xylose. The latter ones, and other reactions used in the differentiation of *Listeria* spp., are listed in the table 1.

Characteristic	Result for:				
	L. monocytogenes	*L. innocua*	*L. ivanovii*	*L. welshimeri*	*L. seeligeri*
β-Hemolysis	+	−	+	−	+
CAMP test (*S. aureus*)	+	−	−	−	+
CAMP test (*R. equi*)	−	−	+	−	−
Acid production from:					
α-Methyl-D-mannoside	+	+	−	+	−
Rhamnose	+	v[a]	−	v	−
Xylose	−	−	+	+	+
Mouse virulence	+	−	+	−	−

Table n. 1: Differentiation of *Listeria* spp.

The incidence of cryptic plasmids in *L. monocytogenes* strains is low, ranging from 0 to 20% (Fistrovici and Collins, 1990; Perez-Diaz et al., 1982). This may be due to the use of acriflavine, a known plasmid-curing agent, in the isolation media. Recently, a 37-kbp plasmid carrying genes for resistance to chloramphenicol, erythromycin, streptomycin, and tetracycline was isolated from a clinical strain of *L. monocytogenes* (Poyart-Salmeron et al., 1990).

The plasmid was self-transferable to other *L. monocytogenes* strains (Farber and Peterkin, 1991).

1.2.2 Taxonomy

Although *L. monocytogenes* was classified for a time by Bergey's Manual of Determinative Bacteriology in the family Corynebacteriaceae (Stuart and Pease, 1972), it is listed in the latest edition of Bergey's, together with *Lactobacillus*, *Erysipelothrix*, *Brochothrix*, and other genera, in a section entitled Regular, Nonsporing Gram-Positive Rods (Seeliger and Jones, 1986). Both the intra and intergeneric taxonomy of bacteria of the genus *Listeria* have been problematical for a number of years. *L. monocytogenes* was the only recognized species within the genus until 1961; *L. denitrificans*, *L. grayi*, and *L. murrayi* were added to the genus in 1961, 1966, and 1971, respectively. All serovar 5 strains showed a strong p-hemolysis and were proposed as a separate species, *L. bulgarica* (Rocourt et al., 1982).

This species was officially named *L. ivanovii* in 1984. Nonpathogenic strains of *L. monocytogenes* belonging to serovar 6 were recognized as new species, *L. innocua*. *L. welshimeri* and *L. seeligeri* were added in 1983 (Seeliger, 1984; Seeliger and Jones, 1940). Reviews on the topic include those by Jones (1975; 1988), Seeliger and Finger (1976), and McLauchlin (1987). Stuart and Pease (1972) concluded from a numerical taxonomic study of 123 strains of *Listeria* and nine other genera, that *Listeria* and *Erysipelothrix* are distinct genera that are not closely related, that *L. denitrificans* is quite different from other *Listeria* strains and that these other *Listeria* strains constituted a single monospecific genus. The numerical taxonomic, DNA base composition, and DNA-DNA hybridization studies of Stuart and Welshimer (1973; 1974) led them to conclude that *L. denitrificans* should be reclassified and to propose that *L. grayi* and *L. murrayi* be transferred to a new genus, *Murraya*, as *M. grayi* and *M. grayi* subsp. murrayi, respectively. The moles percent G+C content of the DNA

of the 19 strains studied varied from 37 to 39, except for that of *L. denitrificans*, which was 56. An extensive numerical taxonomic survey (193 strains, 143 unit characters) was performed on 49 *Listeria* strains, as well as on representatives of the genera *Erysipelothrix*, *Brochothrix*, *Lactobacillus*, *Streptococcus*, *Corynebacterium*, and *Kurthia* (Wilkinson and Jones, 1977). The present taxonomic position of the genus *Listeria* as concluded from these numerical taxonomic and chemical studies, as well as the more recent DNA homology and 16S rRNA cataloging results (Wilhems and Sadow, 1977), is as follows:

(i) it includes the species *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, *L. grayi*, and *L. murrayi*;

(ii) *L. denitrificans* is excluded from the genus and transferred to a new genus, *Jonesia*, as *J. denitrificans*; and

(iii) the genus is closely related to the genus *Brochothrix*; both of these genera occupy a position between *Lactobacillus* and *Bacillus* and are more distantly related to *Streptococcus*, *Lactococcus*, *Enterococcus*, *Staphylococcus*, *Kurthia*, *Gemella* and *Erysipelothrix*.

1.2.3 Antigenic Structure

The biochemistry of the cell structure of *L. monocytogenes* and other *Listeria* spp. was studied by Fiedler (1988), who proposed a macromolecular model of the organization of the *Listeria* cell wall.

Electron micrographs of the cell wall showed it to be that typical of gram-positive bacteria, i.e., a thick homogeneous structure surrounding the cytoplasmic membrane and without the outer membrane characteristic of gram-negative bacteria. Isolated dry cell walls are composed of about 35% peptidoglycan, consisting of cross-linked meso-diaminopimelic acid. The remaining carbohydrate consists of cell wall teichoic acids, which are polymers covalently linked to a specific site on the peptidoglycan. They are usually composed of

glycerol or ribitol, neutral sugars, N-acetylamino sugars, and phosphate. Structurally, two types of cell wall teichoic acids exist amongst *Listeria* serotypes. In the first, ribitol residues are covalently linked by phosphodiester bonds between C-1 and C-5. and are sometimes found with N-acetylglucosamine substituted at C-2; this type is found associated with serotypes 1/2a, b, and c, 3a, b, and c, and 7. In the second, N-acetylglucosamine is integrated into the chain; this type is found associated with serotypes 4a, b, and d. *Listeria* cell walls also consistently contain lipoteichoic acids, in which a glycolipid moiety, such as a galactosyl-glucosyl-diglyceride, is covalently linked to the terminal phosphomonoester of the teichoic acid. This lipid region anchors the polymer chain to the cytoplasmic membrane. These lipoteichoic acids resemble the lipopolysaccharides of gram-negative bacteria in both structure and function, being the only amphipathic polymers at the cell surface. The serovars of *L. monocytogenes* (Table n.2) were classified by Paterson (1940) and later modified by Seeliger (1958) and Donker-Voet (1972). A revision has recently been proposed by Garcia et al. (1990), who found factor IX in some strains of serovar 4b.

Designation		O antigens										H antigens		
Paterson (322)	Seeliger (372) and Donker-Voet (105)													
1	1/2a	I	II	(III)								A	B	
	1/2b	I	II	(III)								A	B	C
2	1/2c	I	II	(III)									B	D
3	3a		II	(III)	IV							A	B	
	3b		II	(III)	IV					(XII)	(XIII)	A	B	C
	3c		II	(III)	IV					(XII)	(XIII)		B	D
4	4a			(III)		(V)		VII		IX		A	B	C
	4ab			(III)		V	VI	VII		IX	X	A	B	C
	4b[a]			(III)		V	VI					A	B	C
	4c			(III)		V		VII				A	B	C
	4d			(III)		(V)	VI		VIII			A	B	C
	4e			(III)		V	VI		(VIII)	(IX)		A	B	C
	7			(III)							XII XIII	A	B	C

Table n.2: Serovars of *L. monocytogenes*

1.2.4 Mechanisms of Virulence

Many factors affecting the pathogenicity of *L. monocytogenes* its capacity for intracellular growth, iron compounds, catalase and superoxide dismutase, surface

components, hemolysins-have been proposed over the years, indicating that its virulence is multifactorial. The virulence of the organism may be affected by its growth temperature.

Growth of *L. monocytogenes* at a reduced temperature (4°C) increased its virulence in intravenously inoculated mice, although it did not seem to affect mice which had been infected orally. This phenomenon may increase the virulence of the organism in refrigerated foods (Farber and Peterkin, 1991).

1.2.5 Iron Compounds

Iron compounds reduced the dose that killed 50% of mice and improved the in vitro growth of the organism, suggesting a possible involvement of host iron metabolism in the infection process. The synthesis of the *L. monocytogenes* hemolysin increases with decreased iron concentration in the growth medium, perhaps with the result in vivo of increased lysis of erythrocytes as a source of iron. Conversely, as discussed below, superoxide dismutase activity is increased by a higher iron concentration in the medium. A protein of ca. 10,000 Da present in *L. monocytogenes* culture supernatants was found to mobilize iron from transferrin. It requires NADH, flavin mononucleotide, and Mg2' as cofactors. The organism binds Fe(II) and also ferric citrate and does not take iron up from ferric ferroxamine, ferric EDTA, or FeCl3. This suggests that iron is acquired principally as the ferrous ion, but that a citrate-inducible uptake system also exists (Farber and Peterkin, 1991).

1.2.6 Attachment and Intracellular Growth

Many pathogenic bacteria have the ability to invade host tissues by inducing their own endocytosis, with subsequent transport across normally protective barriers. This phenomenon, called parasite-directed endocytosis, seems to be operative in the attachment and entry of *L. monocytogenes* into intestinal cells and macrophages (McGeeet al., 1988; Racz et al., 1972).

Endocytosis was demonstrated with the human colon carcinoma cell line Caco-2,

which expresses enterocytic differentiation (Gaillard et al., 1987).

In the presence of cytochalasin D, a drug which inhibits microfilament function and hence endocytosis, bacterial entry was inhibited. However, electron micrographs showed the presence of the bacteria inside vacuoles. In contrast, non virulent *Listeria* spp. were not able to induce their own phagocytosis. The presence of a parasite-directed endocytosis of the organism in a mouse embryo fibroblast cell line was confirmed by Kuhn et al. (1988). The uptake of a virulent, hemolytic strain of the organism was inhibited by cytochalasin B. Strains of other *Listeria* species, including the hemolytic a virulent *L. seeligeri* and the strongly hemolytic *L. ivanovii*, did not penetrate the fibroblast cells, even though *L. ivanovii* is pathogenic in mice. A virulent strain of the organism which bound to the cells of a hepatocarcinoma cell line having a well-characterized ct-D-galactose receptor was found to possess a surface ct-Dgalactose residue (Cowart et a., 1990). This residue was lacking in two non virulent strains. The binding was abolished by pretreatment of the cell line with the sugar or with neuraminidase. The authors proposed that the mechanism of attachment of virulent *L. monocytogenes* cells to eucaryotic cells is mediated by the interaction of the surface sugar in the microbial cell with the eucaryotic galactose receptor. The entry of the organism into macrophages does not seem to depend on listeriolysin 0. Lack of listeriolysin synthesis in transposon-induced non hemolytic (Hly-) mutants of *L. monocytogenes* did not reduce the entry of these organisms into mouse peritoneal macrophage cells, although their subsequent survival was reduced significantly (Kuhn et al.,1988). The Hly- mutants were demonstrated to be a virulent in the mouse pathogenicity test, in contrast to the parent strain and the Hly+ mutant. Although the Hly- mutants were taken up by the mouse spleen cells, they failed to multiply and were eliminated from the animals within 1 day (Katharius et al., 1987). Kuhn et al. (1988) concluded that the hemolysin is required for the intracellular survival of the organism, but not its initial entry. Later, Kuhn and Goebel (1989) identified a major

extracellular protein apparently involved in the entry of the organism. Hly- mutants lacking this 60,000-Da protein (p60) lost their ability to invade mouse fibroblast cells and formed long chains of bacterial cells. These disaggregated to normalsized single cells, which again showed invasiveness, when incubated with p60 at 37°C. Laboratory strains of the organism which had been stored on synthetic media showed a variable ability to invade intestinal epithelial cells. This invasive ability can be enhanced by animal passage. The requirement of hemolysin for intracellular growth was confirmed by Portnoy et al. (1988), who developed transposon Tn9O6 mutants which were nonhemolytic, lacked a secreted 58,000-Da protein, and were a virulent. These mutants were defective in intracellular growth. Revertants were hemolytic, secreted the 58,000-Da protein, were virulent, and were able to grow intracellularly. This intracellular growth was demonstrated in cell lines of mouse bone marrow macrophages J774, primary mouse fibroblasts CL7, and human epithelial cells Henle 407. Intracellular survival and growth of *L. monocytogenes* were demonstrated by Mackaness (1962), using electron microscopy. There are two aspects of intracellular survival-the virulence of the *L. monocytogenes* strain and the state of activation of the macrophages.

Among the virulence factors, secretion of the hemolysin seems to be crucial for growth of *L. monocytogenes* in host tissues. Following phagocytosis of the organism, the membrane surrounding the phagosome undergoes cytolysis, presumably mediated by the hemolysin, allowing growth within the cytoplasm (Portnoy et al., 1988). Within 2 h of infection, actin filaments coat the *Listeria* cells and then become reorganized to form polar tails, which seem to be associated with intracellular movement and intercellular spread.

Nonvirulent mutants of *L. monocytogenes* did not move intracellularly, although actin polymerization was induced. The actin coat was not reorganized, and the bacterial cells did not spread. The use of the bacterial protein synthesis inhibitor chloramphenicol showed that the material inducing actin assembly is secreted by the *Listeria* cell, and not by the

macrophage. Thus, the infecting organism can spread from cell to cell, apparently bypassing the humoral immune system of the host. The organism has even been found within cell nuclei, where it may be protected from cellular enzymes. The organism seems to stimulate host cell actin assembly in a directional manner, leading to its rapid movement through the cytoplasm.

Cytochalasin D treatment prevents the formation of the actin filaments, and bacterial intra- and intercellular movement stops. Donnelly et al. (1987) developed a useful in vitro system to study intracellular growth, by using bovine phagocytes harvested from mastitic milk. Once ingested, the organism was resistant to killing by the phagocytes. Czuprynski et al. (1989), on the other hand, demonstrated the ability of bovine phagocytes (blood polymorphonuclear leukocytes, monocytes, and milk leucocytes) to ingest the organism, produce an oxidative response, and kill the intracellular listeriae. When tested with human neutrophils, *L. monocytogenes* F5380, Scott A, Murray B, and EGD were more resistant to killing when grown at 4 than at 37°C.

This decreased killing did not appear to be related to poor ingestion by the neutrophils (Farber and Peterkin, 1991).

1.2.7 Defense against Activated Phagocytes

Facultative intracellular pathogens such as *L. monocytogenes* must possess means of overcoming the nonspecific immune responses mediated by activated phagocytes. The organism survives inside nonactivated cells of the mononuclear phagocyte system, but is killed in activated macrophages. The formation of a toxic free radical, superoxide (01-), is an important part of the sequence in the phagocytic killing of bacteria. The presence of bacterial superoxide dismutase offers a defense against this toxic molecule and hence is a possible virulence factor of the organism.

The virulence of five strains of the organism as measured by the 50% lethal dose was also lower in catalase positive strains, and the 50% lethal dose roughly paralleled the

superoxide dismutase activity. Increased 02 consumption and catalase activity during successive passages of *L. monocytogenes* (strains 1/2a and 4b) in monkey kidney epithelial cells was correlated to intracellular multiplication of the bacterial cells. Dallmier and Martin (1988) demonstrated that the strains with the highest catalase activity also had the highest superoxide dismutase activity.

Bortolussi et al. (1987) studied the sensitivity of *L. monocytogenes* to oxidative antibacterial agents such as the hydroxyl radical, H202, and hypochlorous acid, which may be present in phagocytic cells. They found that the organism is resistant to these products during log phase growth when the catalase concentration is higher than in the stationary phase, perhaps contributing to its intracellular survival (Farber and Peterkin, 1991).

1.2.8 Hemolysins

The hemolysin of *L. monocytogenes* is recognized as a major virulence factor, and its secretion is essential for promoting the intracellular growth and T-cell recognition of the organism.

The hemolysin, designated listeriolysin 0 (analogous to streptolysin 0 [SLO]) was first isolated from *L. monocytogenes* culture supernatants and shown to be a sulfhydryl (SH)-activated cytolysin, sharing properties with other proteins of this group, such as SLO. Hof and Hefner (1988) demonstrated that only *L. monocytogenes* and *L. ivanovii,* both of which possess a ,-hemolysin, were able to multiply within mice after intravenous injection.

All strains of *L. innocua* and *L. welshimeri,* both non hemolytic species, were avirulent. *L. seeligeri,* however, is weakly hemolytic but avirulent. In a recent report on the hemolysins of the genus *Listeria,* it was shown that all strains of *L. monocytogenes* examined produced listeriolysin 0 (molecular mass, 60,000 Da). *L. ivanovii* and *L. seeligeri* strains also produced thiol-dependent exotoxins, at about 10 times and 1/10 the level respectively, as that found in *L. monocytogenes.* Hemolysin was not found in *L. innocua* or *L. welshimeri* strains.

A second hemolysin, present in some *L. monocytogenes* strains and immunologically distinct from listeriolysin 0, was first reported by Parrisius et al. (1986). Two types of hemolysins were identified in clones from an *L. monocytogenes* gene bank constructed in Escherichia coli. The first was a 23,000-Da protein, possibly the CAMP factor, which was not SH activated and did not cross-react with antilisteriolysin or anti-SLO antibodies. The other crossreacted with anti-SLO, but activation by SH groups was not tested. Vicente et al. (1985; 1987) identified 12 recombinants expressing P-hemolytic activity after the cloning of *L. monocytogenes* genomic DNA into E. coli host cells. Deletions of one of these clones resulted in the preparation of a stable hemolytic clone with an 8.3-kbp insert. Clones whose hemolytic activity was detectable only after sonication were prepared by further subcloning. Gel filtration of the sonicated preparation led to the elution of two peaks of hemolytic activity, corresponding to proteins of 22,000 and 48,000 Da, suggesting the existence of two hemolysins. Genetic evidence of an additional hemolytic determinant to hlyA was obtained from hemolytic recombinants of an *L. monocytogenes* gene bank by restriction mapping and hybridization to Southern blots. *L. ivanovii* also secretes two cytolytic factors. One is a thiol-activated hemolysin of 61,000 Da, termed ivanolysin 0, and the other is a 27,000-Da sphingomyelinase C found to be involved in the activity of the CAMP factor (Farber and Peterkin, 1991).

1.2.9 Biochemistry of hemolysin

Most of the work on the purification and characterization of listeriolysin has been done by Seeliger's and Goebel's groups at the University of Wurzburg. Listeriolysin from *L. ivanovii* was isolated in its membrane-associated form and shown to possess properties similar to those of SLO.

The listeriolysin within the membranes generated large transmembrane pores, which are probably related to the cytolytic properties of this molecule. Listeriolysin isolated from

these membranes, with a monomeric molecular mass of 55,000 to 60,000 Da, was used as the antigen for the preparation of rabbit polyclonal antibodies. Immunoblots of membrane-bound listeriolysin of 28 ß-hemolytic *L. monocytogenes* strains with these antibodies led to the unexpected finding that only 2 strains produced a positive reaction. This suggested the production of at least two immunologically distinct hemolysins by human pathogenic *Listeria* strains. The authors proposed that the SLO-related toxin (listeriolysin 0) be named a-listeriolysin and that the other(s) be named P-listeriolysin.

Listeriolysin 0 was purified to homogeneity from a medium containing peptone and yeast extract, which had been treated with a chelating resin (Chelex). The resulting 20-fold increase in toxin production was presumably due to the very low iron concentration resulting from the use of the chelate. The lytic activity of this protein (molecular mass, 60,000 Da) was inhibited by cholesterol and oxidizing agents, was activated by thiols, and showed antigenic cross-reactivity with SLO. The in vitro inactivation by cholesterol is thought to be due to competitive binding with the membrane-binding site of listeriolysin 0, in common with other SH-activated cytolysins.

There is evidence that different domains are involved in cytolytic activity and cholesterol binding. A truncated listeriolysin 0 lacking a 48-amino-acid C-terminal oligopeptide lacked hemolytic activity but still bound to the membrane receptor cholesterol.

Listeriolysin 0 differed from these toxins (e.g., pneumolysin, perfringolysin, alveolysin, SLO), however, in that its optimum pH was 5.5 and it was inactive at pH 7.0. Its activity was restored by again lowering the pH to 5.5. The authors suggest that this optimization of its lytic activity in an acidic environment such as exists in macrophages might promote intracellular growth of the organism. It has been demonstrated that under conditions of stress such as heat shock or oxidative stress, at least five heat shock proteins are coinduced with listeriolysin 0 in *L. monocytogenes* strains, but not in the other *Listeria* species.

Hemolysins from *L. monocytogenes* and *L. ivanovii* were characterized and partially sequenced (Goebel et al., 1988). They showed the characteristics typical of listeriolysin 0, namely activation by SH reagents, inhibition by cholesterol, cross reactivity with SLO antibodies, and molecular mass of 58,000 Da. In *L. ivanovii*, a protein with a molecular mass of 24,000 Da copurified with this protein and was separated from it by gel filtration in the presence of SDS. This smaller protein was strongly hemolytic against sheep erythrocytes when combined with culture supernatants from *Rhodococcus equi*, and not with supernatants from *S. aureus*. It may therefore represent the *L. ivanovii* CAMP factor. Determination of the N-terminal sequences of the 58,000- and 24,000-Da proteins showed no homology with the N termini of other SH-activated cytolysins. Listeriolysin 0 is secreted by all virulent strains of *L. monocytogenes*, but it could not be demonstrated in the supernatants of *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. grayi*, and *L. murrayi* by cross reaction with anti-listeriolysin 0 or anti-SLO antibodies (Goebel et al., 1988; Farber and Peterkin, 1991).

1.2.10 Genetics of Hemolysin

In further attempts to identify the role of hemolysin in the virulence of *L. monocytogenes*, transposon mutagenesis was used to inactivate the genetic determinant for hemolysin production.

Three nonhemolytic (Hly-) transconjugants and a hemolytic (Hly+) transconjugant were chosen from mutants produced by using transposon Tn9O6 in matings with a serotype 1/2a *L. monocytogenes* strain. The nonvirulent Hly- mutants either lacked the 58,000-Da extracellular protein (listeriolysin 0) or produced a truncated protein of 49,000 Da. Hly+ revertants regained the hemolytic phenotype, virulence, and production -of the 58,000-Da protein (Katharius et al., 1987).

Gene complementation studies were used by Cossart et al. (1944) to exclude the hypothesis that a polar effect of the transposon insertion was causing the production of the

Hly- mutants. A transposoninduced Hly- mutant was generated, and the insertion was localized in hlyA by DNA sequence analysis. The mutant was transformed with a plasmid carrying only hlyA to a stable, hemolytic phenotype identical to that of the wild type.

Transposon mutagenesis with Tn1545 resulted in the production of an Hly- mutant which produced a 52,000-Da SH-dependent hemolysin, lacking the COOH-terminal portion of listeriolysin 0, with an abnormal regulation by iron.

It was not possible to demonstrate a direct relationship between virulence and the amount of hemolysin produced. By using a hyperhemolytic (Hly++) strain, which had a titer of 96 hemolytic units compared with 12 units in the parent strain, Kathariou et al. (1988) demonstrated increased levels of production of a protein of 58,000 Da in the Hly+ + strain.

Despite the increased hemolysin production, virulence-as measured by the number of cells required to infect, number of cells isolated from the spleen during infection, and time course to death-remained unaffected.

Transposon mutagenesis has also been used to prepare Hly- mutants useful in studying the sequence of the hemolysin determinant of this organism. The conjugative 26-kb transposon Tn1545, encoding kanamycin, tetracycline, and erythromycin resistance, was transferred with a frequency of 10-8 to *L. monocytogenes* NCTC 7973, a hemolytic virulent strain. The resulting nonhemolytic mutant also was nonvirulent to mice. The ability to infect mice and to grow in spleen and liver cells was restored by spontaneous loss of the transposon.

The Hly- mutant secreted a truncated protein of 52,000 Da, which was detected by immunoblotting with an antiserum raised against listeriolysin 0, thus demonstrating the insertion of TnJS45 in the structural gene for this protein. The insertion region of the transposon was then cloned and sequenced. The transposon had inserted in an open reading frame (ORF). The deduced amino acid sequence of this ORF revealed homology with SLO and pneumolysin. DNA-DNA hybridization showed that *L. monocytogenes* is the only

Listeria species in which this hlyA sequence is presen. The hlyA gene was cloned into *Bacillus subtilis* host cells, which then expressed hemolysin and were able to grow intracellularly. The change of a common bacterium into a virulent organism by cloning of an *L. monocytogenes* hemolysin determinant was also observed in the ß-hemolytic clones of an *L. monocytogenes* gene bank in *E. coli*. These clones were lethal to mice, whereas nonhemolytic clones were not. The significance of the hemolysin is an essential virulence factor of the organism and the only bacterial gene product known to be absolutely required for intracellular growth. The hlyA gene region has been studied to learn how the gene is regulated and whether silent copies of it exist in nonhemolytic species. The 5' adjacent regions have sequences which show homology to *L. ivanovii* and *L. seeligeri*, but the downstream regions appear specific to *L. monocytogenes*. A spontaneous 450-bp deletion located 1.6 kbp upstream from an intact hlyA gene resulted in the production of a nonhemolytic, avirulent mutant, indicating an area involved in controlling the expression of the gene. The mutant had its hemolytic activity restored by the introduction of a recombinant plasmid expressing a 27-kDa protein. The gene expressing this polypeptide, prfA, positively regulates transcription of the hlyA gene. Sequence analysis of the gene region revealed the presence of two ORFs. ORF D is located downstream from hlyA, and ORF U is located upstream and in the opposite direction; hlyA and ORF U are transcribed in opposite directions from promoters which are adjacent. These two promoter regions are separated by a 14-bp palindromic sequence. This palindrome was also found upstream of the ORF D promoter, suggesting that all three genes are similarly regulated. The ORF located immediately downstream of hlyA was sequenced, and its putative amino acid translation product was deduced. The amino acid sequence was highly similar to that of a family of secreted metalloproteases, of which the *Bacillus thermolysin* is the prototype. The gene, mpl, was species specific to *L. monocytogenes* (Farber and Peterkin, 1991).

1.2.11 Biofilm

The term biofilm was created to describe the sessile form of microbial life, characterized by adhesion of microorganisms to biotic or abiotic surfaces, with consequent production of extracellular polymeric substances (Nicolaev and Plakunov, 2007). Microbial adhesion and biofilms are of great importance for the food industry and occur on a high variety of food contact surfaces (Marquest et al., 2007). In food processing industries, surfaces of stainless steel equipment and utensils are recognized as the major microbial adhesion and biofilm formation sites (Chmielewski and Frank, 2003). Surface-adhered microbial cells contaminate food products during the processing.

This ability of transferring microorganisms through contact with food is termed biotransfer potential. Viable microorganisms adhered to surfaces will present a biotransfer potential even if the number of present cells is low or if it varies within a particular area (Midelet and Carpentier, 2004). Several microorganisms are capable of participating in the adhesion processes and biofilm formation. In the food industry, these microorganisms can be classified as spoilage and pathogenic. Among the pathogenic microorganisms, *L. monocytogenes* is one of the most outstanding. This bacterium is an emergent pathogen of ubiquitous distribution in nature, surviving under adverse environmental conditions.

Developing in different substrates, it is capable of colonizing biotic and abiotic surfaces (Gandhi and Chikindas, 2007; Pan et al., 2006). Studies have shown the capacity of *L. monocytogenes* to persist in the environment for years (Lunden et al., 2002; Senczek et al., 2000). Researches on the presence of *L. monocytogenes* on the surface of equipment and utensils, report its occurrence in meat and dairy processing industries (Chambel et al., 2007; Cruz et al., 2008; Lopez et al., 2008). According to Chae *et al.* (2006), the occurrence of foodborne outbreaks as well as sporadic cases caused by this bacterium, can be attributed to its increased ability of surviving in food processing environments through biofilm formation.

Thus, the high risk of food contamination by sessile cells of *L. monocytogenes*, with consequent infection dissemination makes it necessary to develop control strategies aimed to delay, reduce, or even eliminate the accumulation of this bacterium on industrial surfaces.

According to Oliveira *et al.* (2007), it has been recognized that a greater understanding of the interaction between microorganisms and food processing surfaces is required to control these problems. The association of *L. monocytogenes* to surfaces has been mainly analyzed in the laboratory. However, such studies still need to be standardized, since they are difficult to carry out *in situ*, in food processing environments. The difficulty found in investigating microbial biofilms in nature and the precarious experimental conditions found in most laboratories led to the development of different experimental models of biofilm formation *in vitro*. These systems allow the study of biofilms under defined and controlled conditions and are necessary for the execution of reproducible experiments (De Oliveira et al., 2010).

1.2.12 ListexTM P100 for efficient biocontrol

Many foods can serve as vehicles for this pathogen, *Listeria* was often isolated from ready-to-eat (RTE) foods, such as milk and cheeses, cold-cut meats, smoked fish, seafood and vegetables or, in general, all those foods that are consumed without a final bactericidal processing step. Since the preservation methods applicable to food RTE often seem insufficient to prevent contamination and growth of *Listeria*, new approaches are needed.

Bacteriophages are natural enemies of bacteria; they are specific species and they don't interfere with the indigenous microflora of the food. They are widely distributed in the environment, in food and therefore they should not harm the consumers' health. Due to these characteristics bacteriophages are candidates to be a solution for the reduction and control of *L. monocytogenes*.

The concept of fighting pathogens in food by means of phages can be addressed at all stages of production in the classic "from farm to fork" approach throughout the entire food chain:

- To prevent or reduce colonization and diseases in livestock (phage therapy).
- To decontaminate carcasses and other raw products, such as fresh fruit and vegetables, and to disinfect equipment and contact surfaces (phage biosanitation and biocontrol).
- To extent the shelf life of perishable foods (biopreservation).

Bacteriophages should also be considered in hurdle technology in combination with different preservation methods (Figure n.1) (Erginkaya et al., 20011; Keary et al., 2013; Monk et al., 2010).

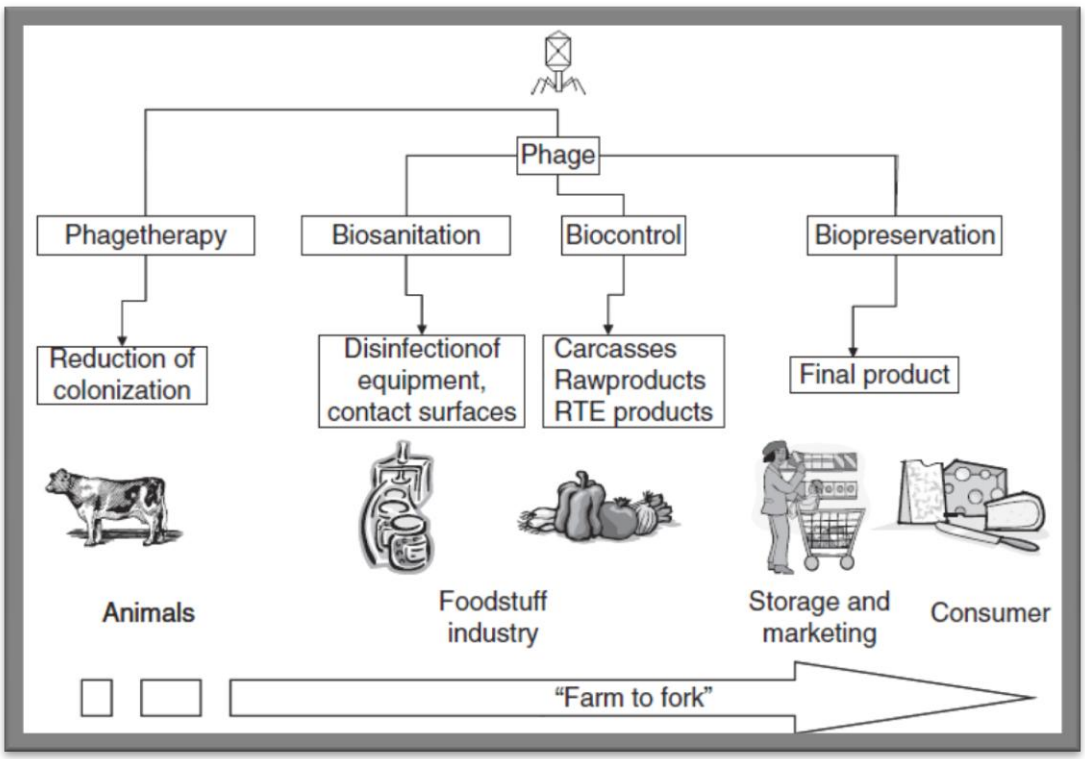


Figure n.1: Functions of bacteriophages

Several products based on bacteriophages are commercialized. In August 2006, the Food and Drug Administration (US FDA, 2006) approved the use of a preparation consisting

of a pool of six different bacteriophages purified to be used as an antimicrobial agent against *L. monocytogenes* in ready-to-eat food (RTE), in meat and in poultry. The product is able to act against 170 different strains of *Listeria monocytogenes*; studies in animals have shown that its use does not cause problems for the consumer's health. The document marks the first time in which the FDA has regulated the use of bacteriophages in food products, classifying them as additives. In the food industry, the EBI (European Bioinformatics Institute) Food Safety recently marked ListexTM P100 for controlling *Listeria monocytogenes*, this product contains the bacteriophage P100. Various studies have tested the use of ListexTM P100 on different types of products and in different environmental conditions. In all the conditions it is confirmed the effectiveness of the bacteriophage for the reduction and control of *L. monocytogenes* (Marsden et al., 2011; Soni and Nannapapeni, 2010). The aim of the study conducted by Chibeu et al. (2013) was to verify the effectiveness of the commercially available anti-*Listeria* phage preparation LISTEX™ P100 in reducing *Listeria monocytogenes* on ready-to-eat (RTE) roast beef and cooked turkey in the presence or absence of the potassium lactate (PL) and sodium diacetate (SD) chemical antimicrobials. Sliced RTE meat cores at 4 and 10°C were inoculated with cold-adapted *L. monocytogenes* to result in a surface contamination level of 10^3cfu/cm^2. LISTEX™P100 was applied at 10^7 pfu/cm^2 and samples taken at regular time intervals during the RTE product's shelf life to enumerate viable *L. monocytogenes*.

The effectiveness of the product LISTEX™P100 is proven because all the samples had a considerable reduction in *L. monocytogenes*. The phage reduces considerably the pathogen both on products stored at 4 ° C than at 10 ° C for all 28 days of shelf life.

During the storage period of the cooked turkey and roast beef samples, it was possible to recover infective phage LISTEX™P100 particles at a concentration similar to that initially inoculated on the sample on day 0. This was proof that the phage remained stable in these

food matrices during the entire storage period. It has been suggested that the re-growth of bacterial cells in phage-treated food can be attributed to inability of phage particles to reach the bacterial targets in the food matrix leading to the bacteria multiplying in protected niches.

In conclusion, the phages such as LISTEX^{TM}P100 in the presence of chemical inhibitors PL and SD provide an effective hurdle which can be used to enhance safety in RTE roast beef and cooked turkey contaminated with *L. monocytogenes* (Chibeu et al., 2013).

1.3 EUROPEAN FOOD LAW

The spread of the *L. monocytogenes* almost ubiquitous, however, connected to a reduced number of cases of disease humans, has led, as a result of extensive studies which took into account the costs of support to get to ensure the absence of the pathogen in the food chain than the public health benefits to identify a threshold of dangerousness. The Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) issued an opinion on *L. monocytogenes*. which has recommended it be an objective concentration of the pathogen in food below 100 cfu/g. The Scientific Committee on Food agreed with these recommendations in the opinion of 22 June 2000. The scientific indications have become standard reference for all countries of the EU, with the enactment by the European Commission's Regulation (EC) of 15 November 2005 n. 2073 and subsequent amendments on microbiological criteria for food products. This Regulation aims to ensure food safety for consumers through the control of certain microbiological criteria.

These criteria are divided into two categories:

- Food safety criteria, which define the acceptability of a food or a game products are applicable to products placed on the market. If a food does not meet the microbiological requirements laid down, the game can't be considered safe for human consumption and it will be necessary to withdraw or recall the product, as determined by Regulation (EC) No. 178/2002;

- Process hygiene criteria, which are parameters (values of microbial specific or generic) that serve to define the critical limits of food business operators and whether the process works hygienically acceptable or not. These criteria do not usually apply to finished products in the markets but they set only indicative values to be considered for the evaluation of the process, exceeded which it is necessary to apply the corrective action, to maintain the hygiene of production.

Although microbiological criteria should be used by food operators as management practices to guarantee the food safety, they are also applied to samples for Official Controls.

Particular importance is covered by the information included in Annex I to Regulation (EC) No. 2073/2005 and subsequent amendments (frequency and methods of sampling, methods of analysis, limits of acceptability, actions subsequent to obtaining non-compliant results). Article 5, paragraph 4 of Regulation (EC) 2073/2005 also states that: "If the aim of the testing is to specifically assess the acceptability of a certain batch of foodstuffs or a process, the minimum condition required is respect sampling plans in Annex I". *L. monocytogenes* is discussed in Chapter 1.

The detection of *L. monocytogenes* according to the food safety criteria is expected only in the following food categories:

1.1 RTE foods or infants and for special medical purposes;

1.2 RTE foods suitable for the growth of *L. monocytogenes*, other than for the infants and for special medical purposes;

1.3 RTE foods not suitable for the growth of *L. monocytogenes*, other than those for infants and for special medical purposes.

For the purposes of this Regulation:

- "RTE foods means food intended by the producer or the manufacturer for human consumption direct, without the need for cooking or other treatment to eliminate or reduce bacteria to an acceptable level;

- Infant formulas means food specifically intended for infants, as defined by Directive 91/321 / EEC;

- Foods for special medical purposes: dietary foods for special medical purposes, as defined Directive 1999/21 / EC. "

The acceptability limits are different depending on whether it is:

- "RTE foods that are able to support the growth of *Listeria monocytogenes*" all foods with a shelf-life

- or more than 5 days and with
- or pH > 4.4;
- or Aw > 0.92;
- or pH > 5.0 in combination with Aw > 0.94;

as indicated in note. 8, Chapter 1 of Annex I to Regulation (EC) 2073/2005 as amended, or

- "RTE foods NOT to support the growth of *L. monocytogenes*" the products with a shelf-life

- or less than 5 days;
- or pH ≤4,4;
- or Aw ≤0,92;
- or in combination with pH ≤5,0 Aw ≤0,94.

Other types of products can also belong to this category, provided there is a scientific justification, as indicated in note. 8, Chapter 1 of Annex I to Regulation (EC) No. 2073/2005 and Subsequent amendments.

The criteria are applied only to products placed on the market during their shelf-life. "Shelf-life" means "the period corresponding to the time preceding the date of minimum durability or the expiration date, as defined respectively in Articles 9 and 10 of Directive 2000/13 / EC."

These food safety criteria are not applied to some products that exclude the ability of growth of the pathogen, even if they are ready for consumption without further cooking. These products are also identified in Note 4 to the bottom of Chapter 1 of Annex I to Regulation (EC) 2073/2005 and subsequent amendments.

The food safety criteria for the detection of *L. monocytogenes* are not required for the following foods:

- subjected to heat treatment or other processing that guarantee the effective elimination of *L. monocytogenes*, when recontamination is not possible after this treatment (for example, the products subjected to heat treatment at the time of final packaging);

- Fruits and vegetables that are fresh, uncut and unprocessed, excluding sprouted seeds;
- Bread, biscuits and similar products;
- Water, soft drinks, beer, cider, wine, spirits and similar products bottled or packed;
- Sugar, honey and confectionery, including products based on cocoa and chocolate;
- Live bivalve mollusks;
- Salt. "

Where the frequencies of sampling and the analysis are not specified, the food business operator has to establish them on the basis of its system of hazard prevention and risk management (self-control system), and he has also to provide an explanation regarding to their efficacy according to the guarantee of security required by law. Sampling plans conducted in accordance with Regulation (EC) No. 2073/2005 and subsequent amendments must be included in the procedures for validation and verification of the plan of self-control of food businesses and the sampling frequency, where not provided by Annex I, must be defined in the context of procedures themselves. The number of sampled units can be reduced if the operator can document the effective application of procedures based on HACCP principles in accordance with Article. 5, paragraph 3 of the Regulation (EC) No. 2073/2005 and

subsequent amendments excluding the cases where the food business operator should evaluate the acceptability of a batch of products and the dose of histamine in certain fishery products.

Article 5, paragraph 2 of Regulation (EC) No. 2073/2005 also requires that "Food business operators manufacturing ready-to-eat foods, which may pose a *L. monocytogenes* risk for public health, shall sample the processing areas and equipment for *L. monocytogenes* as part of their sampling scheme".

The operator must have procedures for the handling of the sampled units and for the sending to the laboratory for analysis.

According to art. 9 of Regulation (EC) No. 2073/2005, the results of all the samples must be assessed by a "trend analysis" in order to take appropriate action if the food business operators observe a trend towards unsatisfactory results.

"When the results of testing against the criteria are unsatisfactory, the food business operators shall take corrective actions defined in their HACCP-based procedures and other actions that are necessary to protect the health of consumers. In addition, they shall take measures to find the cause of the unsatisfactory results in order to prevent the recurrence of the unacceptable microbiological contamination. Those measures may include modifications to the HACCP-based procedures or other food hygiene control measures in place (procedures GMP, GHP, SOPs, HACCP). When testing against food safety criteria provides unsatisfactory results, the product or batch of foodstuffs shall be withdrawn or recalled in accordance with Article 19 of Regulation (EC) No 178/2002" (art. 7, paragraph 2 of Regulation (EC) no. 2073/2005).

Regulation (EC) No. 2073/2005 and subsequent amendments does not provide for the food business operators obligation to assess the presence of *L. monocytogenes* in foods "not ready to eat" and therefore it would be appropriate to limit the detection of the pathogen to foods that are intended to cooking or not bearing the label information relating to this

requirement during the Official Controls. Criteria provided from O.M. 07.12.93 are applied for products to be consumed after cooking.

The Ordinance of the Ministry of Health of 10.11.1978, to the point V (added Ministerial Ordinance 7.12.1993), provides the detection of *L. monocytogenes* in bulk or prepackaged foods destined to be consumed after cooking or that bear on the packaging the dictate to be eaten after cooking (except milk and milk products). Specific categories identified for the quantitative research of *L. monocytogenes,* through MPN method, are: raw foods not submitted to thermal process, frozen foods and cooked or pasteurized foods (Ce.I.R.S.A., 2014).

1.3.1 Official Controls

The "sampling" is one of the most important instruments of Official Control. Despite the European Commission, by Regulation (EC) No. 882/2004 as amended, it was proposed to harmonize the system of controls on food safety between the different European Countries, large differences remain between Countries.

In Italy, the situation is very complex because the Official controls are planned by Regions and Provinces with consequent different peculiarities often linked to local factors.

The main differences caused by several interpretation lead to the following models:

- Official Control is limited to verify that the food business operator performs properly the provided samples and that he manages the results;

- The Official Control verifies that the food business operator performs properly the provided samples and that he manages the results by performing some official samples for the assessment of compliance with the food safety criteria set out in Chapter 1 of Annex I to Regulation (EC) No. 2073/2005 and subsequent amendments or other criteria established by the Community or National rules.

Most of the European Countries operate according to the first model, restricting the official sampling only to the risk cases, while Italy, particularly in Piedmont, has chosen the second model.

Therefore, monitoring plans are yearly defined for verification of the expected parameters of the Reg. 2073/2005 involving a number of samples taken for the Official Control and sent to the laboratories of the Institutes of Experimental Animal Disease Prevention (IZS) with jurisdiction for the execution of the analysis.

The monitoring plans generally provide:

- Sampling in the plants under the control of the Authority to confirm and validate the results of self-control plan of the food business operator;

- Sampling in the markets to verify the compliance with the food safety criteria of the products offered for sale.

Most food safety criteria have no problems of interpretation, but the detection and the enumeration of *L. monocytogenes* linked to the type of food (RTE food, suitable or not for the growth of the pathogen) have sometimes created difficulties in assessing the analytical result and in defining the measures, preventive and punitive, by adoption.

It is reported that there are critical issues for the detection of *L. monocytogenes* during the shelf life of food products both using the Reg. n. 2073/2005 because it states that the product should be taken during its commercial life, but it is a difficult concept to be applied to a bulk product which is typically highly perishable and not showing labeling and use by date, and using the OM 07/12 / 93, applicable to products under the control of the producer.

If the sampling is positive to the presence of the pathogen, the Competent Authority will carry out the necessary checks and requests to the food business operators. In the case of product taken from an OSA resident in the territory of the ASL, the Competent Authority must be sure that the operator identifies the causes of the contamination and that he takes

appropriate steps to reduce the risks to the consumer if the product is already marketed and remove the causes in order to prevent a recurrence of the problem. In this phase it is important to focus on the operative practice and not on the documentation and it may be useful to provide guidance on the production process.

However, the documentation regarding the different production phases, the monitoring of critical control points, the traceability and others is very important and the food business operator has to archive the documentation commensurate with the size and the type of plant as established by Regulation (EC) no. 852/2004.

It is also important to note that the HACCP and Good Practices of Hygiene adopted by the food business operator, even when applied in the best way, are able to reduce the risks to the consumer but not to eliminate them (there is no risk "zero" but the acceptable risk). For this reason the European legislation has established precise obligations that require the presence of traceability procedures by which the OAS should prove to be able to withdraw and recall any non-conforming products. Thus, the assessment of the responsibility of the Competent Authority should focus on finding the actual deficiencies that may have caused the accident.

If the product is prepackaged, the main controls have to be done by the Competent Authority in the manufacturing plant, also regarding the assessment of liability according to Article 19 of Law 283/1962 (Ce.I.R.S.A., 2014).

1.3.2 Information for the Authority Regarding Contaminated Product

Given the complexity of the regulatory framework, it is extremely important that the Competent Authority in the presence of non-compliance provide to the Judicial Authority all relevant information to the later stages of the proceedings. In particular you must indicate in the notes attached to the note:

- The characteristics of the food: the pH and Aw, taken from the test report of the official control laboratory or documents from the manufacturer, or even on bibliographic basis; these data will allow, with the expiration date, or the time of minimum durability an objective classification of the product;

- The instructions provided on the label, on signboards at the point of sale or the statements of the service staff on the intended use and on the shelf life at the store;

- The data may be available from the manufacturer (outcomes of previous samples, other official samples, etc ...) including assessments in the HACCP plan regarding the possibility of the development and growth of *L. monocytogenes* or evidence of shelf-life or challenge test supplied by the manufacturer or by importer after official request from the Competent Authority;

- A final decision of the Competent Authority on the applicable limit and motivations (eg. the food for its characteristics, expire date to 30 days, pH = 6.2 and Aw = 0.97, is a suitable substrate for the growth of the pathogen and the producer doesn't have documentation to guarantee the presence of *L. monocytogenes* is less than 100 cfu/g at the end of shelf-life);

- If the measures taken and the documentation produced by the company are not sufficient in the opinion of the competent authority, the activities resulting from this positivity must be performed with procedures of the Judicial Police (Ce.I.R.S.A., 2014).

1.4 SHELF-LIFE OF RTE FOOD IN RELATION TO L. MONOCYTOGENES

Regulation (EC) No. 2073/2005 (as amended, referred to hereafter as 'the Regulation') includes limits for the number of *L. monocytogenes* in RTE food and requires you to be able to demonstrate these are not exceeded. *L. monocytogenes* must be absent in RTE food intended for consumption by infants or for special medical purposes. Under the Regulation a RTE food or ingredient with a shelf life of less than 5 days is considered to be unable to support the growth of *L. monocytogenes*.

However, in practice since such foods may contain ingredients that support growth of *L. monocytogenes*, in these case you must have evidence to demonstrate that the limit of 100 cfu/g will not be exceeded, otherwise *L. monocytogenes* must be absent.

In addition, Article 14 of Regulation (EC) No. 178/2002 on 'General Food Law' states that "Food shall not be placed on the market if it is unsafe. Food shall be deemed to be unsafe if it is injurious to health or unfit for consumption". Setting shelf life requires taking into full consideration all chemical parameters, all microorganisms in addition to *L. monocytogenes*, and the intended consumer.

1.4.1 Requirements for the Safe Manufacture of RTE Food

The manufacture of RTE food requires a particularly high standard of hygienic preparation.

The following prerequisites must be in place and followed:

1. Good Manufacturing Practices (GMP) and hygiene including:

- Effective equipment cleaning and disinfection systems
- Premises hygiene
- A high standard of personal hygiene
- Ingredients from reputable suppliers

2. Procedures based on Hazard Analysis & Critical Control Point (HACCP) principles, including separation between RTE and non-RTE food (e.g. cooked meat and raw meat) and associated equipment and personnel. A system must be in place to check and review the effectiveness of HACCP based procedures and hygiene, and records kept of these data.

Relevant guidance and Industry Guides will provide further information (Guidance for food business operators, 2010).

1.4.2 Establishing Shelf-Life

The Regulation says that RTE foods must not exceed the limit of 100 cfu/g for *L. monocytogenes* at any point during their shelf life (except those intended for infants or particular medical purposes, which must not contain *L. monocytogenes*). Otherwise *L. monocytogenes* must be absent at the point of manufacture. If you apply the 100 cfu/g limit you must have evidence for each product to show that *L. monocytogenes* does not exceed 100 cfu/g throughout the shelf life. This evidence must be based upon shelf life studies which should initially consist of information on the specific composition for your own product (i.e. physical and chemical characteristics, including packaging) and consultation with relevant scientific literature. If the results of these studies give sufficient confidence that *L. monocytogenes* will not grow in your product no further studies are needed. However, if your results do not give sufficient confidence additional studies will be necessary. Such studies may include one or more of the following:

- Historical data,
- Predictive microbiology,
- Specific laboratory shelf life studies, i.e. durability studies, challenge testing FBOs can collaborate in conducting these studies. FBOs must keep documentation of shelf life studies and verification as part of GMP and HACCP procedures.

Taking each of the above in turn:

a. Product Characteristics and Scientific Literature and Research Data

Product characteristics such as pH, Aw (water activity), salt concentration and/or concentration of chemical preservatives affect *L. monocytogenes* survival and growth within a food, as the way that these products are packed, does and the time and temperature of storage.

You must establish these characteristics for your product as these are important factors in influencing the survival and growth of *L. monocytogenes*. This must be done under the conditions in which your product is normally produced, packed and stored. If you do not have access to your own in-house expertise for this then you should contact research organizations and/or laboratories that can help you to understand and gather the necessary information.

It is important to understand the formulation of your food. In the case of a multicomponent food such as a quiche the highest pH and aw value within the food must be known throughout its shelf life.

Another consideration is whether the food is an emulsion, e.g. mayonnaise, margarine, butter. For these types of foods, Aw and pH measuring will be difficult and will vary throughout the food. Where necessary seek specific expert advice.

Determining the characteristics of your product will then allow you to determine whether *L. monocytogenes* will grow in your product.

Foods are not considered to support the growth of *L. monocytogenes* if:

- pH is less than or equal to 4.4, or
- aw is less than or equal to 0.92, or
- pH is less than or equal to 5.0 with the aw being less than or equal to 0.94

If these parameters are used to demonstrate that the food will not support the growth of *L. monocytogenes* then

• these are critical control points and must be monitored as part of HACCP, and

• further shelf life studies are not required in relation to *L. monocytogenes*

If there is clear scientific evidence that your food cannot support the growth of *L. monocytogenes* the legislated limit of 100 cfu/g throughout shelf life applies.

If scientific evidence is not available, further evidence as set out in the following sections will be necessary to justify the shelf life.

However, the FBO is responsible for the production of safe food under EU law (Guidance for food business operators, 2010).

b. Historical Data

FBOs have a legal obligation under food safety legislation to maintain key records including the safety of foods placed on the market. Historical data comprise records specific to your premises and your foods, built up over a period of time.

Historical data (including end product testing on the day of production and/or end of life) can be used as evidence that a food will not exceed the limit of 100 cfu/g during its shelf life.

Historical data on levels of *L. monocytogenes* in existing RTE foods at the start and/or end of shelf life can be used to assess its growth potential and confirm that the assigned shelf life is appropriate. It can also be applied to similar RTE foods with comparable intrinsic characteristics (pH, aw, microflora, etc.) produced under practically identical conditions. These should be specific to your premises and your foods; however collaboration between FBOs is acceptable under certain circumstances.

Data should include:

• Information from HACCP and monitoring checks, including:

- Process validation, verification and monitoring (e.g. temperature, time, pH and aw)
- Ingredients traceability and microbiological quality testing including for hygiene indicator organisms and/or *L. monocytogenes*
- Sampling for *Listeria* species and appropriate hygiene indicator organisms from processing areas and equipment (to demonstrate the efficacy of factory hygiene and cleaning regimes)
- Final product testing for *L. monocytogenes* for example on the day of production and/or at the end of shelf life to verify effective functioning of the HACCP system and durability verification

• Shelf life evaluation

Detection of *Listeria* species from ingredients, the product or the environment, particularly food contact surfaces after cleaning, requires documented investigation and follow-up remedial hygienic action carried out and documented.

Protocols for shelf life evaluation (e.g. Evaluation of Product Shelf life for Chilled Foods3) are available and provide a basis for historical data sets.

Historical data can provide the best evidence to demonstrate consistent control of the level of *L. monocytogenes* in a particular food.

If there are insufficient historical data, carrying out additional actions as set out in the following sections will be necessary to justify shelf life, otherwise you must demonstrate that *L. monocytogenes* is absent at the end of manufacture until such data have been gathered.

The level of confidence increases with the size of the data set, i.e. the more product units that have been tested the more reliable the historical data becomes. However, it is not possible to recommend a specific amount of data since this will be a risk-based approach

dependent on the varying manufacturing processes and the nature of the food (Guidance for food business operators, 2010).

c. Predictive microbiology (modelling)

Where additional studies are needed, predictive microbiological modelling is expected to be the most commonly used approach to confirm the assigned shelf life.

By inputting key physicochemical factors of your food (e.g. pH, aw/salt) and historical data into a predictive microbiological model (computer programme) it is possible to obtain an indication of potential growth of certain key organisms including *L. monocytogenes*.

Predictive microbiological models are freely available on the internet, e.g. ComBase (http://www.combase.cc). These are useful tools to provide additional confidence in the assigned shelf life. However, they have limitations (e.g. lack of uniformity throughout foods) and must therefore be used with caution and only used by trained and experienced personnel who can help you interpret the results (Guidance for food business operators, 2010)..

d. Specific laboratory shelf life studies

There are microbiological procedures used for determining the growth of *L. monocytogenes* using durability studies and/or challenge tests.

Both methods have limitations as described below (Guidance for food business operators, 2010).

i) Durability Studies

Durability studies evaluate the growth of *L. monocytogenes* in a naturally contaminated food during its storage under reasonably foreseeable conditions.

The EC has defined a protocol for durability studies (EC, 2008). However, since this protocol requires low levels of *L. monocytogenes* to be naturally and consistently present in

batches of the food being studied, the number of foods to which this can be applied is limited (Guidance for food business operators, 2010).

ii) Challenge Tests

Challenge testing is in practice only used if the following methods of assessing safety/stability of the food have not been or cannot be carried out:

- Data on product characteristics
- Historical data
- Predictive microbiology
- Specific laboratory shelf life studies, i.e. durability studies

Challenge tests aim to provide information on the behavior of *L. monocytogenes* artificially introduced into a food before storage under given conditions in a laboratory environment. The EC has defined a protocol for challenge testing (EC, 2008). This protocol involves inoculating the food with a specific cocktail of *L. monocytogenes* to a defined level within the food and measuring any subsequent changes in this level over the anticipated shelf life under worst case chilled conditions.

Because of the complexity of the procedure this protocol demands specialist laboratory expertise.

Other protocols may be acceptable to UK enforcement officers, but their applicability to the intracommunity trade will need to be established with the recipient EU country before conducting a trial (Guidance for food business operators, 2010).

iii) Shelf Life Evaluation

Shelf-life evaluation is a practical approach which can be carried out using established protocols, e.g. CCFRA (Campden BRI, 2004) which does not require pathogens to be present. These protocols give useful guidance on the major considerations to be taken into

account before launching a new or reformulated product onto the market. As these tests do not involve inoculation of the foods they rarely isolate pathogens. Data and information generated from such protocols contribute to historical data (Guidance for food business operators, 2010).

e. Collaboration between food businesses

Each FBO needs to validate that growth data they are using is applicable to their own product and process. Caution should be taken if sharing environmental data. With the provisos set out below FBOs may collaborate in conducting the studies set out in section 6, either between different sites within the same company or different companies, e.g. through a trade association.

The FBO should be able to demonstrate to an enforcement officer that the products and the processing of the products for which the data are being shared are similar. For example:

- For these studies to be valid the products being compared should have the same characteristics (pH, aw, salt content, concentration of preservatives, type of packaging, associated microflora or any other characteristic important for the survival and growth of *L. monocytogenes*), and;
- The production process and storage conditions of the products should be similar.

It must be noted that different production areas will have different potential for contamination; however products may have the same potential for growth of *L. monocytogenes* if contaminated.

If the products are not similar, the FBO should be able to show how they are different and what effect those differences have on the survival and growth of *L. monocytogenes* (Guidance for food business operators, 2010).

1.4.3 Practical Application of Shelf-Life

1.4.3.1 New start-up (new food production facility)

Recommendations:

a) Ensure that requirements for the safe manufacture of RTE foods are in place.

b) Purchase ingredients from a reputable source, obeying usage and storage instructions provided, in particular the Use By date. See checklist for buying ingredients if in doubt.

c) Review the ingredients and determine the control for *L. monocytogenes* in place for each (including shelf life), using the supplier's information as necessary. Note that data are product-specific and are only valid for the supplier from which they are gathered. If there is no further processing of ingredients then shelf life of the finished product must not exceed that of the shortest shelf life ingredient incorporated, e.g. where a product contains ingredients that have a shelf life of between 5 and 10 days the shelf life of the product must be no more than 5 days.

d) Consider any changes to the ingredients that may occur when they are mixed or assembled, i.e. changes to the individual ingredient characteristics, and determine whether this impacts on the continuing efficacy of *L. monocytogenes* controls, which may change the usable shelf life. This may require expert guidance. Consider any changes to the microbial loading or characteristics of the ingredients that may occur when they are handled, processed, mixed or assembled, i.e. cooking, heating, cooling, freezing, thawing and any potential cross contamination.

e) Set up a system to monitor the controls on raw materials, focusing on high risk ingredients.

f) Start an environmental microbiological monitoring programme for the production area, as a minimum check for *Listeria* species, swabbing areas that have the greatest risk of contamination, e.g. slicing equipment.

g) Ensure that any detection of *Listeria* species in the food or environment is investigated and follow-up remedial action carried out and documented.

h) Set up a system to monitor *L. monocytogenes* in the finished product, to verify effective functioning of the HACCP system and for durability verification to demonstrate that 100 cfu/g is not exceeded during the shelf life.

i) Gather data to substantiate that the limit of 100 cfu/g is unlikely to be exceeded at the end of shelf life. Whilst building up such data collect data to demonstrate that you have implemented effective HACCP-based procedures and that *L. monocytogenes* is unlikely to be present at the end of manufacture. See section 6. If you have any doubt as to the validity of this data seek expert advice.

j) Review collated raw material, finished product and environmental data on an ongoing basis to ensure controls are in place.

1.4.3.2 New product (produced in an existing facility with GMP & GHP)

Recommendations:

a) Ensure that any changes in raw materials, product characteristics, suppliers, equipment or processes are fully considered through the HACCP plan.

b) Implement points above as per a new start-up.

c) Historical data (e.g. environmental monitoring) gathered from existing production of similar products with comparable intrinsic characteristics (e.g. pH, aw) may now assist in demonstrating the efficacy of controls and shelf life (Guidance for food business operators, 2010).

1.5 CHALLENGE TESTS

Challenge tests aim to provide information on the behaviour of *L. monocytogenes* that have been artificially inoculated into a food or foodstuffs, under given storage conditions. They may take into account the variability of the foodstuffs (by using different batches) and the specific contamination of the food (by inoculating strains isolated from the food).

However, the level of contamination, the heterogeneity of the contamination and physiological state of the bacteria are difficult to mimic in a challenge test study. Challenge tests can be performed with two different objectives: either (1) assessment of the growth potential (i.e. the ability of *L. monocytogenes* to grow in the food), or (2) estimation of the growth parameters (e.g. maximum growth rate) (SANCO, 2008).

1.5.1 Challenge Tests Assessing Growth Potential

A microbiological challenge test assessing a growth potential ( ) is a laboratory-based study that measures the growth of *L. monocytogenes* in artificially contaminated food stored under foreseeable conditions of transportation, distribution and storage. A microbiological challenge test must reflect conditions that might realistically be expected to occur throughout the cold chain, including storage conditions after production until consumption.

The growth potential (δ) is the difference between the $\log_{10}$ cfu/g at the end of the test and the $\log_{10}$ cfu/g at the beginning of the test. The experimental results relating to may show a wide dispersion, notably because the lag phase is included.

δ depends on many factors, the most important being:

- the inoculated strain(s),
- the physiological state of the inoculated strain(s),
- intrinsic properties of the food (e.g. pH, NaCl content, a_w, nutritional content, associated microflora, antimicrobial constituents),
- extrinsic properties (e.g. time-temperature profile, gas atmosphere).

Among these factors, temperature may be expected to have the greatest influence on the growth of *L. monocytogenes* in a given food type. In the frame of the application of the Regulation (EC) No. 2073/2005, it can be used:

- to classify a food:

- when > 0.5 $\log_{10}$ cfu/g, the food is classified into Ready-to-eat foods able to support the growth of *L. monocytogenes* other than those intended for infants and for special medical purposes (category 1.2),
- when 0.5 $\log_{10}$ cfu/g, the food is classified into Ready-to-eat foods unable to support the growth of *L. monocytogenes* other than those intended for infants and for special medical purposes (category 1.3),

- to quantify the behaviour of *L. monocytogenes* in a food of category 1.2 according to defined reasonably foreseeable conditions between production and consumption,

- to permit calculation of a concentration of *L. monocytogenes* at point of production, that should not lead to the level of 100 cfu/g being exceeded at the end of shelf-life.

The main advantages of this method are: (i) that it is relatively simple to implement and (ii) that results can be directly used (see above). Its drawback is the lack of flexibility in the interpretation: the results are only valid for the studied food along the studied conditions, so that new experiments have to be performed each time there is a change (e.g. the receipt is changed, different time-temperature profiles are used,). Also, the growth potential covers a generally long period of time (e.g. the whole shelf life) and then cannot be used to predict growth during a limited part period of time (SANCO, 2008).

1.5.2 Challenge Tests Assessing the Maximum Growth Rate

The drawbacks of the previous approach can be solved by combining predictive microbiology models and challenge tests assessing μ_{max} (growth rates). These experiments are more expensive and time-consuming than the challenge tests assessing growth potential. They

are restricted to cases in which predictive microbiology can be applied, by laboratories trained in predictive microbiology. A microbiological challenge test assessing the maximum growth rate is a laboratory-based study that measures the rate of growth of *L. monocytogenes* in an artificially contaminated food stored at an appropriate temperature. The temperature used for the experiment is not (necessarily) the one used for predictions since it is possible to predict growth at another temperature than the one tested, or along a time-temperature profile chosen to be representative of the foreseeable conditions of transportation, distribution and storage.

Once the test has been performed, the maximum growth rate (μ_{max}) of the studied *L. monocytogenes* strain at the studied temperature is calculated from the growth curve. In the exponential growth phase, plotting the natural logarithm of cell number against time produces a straight line. The slope of this line is the μ_{max}. It is expressed, for our purpose, in day^{-1}.The maximum growth rate is an important parameter of the growth curve which depends on:

- the inoculated strain(s),
- intrinsic properties of the food (e.g. pH, NaCl content, a_w, nutritional content, associated microflora, antimicrobial constituents),
- extrinsic properties (e.g. temperature, gas atmosphere).

Then, using for example an equation suggested in the present document, it is possible to extrapolate this μ_{max} at a temperature to predict other μ_{max}-values at other temperatures in the same food.

Such microbiological challenge tests allow:

- an estimation of the concentration of *L. monocytogenes* at a given day of the shelf-life if the initial concentration is known,
- an estimation of the maximum allowable concentration of *L. monocytogenes* in a food that may be present on the day of production, in order to comply with the limit of 100 cfu/g at the end of shelf-life (SANCO, 2008).

1.5.3 Durability Studies

Durability studies allow an evaluation of the growth of *L. monocytogenes* in a naturally contaminated food during its storage according to reasonably foreseeable conditions. Durability studies may be considered more realistic than a challenge test for individual foods, as the contamination is naturally occurring. The interpretation of the results of durability studies can be difficult because of the low probability of testing a contaminated unit, the very low number of *L. monocytogenes* initially present and the heterogeneity of the distribution in the food. In these situations it may be necessary to use challenge tests to collect the information necessary to establish shelf-life and ensure compliance of <100 cfu/g at the end of the shelf-life of the product.

Durability studies can be used when *L. monocytogenes* is routinely detected in the tested food at the end of manufacturing (SANCO, 2008).

1.5.3.1 Selection of appropriate microbiological procedures

The choice of the tests to be implemented should be done by the FBO, with the collaboration of the laboratory who will conduct them. The choice should be led by the information to be obtained, as illustrated in Figure 2. Some basic rules are suggested below for this choice:

- Challenge tests for assessing are likely to be the "first intention" tests in most cases, especially to differentiate between products able or not to support growth of *L. monocytogenes*.

- Implementing challenge tests for assessing μ_{max} should mostly be regarded as "second intention" tests, in specific cases in which it is expected that the additional information that they could provide may be useful. Basic knowledge about predictive microbiology is necessary to interpret results.

- Durability studies are particularly appropriate when the prevalence of *L. monocytogenes* is high (SANCO, 2008).

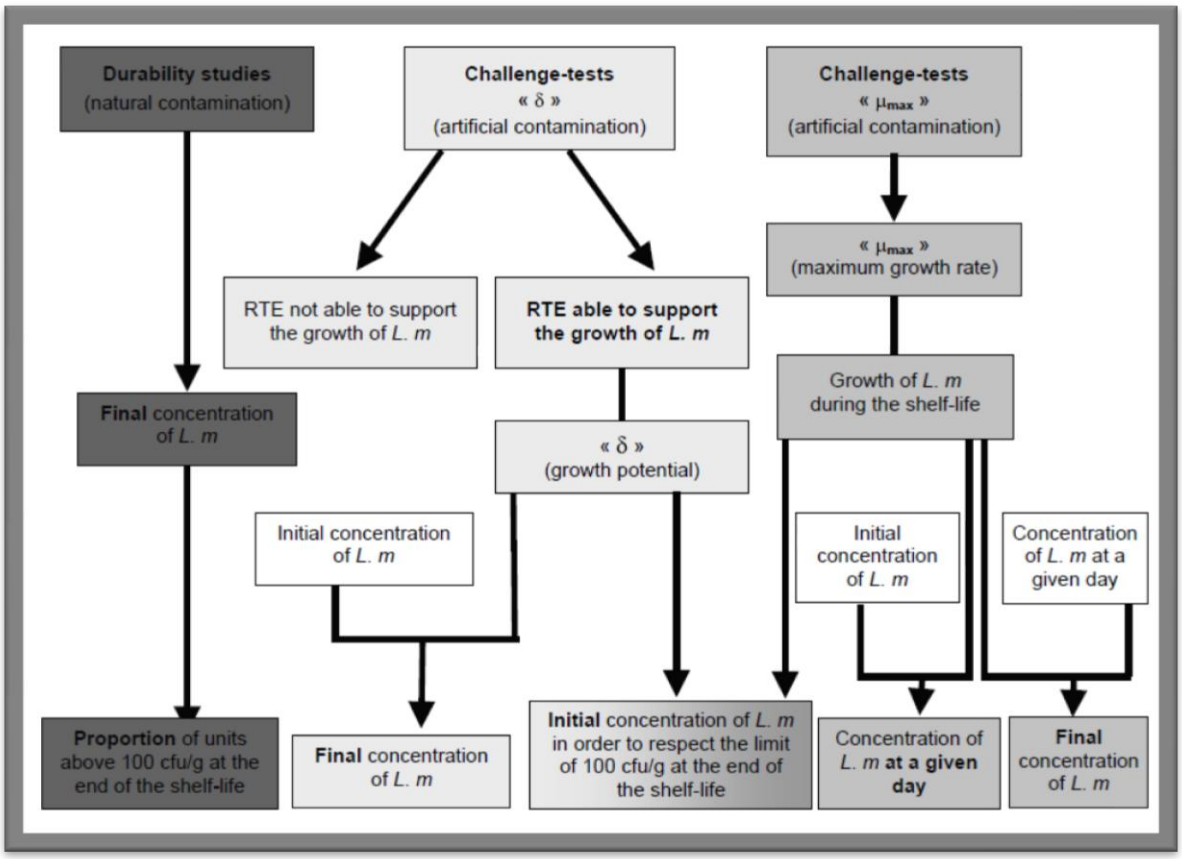


Figure n.2: Data obtained from challenge tests and durability tests

2.Aim

Good manufacturing practices, appropriate cleaning, sanification and hygiene programs and effective temperature control throughout the food production, distribution and storage chain are required for prevention of contamination or inhibition of growth of *L. monocytogenes* to levels exceeding 100 cfu/g in foods that may pose a *L. monocytogenes* risk (EFSA,2013).

The aim of the survey is to estimate the prevalence of *Listeria* spp. in the following RTE food categories, in surface sponges before production and in samples selected on a random basis during production. The sample set is composed by cured meat and cooked hams under vacuum packaged and cured meat and cooked hams sliced in MAP trays.

Specific objectives were the following:

- estimation of the prevalence of *Listeria* spp. in the surveyed RTE foods,
- analysis of the qualitative and quantitative survey test results,
- analysis of factors related to the prevalence of contaminated foods,
- development of challenge tests for the microbial growth of *L. monocytogenes* food safety criteria in foods.

3.Materials and Methods

3.1 SAMPLES SET

All the sampled products were made of pork or beef and they were collected in Northern Italy from April to November 2014.

Half of the sampled meat products were sold in sliced MAP (Modified Atmosphere Packaged) form and the remaining part were sold under vacuum packages. For each type of sample the production surfaces were analyzed using sterile sponges.

The samples set was composed by:

- Under vacuum packaged products:
    - 160 of Dry-cured Ham;
    - 160 of Salami;
    - 160 of Pancetta;
    - 160 of Coppa;
    - 160 of Bresaola;
    - 160 of Cooked Ham;
    - 160 of Mortadella;
    - 160 sponges before production of Dry-cured Ham;
    - 160 sponges before production of Salami;
    - 160 sponges before production of Pancetta;
    - 160 sponges before production of Coppa;
    - 160 sponges before production of Bresaola;
    - 160 sponges before production of Cooked Ham;
    - 160 sponges before production of Mortadella;

- MAP packaged products:
    - 160 of Dry-cured Ham;
    - 160 of Salami;
    - 160 of Pancetta;
    - 160 of Coppa;
    - 160 of Bresaola;
    - 160 of Cooked Ham;
    - 160 of Mortadella;
    - 160 sponges before production of Dry-cured Ham;
    - 160 sponges before production of Salami;
    - 160 sponges before production of Pancetta;
    - 160 sponges before production of Coppa;
    - 160 sponges before production of Bresaola;
    - 160 sponges before production of Cooked Ham;
    - 160 sponges before production of Mortadella;

Upon sampling, surveyed meat products were transported to the laboratories, where they were kept refrigerated until analysis.

3.2 SAMPLING

Before carrying out the microbiological analysis it is necessary that you homogenize the product using a solvent and applying a mechanical force, by means of stomacher, without damaging microbial forms exist.

The sampling is done in a sterile environment, working under a laminar flow hood, near a burner and equipment strictly sterile to avoid external contamination that would invalidate the analysis.

The procedures for sample preparation vary according to the nature of the product:

• If liquid, it does not include any preparation. The sample is used as such and subsequently aliquoted, in a sterile bag, in the respective broth in a ratio of 1:10;

• if in solid form must be weighed a representative quantity of the sample (25 grams), in a sterile bag, diluted in the respective broth in a ratio of 1:10 and subsequently homogenized using a stomacher (a device with pedals that with movement crush the sample by determining the shredding).

3.3 DETECTION OF LISTERIA MONOCYTOGENES

Detection of *L. monocytogenes* was performed according to EN ISO 11290-1:1996 amended in 2004 (Diagram n.1).

The method consists of a double enrichment in Half Fraser and Fraser selective broths. The initial incubation in Half Fraser broth is carried out for 24 hours at 30 °C. The second step of the enrichment is carried out in Fraser broth for 48 hours at a temperature of 37 °C. Half Fraser broth contains half the concentration of nalidixic acid and acriflavin of that found in Fraser broth.

Cultures obtained in Half Fraser and Fraser broths are plated out on two selective solid media: Agar *Listeria* according to Ottaviani and Agosti and an additional selective medium of your choice. After two days of incubation, the colonies of presumptive *L. monocytogenes* or *Listeria* species, are sub-cultured and confirmed by means of appropriate morphological and biochemical tests described in the Standard and reported here.

The theoretical limit of sensitivity of the EN ISO 11290-1 method for the detection of *L. monocytogenes* in food is one cell in 25g or ml samples.

The relative level of detection (LOD_{50}) is the smallest number of cultivable microorganisms that can be detected in the sample in 50 % of occasions by the alternative and reference methods. In 2012, many validation studies of rapid commercial methods performed in comparison to the Standard method were available from AFNOR Certification for the detection of *L. monocytogenes* in food and environmental samples. According to these studies, the standard method shows a LOD that is generally below 1, comprised between of 0.4 and 1.7 cfu/25 g for meat, between 0.3 and 1.3 for seafood products and between 0.3 and 1.2 cfu/25 g for dairy products.

The validation study of the revised Nordic Committee on Food Analysis (NMKL) method N°136, very similar to the EN ISO 11290-1 Standard, allowed to better define the

performance characteristics of the method. The sensitivity values of the detection method were 98.6 %, 97.2 % and 98.6 %, respectively, for brie cheese made from pasteurized milk, hot smoked salmon and cooked vacuum-packed ham, and the specificity values were respectively 94.4 %, 100 % and 100 % for the same products.

It is believed that the double enrichment may allow overgrowth of *L. monocytogenes* by *L. innocua* in samples where both species are present. Indeed, each of the species within the genus *Listeria* can be isolated from food. From a practical perspective, the overgrowth by a non-pathogenic species of *Listeria* may mask the presence of low numbers of *L. monocytogenes* in the original food sample, and result in false-negative results (EFSA, 2013).

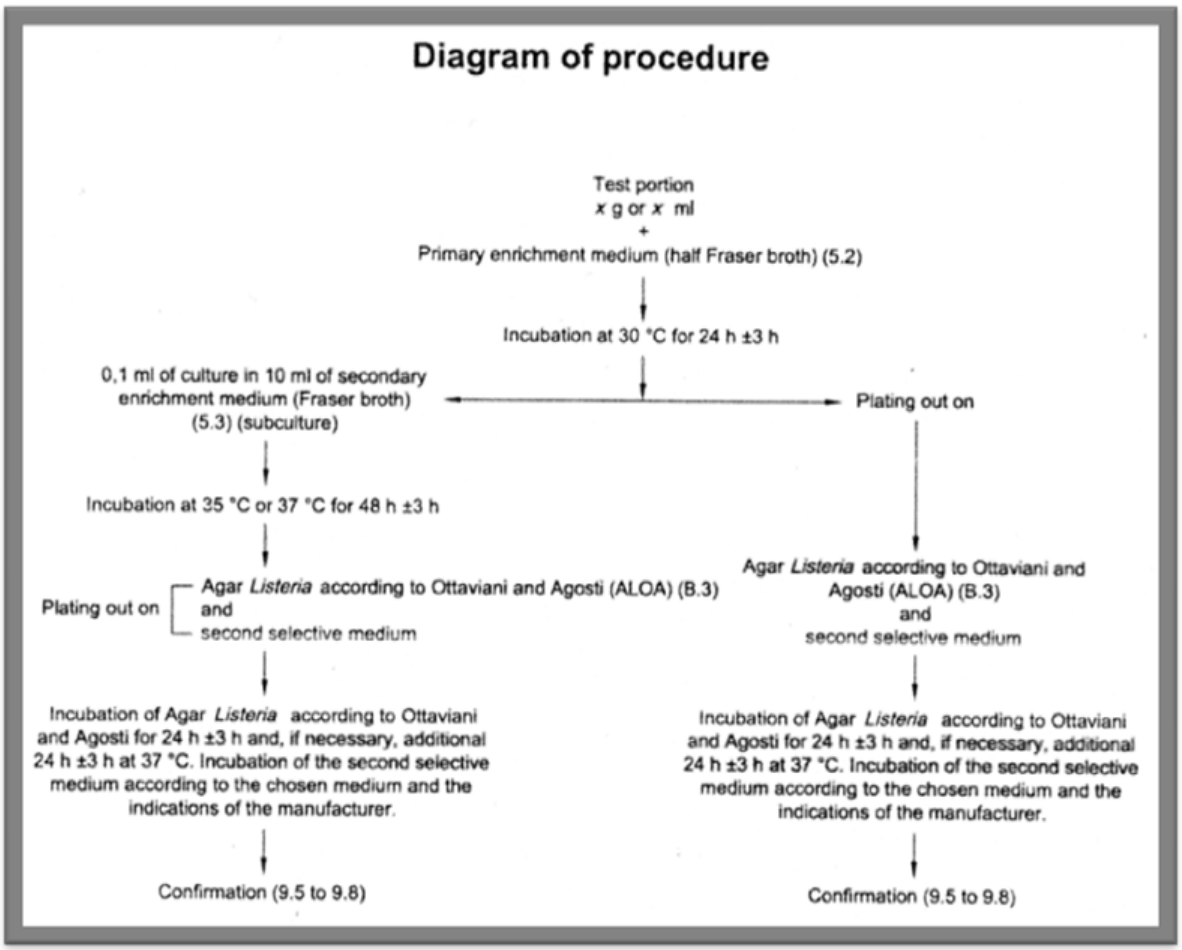


Diagram n.1: Detection of *L. monocytogenes* ISO 11290-1

3.4 ENUMERATION OF LISTERA MONOCYTOGENES

The enumeration of *L. monocytogenes* was performed according to EN ISO 11290-2:1998 and its modification EN ISO 11290-2:1998/A1:2004 (Diagram n.2).

The sample is decimally diluted in an appropriate diluent (buffered peptone water or half Fraser broth base without selective agents) and subsequently homogenized. A specified volume of this initial suspension and/or of subsequent decimal dilutions is surface-plated on Agar *Listeria* according to Ottaviani and Agosti. After appropriate incubation, the colonies of presumptive *L. monocytogenes* are counted, sub-cultured and confirmed by means of appropriate morphological and biochemical tests described in the Standard. The calculation of the *L. monocytogenes* contamination level is carried out according to the number of confirmed colonies.

According to the expected low contamination levels, it was advised to plate 1 ml of the initial suspension in duplicate on three 90-mm plates (or one plate of 140 mm diameter), as indicated in the Standard, in order to increase theoretical limit of sensitivity to10 cfu/g.

The theoretical limit of sensitivity of the EN ISO 11290-2 method for the enumeration of *L. monocytogenes* in food is 10 cfu/g when spreading 1 ml of the decimally diluted sample on three 90-mm plates (or one plate of 140 mm diameter).

According to ISO 7218:2007 Standard:

- the limit of detection of the method is 10 cfu/g (when spreading 1 ml of the initial food suspension).
- the theoretical limit of quantification is then 40 cfu/g (four times the limit of detection). Below this value, the microorganism cannot be reliably quantified, though its presence may be reported.

- under 100 cfu/g (which correspond to 10 colonies when spreading 1 ml of the initial food suspension) the result has to be expressed as an estimated result or its measurement uncertainty has to be specified.

Moreover, a contamination level of about 100 cfu/g (when spreading in duplicate 1 ml of the initial food suspension) is also associated with a quite elevated 95% confidence interval (up to 60 to 150 cfu/g according to ISO 7218:1996).

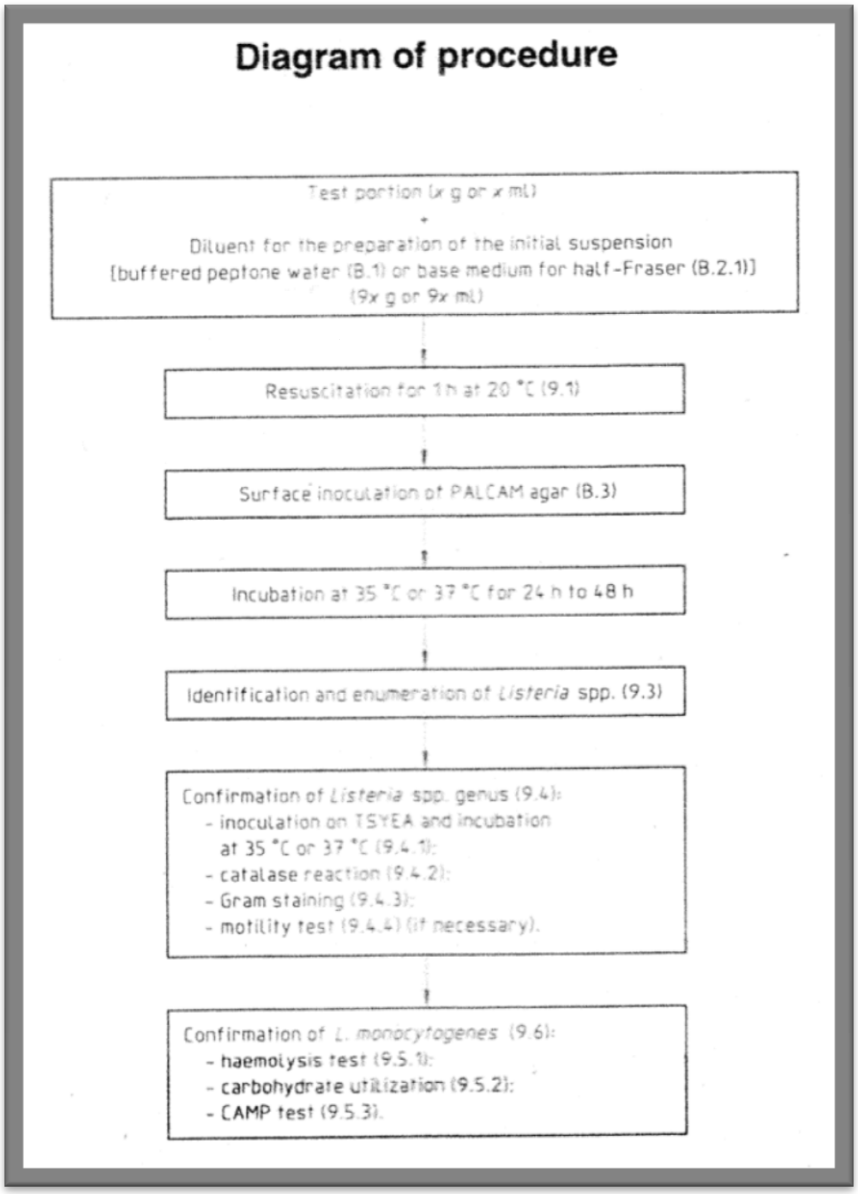


Diagram n.2: Enumeration of *L. monocytogenes* ISO 11290-2

3.5 BROTHS AND AGAR USED FOR THE DETECTION OF L. MONOCYTOGENES

3.5.1 Fraser Broth

A secondary selective diagnostic enrichment medium for the isolation of *Listeria* spp. from food and environmental specimens. It contains ferric ammonium citrate and lithium chloride. Blackening of the medium is presumptive evidence of the presence of *Listeria*. Contrary to early indications, cultures which do not blacken cannot be assumed to be Listeria-free. All Fraser Broth enrichment cultures should be subcultured to plating medium. Fraser Broth has proven to be remarkably accurate in detecting *Listeria* spp. in food and environmental samples.

All *Listeria* spp. hydrolyze aesculin to aesculetin. Aesculetin reacts with ferric ions which results in blackening. Another possible advantage to the addition of ferric ammonium citrate is that it has been shown that ferric ions enhance the growth of *L. monocytogenes.*

Lithium chloride is included in the medium to inhibit the growth of enterococci which can also hydrolyze aesculin. Care must be taken when using Fraser Broth with DNA probe methodology because the high salt content of the medium may have an inhibitory effect on detection.

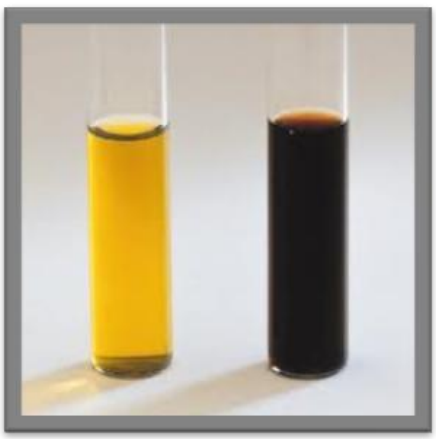


Figure n.3:Blanking of Fraser Broth cause by *L. monocytogenes*

Half Fraser Broth is a modification of Fraser Broth which contains half of the concentration of nalidixic acid and acriflavin hydrochloride to aid in the recovery of stressed cells. Half Fraser Broth is used as the primary enrichment broth in the ISO methodology for the detection of *Listeria* spp. (Figure n.3) (OXOID, 2014).

3.5.2 Agar Listeria acc. to Ottaviani & Agosti: ALOA

ALOA agar is a pre-prepared selective and differential medium for the isolation of *Listeria* spp. from food samples and for the presumptive identification of *L. monocytogenes*. To minimise the growth of contaminating organisms, lithium chloride and a balanced antimicrobial mixture are employed.

The incorporation of the chromogenic substrate X-glucoside for the detection of beta-glucosidase demonstrates the presence of *Listeria* spp., whilst the detection of a specific phospholipase C enzyme produced by pathogenic *Listeria* spp. including *L. monocytogenes* is also achieved. *Listeria* spp. grow on this medium producing blue/ green colonies, with pathogenic species producing similar coloured colonies surrounded by a characteristic opaque halo after 24 hours incubation at 37oC . Non-*Listeria* spp. produce white colonies (Figure n.4) (MICROGEN, 2014).

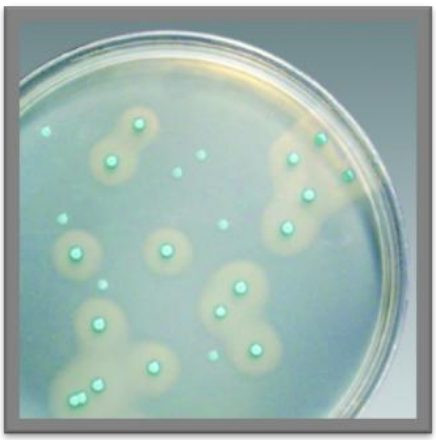


Figure n.4: Colonies of *L. monocytogenes* on ALOA plate

3.5.3 Listeria Oxford agar base: OXFORD

It is a selective agar to identify the growth of *L. monocytogenes.* The OXFORD Agar is composed by different substances that forbid the growth of other bacteria by color indicator as aesculin and iron citrate. *L. monocytogenes* hydrolyses aesculin and produce a black halo caused by phenolic compounds (Figure n.5).

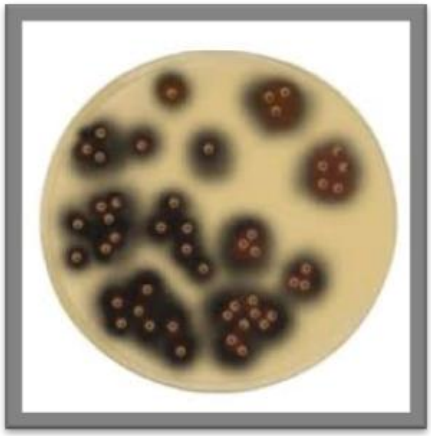


Figure n.5: Colonies of *L. monocytogenes* on OXFORD Agar plate

3.6 CONFIRMATION OF LISTERIA SPP.

For confirmation tests almost five colonies presumed to be *L. monocytogenes* have to be taken from each plate of selective medium and streaked onto the surface of pre-dried plates of Tryptone Soya Yeast Extract Agar (TSYEA). After 24 hours of incubation the typical colonies should be 1-2mm of diameter, convex, colorless and opaque with an entire edge (EN ISO 11290-1/2).

3.6.1 Catalase reaction

An isolated colony has to be suspended in a drop of hydrogen peroxide solution on a side of the TSYEA plate; the immediate formation of gas bubbles indicates a positive reaction (figure n.6) (EN ISO 11290-1/2).

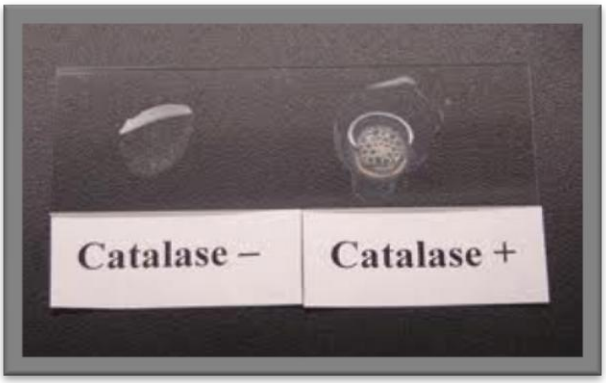


Figure n.6: Catalase reactions

3.6.2 Gram staining

Listeria spp. are revealed as Gram-positive slim, short rods after the Gram stain on a separated colony (Figure n.7) (EN ISO 11290-1/2).

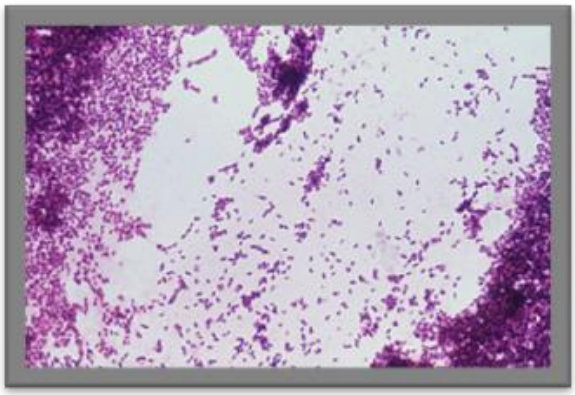


Figure n.7: Gram staining of *L. monocytogenes*

3.6.3 Motility test

After 24 hours of incubation in TSYE broth, a cloudy medium could be observed in the bottom of the tube. Observing a drop of the medium to the microscope *Listeria* spp. appears as slim, short rods with rapid tumbling motility. Cocci, large rods or rods with swimming motility are not *Listeria* spp. (Figure n.8) (EN ISO 11290-1/2).

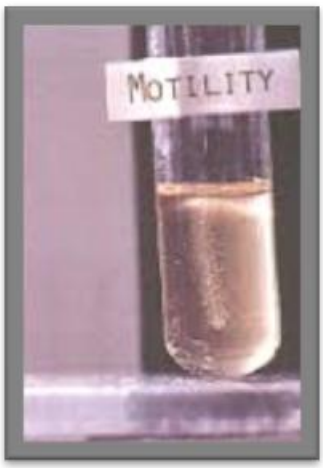


Figure n.8: Motility of *L. monocytogenes*

3.7 CONFIRMATION OF LISTERIA MONOCYTOGENES

If the morphological and physiological characteristics and catalase reaction are indicative of *Listeria* spp. it is necessary to proceed with the other tests (EN ISO 11290-1/2).

3.7.1 Haemolysis test

A colony has to be plated on the sheep blood agar using a wire and simultaneously also the positive (*L. monocytogenes*) and the negative (*L. innocua*) controls have to be plated to observe the different reactions. After incubation at 37°C for 24 h *L. monocytogenes* show narrow, clear, light zones (ß-haemolysis) while *L. innocua* show no clear zone around the stab (EN ISO 11290-1/2).

3.7.2 Carbohydrate fermentation

A colony diluted in a carbohydrate fermentation broth gives a positive reaction if the red color of the broth becomes yellow (Figure n.9) (EN ISO 11290-1/2).

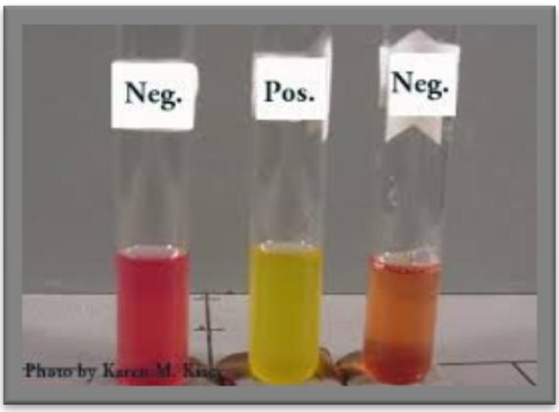


Figure n.9: Carbohydrate fermentation

3.7.3 CAMP test

Staphylococcus aureus and *Rhodococcus equi* cultures have to be streaked in single lines across the sheep blood agar plate so that the two cultures are parallel and diametrically opposite. The stain test has to be streaked in a similar fashion at right angles to these cultures

so that the test culture and *S. aureus* and *R. equi* cultures do not touch but at their closest are about 1 mm to 2 mm apart. The control cultures are *L. monocytogenes*, *L. innocua* and *L. ivanovii*. An enhanced zone of ß-haemolysis at the intersection of the test strain with each of the cultures of *S. aureus* and *R. equi* is considered to be a positive reaction, it seems as a wide (5 mm to 10 mm) "arrow-head" of haemolysis. The reaction is considered as negative if a small zone of weak haemolysis extends only about 1mm at the intersection of the test strain with the diffusion zone of the *R. equi* culture. A positive reaction with *S. aureus* appears as a small zone of enhanced haemolysis extending only about 2 mm from the test strain and within the weakly haemolytic zone due to growth of the *S. aureus* culture. Large zones of haemolysis do not occur in the area of *S. aureus* and *L. monocytogenes* (Figure n.10) (EN ISO 11290-1/2).

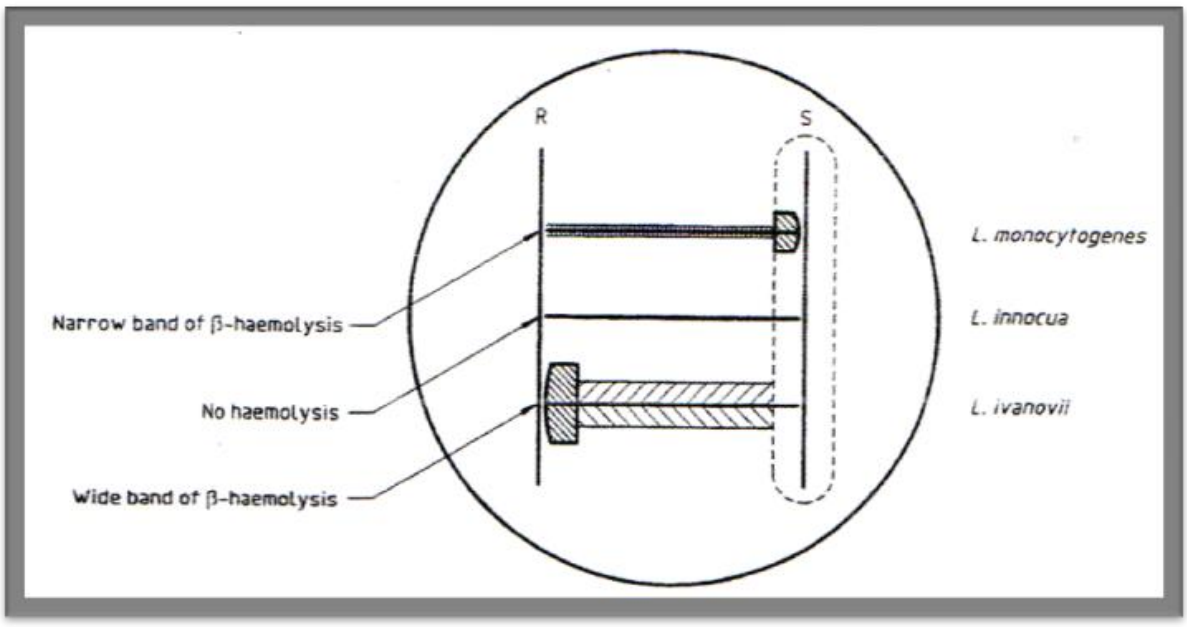


Figure n.10: CAMP test

3.7.4 API LISTERIA

API LISTERIA is a standard method to identify the various species of *Listeria*.The gallery is composed by 10 micro tubes containing different dried substrate to take over the enzymatic and fermentative activities of carbohydrates. The results depend by the color toning of different micro tubes after incubation at 37°C for 24h (Figure n.11) (EN ISO 11290-1/2).

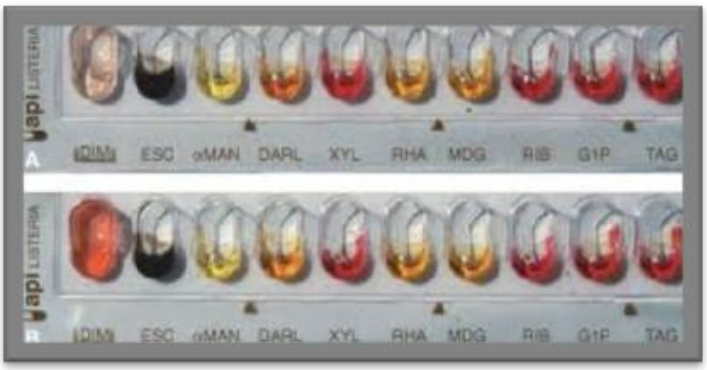


Figure n.11: API LISTERIA

3.8 INTERPRETATION OF MORPHOLOGICAL AND PHYSIOLOGICAL PROPERTIES AND OF THE BIOCHEMICAL REACTIONS

All *Listeria* spp. are small, Gram-positive rods that demonstrate motility. They are catalase positive. *L. monocytogenes* are distinguished from other species by the characteristics listed in the table n.3 (EN ISO 11290-1/2).

Species	Haemolysis	Production of acid		CAMP test	
		Rhamnose	Xylose	*S. aureus*	*R. equi*
L. monocytogenes	+	+	−	+	−
L. innocua	−	V	−	−	−
L. ivanovii	+	−	+	−	+
L. seeligeri	(+)	−	+	(+)	−
L. welshimeri	−	V	+	−	−
L. grayi subsp. *grayi*	−	−	−	−	−
L. grayi subsp. *murrayi*	−	V	−	−	−

V: variable reaction
(+): weak reaction
+: > 90 % of positive reactions
−: no reaction

NOTE — There exist rare strains of *L. monocytogenes* which do not show β-haemolysis or a positive reaction to the CAMP test under the conditions described in this part of ISO 11290.

Table n.3: Reactions for the identification of *L. monocytogenes*

Strains which are considered to be *L. monocytogenes* may be sent to a recognized *Listeria* reference laboratory for serological or lysogenic typing.

3.9 CHALLENGE TEST

To conduct challenge tests in order to assess the growth potential, the following factors, at least, must be taken into consideration:

- product characteristics,
- shelf-life of the product,
- number of batches,
- choice of the strain(s),
- preparation of the inoculum,
- preparation and inoculation of the test units,
- storage conditions,
- measurement of physical-chemical characteristics,
- microbiological analysis,
- calculation of the growth potential.

Product characteristics at the end of the production must be described and must be representative of the variability of the characteristics of the food. These characteristics should include both intrinsic and extrinsic properties:

- physical-chemical characteristics such as pH, a_w, salt content, preservative concentration;
- associated microflora (total count) or specific microflora (e.g. lactic acid bacteria, *Pseudomonas*,)
- packaging conditions (air, vacuum packaging, modified atmosphere packaging).

Three different batches of the same product have to be tested at least. Perform microbial challenge tests with a mixture of at least 3 strains to account for variations in growth among the strains. Among the selected strains one must be a reference strain (ATCC, NCTC, CIP or equivalent). The other strains must be isolated from the same or a similar food matrix. Before the implementation of the challenge test, conduct prior trials to determine the time that is necessary to reach the stationary phase. Subculture each strain in a medium (e.g.

Tryptone Soy Broth (TSB) or Brain Heart Infusion (BHI)) and at a temperature (37°C) favourable to optimal growth of *Listeria monocytogenes*, for a sufficient time for the organism to reach the beginning of the stationary phase. This first subculture is mainly aimed at getting the cells in the same physiological state. Prepare a second subculture and incubate it at a temperature close to the temperature of the product, in order to adapt the strain to the storage condition of the product. Incubate this culture for a sufficient time for growth of the strains to late exponential phase or early stationary phase. Combine in equal quantity the cultures from each of the 3 strains at the same concentration. Prepare successive dilutions of the mixed culture in physiological water to obtain a concentration in the foodstuff similar to the one that might be realistically expected to occur naturally in the foodstuff. Check the inoculum level on Tryptone Soy Agar (TSA). Test units may be used to detect and/or enumerate *L. monocytogenes* occurring naturally in the foodstuff, these blank samples are not inoculated. Even if *L. monocytogenes* is present in the "blank samples", the result of the challenge test is valid. It provides the additional information that naturally occurring *L. monocytogenes* strain(s) at realistic levels were present in addition to the added mixture of strains. For determining the physical-chemical characteristics and the concentration of the microflora, do not inoculate the test units with *L. monocytogenes* but inject instead sterile physiological water. The determination of the physical-chemical characteristics and associated microflora are necessary in order to compare the products submitted to challenge testing to the products routinely produced by the factory . Moreover, the determination of the concentration of the associated microflora can bring some information about possible interactions between *L. monocytogenes* and associated microflora. Such interactions may influence the growth of *L. monocytogenes*.

The challenge test may be performed on either a part or the whole of the commercial unit of the foodstuff. If the food is composed of several parts, the part which will be most

likely contaminated with *L. monocytogenes* (e.g. the filling of a sandwich) must be the artificially contaminated. The distribution of the inoculum in the food shall mimic the plausible distribution of *L. monocytogenes* in the foodstuff, which may or may not be uniform. The inoculation must be as effective as possible at simulating natural contamination conditions and maintaining the intrinsic properties of the foodstuff. In order to minimize changes to the physicalchemical properties, the inoculum should not exceed 1% of the volume of the test unit, otherwise it can seriously effect the intrinsic properties of the product and thus the growth characteristics of the inoculum. Ensure that the method of inoculation does not change the gaseous composition within the food pack, and that the gas composition within the inoculated pack during incubation is identical to the composition that would be expected to be found in a similar uninoculated pack. This may be achieved by inoculating through a cover or septum which immediately seals after the inoculating device is removed, thus maintaining correct gas condition, or by unpacking the commercial unit, inoculating the product, then repacking in a way that ensures that the gas condition is identical to that in an unopened pack.

Inoculate the foodstuff or the specific part suspected to be contaminated in a manner to mimic as closely as possible the expected natural contamination.

This can be done as follows:

- in depth: for food considered to be homogeneous (e.g. ground foodstuffs) or prepared by mixing several materials (e.g. mixed salad),

- or at the surface: to mimic contamination of a specific part during process (e.g. smoked salmon contaminated during slicing).

Target the contamination level at 50 cfu/g, which should not exceed 100 cfu/g.

The storage (incubation) conditions applied during challenge testing must comply with the conditions at which the product is most likely to be subjected in normal use, until its final

consumption. This should include the typical temperature range at which the product is to be transported, distributed and stored (SANCO, 2008; FDA, 2014).

This is a critical part of any challenge test. It is the responsibility of the FBO and laboratory to work together to ensure that the storage (incubation) conditions used are realistic, and understanding the fact that, proper storage temperatures are not always maintained throughout the cold chain (production to consumption). Therefore, challenge tests must consider the use of abuse temperature(s) as well. According to Annex I of Regulation No. 2073/2005, the reference detection and enumeration method for *L. monocytogenes* is the standard method EN ISO 11290-1, -2, amended.

4. Results

4.1 LISTERIA SPP. IN UNDER VACUUM PACKAGED PRODUCTS

All the samples, 1120 under vacuum packaged products and 1120 sponges before production, were analyzed according to EN ISO 11290-1:1996 amended in 2004. The results are reported in the table below (Table n.4).

Under vacuum packaged products	**N. of samples**	***Listeria* spp.**	***L. monocytogenes***
Sponges before production of Dry-cured Ham	160	2	0
Dry-cured Ham	160	0	0
Sponges before production of Salame	160	4	0
Salame	160	0	0
Sponges before production of Pancetta	160	2	0
Pancetta	160	0	0
Sponges before production of Coppa	160	6	0
Coppa	160	0	0
Sponges before production of Bresaola	160	4	0
Bresaola	160	0	0
Sponges before production of Cooked Ham	160	0	0
Cooked Ham	160	0	0
Sponges before production of Mortadella	160	0	0
Mortadella	160	0	0
Total	**2240**	**18**	**0**

Table n.4: Detection of *Listeria* spp. in under vacuum packaged products

L. monocytogenes was absent in 25g for all the samples set both in the products and on the environmental surfaces before production. *Listeria* spp. was isolated in 25g in 18 out of 1120 samples (1.6%) of environmental surfaces only from floor drains.

Though the enumeration method does not include the count of *Listeria* spp., they were always detected inferior to 10 cfu/g in all 18 samples.

4.2 DETECTION OF LISTERIA SPP. IN MAP PACKAGED PRODUCTS

All samples, 560 MAP packaged products and 1120 sponges before production, were analyzed according to EN ISO 11290-1:1996 amended in 2004. The results are reported in the table below (Table n.5).

MAP packaged products	N. of samples	*Listeria* spp.	*L. monocytogenes*
Sponges before production of Dry-cured Ham	160	8	0
Dry-cured Ham	160	0	0
Sponges before production of Salame	160	10	0
Salame	160	0	0
Sponges before production of Pancetta	160	12	0
Pancetta	160	0	0
Sponges before production of Coppa	160	16	0
Coppa	160	0	0
Sponges before production of Bresaola	160	14	0
Bresaola	160	0	0
Sponges before production of Cooked Ham	160	0	0
Cooked Ham	160	0	0
Sponges before production of Mortadella	160	0	0
Mortadella	160	0	0
Total	**2240**	**60**	**0**

Table n.5: Detection of *Listeria* spp. in MAP packaged products

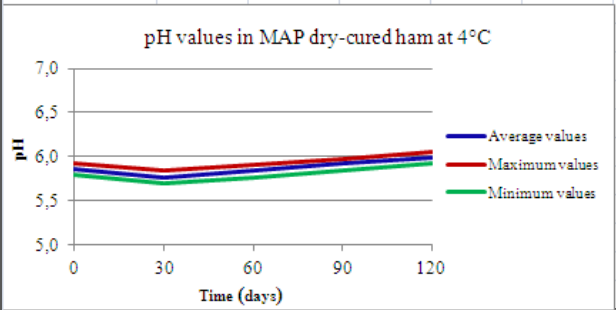
L. monocytogenes was absent in 25g for all the samples set both in the products and on the environmental surfaces before production. *Listeria* spp. was isolated in 25g in 60 out of 1120 samples (5.4%) of environmental surfaces only from floor drains.

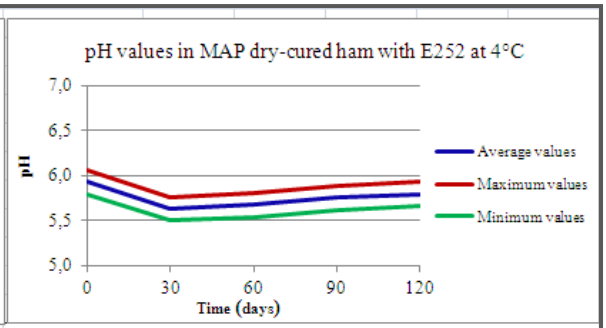
Though the enumeration method does not include the count of *Listeria* spp., they were always detected in a level inferior to 10 cfu/g in all 60 samples.

4.3 CHALLENGE TEST OF DRY-CURED HAM

The aim of the study was to evaluate the growth of *L. monocyt*ogenes in dry-cured hams with and without preservatives (E252). The products were contaminated, sliced and stored at different temperatures (4°C, 10°C, 15°C and 20°C) for 120 days. The different dry-cured hams were analyzed for pH, Aw and *L. monocyt*ogenes after 0, 30, 60, 90 and 120 days (T0, T30, T60, T90 and T120).

The values of pH at 4°C are reported in the graphic n.1.





pH values in MAP dry-cured ham at 4°C

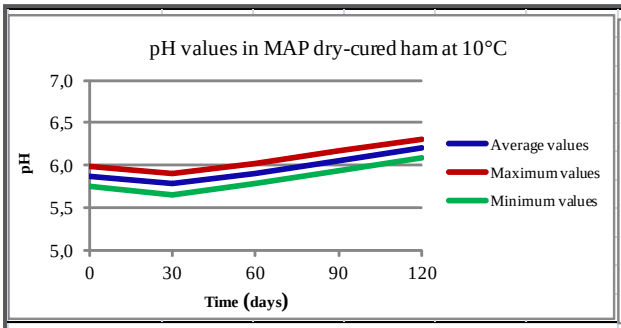
Time (days)	0	30	60	90	120
Average values	5,86	5,77	5,84	5,92	5,99
Maximum values	5,92	5,84	5,91	5,98	6,06
Minimum values	5,79	5,70	5,77	5,85	5,92

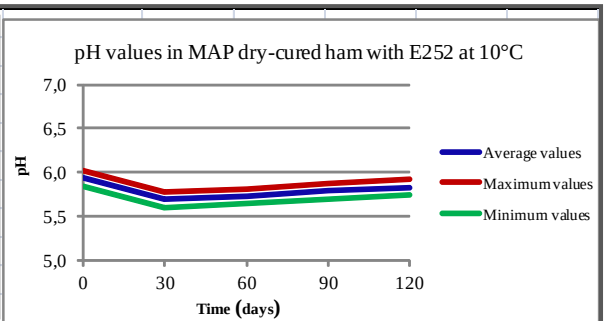
pH values in MAP dry-cured hamwith E252 at 4°C

Time (days)	0	30	60	90	120
Average values	5,94	5,63	5,68	5,76	5,80
Maximum values	6,07	5,76	5,81	5,89	5,93
Minimum values	5,80	5,50	5,54	5,62	5,67

Gaphic n.1: Values of pH at 4°C

The values of pH at 10°C are reported in the graphic n.2.





pH values in MAP dry-cured ham at 10°C

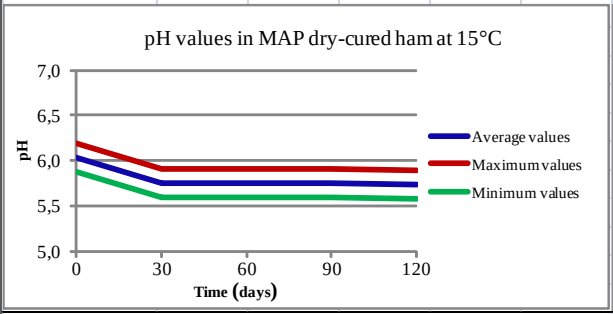
Time (days)	0	30	60	90	120
Average values	5,87	5,78	5,90	6,05	6,20
Maximum values	5,99	5,90	6,02	6,17	6,31
Minimum values	5,75	5,66	5,78	5,93	6,08

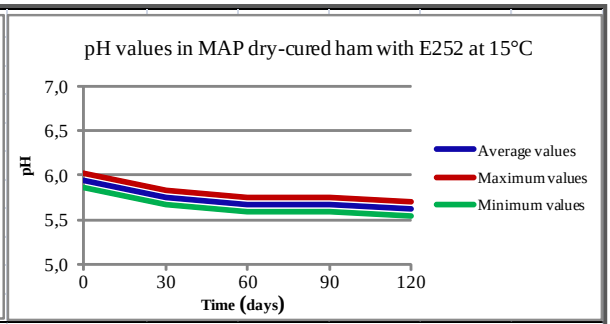
pH values in MAP dry-cured ham with E252 at 10°C

Time (days)	0	30	60	90	120
Average values	5,94	5,69	5,73	5,79	5,83
Maximum values	6,02	5,78	5,81	5,88	5,92
Minimum values	5,85	5,60	5,64	5,70	5,74

Gaphic n.2: Values of pH at 10°C

The values of pH at 15°C are reported in the graphic n.3.

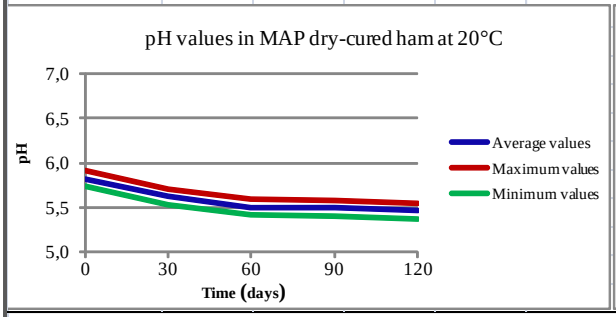


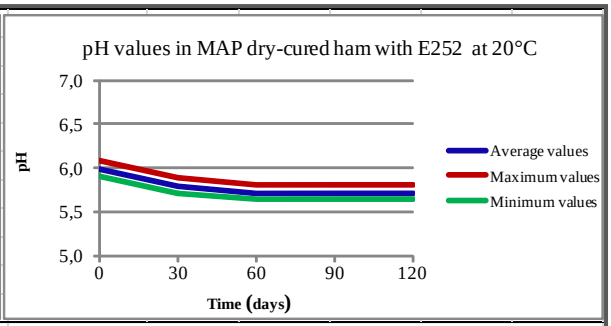


pH values in MAP dry-cured ham at 15°C						pH values in MAP dry-cured ham with E252 at 15°C					
Time (days)	**0**	**30**	**60**	**90**	**120**	**Time (days)**	**0**	**30**	**60**	**90**	**120**
Average values	6,04	5,75	5,75	5,75	5,74	**Average values**	5,94	5,75	5,67	5,67	5,62
Maximum values	6,20	5,91	5,91	5,91	5,90	**Maximum values**	6,02	5,83	5,75	5,75	5,70
Minimum values	5,88	5,59	5,59	5,59	5,58	**Minimum values**	5,86	5,67	5,59	5,59	5,54

Graphic n.3: Values of pH at 15°C

The values of pH at 20°C are reported in the graphic n.4.



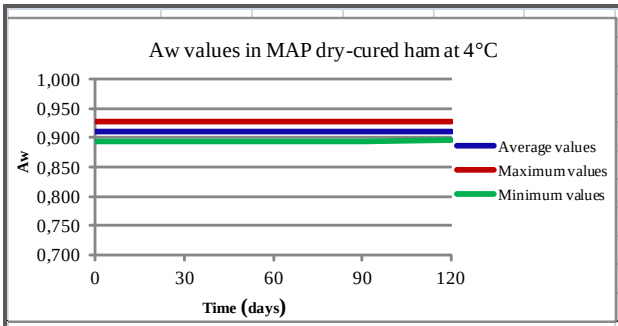


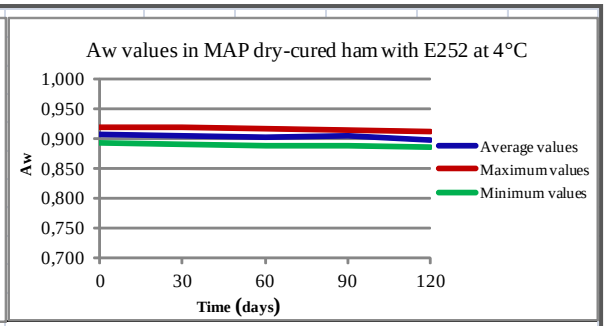
pH values in MAP dry-cured ham at 20°C						pH values in MAP dry-cured ham with E252 at 20°C					
Time (days)	**0**	**30**	**60**	**90**	**120**	**Time (days)**	**0**	**30**	**60**	**90**	**120**
Average values	5,82	5,62	5,50	5,49	5,46	**Average values**	5,99	5,80	5,72	5,72	5,72
Maximum values	5,91	5,71	5,59	5,58	5,55	**Maximum values**	6,08	5,89	5,81	5,81	5,81
Minimum values	5,73	5,53	5,41	5,40	5,37	**Minimum values**	5,91	5,72	5,64	5,64	5,64

Graphic n.4: Values of pH at 20°C

The average values of pH in both dry-cured hams at 4°C and at 10°C were constant for all the 120 days, also considering a standard error of 0,10. The average values of pH decreased in both products stored at 15°C and at 20°C during the shelf-life. If we consider only pH, the values in the graphics n. 1, 2, 3 and 4 could allow the growth of *L. monocytogenes*.

The values of Aw at 4°C are reported in the graphic n.5.





Aw values in MAP dry-cured ham at 4°C

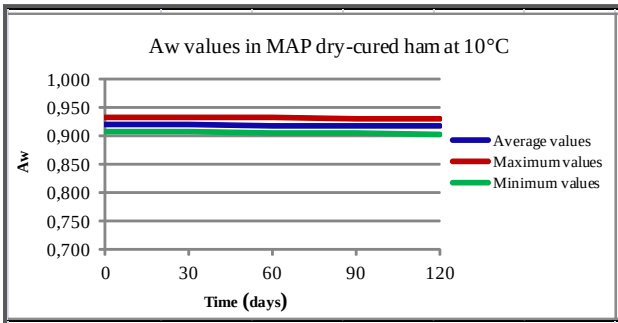
Time (days)	0	30	60	90	120
Average values	0,911	0,912	0,912	0,912	0,912
Maximum values	0,928	0,928	0,928	0,928	0,928
Minimum values	0,895	0,895	0,895	0,895	0,896

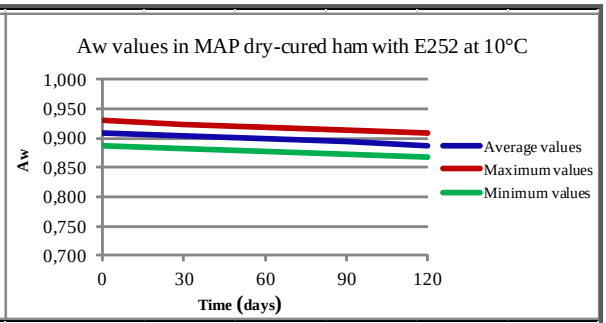
Aw values in MAP dry-cured hamwith E252 at 4°C

Time (days)	0	30	60	90	120
Average values	0,906	0,904	0,902	0,904	0,898
Maximum values	0,920	0,918	0,916	0,914	0,912
Minimum values	0,893	0,891	0,889	0,887	0,885

Graphic n.5: Values of Aw at 4°C

The values of Aw at 10°C are reported in the graphic n.6.





Aw values in MAP dry-cured ham at 10°C

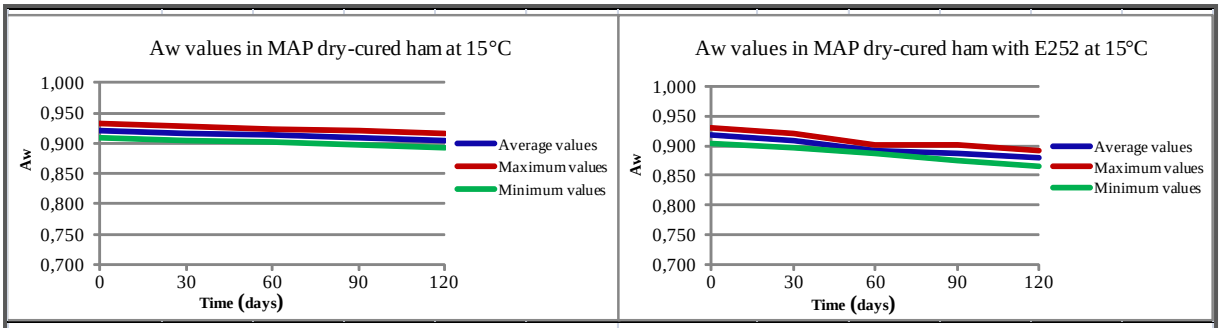
Time (days)	0	30	60	90	120
Average values	0,921	0,920	0,919	0,918	0,917
Maximum values	0,934	0,933	0,932	0,931	0,930
Minimum values	0,908	0,907	0,906	0,905	0,904

Aw values in MAP dry-cured ham with E252 at 10°C

Time (days)	0	30	60	90	120
Average values	0,909	0,904	0,898	0,893	0,888
Maximum values	0,930	0,924	0,919	0,914	0,909
Minimum values	0,888	0,883	0,877	0,872	0,867

Graphic n.6: Values of Aw at 10°C

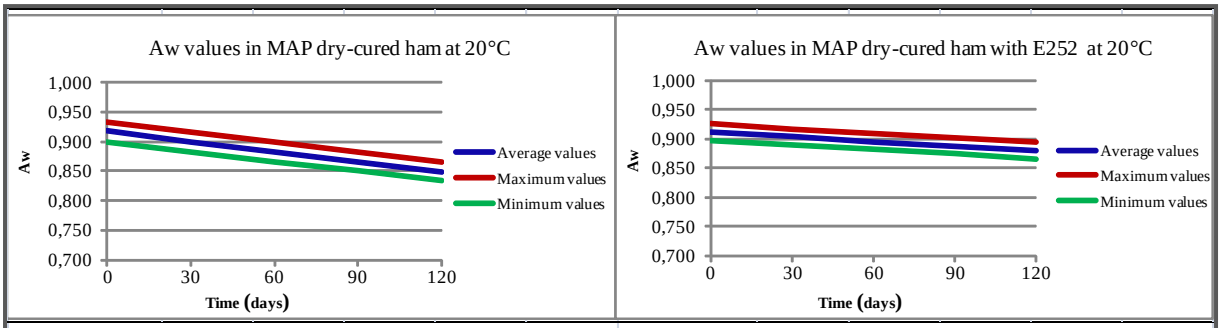
The values of Aw at 15°C are reported in the graphic n.7.



Aw values in MAP dry-cured ham at 15°C						Aw values in MAP dry-cured ham with E252 at 15°C					
Time (days)	**0**	**30**	**60**	**90**	**120**	**Time (days)**	**0**	**30**	**60**	**90**	**120**
Average values	0,920	0,917	0,913	0,909	0,905	**Average values**	0,918	0,908	0,893	0,888	0,879
Maximum values	0,932	0,928	0,924	0,921	0,917	**Maximum values**	0,930	0,920	0,901	0,901	0,891
Minimum values	0,909	0,905	0,901	0,897	0,893	**Minimum values**	0,905	0,896	0,886	0,876	0,866

Graphic n.7: Values of Aw at 15°C

The values of Aw at 20°C are reported in the graphic n.8.

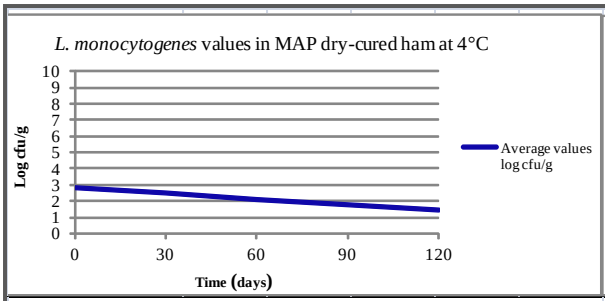


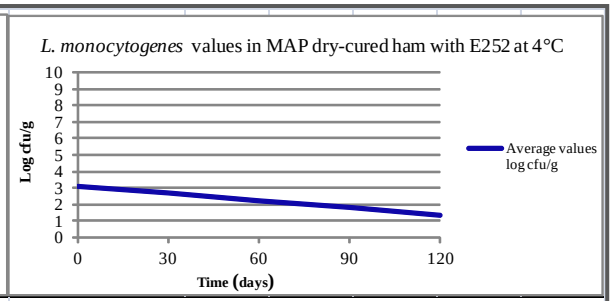
Aw values in MAP dry-cured ham at 20°C						Aw values in MAP dry-cured ham with E252 at 20°C					
Time (days)	**0**	**30**	**60**	**90**	**120**	**Time (days)**	**0**	**30**	**60**	**90**	**120**
Average values	0,917	0,900	0,883	0,866	0,849	**Average values**	0,911	0,903	0,895	0,888	0,880
Maximum values	0,933	0,916	0,899	0,882	0,865	**Maximum values**	0,925	0,917	0,909	0,901	0,894
Minimum values	0,900	0,883	0,866	0,850	0,833	**Minimum values**	0,897	0,889	0,882	0,874	0,866

Graphic n.8: Values of Aw at 20°C

The values of Aw did not allow the growth of the pathogen. The average values of Aw in both dry-cured hams at 4°C and 10°C were constant for all the 120 days. The average values of Aw decreased in both products stored at 15°C and 20°C during the shelf-life.

The values of *L. monocytogenes* at 4°C are reported in the graphic n.9.

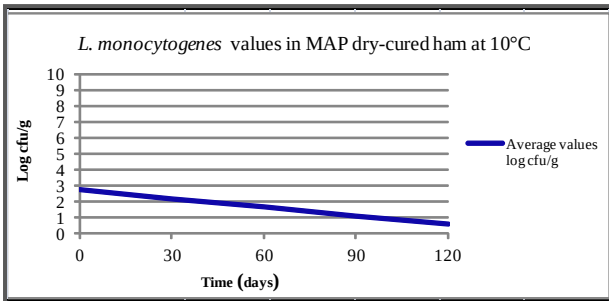


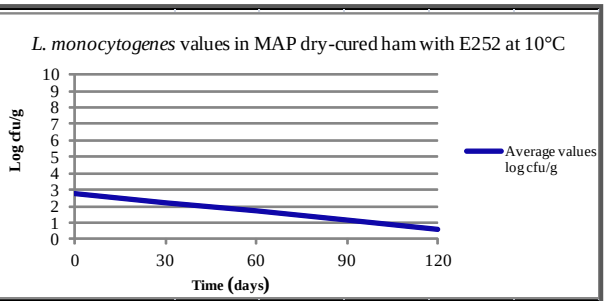


***L. monocytogenes* values in MAP dry-cured ham at 4°C**						***L. monocytogenes* values in MAP dry-cured hamwith E252 at 4°C**					
Time (days)	**0**	**30**	**60**	**90**	**120**	**Time (days)**	**0**	**30**	**60**	**90**	**120**
Average values log cfu/g	2,814	2,476	2,136	1,800	1,462	**Average values log cfu/g**	3,089	2,656	2,222	1,789	1,356
Average values cfu/g	651	299	137	63	29	**Average values cfu/g**	1228	453	167	62	23

Graphic n.9: Values of *L. monocytogenes* at 4°C

The values of *L. monocytogenes* at 10°C are reported in the graphic n.10.

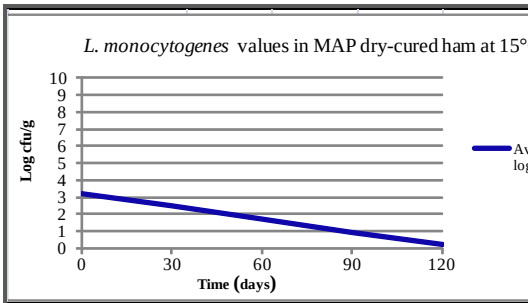


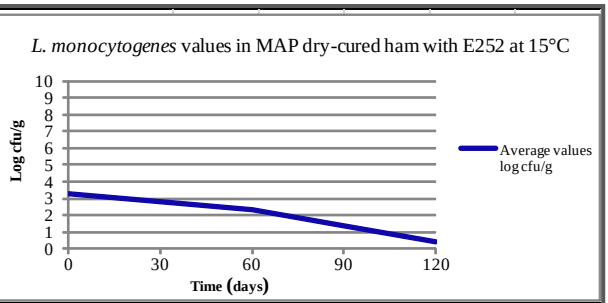


***L. monocytogenes* values in MAP dry-cured ham at 10°C**						***L. monocytogenes* values in MAP dry-cured ham with E252 at 10°C**					
Time (days)	**0**	**30**	**60**	**90**	**120**	**Time (days)**	**0**	**30**	**60**	**90**	**120**
Average values log cfu/g	2,741	2,203	1,664	1,126	0,588	**Average values log cfu/g**	2,800	2,245	1,690	1,135	0,581
Average values cfu/g	551	160	46	13	4	**Average values cfu/g**	631	176	49	14	4

Graphic n.10: Values of *L. monocytogenes* at 10°C

The values of *L. monocytogenes* at 15°C are reported in the graphic n.11.

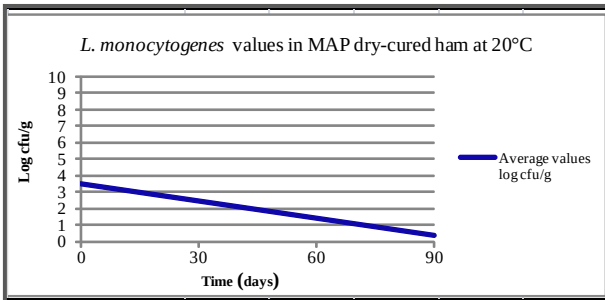


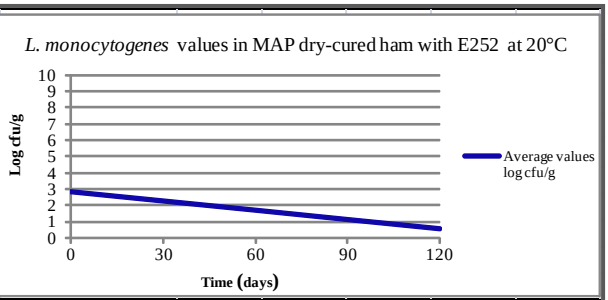


***L. monocytogenes* values in MAP dry-cured ham at 15°C**						***L. monocytogenes* values in MAP dry-cured ham with E252 at 15°C**					
Time (days)	**0**	**30**	**60**	**90**	**120**	**Time (days)**	**0**	**30**	**60**	**90**	**120**
Average values log cfu/g	3,229	2,472	1,715	0,958	0,201	**Average values log cfu/g**	3,320	2,829	2,339	1,358	0,377
Average values cfu/g	1694	296	52	9	2	**Average values cfu/g**	2088	675	218	23	2

Graphic n.11: Values of *L. monocytogenes* at 15°C

The values of *L. monocytogenes* at 20°C are reported in the graphic n.12.





***L. monocytogenes* values in MAP dry-cured ham at 20°C**						***L. monocytogenes* values in MAP dry-cured ham with E252 at 20°C**					
Time (days)	**0**	**30**	**60**	**90**	**120**	**Time (days)**	**0**	**30**	**60**	**90**	**120**
Average values log cfu/g	3,460	2,430	1,400	0,371		**Average values log cfu/g**	3,790	2,740	1,690	0,640	0,010
Average values cfu/g	2885	269	25	2		**Average values cfu/g**	6170	550	49	4	1

Graphic n.12: Values of *L. monocytogenes* at 20°C

The level of contamination of *L. monocytogenes* decreased during the shelf-life of dry-cured hams with and without preservatives (E252) at all the different temperatures of storage. The values of the pathogen were similar for both products, there were no relevant differences between the samples.

4.4 CHALLENGE TEST OF SALAMI

The aim of the study was to evaluate the growth of *L. monocyt*ogenes in salami.

The product was divided into 4 batches, contaminated, sliced, MAP packaged and stored at 4°C for 120 days. The different batches were immediately analyzed for pH and Aw and after 0, 60, 90 and 120 days (T0, T60, T90 and T120) tested for the detection and the enumeration of *L. monocyt*ogenes.

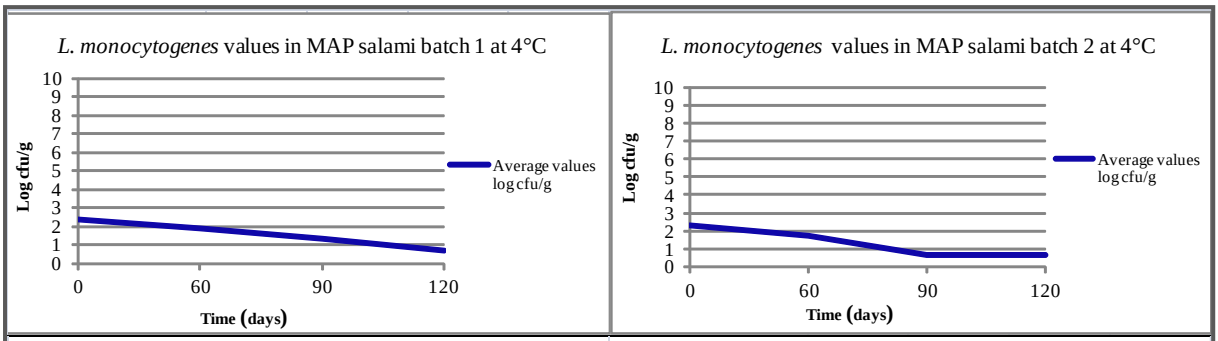
The average values of pH and Aw are reported in the table n.6.

Method	Average values ± standard deviation			
	Batch 1	Batch 2	Batch 3	Batch 4
pH	5,05 ±0,03	5,15 ± 0,06	5,10 ± 0,04	5,08 ± 0,04
Aw	0,928 ±0,000	0,940 ±0,00	0,940 ±0,00	0,931±0,00

Table n.6: Average values of pH and Aw

The values of pH and Aw detected at T0 allowed the growth of the pathogen, therefore the salami could be a "suitable substrate" for the growth of *L. monocyt*ogenes.

The values of *L. monocytogenes* in MAP salami batches 1 and 2 at 4°C are reported in the graphic n.13.

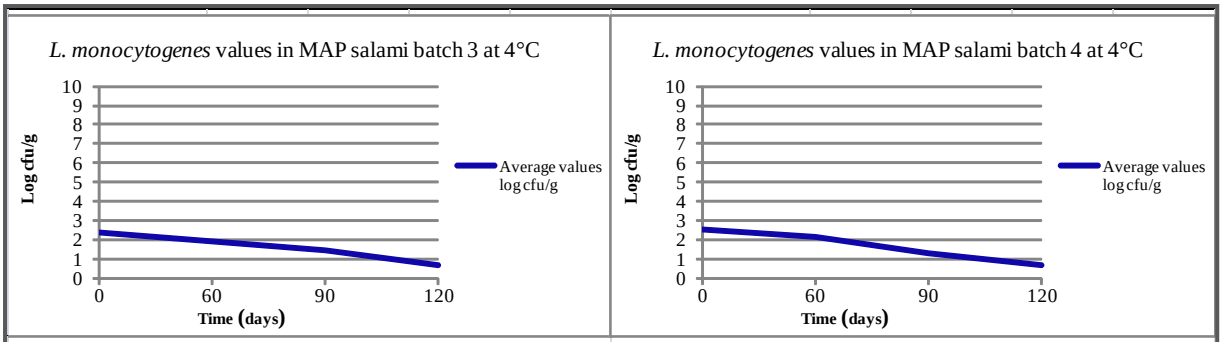


L. monocytogenes values in MAP salami batch 1 at 4°C				
Time (days)	0	60	90	120
Average values log cfu/g	2,380	1,900	1,300	0,700

L. monocytogenes values in MAP salami batch 2 at 4°C				
Time (days)	0	60	90	120
Average values log cfu/g	2,320	1,700	0,700	0,700

Graphic n.13: Values of *L. monocytogenes*

The values of *L. monocytogenes* in MAP salami batches 3 and 4 at 4°C are reported in the graphic n.14.



***L. monocytogenes* values in MAP salami batch 3 at 4°C**

Time (days)	0	60	90	120
Average values log cfu/g	2,410	1,950	1,480	0,700

***L. monocytogenes* values in MAP salami batch 4at 4°C**

Time (days)	0	60	90	120
Average values log cfu/g	2,520	2,110	1,300	0,700

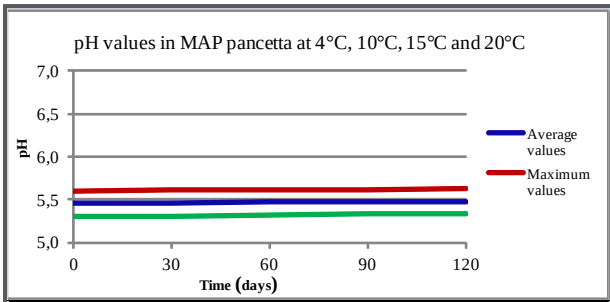
Graphic n.14: Values of *L. monocytogenes*

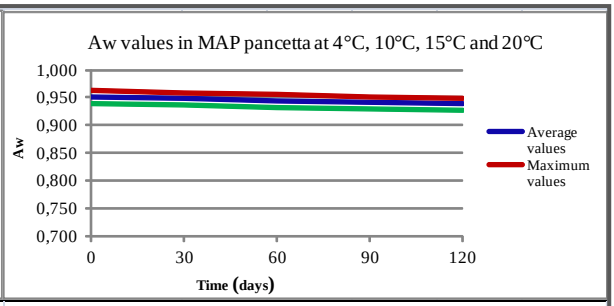
L. monocytogenes decreased during the shelf-life of the samples stored at 4°C. The results obtained were similar for all the 4 batches, therefore the decrease of the pathogen was constant and linear during the shelf-life at 4° C.

4.5 CHALLENGE TEST OF PANCETTA

The aim of the study was to evaluate the growth of *L. monocyto*genes in sliced artificially contaminated pancetta. The contaminated products were sliced, MAP packaged and stored at different temperatures (4°C, 10°C, 15°C and 20°C) for 120 days. The samples were analyzed during their shelf-life for pH, Aw and *L. monocyto*genes after 0, 30, 60, 90 and 120 days (T0, T30, T60, T90 and T120).

The values of pH and Aw at 4°C, 10°C, 15°C and 20°C are reported in the graphic n.15.





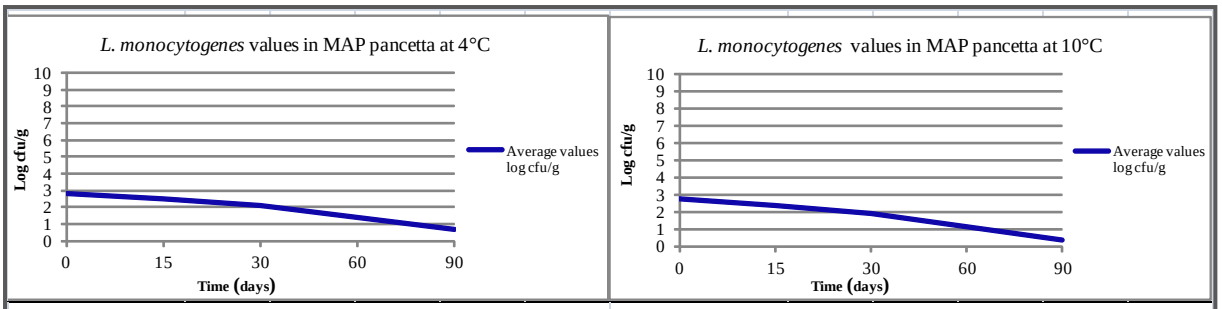
pH values in MAP pancetta						Aw values in MAP pancetta					
Time (days)	**0**	**30**	**60**	**90**	**120**	**Time (days)**	**0**	**30**	**60**	**90**	**120**
Average values	5,46	5,46	5,47	5,47	5,48	**Average values**	0,951	0,948	0,944	0,941	0,938
Maximum values	5,60	5,61	5,61	5,62	5,63	**Maximum values**	0,962	0,959	0,956	0,952	0,949
Minimum values	5,31	5,31	5,32	5,33	5,33	**Minimum values**	0,939	0,936	0,933	0,930	0,926

Graphic n.15: Values of pH and Aw at 4°C, 10°C, 15°C and 20°C

The values of pH and Aw detected at T 0 allowed the growth of the pathogen, therefore the pancetta samples could be a "suitable substrate" for the growth of *L. monocyto*genes.

The average value of pH was constant at all the different temperatures during the shelf-life of the samples. The values of Aw decreased during the shelf-life without relevant differences between the storage temperatures.

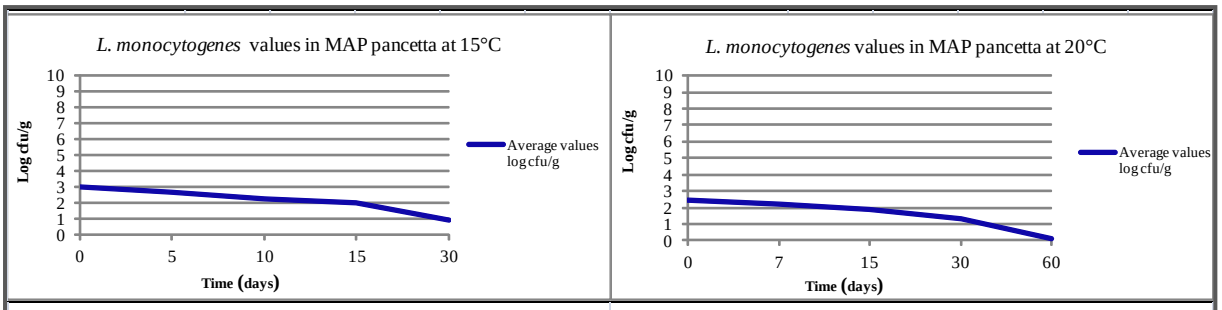
The values of *L. monocytogenes* in MAP pancetta at 4°C and 10°C are reported in the graphic n.16.



L. monocytogenes values in MAP pancetta at 4°C						*L. monocytogenes* values in MAP pancetta at 10°C					
Time (days)	**0**	**15**	**30**	**60**	**90**	**Time (days)**	**0**	**15**	**30**	**60**	**90**
Average values log cfu/g	2,848	2,491	2,133	1,418	0,703	**Average values log cfu/g**	2,739	2,345	1,951	1,162	0,374
Average values cfu/g	705	309	136	26	29	**Average values cfu/g**	548	221	89	15	2

Graphic n.16: Values of *L. monocytogenes* in MAP pancetta at 4°C and 10°C

The values of *L. monocytogenes* in MAP pancetta at 15°C and 20°C are reported in the graphic n.17.



L. monocytogenes values in MAP pancetta at 15°C						*L. monocytogenes* values in MAP pancetta at 20°C					
Time (days)	**0**	**5**	**10**	**15**	**30**	**Time (days)**	**0**	**7**	**15**	**30**	**60**
Average values log cfu/g	2,989	2,655	2,321	1,998	0,987	**Average values log cfu/g**	2,490	2,218	1,907	1,324	0,158
Average values cfu/g	974	452	210	97	10	**Average values cfu/g**	309	165	81	21	1

Graphic n.17: Values of *L. monocytogenes* in MAP pancetta at 15°C and 20°

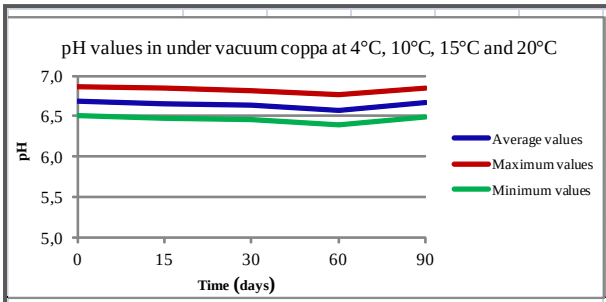
L. monocytogenes decreased during the shelf-life of the samples stored at 4°C. We can observe that the reduction of the pathogen was constant also for the other samples stored at the different temperatures of thermal abuse. Moreover the pathogen was only detected in the pancetta samples stored at 15°C until 30 days, while it was present in the samples stored at 20°C until 60 days.

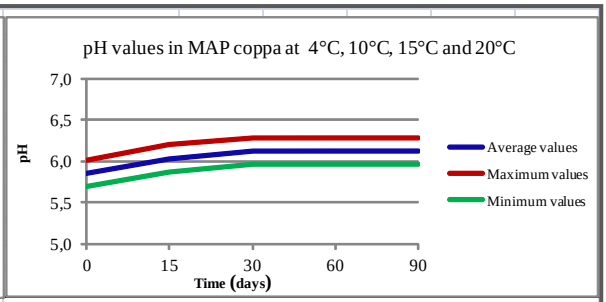
4.6 CHALLENGE TEST OF COPPA

The aim of the study was to evaluate the growth of *L. monocyto*genes in artificially contaminated under vacuum packed coppa and artificially contaminated sliced MAP packed coppa.

The contaminated products were stored at different temperatures (4°C, 10°C, 15°C and 20°C) for 120 days. The products were analyzed during their shelf-life for pH, Aw and *L. monocyto*genes after 0, 15, 30, 60, 90 and 120 days (T0, T30, T60, T90 and T120).

The values of pH at 4°C, 10°C, 15°C and 20°C are reported in the graphic n.18.





pH values in under vacuum coppa

Time (days)	0	15	30	60	90
Average values	6,68	6,66	6,63	6,58	6,67
Maximum values	6,86	6,84	6,81	6,76	6,85
Minimum values	6,51	6,48	6,46	6,4	6,49

pH values in MAP coppa

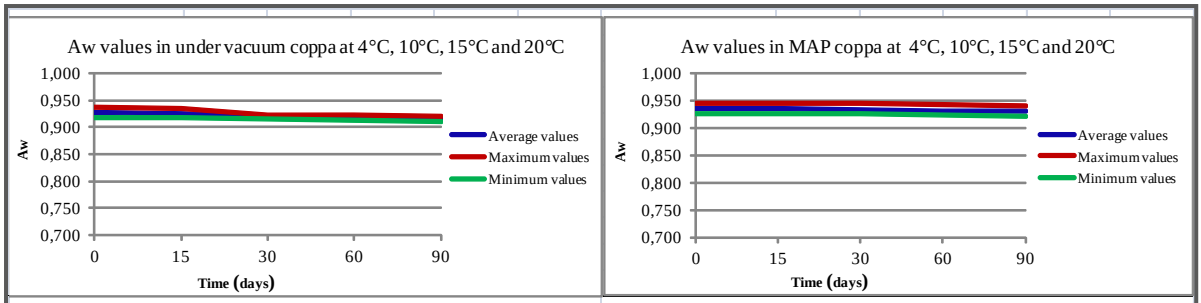
Time (days)	0	15	30	60	90
Average values	5,85	6,03	6,13	6,13	6,13
Maximum values	6,01	6,20	6,29	6,29	6,29
Minimum values	5,69	5,87	5,97	5,97	5,97

Graphic n.18: Values of pH at 4°C, 10°C, 15°C and 20°C

The average value of pH was almost constant at all the different storage temperatures during the shelf-life of the under vacuum packaged samples. While the pH values increased from 5,85 at T0 to 6,13 at T90 for the sliced MAP packaged samples.

The pH values detected for both samples did not guarantee the inability of growth of the pathogen, therefore the coppa samples could be a "suitable substrate" of growth for *L. monocyto*genes.

The values of Aw at 4°C, 10°C, 15°C and 20°C are reported in the graphic n.19.



Aw values in under vacuum coppa

Time (days)	0	15	30	60	90
Average values	0,928	0,926	0,920	0,919	0,916
Maximum values	0,938	0,935	0,924	0,923	0,920
Minimum values	0,918	0,917	0,916	0,914	0,912

Aw values in MAP coppa

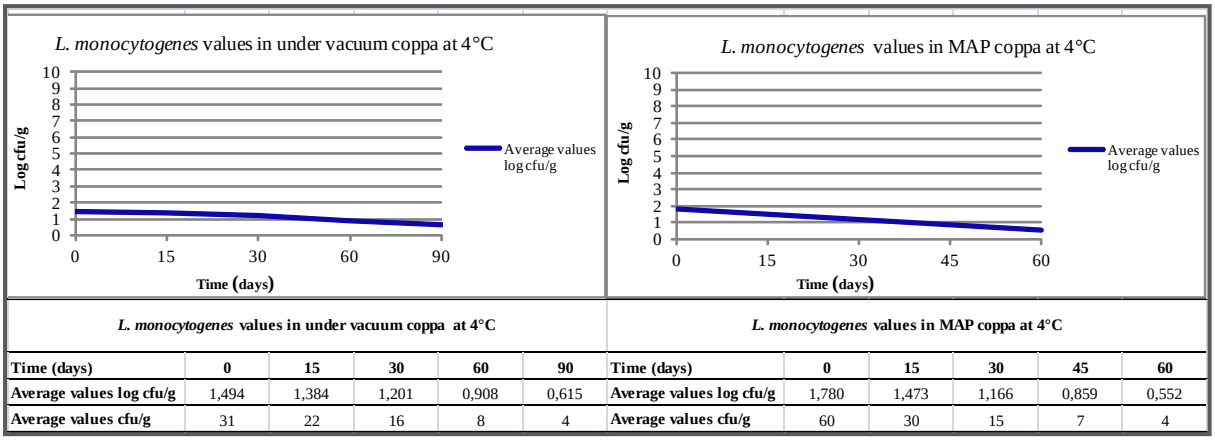
Time (days)	0	15	30	60	90
Average values	0,936	0,935	0,934	0,932	0,930
Maximum values	0,946	0,945	0,944	0,942	0,940
Minimum values	0,927	0,926	0,925	0,923	0,921

Graphic n.19: Values of pH at 4°C, 10°C, 15°C and 20°C

The values of Aw detected for the under vacuum packed samples and for sliced MAP packed samples decreased during the shelf-life without relevant differences between the storage temperatures.

The values of pH and Aw detected allowed the growth of the pathogen, therefore the coppa samples could be a "suitable substrate" for the growth of *L. monocyt*ogenes.

The values of *L. monocytogenes* in under vacuum packed coppa and in sliced MAP packed coppa at 4°C are reported in the graphic n.20.



***L. monocytogenes* values in under vacuum coppa at 4°C**

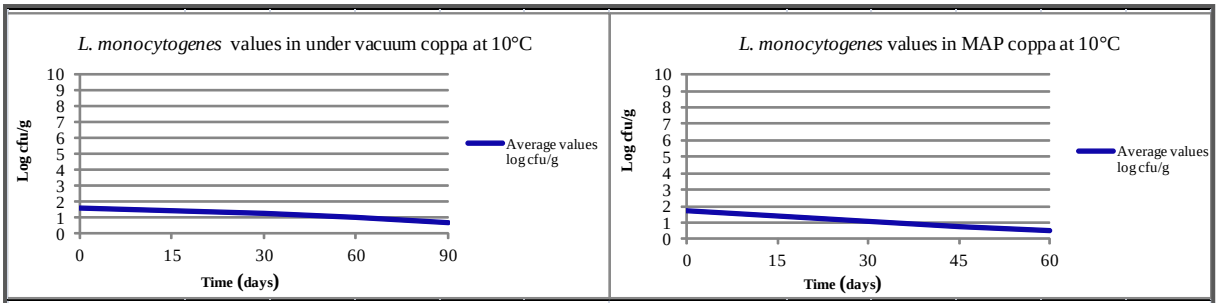
Time (days)	0	15	30	60	90
Average values log cfu/g	1,494	1,384	1,201	0,908	0,615
Average values cfu/g	31	22	16	8	4

***L. monocytogenes* values in MAP coppa at 4°C**

Time (days)	0	15	30	45	60
Average values log cfu/g	1,780	1,473	1,166	0,859	0,552
Average values cfu/g	60	30	15	7	4

Graphic n.20: Values of *L. monocytogenes* at 4°C

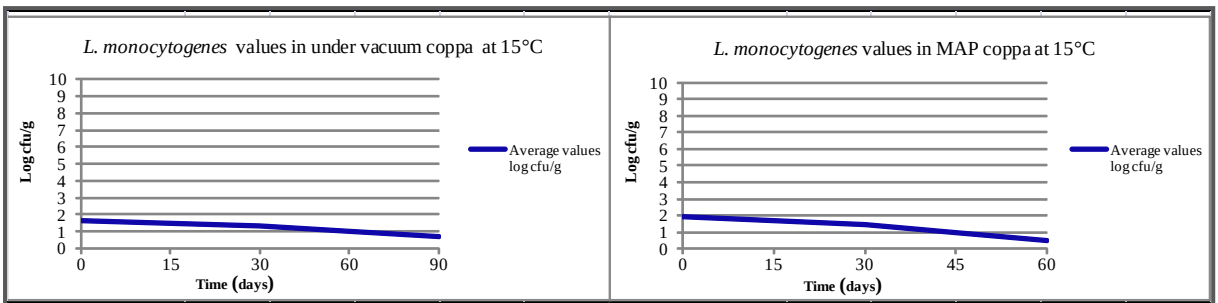
The values of *L. monocytogenes* in under vacuum packed coppa and in sliced MAP packed coppa at 10°C are reported in the graphic n.21.



***L. monocytogenes* values in under vacuum coppa at 10°C**						***L. monocytogenes* values in coppa at 10°C**					
Time (days)	**0**	**15**	**30**	**60**	**90**	**Time (days)**	**0**	**15**	**30**	**45**	**60**
Average values log cfu/g	1,629	1,470	1,311	0,993	0,675	**Average values log cfu/g**	1,730	1,418	1,106	0,794	0,482
Average values cfu/g	43	30	20	10	5	**Average values cfu/g**	54	26	13	6	3

Graphic n.21: values of *L. monocytogenes* at 10°C

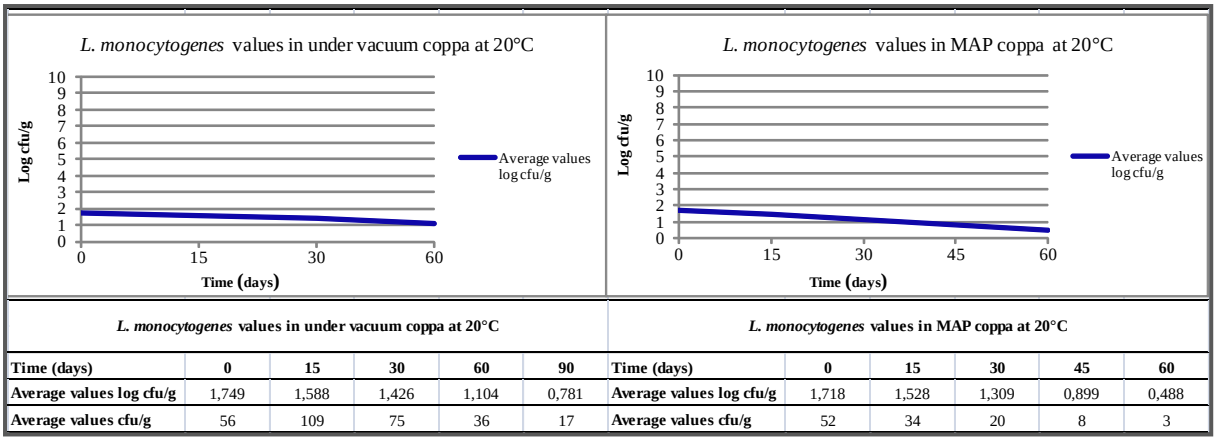
The values of *L. monocytogenes* in under vacuum packed coppa and in sliced MAP packed coppa at 15°C are reported in the graphic n.22.



***L. monocytogenes* values in under vacuum coppa at 15°C**						***L. monocytogenes* values in MAP coppa at 15°C**					
Time (days)	**0**	**15**	**30**	**60**	**90**	**Time (days)**	**0**	**15**	**30**	**45**	**60**
Average values log cfu/g	1,645	1,489	1,332	1,020	0,707	**Average values log cfu/g**	1,921	1,696	1,440	0,958	0,477
Average values cfu/g	44	31	21	10	5	**Average values cfu/g**	83	50	28	9	3

Graphic n.22: Values of *L. monocytogenes* at 15°C

The values of *L. monocytogenes* in under vacuum packed coppa and in sliced MAP packed coppa at 20°C are reported in the graphic n.23.



***L. monocytogenes* values in under vacuum coppa at 20°C**						***L. monocytogenes* values in MAP coppa at 20°C**					
Time (days)	**0**	**15**	**30**	**60**	**90**	**Time (days)**	**0**	**15**	**30**	**45**	**60**
Average values log cfu/g	1,749	1,588	1,426	1,104	0,781	**Average values log cfu/g**	1,718	1,528	1,309	0,899	0,488
Average values cfu/g	56	109	75	36	17	**Average values cfu/g**	52	34	20	8	3

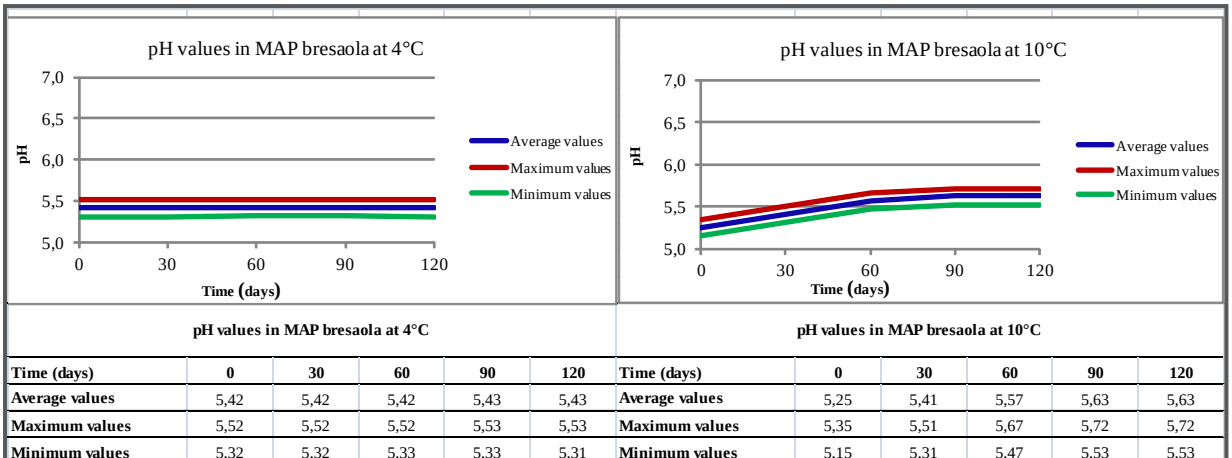
Graphic n.23: Values of *L. monocytogenes* at 20°C

L. monocytogenes decreased during the shelf-life of the under vacuum packed and of the sliced MAP packed samples stored at 4°C. We can observe that the reduction of the pathogen was also constant for the other samples stored at the different temperatures of thermal abuse. Moreover, the pathogen grew in the under vacuum samples stored at all different temperatures until 60 days, while it grew in the sliced MAP packaged samples stored at all different temperatures until 60 days.

4.7 CHALLENGE TEST OF BRESAOLA

The aim of the study was to evaluate the growth of *L. monocyto*genes in artificially contaminated sliced packed bresaola. The contaminated samples were stored at different temperatures (4°C, 10°C, 15°C and 20°C) for 120 days. The samples were analyzed during their shelf-life for pH, Aw and *L. monocyto*genes after 0, 15, 30, 60, 90 and 120 days (T0, T30, T60, T90 and T120).

The values of pH at 4°C, 10°C, 15°C and 20°C are reported in the graphic n.24.



pH values in MAP bresaola at 4°C

Time (days)	0	30	60	90	120
Average values	5,42	5,42	5,42	5,43	5,43
Maximum values	5,52	5,52	5,52	5,53	5,53
Minimum values	5,32	5,32	5,33	5,33	5,31

pH values in MAP bresaola at 10°C

Time (days)	0	30	60	90	120
Average values	5,25	5,41	5,57	5,63	5,63
Maximum values	5,35	5,51	5,67	5,72	5,72
Minimum values	5,15	5,31	5,47	5,53	5,53

pH values in MAP bresaola at 15°C

Time (days)	0	30	60	90	120
Average values	5,39	5,62	5,84	6,07	6,29
Maximum values	5,51	5,74	5,96	6,19	6,42
Minimum values	5,27	5,50	5,72	5,95	6,17

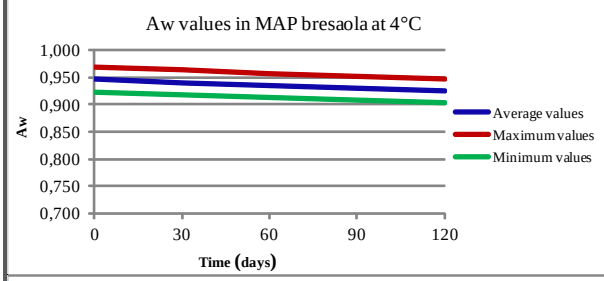
pH values in MAP bresaola at 20°C

Time (days)	0	30	60	90	120
Average values	5,43	5,77	6,11	6,14	6,14
Maximum values	5,61	5,95	6,29	6,32	6,31
Minimum values	5,25	5,59	5,93	5,96	5,97

Graphic n.24: Values of pH at 4°C, 10°C, 15°C and 20°C

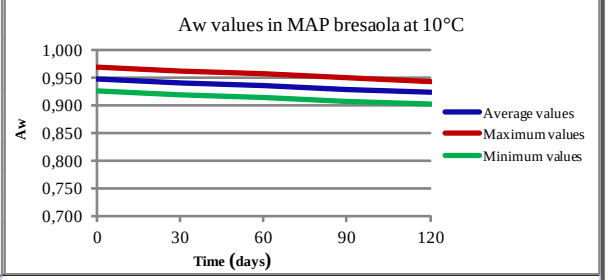
The average values of pH were almost constant during the shelf-life at 4°C storage condition. The pH values of the samples stored at thermal abuse temperatures of 10°C, 15°C and 20°C increased during the shelf-life of the samples.

The values of Aw at 4°C, 10°C, 15°C and 20°C are reported in the graphic n.25



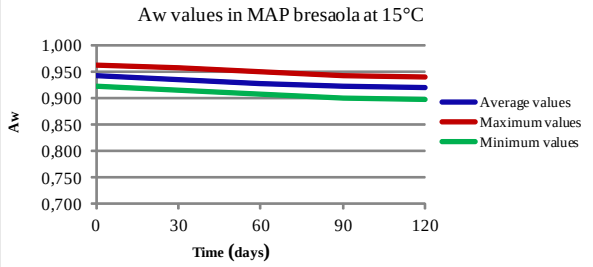
Aw values in MAP bresaola at 4°C

Time (days)	0	30	60	90	120
Average values	0,946	0,941	0,936	0,931	0,925
Maximum values	0,968	0,963	0,958	0,953	0,947
Minimum values	0,924	0,919	0,914	0,909	0,903



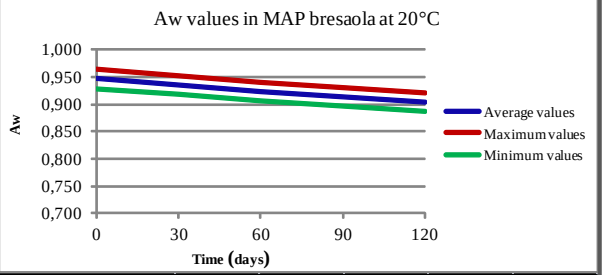
Aw values in MAP bresaola at 10°C

Time (days)	0	30	60	90	120
Average values	0,947	0,941	0,935	0,929	0,923
Maximum values	0,968	0,962	0,956	0,949	0,943
Minimum values	0,926	0,920	0,914	0,908	0,902



Aw values in MAP bresaola at 15°C

Time (days)	0	30	60	90	120
Average values	0,943	0,936	0,929	0,922	0,920
Maximum values	0,964	0,957	0,950	0,943	0,941
Minimum values	0,922	0,915	0,908	0,901	0,899



Aw values in MAP bresaola at 20°C

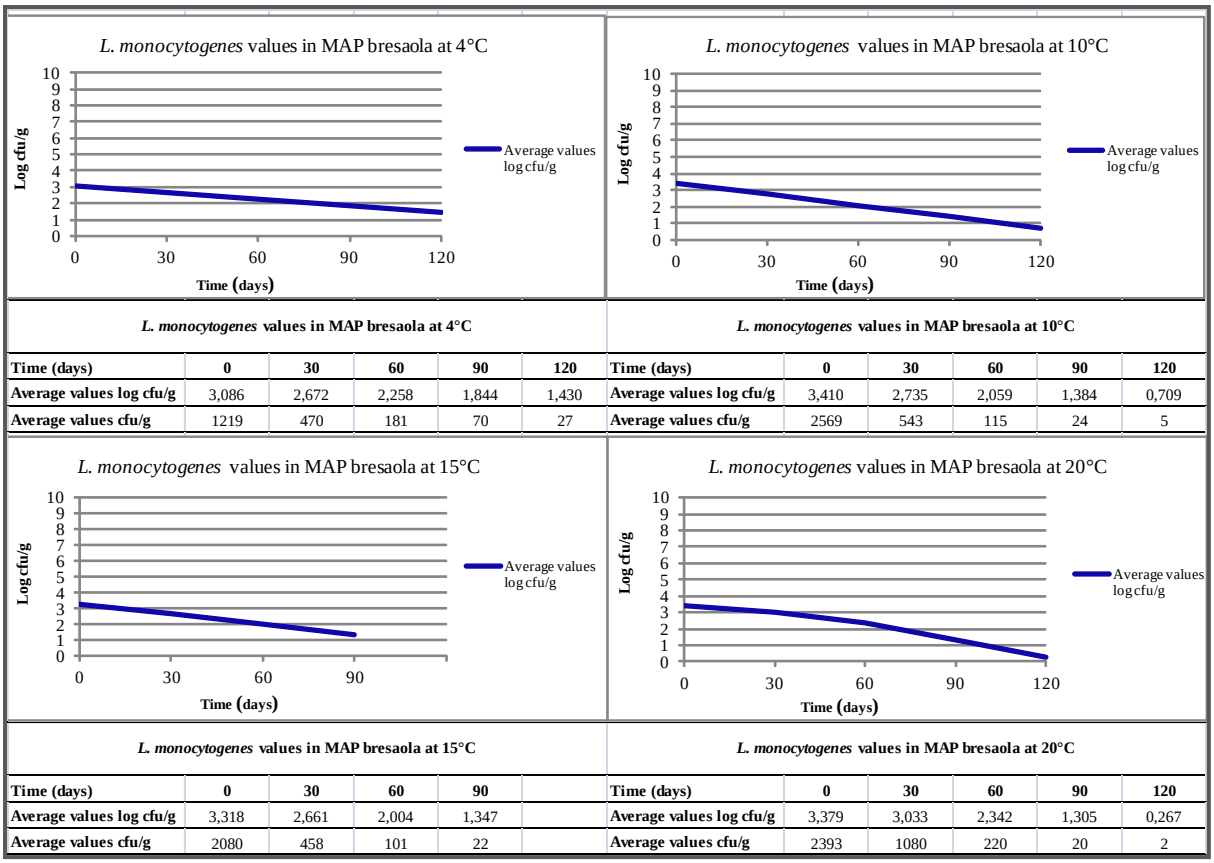
Time (days)	0	30	60	90	120
Average values	0,946	0,935	0,924	0,914	0,903
Maximum values	0,963	0,952	0,941	0,931	0,920
Minimum values	0,929	0,918	0,907	0,897	0,886

. Graphic n.25: Values of Aw at 4°C, 10°C, 15°C and 20°C

The values of Aw detected for the sliced bresaola samples decreased during their shelf-life without relevant differences between the storage temperatures.

The values of pH and Aw detected allowed the growth of the pathogen in the product analyzed, therefore the bresaola samples could be a "suitable substrate" for the growth of *L. monocyt*ogenes.

The values of *L. monocytogenes* at 4°C, 10°C, 15°C and 20°C are reported in the graphic n.26.



***L. monocytogenes* values in MAP bresaola at 4°C**

Time (days)	0	30	60	90	120
Average values log cfu/g	3,086	2,672	2,258	1,844	1,430
Average values cfu/g	1219	470	181	70	27

***L. monocytogenes* values in MAP bresaola at 10°C**

Time (days)	0	30	60	90	120
Average values log cfu/g	3,410	2,735	2,059	1,384	0,709
Average values cfu/g	2569	543	115	24	5

***L. monocytogenes* values in MAP bresaola at 15°C**

Time (days)	0	30	60	90
Average values log cfu/g	3,318	2,661	2,004	1,347
Average values cfu/g	2080	458	101	22

***L. monocytogenes* values in MAP bresaola at 20°C**

Time (days)	0	30	60	90	120
Average values log cfu/g	3,379	3,033	2,342	1,305	0,267
Average values cfu/g	2393	1080	220	20	2

Graphic n.26: Values of *L. monocytogenes* at 4°C, 10°C, 15°C and 20°C

L. monocytogenes decreased during the shelf-life of the sliced MAP packaged bresaola samples stored at 4°C. We can observe that the reduction of the pathogen was constant also for the other samples stored at the different temperatures of thermal abuse.

4.8 CHALLENGE TEST OF COOKED HAM

The aim of the study was to evaluate the growth of *L. monocyt*ogenes in sliced MAP packed cooked ham. The samples were contaminated with an 10cfu/g inoculum of *L. monocyt*ogenes and stored at 4°C for 40 days. The samples were analyzed daily during their shelf-life for the detection and enumeration of the pathogen (Table n.7).

Time of analysis	Storage conditions	Ufc/g	Time of analysis	Storage conditions	Ufc/g
1	Sliced cooked ham stored at 4°C	**6**	21	Sliced cooked ham stored at 4°C	**10**
2	Sliced cooked ham stored at 4°C	**5**	22	Sliced cooked ham stored at 4°C	**10**
3	Sliced cooked ham stored at 4°C	**7**	23	Sliced cooked ham stored at 4°C	**5**
4	Sliced cooked ham stored at 4°C	**5**	24	Sliced cooked ham stored at 4°C	**5**
5	Sliced cooked ham stored at 4°C	**5**	25	Sliced cooked ham stored at 4°C	**5**
6	Sliced cooked ham stored at 4°C	**4**	26	Sliced cooked ham stored at 4°C	**5**
7	Sliced cooked ham stored at 4°C	**6**	27	Sliced cooked ham stored at 4°C	**6**
8	Sliced cooked ham stored at 4°C	**5**	28	Sliced cooked ham stored at 4°C	**8**
9	Sliced cooked ham stored at 4°C	**5**	29	Sliced cooked ham stored at 4°C	**5**
10	Sliced cooked ham stored at 4°C	**5**	30	Sliced cooked ham stored at 4°C	**5**
11	Sliced cooked ham stored at 4°C	**5**	31	Sliced cooked ham stored at 4°C	**5**
12	Sliced cooked ham stored at 4°C	**5**	32	Sliced cooked ham stored at 4°C	**6**
13	Sliced cooked ham stored at 4°C	**5**	33	Sliced cooked ham stored at 4°C	**10**
14	Sliced cooked ham stored at 4°C	**4**	34	Sliced cooked ham stored at 4°C	**<4**
15	Sliced cooked ham stored at 4°C	**7**	35	Sliced cooked ham stored at 4°C	**5**
16	Sliced cooked ham stored at 4°C	**4**	36	Sliced cooked ham stored at 4°C	**<4**
17	Sliced cooked ham stored at 4°C	**15**	37	Sliced cooked ham stored at 4°C	**5**
18	Sliced cooked ham stored at 4°C	**5**	38	Sliced cooked ham stored at 4°C	**<1**
19	Sliced cooked ham stored at 4°C	**5**	39	Sliced cooked ham stored at 4°C	**<4**
20	Sliced cooked ham stored at 4°C	**7**	40	Sliced cooked ham stored at 4°C	**<1**

Table n.7: *L. monocyt*ogenes in artificially contaminated cooked ham

L. monocytogenes did not grow in artificially contaminated sliced cured ham during 40 days of shelf-life at 4°C. The maximum growth value of the pathogen was 15 cfu/g after two weeks of storage. The level of contamination of *L. monocytogenes* was lower than 100 ufc/g.

4.9 CHALLENGE TEST OF MORTADELLA

The aim of the study was to evaluate the growth of *L. monocyt*ogenes in sliced MAP packed mortadella. The samples were contaminated with 10cfu/g inoculum of *L. monocyt*ogenes and stored at 4°C for 40 days. The samples were daily analyzed during their shelf-life for the detection and the enumeration of the pathogen (Table n.8).

Time of analysis	**Storage conditions**	**Ufc/g**
1	Sliced mortadella stored at 4°C	**8**
2	Sliced mortadella stored at 4°C	**14**
3	Sliced mortadella stored at 4°C	**22**
4	Sliced mortadella stored at 4°C	**28**
5	Sliced mortadella stored at 4°C	**34**
6	Sliced mortadella stored at 4°C	**48**
7	Sliced mortadella stored at 4°C	**55**
8	Sliced mortadella stored at 4°C	**64**
9	Sliced mortadella stored at 4°C	**71**
10	Sliced mortadella stored at 4°C	**82**
11	Sliced mortadella stored at 4°C	**94**
12	Sliced mortadella stored at 4°C	**102**

Table n.8: *L. monocyt*ogenes in artificially contaminated mortadella

The detection and the enumeration of *L. monocytogenes* were stopped after 12 day because the pathogen grew more than 100 cfu/g.

5.Discussions and Conclusion

The presence of *L. monocytogenes* and *Listeria* spp. in the food industry is very common.

Moreover *L. monocytogenes* is an important foodborne pathogen with a persistent ability to form biofilm matrices in the food processing environments. The persistence of *L. monocytogenes* biofilms on food and nonfood contact surfaces is the major attribute facilitating this pathogen's environmental spread and subsequent contamination of ready-to-eat food products. In a food processing plant, the common sites for *L. monocytogenes* isolations are floor drains, conveyor belts, stainless steel equipment surfaces, product transportation racks, and cold rooms. Prevailing conditions on these sites such as ample water content and food residues further provide a unique opportunity for pathogenic *L. monocytogenes* to form biofilm matrix and reside therein.

Though the adherence strength of different *L. monocytogenes* strains varies, the majority of *L. monocytogenes* strains of different serotypes are able to produce biofilm.

Antimicrobial agents have been tested for their efficacy against *L. monocytogenes* biofilms: alkaline and acid electrolyzed water, octenidine hydrochloride, quaternary ammonium compounds, sodium hypochlorite and hydrogen peroxide. These findings report a 2-4 log reduction in *L. monocytogenes* populations biofilms and indicate that although sanitizer compounds aid in biofilm removal, the degree of biofilm removal depends on factors such as biofilm age, the surface on which biofilm is formed, and the substrate in which the biofilms were produced. In addition, repeated exposure to single disinfecting agents could also confer subsequent insensitivity to *L. monocytogenes* cells.

Another promising approach to control and eradicate biofilm formation is the use of bacteriophages as antibacterial agents. Bacteriophages are viruses that infect bacterial using

cells specific mechanisms for a target genus, serotype, or strain. Bacteriophages are ubiquitous in nature, and as many as 108 phage particles can be isolated, for example, from 1g of soil or water. Phages have also been isolated from several food products such as meat, dairy, and vegetable products. All phage are obligate parasites, meaning that they rely on a specific host for propagation. For biocontrol strategies, lytic phages that have the ability to rapidly lyse bacterial cells without integration into bacterial DNA are recommended (Garcia et al., 2008; Hangens et al., 2008; McIntyre et al., 2007; Sharma, 2013).

Recently, the U.S. Food and Drug Administration approved the anti-listerial phage-based product, Listex P100, as GRAS (Generally Recognized As Safe) for all food product, at levels not to exceed 10^9 pfu/g. In Europe and Asia, food safety legislation regarding the use of phages as biocontrol agents is largely undefined. Debate continue in Europe as to whether such preparation should be used and whether they should be considered as processing aids or food additives, although the recent opinion of the European Food Safety Authority has helped to clarify the issues in Europe. In particular, EFSA says that the safety features of phage should be assessed "case by case" (EFSA, 2006; EFSA, 2012). However, in Europe, nowadays, the use of phage can take place exclusively on contact surfaces and environments, not directly on the product (Soni and Nannapaneni, 2010).

The evidence from the numerous research studies conducted on biocontrol of *L. monocytogenes* suggest that there is undoubtedly a role for the strategic and intelligent use of biopreservation strategies in the control of this organism in ready to eat foods. However, similar to other control measures, biopreservation does not offer a “one size fits all” solution to pathogen control, and applications need to be designed on a “case by case” basis. In addition, biological preservation can only be considered as an additional processing parameter for improved safety and quality assurance of a food. It should not replace proper controls and hygiene practices (Holk and Berg, 2009; Kalkan et al., 2011; Mahony et al., 2011; Marsden et al.,2011; Sanna et al., 2012).

The results of the analysis studied in this survey are very satisfactory because the absence of *L. monocytogenes* was confirmed in all the samples and only few superficial samples were positive to the detection of *Listeria* spp. The presence of the species of *Listeria* was only detected on the floor drains and they was never detected on conveyor belts, stainless steel equipment surfaces, product transportation racks, cold rooms and in the products. The daily GMPs and the sanification programs are able to guarantee the absence of *L. monocytogenes* both on the surface of the production plant and in the RTE foods. The food business operators can demonstrate the absence of the pathogen if hygiene process criteria is in compliance with the Regulation (EC) No. 2073/2005.

In accordance with Article 4 of Regulation (EC) No. 852/2004 the food business operators are required to comply with the microbiological criteria listed in Regulation (EC) No. 2073/2005 of the Commission of 15 November 2005 on microbiological criteria for foodstuffs, which took effect from 1 January 2006.

In particular, the cooked and cured meat falls under the category 1.2 "Prepared meals that are able to support the growth of *Listeria monocytogenes*, other than those intended for infants and for special medical purposes". For these products *L. monocytogenes* shall be absent in 5 units from 25 g of sample, unless there is evidence that at the end of the shelf life of the product the pathogen is not able to overcome the threshold of 100 cfu/g, always in the 5 united sample.

Contamination of meat products "Ready-To-Eat" (RTE) by *L. monocytogenes* is mainly during the later stages of the primary processing, and it is common practice to the consumption of these products without further preparation.

5.1 DRY-CURED HAM

Dry-cured ham has always been regarded as a safe product. Long ripening times, refrigeration temperatures during the first processing stages, salt presence, constant a_w lowering are all parameters which should constitute a sanitary warranty for the finished product. Few experimental surveys are been conducted to validate ham processing procedure but from literature data it can be inferred that *L. monocytogenes* might undergo a drastic quantitative reduction during the various processing stages even if present in raw material (Reynolds et al., 20011; Portocarrero et al., 2002). Dry-cured ham is an example of product which may undergo a cross contamination at the end of the ripening period, when it is cut into pieces, sliced and MAP packed. If *L. monocytogenes* is present on the surfaces of the production plants, the pathogen may be transferred to the product during the cutting processes. Ready-to-eat products include several control programs in the HACCP and SOP plans in order to prevent the growth and the diffusion of the pathogen and forbidden its possibility to create biofilms. In the USDA/FSIS on "*Listeria* Risk Assessment" it is indicated that the use and/or the combination of several methods for *L. monocytogenes* control in RTE products exposed to the environment after inactivation treatment have a synergistic effect on risk reduction (FDA-FSIS, 2003). The absence of the pathogen on the surfaces of the plant before production demonstrate that good manufacture practices and sanification programs guarantee the food safety until the end of the shelf life of the product, as tested by the results of this survey. Pathogen behavior during commercialization can be strictly correlated to storage temperature and the physic-chemical characteristics of dry-cured ham; this is the reason why cold storage of pre-sliced ham and under-vacuum ham pieces is commonly recommended. In addition, previous studies have emphasized the necessity for assigning to slicing only hams with aw ≤91 (Grisenti et al., 2004). Predictive models were also developed for *L. monocytogenes* growth at different pH levels, different temperatures and different salt

concentrations. Moreover, the packing in protective atmosphere can be effective for the pathogen control; it has been observed that the use of CO_2 at levels higher than 20% may cause an extension of the lag phase, inhibition of growth and/or slight inactivation. The results of this study show that there is no possibility of the pathogen growing in dry-cured ham packed under protective atmosphere or under-vacuum. Also Grisenti et al. (2004) have controlled the behavior of *L. monocytogenes* in raw hams whole, sliced and packaged in slices in a protective atmosphere. The values of aw of the products ranged between 0.90 and 0.93 and the storage temperatures to which they were maintained equal to 3-8° C and 20° C, and in all the samples examined these researchers found no growth *L. monocytogenes* with the progressive reduction of the bacterial cells, in agreement with this survey.

In 2005, Comi et al. have examined the performance of *L. monocytogenes* in portions of ham vacuum packed and MAP (15% CO2 / 85% N2) part doped sodium lactate/ odium diacetate (1.5% -1%) recording the reduction number of listeria inoculated regardless of activity of preservatives added. In 2009, Barbuti et al. have validated the production process of the ham for the inactivation of *L. monocytogenes*. The results obtained with the experimentation have shown that the production process is able to mature both *L. monocytogenes* that *Salmonella* spp.; the inhibiting factors were how salting initial and steady reduction in a_w tissue. In 2010, Boni et al. have verified the behavior of *L. monocytogenes* during the commercial life of Prosciutto di Modena, assuming recontamination being portioning into slices or later vacuum packaging. Based on the results obtained these researchers have shown that the ham of Modena is not a food that supports the growth of *L. monocytogenes*. This situation occurred both at 5°C, 10°C and 20°C. Also the results of this survey show no growth of the pathogen nor in dry-cured hams with preservatives or without preservatives for almost 90 days, moreover the shelf life of the product could be elongated until 120 day without risk for the consumers.

5.2 SALAMI

The seasoned salami products in our country are microbiologically healthy and stable. This is achieved by the simultaneous combination of different factors identified by Leistner and Gorris (1995) several years ago. The healthiness of dried products is based on the migration of the salt in the meat and the addition of nitrite. The salt decreases the initial activity of the water by inhibiting or delaying the growth of many spoilage microorganisms while favoring the development of Lactic Acid Bacteria (LAB) or LAB starter and staphylococci starter. The addition of nitrite at the beginning of the fermentation process for the stability of the product inhibits the development of *Salmonella* spp. (Poulanne, 1977). The nitrite in the form of nitrous acid undissociated (HNO2) is able to pass the barrier ionic wall of the bacterial cell and disturb the functionality of bacterial enzymes, and then the bacterial growth. The lowering of the pH to values of 5 causes the reduction of nitrite in nitrogen oxide form ($3H2NO2 \rightarrow 2NO + H2O + HNO3$) (Cantoni, 2012).

During the first day of fermentation the growth of bacteria in the mixture of salami uses all the oxygen in the meat and also that incorporated during crushing. This reduces the oxidation-reduction potential (ORP) making added nitrite more effective and blocks the growth of Gram-negative aerobic bacteria (Pseudomonas and others) that are present in fresh meat. After a few days of fermentation the presence of a high number of LAB, they split the natural sugars, or those added, produce lactic acid with a consequent decrease in pH.

The main obstacle (or factor) which promotes the growth of lactobacilli and coagulase-negative staphylococci is the low water activity of salami. The low pH value decreases the water retention capacity of the meat favoring the dehydration of the tissue meaty.

The growth of enterobacteria, such as Salmonella is inhibited by nitrous acid derived from nitrite, from the low oxygen and the water activity (Cantoni, 2012; Poulanne, 1977).

In this regard, the rapid decrease in the a_w below 0.94 has the effect of destroying enterobacteria breaking the wall by the removal of moisture.

Several works have been carried out by Italian and foreign researchers to verify the behavior of *L. monocytogenes* in salami slices (Garofali et al., 2008; Coppet et al, 2007).

All of these researchers have not only demonstrated the inhibitory activity of the mixtures of salami against *L. monocytogenes*, but also the decrease in the number of cells of the microorganism during the maturing or the packaging of the slices in MAP. In particular Grisenti et al. (2009) were able to demonstrate that *L. monocytogenes* does not develop in seasoned salami that are of value to aw from 0.92 to 0.95 and pH values from 5.1 to 5.7. This inhibition was observed both at temperatures of normal refrigeration (+4 to + 8°C) or at room temperature of 15°, 21° and 25°C.

The surveys reported above are in agreement with the results obtained from this study. *L. monocytogenes* was not found nor on the surface of production or in the finished product, under vacuum or MAP packed. Therefore I can assume that also the salami is not a substrate dangerous for the growth of the pathogen although a_w and pH would place the product among those considered "suitable substrate" for growth of *L. monocytogenes*. Several actions can be assumed to prevent the contamination such as the quality of raw meat, the seasoning process until to obtain a reduction in weight of 25% and a correct acidification process. Moreover sanification and cleaning procedures and good manufacture practices have to be effected to limit the risk of cross contamination.

5.3 PANCETTA, COPPA AND BRESAOLA

Grisenti et al. (2008) evaluated the possible growth of *L. monocytogenes* in whole and sliced Pancetta under vacuum and MAP packaged during the life of the product. The researchers did not detect any increase of *L. monocytogenes*, conversely they observed a drastic reduction of the cells at 4°C, 8°C and 15°C. For this product the inhibiting factors are due the presence of nitrite and probably also to the free fatty acids cause by lipolysis during ripening and shelf-life period. The authors confirm the inability of the pathogen to grow although a_w and pH would place the product among those considered "suitable substrate" for growth of *L. monocytogenes*. The same data are confirmed in this survey.

Coppa was the other product examined in this study. There is no bibliography concerning the detection of *Listeria* spp. in Coppa packed under vacuum or sliced MAP packed. The results of analysis were satisfactory also in this case because both the surface of slicing equipment and the finished products reported the absence of *L. monocytogenes*. The product to be considered safe and to be in compliance with the law can be contaminated with maximum 100 cfu/g of *L. monocytoges* if pH $\leq$ 5.0 and aw $\leq$0.94. Coppa has a average value of pH and a_w equal to 6.0 and 93, respectively therefore it is considered "suitable substrate" for growth of *L. monocytogenes* and it should be absent in the product until the end of its shelf life. The challenge tests demonstrate the inability of the pathogen to grow at +4°C and also at +8°C both in whole and sliced Coppa for 120 days indeed it has been possible to observe a reduction of the pathogen. Therefore the challenge test is in contrast with the predictive models and it is demonstrated that the coppa is not a "suitable substrate" for growth of *L. monocytogenes*.

The evaluation of the shelf-life of Bresaola under vacuum packed and sliced MAP has however shown that this type of food during the storage time planned was not able to support the growth of *L. monocytogenes* neither at +4°C, the optimal storage conditions, nor at 10 ° C

in situations of thermal abuse. Among the intrinsic factors that affect the microbiological stability of food products must remember the pH and the Aw; observation of the values obtained, however, have not been highlighted significant changes or trends, such as to justify the reduction of the pathogen; on the contrary, the population of mesophilic lactobacilli, present in high concentrations in the product at the end of maturation, may, instead, have a role in influencing the performance of regard of *L. monocytogenes*. Several scientific works, in fact, have demonstrated how the lactic acid bacteria have the ability to produce a wide range of bacteriocins namely bacterial protein molecules characterized by antimicrobial activity. The synthesis of batteriocinica might suggest a possible involvement of these substances both in defense mechanisms (preventing the invasion, by other bacterial strains, within the habitat of the bacterium producer), and offensive, implementing strategies invasive in a particular ecological niche (Miller and Bassler, 2001). Among bacteriocins, nisin has proved active and effective against a wide range of Gram positive bacteria, including *L. monocytogenes* (Martinez and Rodriguez, 2005). Also several authors such as Cantoni et al. (2006), Frustoli et al. (2007) and Miraglia et al. (2009) have excluded the possibility of survival of *L. monocytogenes* during the production process but it is necessary to consider the possibility of recontamination of the product during the slicing and the behavior of *L. monocytogenes* during shelf life. The results obtained in this survey have shown the reduction of the pathogen during the life of the product thus ensuring the food safety criteria. The conclusions of challenge tests indicate the inability of growth of *L. monocytogenes* and its constant inactivation during storage and therefore the results should be attributed to the chemical and physical characteristics of the product (salt, aw and nitrite) in addition to the competition exerted by lactic acid that developed in packs. The challenge test is in contrast with the predictive models and it is demonstrated that the bresaola is not a “suitable substrate” for growth of *L. monocytogenes*.

5.4 COOKED HAMS AND MORTADELLA

L. monocytogenes contaminated in meat could be virtually eliminated during the cooking step of RTE meats processing. Therefore, *L. monocytogenes* contamination in RTE meats is primarily due to post-cooking contamination. Post-package decontamination methods such as in-package thermal pasteurization and irradiation, and formulating meat products with antimicrobial additives are common approaches to control of *L. monocytogenes* in RTE meat. Depending on whether there are post-lethality treatments and growth inhibitors, the susceptibility of RTE foods to L. monocytogenes contamination varies. To effectively control *L. monocytogenes* in RTE foods, the FSIS published a final rule that stipulates 3 alternatives:

1. Using both a post-lethality treatment and a growth inhibitor for *Listeria* on RTE products. Establishments opting for this alternative will be subject to FSIS verification activity that focuses on the post-lethality treatment effectiveness. Sanitation is important but is built into the degree of lethality necessary for safety as delivered by the post-lethality treatment.
2. Using either a post-lethality treatment or a growth inhibitor for *Listeria* on RTE products. Establishments opting for this alternative will be subject to more frequent FSIS verification activity than for Alternative 1.
3. Using sanitation measures only. Establishments opting for this alternative will be targeted with the most frequent level of FSIS verification activity. Within this alternative, FSIS will place increased scrutiny on operations that produce hot dogs and deli meats. In a 2001 risk ranking, the FSIS and the Food and Drug Administration identified these products as posing a relative high risk for illness and death (FSIS 2003b). Therefore, it is to the manufacturer's advantage to take measurements for reducing *L. monocytogenes* contamination in food.

The cooked ham and Mortadella portioned, ready in trays already sliced are the most "suitable substrate" for growth of *L. monocytogenes*. Although this product contains some salts like NaCl, nitrites, which have antimicrobial activity, they are not able to inhibit *L. monocytogenes* during the storage at refrigeration temperatures. Several authors observed growth of *L. monocytogenes* in different meaty products after 6 weeks to 4.4 ° C, regardless of the level of initial inoculum. The same protective atmosphere packaging (MAP), also in combination with the refrigeration temperature, was not able to control the growth of *L. monocytogenes* (Bersot et al., 2001;2008).

The results of Challenge test of cooked ham show that there is no growth of the pathogen after 40 days of analysis. The cooked hams are a suitable substrate for *L. monocytogenes* therefore the sanification and cleaning actions, the Good Manufacturing Practice and the HACCP plan have to be more restrictive. The slicing process is done in clean room more selective than those of cured meat because the cross contamination can't be stopped during the shelf life neither by the characteristics of the product nor by modified atmosphere. The shelf life of cooked hams is shorter than that of cured meat to guarantee the food safety for the consumer. The percentage of modified atmosphere is 50% CO2 + 50% N2.

The experimentation has confirmed that the mortadella is a "suitable substrate" for the growth of *L. monocytogenes*, as reported in literature (Samelis et al., 2001, Finazzi et al. 2008).

The packaging in MAP using 30% of CO_2 slows the growth of the microorganism but not sufficiently to ensure that at the end of shelf-life attributed to the product contains a concentration of the pathogen of less than 100 cfu/g, starting from a initial concentration of 1/25 cfu/g.

In fact, the experimental data obtained show that at 4°C that the limit is reached in 12 days. The technical characteristics for the production of Mortadella indicate how critical control point, for the microbiological risk against *L. monocytogenes*, is the slicing step.

The packaging must take place in clean rooms with the respect of good manufacturing practices, hygiene environmental, equipment and materials in contact with food. In fact, the accidental contamination of the product, at this stage, does not ensure the safety for the final consumer; conversely, the absence of the pathogen in the environment of packaging and on the surface of Mortadella makes the product fail relatively *L. monocytogenes*, regardless of storage temperature.

The actions of the under vacuum packaging against microorganisms are inhibition and selection, the most important is the first one because it is particularly marked on aerobic germs, increase in the lag phase, reduces the speed of propagation of bacteria and limits the maximum cell density (number germs in stationary phase).

The MAP packaging consists in the extraction of air from the package with its replacement with gases such as O2, CO2 and N2 in different proportions depending on the product. The gas active against aerobic spoilage bacteria is that the CO2 dissolves in the water in the fat tissue and lowering the pH of the meat product. For cured meat the typically concentrations used are 30% CO2 + 70% N2.

Foods are heterogeneous and dynamic ecosystems and the understanding and application of the principles of "microbial ecology" to food systems is of fundamental importance for the control of their quality and microbiological safety.

In conclusion, the data obtained, about the dynamics of *L. monocytogenes* in cured meat show the reduction of the pathogen within the shelf-life of the product even if the products are considered "suitable substrates" for the growth of the pathogen.

This study highlighted the importance of GMP, sanification actions and control of critical controls point during production and slicing of cooked meat because a cross contamination could not guarantee the respect of the limits prescribed by the Regulation (EC) No. 2073/2005 and not guarantee the consumer's safety.

This survey provides the FBO data acquisition expendable in ensuring consumer's safety and respect for the limits of the hygiene process and food safety criteria in RTE food in compliance with European law.

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Finalmente la fine…

Prometto alla mia famiglia che questa è l'ultima… ultimi mesi di ansie e delirio, giuro!

Sono stanca e stremata mentre cerco di scrivere questi ultimi ringraziamenti, ma estremamente felice di portare a termine un percorso che ha segnato sicuramente la mia vita.

Di tutti gli avvenimenti voglio ricordare solo quelli che mi hanno scaldato il cuore e fatto ridere, perché solo le cose belle meritano di rimanere nella memoria:

ricordo gli occhi infinitamente azzurri di Ele il primo giorno in laboratorio, per capire dove avrei trascorso i sei mesi di tirocinio che poi si sono trasformati in anni, da quel giorno è stato amore puro, non abbiamo mai smesso di essere amiche, anche se ora siamo lontane, grazie per la calma e la dolcezza che non mi fai mai mancare!

ricordo dei ricci ribelli e un viso severo, sempre in laboratorio, che dopo poco si sono trasformati in risate, chiacchierate, confessioni, lontananza e profonda amicizia… il tuo matrimonio, Virginia, il mio matrimonio senza di te e la mattina dopo su skype per farti vedere come stavo con il mio tanto desiderato abito da sposa… Lori amica mia grazie!

il ricordo più recente Ali sei sicuramente tu… desideravo il tuo arrivo come ben sai anche se all'inizio mi prendevi per pazza e ho dovuto sudare per meritarmi ma la tua amicizia… ma le sere passate insieme, le cene prima delle sette al giapponese, i viaggi a Pavia, sezionare e digerire cuori, le lezioni di stile, i leoncini bianchi alla tv prima di andare a lavoro, la mia compagna di ufficio, la mia amica pata… ti adoro

ricordo anche te cara Ila… pochi mesi e vi ho abbandonate… ma chi si scorda le nostre risate, le colazioni, le giornate in PCR, i pranzi con quelle donne splendide col camice bianco che spesso mi hanno fatto da mamme, per non dire da nonne…

ringrazio anche tutti i miei ragazzi del terzo, quarto e quinto anno che per tre anni ho seguito in esercitazioni, orientamento e tirocinio… sentirsi ringraziare per l'impegno e la passione che trasmettevo loro è stata una notevole soddisfazione.

Come sempre grazie alle mie amiche Simo, Vale, Marti, Anna e Valeria presenti nella mia vita come sorelle

Chi è rimasto ancora… Chi ha segnato la mia vita da quando sono nata…

Chi mi ha trasmesso quei valori di onestà, amore e rispetto che sono ben radicati nel mio cuore… Edo e Fiore… che dire… che sono la figlia più fortunata al mondo?si, senza alcun dubbio… papà non potrò mai dimenticare i tuoi occhi la sera che ti abbiamo detto che volevamo sposarci…e ancora prima che saremmo tornati a Monticelli… tu che mi hai accompagnata all'altare… mami, tu la dolcezza e la bontà, tu la bellezza di una donna in carriera e la bellezza magica di mamma, le tue telefonate che mi accompagnano al mattino a lavoro e mi riaccompagnano a casa la sera, voi che per me fareste tutto… grazie per l'amore che non mi fate mai mancare…

Ale gioia mia, le lacrime che ho dovuto trattenere quando ti ho visto vestito da testimone per il matrimonio, bello da togliere il fiato, il mio fratello così silenzioso che con poche parole e grandi gesti sa aprirmi il cuore

Nonno, la fiducia cieca che da anni riponi in me e che a volte ancora mi stupisce, non hai mai dubitato che le mie scelte potessero essere sbagliate e so che, se te l'avessero chiesto, avresti scommesso tutto su di me il primo giorno di lavoro a Mantova…

Vita mia infinita, Nonna, tutto quello che ho imparato e che sono lo devo a te! Un donnino così piccolo con una forza, una determinazione e un' indipendenza che pochi riescono ad immaginare… le tue parole, la forza e il coraggio che mi hai sempre dato… "sempre a testa alta", si nonnina mia, sempre e ovunque come mi hai insegnato!

Amore mio immenso, quanto ti ho trascurato in questi mesi… appena sposati e su due pianeti diversi… io così lontana da dimenticarmi di tutto… perdonami e aspettami… sto tornando a casa… dove tu sei sempre stato… e dove ti sei preso cura di tutto… ti amo…

Grazie infinite alle possibilità che mi sono state date…a un nuovo lavoro che mi fa sentire viva e realizzata, alla possibilità di terminare il dottorato, all'appoggio morale che ho trovato in tutti i miei colleghi e al permesso di trasformare il mio lavoro in questa tesi.

Grazie alle nuove amicizie… a Cri per l'intesa, la complicità e il nostro difenderci a vicenda.... a Silvia per la forza che mi da, per le nostre pazzie e spese folli e per tutto quello che ci legherà nei prossimi anni

Grazie a chi ha sempre creduto in me!