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Development of a post-lethality treatment to reduce the risk of *Listeria monocytogenes* contamination in Ricotta salata cheese

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Abstract

Listeria monocytogenes contamination in Ricotta salata cheese rind is a common finding. In the last year numerous cases of food alert and even of human listeriosis associated with the consume of Ricotta salata have been reported in different Member States of the European Union and other countries. The presence of *L. monocytogenes* in the cheese making plant environment represent an important risk factor in the contamination of ricotta salata (i.e. post-proces contamination). *L. monocytogenes* is mainly localized on the rind of the product. The reduction of the risk associated with environmental contamination can be obtained through the application of post-lethality treatment. The feasibility to inactivate *L. monocytogenes* using water bath heat-treatment under experimental conditions has already been demonstrated by Spanu *et al.,* (2013). The aim of this work was to optimize a water bath heat treatment, which can be used at industrial level, allowing the control of *L. monocytogenes* post-process contamination in Ricotta salata wheels and wedges. For each type of product was tested the efficacy of 9 time-temperature combinations on samples artificially inoculated with *L. monocytogenes*. On the test units were conducted microbiological, physic-chemical and sensorial analysis. The best time-temperature combination would selected taking into account the inactivation level of *L. monocytogenes* (the target was set at 5-log reduction), the economic sustainability of the treatment and the influence on sensory properties of the product.

CHAPTER 1

Introduction

Introduction

1.1 The microorganism

1.1.1 Listeria spp

Listeria monocytogenes is part of a large group of microorganisms of the genus Listeria. The genus *Listeria* is currently comprised of seventeen species. To the "classic" six *Listeria* species described before 1985 (*Listeria monocytogenes, L. innocua, L. welshimeri, L. seeligeri, L. ivanovii* and *L. grayi*) (Rocourt and Buchrieser, 2007) "new" species have been identified in recent years. Since 2009 eleven *Listeria* species were isolated all over the world: *L. rocourtiae* (Leclercq *et al.*, 2010), *L. marthii* (Graves *et al.*, 2010), *L. Fleischmannii* (Bertsch *et al.*, 2012) and *L. weihenstepahnensis* (Halter *et al.*, 2013), *L. aquatica, L. floridensins, L. cornellensis, L. grandensis, L. riparia, L. borriae, L. newyorkensis* (den Bakker *et al.*, 2014; Weller *et al.*, 2015).

Listeriae are short, Gram-positive, non-spore-forming, facultative anaerobic rods. They are catalase positive, oxidase negative and beta-hemolytic. *Listeria* is motile at 20-25 °C but non-motile at 37 °C, because the gene encoding flagella is not transcribed at this higher temperature (Dons *et al.*, 1992; Kathariou *et al.*, 1995). *Listeria* is able to grow in a wide range of temperatures (0-45 °C) (Halter *et al.*, 2013). *Listeria spp* are not able to grow below -1.5 °C, but they can survive at lower temperature. The optimal range for the growth is between 30-37 °C, while temperature above 50 °C is lethal (Rocourt and Buchrieser, 2007).

Among all the seventeen species, the only two species considered pathogenic are *Listeria monocytogenes* and *Listeria ivanovii*. Occasionally *L. seeligeri* and *L. ivanovii* have been involved in human clinical cases occurred in individuals with suppressed immune functions (Rocourt *et al.*, 1987; Cummins *et al.*, 1994; Lessing *et al.*, 1994; Liu 2013). *L. innocua* was initially considered non-pathogenic (hence its name) and non-haemolytic. However, recently has been identified a number of haemolytic *L. innocua* isolates (Rocha *et al.*, 2013). *L. innocua* has been associated with cerebral infection in ewe and in cattle, and a fatal case occurred in an old man was reported in 2003 (Perrin *et al.*, 2003).

Listeria spp are ubiquitous bacteria widely distributed in different natural, urban and suburban environments, including soil, surface water, vegetation, sewage, faces of animal and humans, animal feed, farm environments, food processing environments (Gravani, 1999; McLauchlin *et al.*, 2004; Korsak *et al.*, 2016).

1.1.2 Listeria monocytogenes

Although an organism, later to be identified as *Listeria monocytogenes*, was isolated from cerebrospinal fluid as early as 1918, the characterization of that bacterium did not take place until 1926, by Murray *et al.* It is only since the 1980s that the importance of food in the transmission of listeriosis has been recognised (Encyclopedia of Food Sciences and Nutrition, 2003).

Microscopically, they appear as gram-positive small rods, which can be arranged in short chains. In direct smears, they may be coccoid, and therefore they may be mistaken for streptococci. Longer rods may resemble corynebacteria. Haemolytic activity on blood agar has been used as a marker to distinguish *Listeria monocytogenes* from other *Listeria species*, but it is not a definitive criterion. Further biochemical characterization may be necessary to distinguish among the different *Listeria spp. L. monocytogenes* is catalase positive and oxidase negative (PHE, 2014).

L. monocytogenes presents peritrichous flagella, which give it a characteristic tumbling, motility, occurring only in a narrow temperature range $(20-25°C)$ (Peel *et al.*, 1988).

Listeria monocytogenes can be differentiated into 13 serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7 (Seeliger and Jones, 1986). A large number of phylogenetic and subtyping studies have shown that *L. monocytogenes* isolates form a structured population, composed of divergent lineages. *L. monocytogenes* consists of at least four evolutionary lineages (I, II, III and IV). Lineage I includes the serotypes 1/2b, 3b, 3c and 4b. Lineage II includes the serotypes 1/2a, 1/2c, and 3a. Lineage III and Lineage IV comprise serotypes 4a, 4b and 4c.

The majority of human listeriosis outbreaks have been linked to lineage I serotypes 4b isolates, even though some outbreaks have been caused by lineage I serotype 1/2b isolates and lineage II serotype 1/2a isolates (Orsi *et al.,* 2011). This trend does not

seem to be consistent and the association between lineages and human listeriosis cases may vary by region. Lukinmaa et al., (2003) and Parihar et al., (2008) shown that lineage II serotype 1/2a strains appear to be more common among human listeriosis cases in Northern Europe, instead Jeffers et al., (2001) shown that lineage I are predominant in among human listeriosis cases in USA.

L. monocytogenes is largely distributed in the environment. It has been commonly isolated from surface waters, decaying vegetation, soils, sewage, and silage. *Listeria monocytogenes* can be isolated from mammalian and bird feces, but ruminants are the main carriers of the microorganism. The presence of the bacteria in ruminants is usually associated to silage feeding. *Listeria monocytogenes* can survive in soil for many years and fecal shedding is the principal source of the bacterium in the soil.

1.1.3 Growth of Listeria monocytogenes

Unlike many other foodborne pathogens, *L. monocytogenes* can survive and growth under difficult environmental conditions, that are usually lethal for other microorganisms (Zarei *et al.*, 2012; Sleator *et al.,* 2003). The optimum range of temperature is between 30 and 37 °C. In particular conditions, it can growth also at refrigeration temperatures (between 0 and 4 °C). Despite growth is inhibited below -1.5°C, *L. monocytogenes* can survive at freezing temperature (Sergelidis and Abrahim, 2009).

Listeria monocytogenes presents a good resistance at a wide pH range, between 4.0 and 9.5 (Liu *et. al*, 2005). It appears to be mildly acid-tolerant (Lado and Yousef, 2007). For these reasons, *L. monocytogenes* may survive in the gastric acid environment, cross the intestinal barrier, making it possible the oral infection (Wiedmann *et al.*, 1998). The resistance to low pH values decreases with increasing temperature (Lado and Yousef, 2007).

Listeria monocytogenes is one of the few foodborne pathogens that can grow at a_w values below 0.930 (Johnson *et al.* 1988, Lado and Yousef, 2007). However, conditions for an optimal growth are at $a_w \geq 0.970$ (Ryser and Marth, 1999).

It is clearly demonstrated that *Listeria monocytogenes* is a halophilic bacteria that can growth at NaCl concentration up to 10% and can survive at values of 20% (Sutherland *et al.*, 2003; Seeliger, 1986). Liu *et al.* (2005) proved that *Listeria monocytogenes is* still able to grow in a solution saturated at 40% during incubation for 20h. *Listeria monocytogenes* is aerobic, microaerophilic, facultative anaerobic (Lungu *et al.*, 2009).

1.1.4 Thermal resistance of Listeria monocytogenes

The thermal inactivation of *Listeria monocytogenes* has been questioned following a listeriosis outbreak occurred in 1983, in the USA where pasteurized milk was implicated as the source of infection. On that occasion, controls performed in the dairy plant did not detect any failure in the pasteurization process. Since then many assumptions were made on the possible resistance of *L. monocytogenes* to pasteurization. At first it was speculated

that *Listeria monocytogenes* when incorporated within cells of the immune system, could resist the high-temperature short-time (HTST) pasteurization process (71.7 °C for 15 seconds) (Doyle *et al.*, 1987). However, subsequent studies by Bunning et al (1988) conclusively showed that the intracellular position of the microorganism do not significantly increase the thermal resistance. The heat resistance of *L. monocytogenes* in milk has been reviewed by Mackey and Bratchell (1989), showing for HTST pasteurization of milk a value of $D_{71.7}$ (the decimal reduction time) of 5.2 seconds and a *z* value ranging between 6.1 °C and 7.4 °C. Bradshaw *et al.,* (1985) artificially inoculated the milk with *L. monocytogenes*, and showed that 0.9 seconds at 71.7 °C are needed for one log_{10} reduction. Other studies showed that at 62 °C one log_{10} reduction is reached in a time ranging between 6 and 20 seconds (Donnelly *et al.,* 1987).

Piyasena *et al.,* (1998) developed a predictive model to describe the thermal inactivation, confirming that the HTST pasteurization can ensure at least an 11-log reduction of *L. monocytogenes*.

In raw milk the most probable concentration estimated of *L. monocytogenes* is about 0.1 cfu/ml (Meyer-Broseta *et al.*,2003), therefore these data indicate that there is a substantial margin of safety in the HTST pasteurization process.

1.1.5 Biofilm formation and persistence

Listeria monocytogenes may grow in biofilms that protect them against environmental stress and can be isolated from surfaces even after cleaning and

disinfection. In the food industry, biofilm formation in dairy processing plants is a significant problem. Contamination of dairy products originating from biofilms within the processing environment is common (Simões *et al.,* 2010).

The biofilm matrix is a natural tendency of microorganisms to attach to damp surfaces, in which the microbial cells create an optimal substrate for their survival and replication using all available nutrients. The major matrix components are microbial cells, polysaccharides and water, together with excreted cellular products (Sutherland *et al.,* 2001). Nutrients, metabolites and waste products interchange is ensured by the presence of water channels within the biofilm matrix (Sauer *et al.* 2007; Sutherland, 2001).

The attachment of microorganisms to surfaces and the subsequent biofilm development is regulated by several variables. In general, attachment will occur most promptly on rough surfaces, which are more hydrophobic and coated by surface conditioning films (Simões *et al.,* 2010). Usually the areas where *Listeria* is able to form biofilm easier are wet surfaces such as drains, walls, ceilings, storage tanks, hand trucks, conveyor belts and other sites that can accumulate food residues (Wong, 1998), (Shi and Zhu, 2009; Lee et al., 2017).

Due to the ability to form biofilm, *L monocytogenes* is able to establish into environmental niches (Beresford, 2001). Niches are areas that are generally characterized by high humidity and low temperatures and difficult to clean and to disinfect with normal

procedures (Carpentier *et al.,* 2011; Tompkin, 2002). Within niches, *L. monocytogenes* can survive and multiply for a long time (Unnerstad *et al.,* 1996).

The ability of *L. monocytogenes* to form biofilms can also be affected by the presence of competing microbiota (Tompkin, 2002). Many authors showed that the presence of *Pseudomonas* and *Flavobacterium* spp can increases the ability of *L. monocytogenes* to form biofilm (Sasahara and Zottola, 1993; Bremer *et al.,* 2001). On the contrary, Mariani *et al.* (2011) demonstrated that the presence of *Lactococcus lactis* and other hetero-fermentative lactobacilli could exert an anti-*Listeria* action.

The protective effect provided by the biofilm structure may enhance the resistance of bacterial cells to sanitizing agents (Belessi, Gounadaki, Psomas, & Skandamis, 2011) and from various chemical and physical stresses (Harvey *et al.,* 2006; Chae *et al.,* 2006), creating a permanent source of contamination in the food processing facilities. The persistence of *L. monocytogenes* in food industry environments has been associated with the ability of specific isolates to produce biofilms (Lee *et al.,* 2017). *Listeria monocytogenes* serotypes 1/2a and 1/2c are generally considered more efficient in biofilm formation as compared to strains of serotypes 4b and 1/2b (Nilsson *et al.,* 2011; Harvey *et al.,* 2007; Borucki *et al.,* 2003).

Persistent strains are more able to adhere and to form biofilm as compared to non-persistent strains (Folsom *et al.,* 2006; Lundén *et al.,* 2000; Norwood and Gilmour, 1999).

L. monocytogenes strains when isolated from the same processing plant environment over time can be considered persistent (Carpentier *et al.*, 2011). However, persistence is a loosely defined concept. Some authors define a strain persistent when isolated on at least three sampling dates in a one-year period (Carpentier *et al.*, 2011; Ragimbeau *et al.,* 2002). The main properties that influence the possibility of *Listeria monocytogenes* to persist are the ability to form biofilm, to establish into niches, to grow at low temperatures and to resist to sanitizers (Pan *et al.*, 2006).

1.1.6 Pathogenesis of Listeria monocytogenes and Listeriosis

Listeria monocytogenes is responsible for human listeriosis, a severe foodborne disease occurring as consequence of the ingestion of contaminated food products, namely dairy, meat, fish, and vegetables (Magalhães *et al.*, 2014). Listeriosis, generally considered a sporadic disease, is characterized by a high mortality rate, which makes it one of the most important foodborne disease (Schneider *et al.*, 2009). The Centers for Disease Control and Prevention (CDC) classified the listeriosis as the third leading cause of death from food poisoning. The annual incidence of confirmed listeriosis in the United States is about 0,24 cases per 100.000 population, and it is responsible of an average case-fatality rate of 20-30% (CDC 2016;WHO/FAO 2004; EFSA, 2014; Mead *et al.*, 1999).

Listeriosis is generally acquired through foodborne transmission, except for neonatal infection, which may occur by vertical transmission of *L. monocytogenes* from mother to fetus (Becroft et al. 1971). Cutaneous infections have been seldom reported

among veterinarians and farmers following direct animal contact, particularly involving livestock products of conception (Rocourt and Buchrieser, 2007; Bortolussi and Mailman, 2010; Posfay-Barbe and Wald, 2009).

The majority of human cases of listeriosis occur in individuals who have an underlying condition, which leads to immunity suppression. Categories of most vulnerable patients are children, the elderly, pregnant woman and immunosuppressed. One-third of all human listeriosis cases in the USA occurred during pregnancy (CDC 2013). In general, pregnant women are 12 times more likely than general population to get Listeria infection (Hof, 2003). Pregnant Hispanic women are 24 times more likely than general population to get Listeria infection (CDC 2016). The foetus can contract the infection in utero or, alternatively, the mother can contaminate the respiratory tract of the baby during the passage through the infect birth canal (Allerberger and Wagner, 2010; Schlech, 2000).

Diabetes, alcoholism, liver and kidney diseases, drug addiction are other predisposing conditions for listeriosis (Bortolussi and Mailman, 2010; Schlech, 2000).

Listeria monocytogenes is an intracellular pathogen and several virulence factors have been identified and characterized, including hemolisyn (listerolisyn O), two distinct phospholipases, a protein (ActA) and several internalins (Kathariou *et al.*, 2002). Their study has provided a series of information on the mechanisms employed by this

microorganism to interact with mammalian host cells. The *L. monocytogenes* hemolysin is recognised as a major virulence factor of *L. monocytogenes.*

The infection from *L. monocytogenes* can be grouped into five phases: 1) entry of the bacterium in the host; 2) invasion of cells; 3) phagosomal vacuole lysis; 4) multiplication in cytosol; 5) direct cell to cell diffusion (Jemmi and Stephane, 2006; Vazquez-Boland *et al*, 2001).

Listeriosis can be presented in two different clinical forms: invasive and non-invasive. The non-invasive listeriosis occurs with a self-limited acute febrile gastroenteritis with nausea, vomiting and diarrhoea following high-dose *Listeria* exposure (Doganay, 2003). Because Listeria cannot be detected by routine stool culture, febrile gastroenteritis from *Listeria* infection is underreported since it is occasionally diagnosed outside of outbreak settings. (Allerberger and Wagner, 2010; Vazquez-Boland *et al.*, 2001).

In elder adults and people with immunocompromised conditions, the most common clinical presentations is the invasive form, characterised by sepsis, meningitis, and meningoencephalitis (Allerberger and Wagner, 2009; Barbuddhe *et al.*, 2004). Meningoencephalitis symptoms are fever, intense headache, nuchal rigidity, movement disorders such as tremor, ataxia and seizures disorders (Allerberger and Wagner, 2009; Doganay, 2003). Patience can also experience focal infections, including septic arthritis, osteomyelitis, prosthetic graft infections, and infections of sites inside the chest and

abdomen or of the skin and eye. Symptoms of the invasive form appear 3-70 days after

the exposure (Lecuit, 2007). Among bacterial meningitis *Listeria* account for 11% of all

cases and has the highest mortality rate, close to 22% (Lecuit, 2007). Less commonly,

otherwise healthy young people may also develop invasive listeriosis (CDC 2016).

The foetal infection occurs in the third trimester of pregnancy when the immune

defences are low (Allerberger and Wagner, 2009). The infection during pregnancy can

cause foetal distress, miscarriage, death or premature birth of a severely ill infant and in

the case of contamination during the childbirth, infants can present meningitis (Schlech,

2000). Maternal infection may present as a nonspecific, flu-like illness with fever,

myalgia, backache, and headache, often preceded by diarrhoea or other gastrointestinal

symptoms (Di Maio, 2000); in some cases the infection can be completely asymptomatic

(Doganay, 2003).

The minimum dose required to cause clinical infection in humans has not been

determined. However the high microbial load of foods responsible for epidemic and

sporadic cases of listeriosis (between 10^2 and $10^6 \log_{10}$ cfu/g of ingested product) suggests

that a high number of *L. monocytogenes* cells is necessary to cause infection (Posfay-

Barbe and Wald, 2009; Jemmi and Stephane, 2006; Vazquez-Boland *et al*, 2001). *L.

monocytogenes* infective dose may vary depending upon the pathogenicity and virulence

of the strain involved and the host related risk factors (FDA, 2003). However, these data

should be interpreted with caution, given the long incubation period of invasive listeriosis

and the time normally elapsed between diagnosis and analysis of the food ingested, during which *Listeria* organisms can have multiplied in the patient's refrigerator (Vazquez-Boland *et al.,* 2001; CFSPH, 2005).

1.1.7 Food as a source of Listeria monocytogenes

L. monocytogenes has been isolated from a wide variety of foods of animal and vegetable origin. Numerous studies demonstrated that *Listeria monocytogenes* can contaminate almost all food categories. Listeriosis infection are associated with meat products (Selby et al., 2006), dairy products (Leite et al., 2005), fish products (Jallewar et al., 2007) and vegetables (Crepet et al., 2007).

Whatever the origin of the food, the majority of the cases of listeriosis were associated to RTE (Garrido *et al.,* 2010; Uyttendaele *et al.,* 2009). The RTE are edible as they are, without undergoing any treatment, for example cooking, before their consumption. Some examples of RTE foods include: soft cheeses (including whey cheeses), hot dogs, sandwiches, pâtés/meat spreads, refrigerated smoked seafood products (such as smoked salmon), pre-packed raw vegetables and mixed raw vegetable salad and pre-cut fresh fruits.

A European survey on *Listeria monocytogenes* was carried out in 2010 – 2011 by EFSA, with the aim of estimating the prevalence of *Listeria monocytogenes* in RTE foods at retail. The RTE samples included smoked fish, meat products and soft or semi-soft cheese. Ninety percent of listeriosis cases in the USA were associated to meat and dairy

RTE products (FDA, 2003). The prevalence of *Listeria monocytogenes* in fish samples was 10.3 %, while for meat and cheese samples at the end of shelf-life these prevalence were 2.07 % and 0.47 %, respectively. Among RTE, those characterised by a long shelf life under refrigeration temperatures are more likely to support *L. monocytogenes* survival and growth (Rocourt, 1996).

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

Among RTE foods the Regulation distinguish three categories: RTE foods intended for infants and ready-to-eat foods for special medical purposes; RTE foods able to support the growth of *Listeria monocytogenes*, other than those intended for infants and for special medical purposes; RTE foods unable to support the growth of *Listeria monocytogenes*, other than those intended for infants and for special medical purposes. For the three product categories, the European legislature identified different levels of tolerance. The first category (RTE foods intended for infants and ready-to-eat foods for special medical purposes) where a zero tolerance is applied, has a "presence-absence" criteria. While the second e third categories where a minimum presence of *L.*

monocytogenes is tolerated depending on the characteristics of the product, the microorganism enumeration should not be <100 cfu/g for the all shelf-life.

In USA the Department of Agriculture (USDA) and Food Drug Administration (FDA) applies a "zero-tolerance" policy for *Listeria monocytogenes* in RTE foods. Instead, the Canadian Listeria policy for RTE foods is based on the application of Good Manufacturing Practices (GMP), on the HACCP principle and the control of the risk for *Listeria* contamination was developed using a health risk assessment approach.

1.2 *Listeria monocytogenes* in the dairy sector

Listeria monocytogenes has been associated with a wide variety of dairy products. Among all dairy products, raw-milk cheeses, soft-ripened and blue-veined cheese are the most involved in cases of listeriosis. However, sporadic cases of listeriosis associated with consumption of cheese made from pasteurized milk as consequence of a post process contamination of the product are reported in literature (Cumming *et al.*, 2008; Fleming *et al.*, 1985). In Europe, about half of the outbreaks and sporadic cases of listeriosis have been linked to the consumption of dairy products (Lunden *et al.,* 2004).

1.2.1 Milk

The level of *L. monocytogenes* in raw milk is usually lower than 0.1 cfu/ml but counts may reach 6.4 cfu/ml (Encyclopedia of Food Sciences and Nutrition, 2003, D'Amico and Donnelly, 2010; Vilar *et al.*, 2007; Van Kessel *et al.*, 2004; Meyer-broseta *et al.,* 2003). The sources of contamination of raw milk with *L. monocytogenes* are numerous. *L. monocytogenes* could be, though rarely, the causative agent of mastitis in dairy animals. The microorganism has been recovered from milk of mastitic cows at counts ranging from 10^2 to 10^4 cfu/ml. Prolonged excretion of *L. monocytogenes* in milk and feces of infected animals and asymptomatic carriers has been described. This would lead to build-up of an endogenous pool of *Listeria* in the farm environment, which could be augmented, from other sources such as the use of poor-quality silage, drinking water provided to the animals and feces of feral animals and birds (Vazquez-Boland *et al*, 2001).

Rahim (2014) and Al-Tahiri (2008) showed a prevalence of *L. monocytogenes* in sheep's milk showed ranged between 3.6 % and 10-12.7%. Sheep milk inoculated with *Listeria* spp at concentration of 10^6 cfu/ml can resist to heat treatment at 65 °C for 15 minutes (MacDonald and Sutherland 1993). This is presumably possible for the protective effect of the fat present in sheep's milk; in fact, this does not occur in cows and goat milk. The pasteurization treatment of 72 °C for 15 minutes appears to be effective for the control of *Listeria* in raw milk.

The pasteurization applied for fluid milk, which has become a routine practice in USA and in Europe since the 1950s ensure the total destruction of *Listeria monocytogenes*. Nevertheless, in the last 10 years an increase in the trade and consumption of cow raw (unpasteurized) milk was registered (Van Kessel *et al.,* 2004) posing a potential health threat to consumers.

1.2.2 Dairy products

In dairy products it is uncommon to find *L. monocytogenes* levels greater than 100 cfu/g (EFSA, 2010), however some cases of listeriosis resulting from the consumption of dairy products have been reported (Almeida et al., 2013). The FDA estimates that 35 % of listeriosis outbreaks are linked to the consumption of cheeses, therefore, they have been included in the "FDA Top Ten" riskiest foods report (CSPI, 2009).

In general, the prevalence of *Listeria monocytogenes* contamination in cheeses reported in literature differs considerably depending on the cheese type. The cheese at most risk of *L. monocytogenes* contamination are soft cheese such as Camembert, Brie, Stilton, Gorgonzola and Roquefort. The high water activity (> 0.920) and the pH close to neutral make of soft cheeses an excellent substrate (Rudolf and Scherer, 2001), enhancing the possibilities of survival and growth (Carminati *et al.*, 2004).

1.2.3 Listeria monocytogenes in in the industrial dairy processing environment

Listeria monocytogenes is able to survive and growth in the industrial dairy processing environment, representing a potential source of post-process contamination of dairy products made from pasteurized or thermized milk (Ibba *et al.*, 2013)

There are several ways that allow the microorganism to enter into the food processing environment at any level of the production process (Ryser, 1999).

Some examples of contamination sources are raw materials, equipment and people. If appropriate control measures are not in place *Listeria* can be introduced in the food processing environment through the raw materials and subsequently contaminate foods and equipment (Almeida *et al.*, 2013). *Listeria* can be introduced by employees, visitors that can vehicular the microorganism via shoes, clothing and personal items.

Kells and Gilmour (2004) showed levels of *Listeria monocytogenes* contamination of about 7% in the hands of food production workers. Confirming the

theory that the employers play an important role in the *Listeria* dissemination, Lomonaco *et al.* (2009) detected *Listeria monocytogenes* in the toilet and in the changing rooms.

In addition, also the raw milk contaminated with *Listeria monocytogenes* could represent an important source for the introduction of the microorganism in industrial dairy plants. The prevalence of positive raw milk samples ranges between 3.0 and 6.5% (Vitas *et al.*, 2004; Al-Tahiri and Rewashdeh, 2008).

Sources of contamination can also be represented by the entrance in the dairy plant of materials such as the packaging material or equipment. Furthermore the entrance of semi-finished food can be vehicle for the microorganism.

The pasteurization processes reduce of 3 to 6 log the number of viable *Listeria monocytogenes* cells present in raw milk (ICMSF, 1996). For that reason, the presence of *Listeria monocytogenes* in dairy products obtained by pasteurized milk can be attributed to a post-process contamination originating from the processing environment (Tompkin, 2002; Unnerstad *et al.*, 1996).

The ability to grow at low temperatures, the adaptability to stress conditions (e.g. acidity, alkalinity and high salt concentration) make *Listeria monocytogenes* able to survive in the food processing plants (Lou and Yousef, 1999).

The low temperatures, the high level of humidity and the presence of organic residues are the main characteristics of the cheese processing environments, conditions that are particularly favourable to *Listeria monocytogenes* growth (Tompkin, 2002;

Unnerstad *et al.*, 1996). Moreover, in the processing plant environment the contamination is generally represented by resident strains that colonize specific niches (Blackman and Frank, 1996). The daily cleaning and sanitizing procedures became ineffective when *L. monocytogenes* establish into niches (Tomking, 1999). The ineffectiveness persists even if the cleaning and disinfection operations are implemented (Carpentier and Cerf, 2011). Therefore, it appears to be necessary to eliminate or minimize any conditions predisposing to the formation of *L. monocytogenes* persistence niches (Kornacki, 2012).

1.3 Ricotta salata

1.3.1 Whey Cheeses

Whey cheeses are products obtained by the concentration of whey or by coagulation of whey by heat, with or without the addition of acid. Whey cheeses can are solid, semi-solid or soft products. The process can include the addition of milk, cream, or other milk origin raw materials, before or after coagulation or concentration (Codex Alimentarius, 2011).

The European Decision NO 80/1997 define the whey as the residual product obtained during cheese making or from casein. The whey is rich of high value components such as protein and peptides, lactose, vitamins, minerals and some lipids. His composition can be very variable and depends on numerous factors, including the origin of milk and the technology in the cheese making.

Pintado et al., (2001) estimated that about half of the world whey production is poured out on land or water, representing a major source of pollution. For each kilogram of cheese approximately 9 liters of whey are produced (85-90% of the milk volume). At the global level it is produced about 115 million tons of whey per year and about 47% is not subject to any treatment before being released into the environment.

The processing of sheep whey is widespread in many countries of the Mediterranean basin. Examples of European whey cheeses are Mizithra, Anthotyros and Manouri (Greece), Anari (Cyprus), Requesón (Spain), Requeijao (Portugal), Broccio

(France), Urdă (Balkans region) each with its own characteristic technology. In most of the cases, these are fresh and soft or semi-soft traditional products made in small-scale operations and sell in local markets.

In the last years the whey cheeses, as well as all soft cheeses, have been acquiring an increasing role in the economy of the industrial dairy sector. In fact, if the whey cheeses were previously considered as a waste product of the cheese making industry, they are currently reaching a steady growth of consumer demand. In addition, the large distribution is showing an always-increasing interest for ricotta.

1.3.2 Ricotta types

Ricotta is generally sold as a fresh, unripened grainy cheese, which is white, soft and moist. It has bland taste, or at most semi-sweet, when it is manufactured from fresh, sweet whey (Pintado et al., 2001).

In Italy, numerous types of whey cheese are manufactured, with many differences according to conditions and traditions of the various production areas (Decreto Ministeriale 8 settembre 1999, n.350). All the whey cheese products can be classified according to the whey origin. Ricotta can be manufactured using milks of different species: sheep, buffalo, goat, which can be mixed with milk and/or cream of the same or other species (Mucchetti and Neviani, 2006). Among all the ricotta cheeses manufactured in Italy only two obtained the Protected Designation of Origin (PDO), while other were included in the list of Traditional Food Products (MIPAAF, 2015). A traditional product

is a food product whose methods of processing, of storage and seasoning are time-honoured, similar for all the territory concerned, according to traditional rules, for a period not less than twenty-five years (Laore, Decreto 18 luglio 2000).

The Italian PDO whey cheese are:

- Ricotta Romana PDO: it has a very ancient origin, with historical notes to this product dated to the second century Before Christ. It is produced in Lazio with sheep's whey that can be added with sheep's milk. The PDO was attributed in 2005 (EC Regulation No 737/2005).

- Ricotta di Bufala Campana PDO: it can be produced in the regions of Lazio, Campania, Puglia and Molise. There are two variety of Ricotta di Bufala Campana PDO which differ in the durability: the fresh type, that has a shelf-life of 7 days and the fresh homogenized type that has undergone, in addition to the mechanical homogenization treatment, an heat treatment that prolongs the shelf-life up to 21 days. The PDO was attributed in 2010 (EC Regulation No 634/2010).

Sardinia includes different typologies of sheep's ricotta cheeses regulated by D.M. 8 Settembre 1999 No 350 and from D.Lgs. 173/98. Based on technological characteristics is possible to distinguish:

- *"Ricotta fresca" o "ricotta gentile"*: it is obtained by heating the whey at + 80-82°C, after molding it is stored at refrigeration temperature to allow

the draining of the residual whey. It has a very short shelf-life of about 4-5 days.

- *"Ricotta di colostro ovino"*: obtained by the whey remaining after colostrum cheese making. It is a typical product rich in high nutritional components.

- "*Arescottu spongiau*": It is a particularly worked whey cheese, crushed by hand and dried for at least one month. It is typical in the South-Sardinia.

- *"Ricotta salata"*: the whey cheese after molding is pressed and salted. It is dried for about 10 to 20 days. According to the moulding shape it can be named: "Ricotta Moliterna", "Ricotta Toscanella", "Ricotta Montella".

- *"Ricotta Mustia":* it is very similar to "ricotta salata" but is treated by smoke-curing in the surface.

- *"Ricotta Testa di Morto":* this particular whey cheese is molded and pressed inside of a cloth similar to a gauze, than it is hung to allow the draining of the residual whey. Once extracted from the cloth it has acquired the shape of a ball or a "head". It is dried for about 10 to 20 days.

1.3.3 Production technology

Whey products

Despite the existence of different kinds of Ricotta cheese, the production process is very similar in all the types of ricotta cheeses described above. There are two main production methods: the discontinuous (manual) and the continuous (automatic) methods.

The discontinuous or manual method is the most widely technology used technology, both at artisanal and industrial level. Before the whey, remaining after cheese production is transferred from the polyvalent into large open kettles it is pre-heated at 60-65 °C. Inside the kettle, the whey is then heated by steam flowing in a cavity. The whey heating is conducted under continuous stirring until the temperature reaches 70 °C, when the curd begins to rise and float on the surface of the whey (Mathur and Shahani, 1981). When the curd formation is completed, at + 85-87 ° C in about 5-10 minutes, the heating is blocked and the hot whey is then allowed to rest for ca. 5 minutes. The curd is then scooped using perforated ladles from the surface into plastic baskets. Ricotta cheese is then allowed to drain ca. 6 hours in a cool room and then they are covered with food paper and ice. Usually, 1 kg of Ricotta can be obtained from 15-20 liters of whey (Mills, 1986 da Pintado). The pH adjustment with citric acid is generally not necessary for curd clotting of sheep because its natural acidity allows the outcrop of the ricotta.

The continuous or automatic method uses special equipment that were designed to optimize the production as consequence of the always-increasing demand of Ricotta

cheese from the large distribution. The Rota method, developed in the nineties' (Rota 1990) is based on the pre-mixing of all the ingredients in the whey and on the continuous heating in a steam coil. This technique involves a continuous flow and ends with the conveyance of the curd towards the end of the machine, where perforated tins are positioned and then filled. Another continuous method is the Modler method. The acidified whey is conveyed in a spiral pipe of 4 cm in diameter and 30 meters long, where the formation of curd and the whey separation occurs. The flow volume is 4.5 liter per minute. The whey with a pH of 6.35-6.50, is pasteurized and then heated up to + 85 ° C by direct steam injection. Between the continuous technologies, also ultrafiltration could also be ascribed. This method is based on the separation of the liquid phase from the solid before the curd formation (Pintado *et al,*. 2001).

Ricotta salata

Ricotta salata cheese is a traditional variety of ricotta added with salt which in Sardinia (Italy) is made from the whey remaining after the production of Pecorino Sardo PDO, Pecorino Romano PDO or other hard and soft sheep's milk cheeses. The manufacturing of ricotta salata cheese in Sardinian industrial cheese-making plants follows the traditional batch process (Laore, 2015). It proceeds with the whey filtration and with preheating at 60-70°C by a plate heat exchanger. The whey is then transferred in large open kettles (1,200-1,500 l capacity). Before starting the heating, pasteurized cream or milk can be added to the whey to enrich the solid content. At this point, the whey

is heated to temperature above 85 °C, and held for 30 min. After the flocculated protein

rises to the surface, clots are collected using perforated ladles and transferred into plastic

cylindrical molds. The curd is then pressed to enhance drainage for up to 24 h and

transferred in cold room (10-12°C) for about 10 days. Salting can be made adding directly

sodium chloride to the whey or to the curd during molding or by dry salting or in brine

after molding or during refrigerated storage (Casti *et al,*. 2016). The final moisture level

can vary depending on the use of the product. If it is intended to be used for grating, it is

dried until the moisture content is about 50% or of ca. 55-60% if used as it is. At the end

of production process ricotta has a weigh of about 3 kg. The final product has a main

content of fat and protein respectively of 28-33% and 14-23%. The pH ranges between

6.1 and 6.9 and the water activity ranges between 0.940 and 0.970 (Spanu *et al.,* 2012;

Spanu *et al.,* 2013). Ricotta salata is packaged in shrinking vacuum bags as a hole or after

cutting into wedges and stored at refrigeration temperature (Spanu *et al.,* 2015). Under

the responsibility of food business operator, Ricotta salata is generally attributed a shelf

life, which vary from three weeks up to several months. Ricotta salata can be destined to

various uses, from the industrial confectionery, to the domestic use in salads or as an

ingredient in other dishes without further heating or cooking.

1.3.5 Microbiological profile of ricotta salata cheese

The production technology of ricotta salata, in particular way the whey heating

(ca. 85 °C) which inactivates natural microflora and absence of starter cultures, the

intrinsic properties (high pH and elevated water activity), determine the ability of the product to support the growth of pathogen and spoilage microorganism. The refrigerated storage and the prolonged shelf life allow the possible growth of psychrotrophic pathogenic and spoilage bacteria (Casti *et al.*, 2016). Among pathogen microorganisms able to growth in Ricotta salata cheese at refrigeration temperatures, of particular concern are *Listeria monocytogenes* and psychrotrophic *Bacillus cereus* strains.

Evidence that *L. monocytogenes* contamination of ricotta salata cheese origins from the processing environment and that this product supports the growth of the pathogen during refrigerated storage have been documented (Spanu et al., 2012; Ibba et al., 2013; Spanu et al., 2016). The presence of pathogens contaminants such as *B. cereus* has also been observed. The presence of *B. cereus* in Ricotta salata is a rare finding, with maximum contamination level of ca. 3 $\log_{10}$ cfu/g^{-1} (Cosentino *et al.*, 1997; Fadda *et al.*, 2012; Spanu *et al* 2016) and no cases of *B. cereus* human illness have been associated with the consumption of Ricotta salata cheese (De Santis et al., 2008). Spanu *et al.*, (2016) observed a high contamination level of *B. cereus* (up to 8.33 $\log_{10}$ cfu/g^{-1}) in Ricotta salata in a large number of positive batches (nine) over a limited period of time and considered it as a as an event strictly associated with the late summer and early fall production period.

Ricotta salata for its features is also very susceptible to microbiological spoilage by *Enterobacteriaceae*, yeast, molds and *Pseudomonas* (Casti *et al.*, 2016).

The production of ricotta salata cheese is an open system, which exposes the product to environmental contamination. The origin of contamination can be from raw materials or from the environment and differs from one microorganism to another (Almeida et al., 2013). The production of ricotta salata cheese, especially in the traditional batch manufacturing system, includes manual manipulation of the curd after floating, the exposition to environmental contamination from several food contact surfaces (drainage tables, plastic moulds, pressing equipment, drying shelves) and non-food contact surfaces (floors, drains, walls and ceiling). Contaminants may reach the product by means of aerosol, condenses, dripping or inappropriate practices of operators (Widemann, 2003).

The durability of ricotta salata cheese defined by food business operator is generally of several months under refrigerated storage. The shelf life of ricotta salata cheese depends upon a number of interacting factors other than storage temperature such as packaging conditions, product composition, presence of preservatives and competitive microflora.

1.4 *Listeria monocytogenes* and Ricotta salata cheese

Due to the intrinsic characteristics, the absence of competitive associated microflora, the storage conditions and the numerous post process manipulations, it is now clear that Ricotta salata represent a good substrate for the growth of *L. monocytogenes*.

Spanu *et al.,* (2012) conducted a challenge test aimed to assess the growth potential (δ) of *Listeria monocytogenes* in Ricotta salata. They concluded that Ricotta

salata is able to support the growth of *L. monocytogenes* to level as high as 7.0 $\log_{10}$ cfu/g of rind after two months of refrigerated storage. This represents a serious concern to public health since there is no evidence that the microbiological criteria established by Commission Regulation (EC) No 2073/2005 can be met throughout the shelf-life of the product.

In the last decade were reported numerous cases of food alerts due to the presence of *Listeria monocytogenes* in Ricotta salata cheese. In 1999 an American firm, "Schratter Foods Inc." recalled 2,056 salted ricotta of Sardinian origin, for a positivity to *Listeria monocytogenes*. In 2002, a company in New Jersey recalled from the market 6,700 forms of Ricotta salata, from Sardinia, because contaminated with *Listeria monocytogenes*. In 2007, there was a recall in Georgia (USA) and in 2008 in Montreal. In the same year, an alert has been issued in Germany, for ricotta salata cheese from Sardinia, with contamination levels of 31,000 cfu/g.

In September 2012, a batch of Italian Ricotta salata has been involved in a case of human listeriosis in thirteen US states, which caused twenty hospitalizations and three fatal cases. Ricotta salata cheese, distributed in the USA by the firm "Forever Cheese Inc." were immediately recalled from the market (10 September 2012) and the Food and Drug Administration blocked the import of cheese from the Italian company involved. American authorities, after verifying that it was a listeriosis outbreak, started the investigation and traced back to a Sardinian (Italy) cheese making plant the origin of the

contaminated Ricotta salata. The ricotta salata cheeses were produced in Sardinia but shipped to another plant in Puglia where ricotta wheels were removed from their original packaging, portioned into wedges, repackaged and sold in several European countries (France, Germany, Greece, Belgium and the Netherlands), and north America (USA, Canada and Mexico) and other international markets (Australia, Japan and Egypt).

1.5 Shelf-life determination

With the introduction of the "hygiene package", the food business operator (FBO) becomes responsible for the determination of the shelf life of the foods they place on the market. The Regulation EC No 2073/2005 defines the shelf life of a food as "the period corresponding to the period preceding the "use by" or the minimum durability date". Furthermore, the same Regulation establishes that "the food business operators shall ensure that foodstuffs comply with the relevant microbiological criteria at each stage of food production, processing and distribution, including retail". The article 3 point 2 of the Regulation obliges the food business operators responsible for the manufacture of the product, to conduct studies in order to investigate compliance with the criteria throughout the shelf life. In particular, this applies to ready-to-eat foods that are able to support the growth of *Listeria monocytogenes* and that may pose a *Listeria monocytogenes* risk for public health.

1.5.1 Durability studies and Challenge tests method

In order to determine the shelf life of their products, the FBO can conduct some studies that are indicated in annex 2 of the Regulation EC No 2073/2005. The studies shall include:

- specifications for physico-chemical characteristics of the product, such as pH, aw, salt content, concentration of preservatives and the type of packaging system, taking into account the storage and processing conditions, the possibilities for contamination and the foreseen shelf life;

- consultation of available scientific literature and research data regarding the growth and survival characteristics of the microorganisms of concern.

When necessary, the food business operator shall conduct additional studies, which may include:

- predictive mathematical modelling, using growth or survival data of the micro-organisms of concern in the product;

- tests to investigate the ability of a microorganism experimentally inoculated in the product to grow or survive under the foreseeable storage conditions;

- evaluation of the growth or survival of a microorganisms that may be present in the product during the shelf-life under reasonably foreseeable conditions of distribution, storage and use.

These studies shall take into account the inherent variability linked to the product, the microorganisms in question and the processing and storage conditions. However, the Regulation 2073/2005 does not give any indication on the methodology to use in order to conduct such studies.

In 2008, the European Commission issued a document entitled "Guidance document on *Listeria monocytogenes* shelf life for ready-to-eat foods, under Regulation EC 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs". This document is addressed to the producers of RTE foods. The document aims to guide RTE producers in identifying the *L. monocytogenes* risk in their foods and to provide general principles for the decision on when and which shelf-life studies are needed. The competent authorities may also use the document to verify the implementation of shelf life studies.

Another document entitled "Technical Guidance Document on Shelf-life Studies for *Listeria monocytogenes* in Ready to Eat Foods" was issued by the "European Community reference Laboratory for Listeria monocytogenes" and by the "Agence Francaise de Securitè Sanitarie de Aliments". This technical guidance document is basically intended for laboratories conducting shelf-life studies for *L. monocytogenes* in RTE foods.

These documents describe the principles and procedures to conduct studies on the shelflife of RTE foods with particular regard to *L. monocytogenes*, analyzing applications and limitations of predictive microbiology, of durability studies and challenge tests.

Predictive microbiology

Predictive microbiological models are computer based software packages which allow to predict the behavior of microorganisms in foods under their manufacturing or storing conditions. Trough the use of a number of dedicated software it is possible to run mathematical predictive models which can predict, as examples, the probability of growth of a microorganism in a given food product, estimate the contamination level at a specific storage day, the possible outcomes of an interruption in the cold chain. They represent an useful tool for FBO to understand the interaction between changes in food composition, technology, packaging and storing conditions. Some models are able to predict the microbial growth when the physico-chemical characteristics of the food and the storage temperature are known. Some other models can predict the behavior of microorganisms taking into account the presence of competitive microflora. Several models have been developed and incorporated in many user-friendly software.

There are primary, secondary and tertiary predictive models. The primary predictive models only consider the growth curve of the microorganism, the secondary models establish a common relationship between growth parameters and environmental

factors (pH, aw and NaCl) and tertiary models convert primary models and one or more secondary models in software predictive microbiology.

Predictive microbiology may be useful for the following applications:

- to predict bacterial growth in various conditions;

- to predict the growth probability of micro-organisms in foods;

- to estimate the contamination level at a given day of the shelf-life;

- to test the variability between 2 batches;

- to optimize formulation (additives, pH, salt) to assure the best stability;

- to evaluate the impact of cold chain breaks, and to test different storage scenarios;

- to help to identify Critical Control Points in a process.

In recent years, significant advances have been made in the field of predictive microbiology especially for estimating the growth of *L. monocytogenes* in foods. Mathematical models predicting the growth probability of *L. monocytogenes* can help FBO to categorize their foods.

Despite everything, the predictive models have some limitations, because the mathematical equations cannot reproduce the variability as well as occur in nature. Furthermore, these mathematical models are made on data obtained in labs through the use of solid or liquid growth media, which are not able to reproduce the dynamics and the interactions between the microorganisms and the food.

Durability studies

Through the durability studies it is possible to evaluate the growth of *L. monocytogenes* in a naturally contaminated food. Durability studies are certainly more realistic than other type of shelf life studies since they are based on the natural occurrence of *L. monocytogenes* in the food product. However, they have the limitation that may be difficult to interpret, especially in those situation in which the prevalence of food contamination is low, the level of the contamination is close to the detection limit of the method and when the contamination is not evenly distributed in the food matrix. In addition an elevated number of test units is needed to obtain the data necessary to gain a sufficient confidence level. Therefore, durability studies can be easily used when the microorganism of interest is systematically present in a product. Furthermore, the reliability of the study is related to the amount of available samples and data. Alternatively, the use of other tools, such as challenge tests, may be needed.

Challenge tests

The challenge tests are designed to provide information on the behavior of *Listeria monocytogenes* artificially inoculated in a food before storage under the foreseeable conditions. They consist in the voluntary contaminating of a food with a known quantity of a target microorganism, in order to assess its behavior during the phases of handling and conservation. These tests can be used to assess the growth potential (δ) of a microorganism on a particular food, to estimate the growth parameters (e.g. maximum

growth rate μ_{max}) and to validate a post-lethality treatment in order to inactivate a known concentration of microorganism artificially inoculated in food.

Growth potential (δ)

A microbiological challenge tests that assess the growth potential (δ) is a study able to quantify the growth of *Listeria monocytogenes* in an artificially contaminated food stored under defined conditions for all its shelf-life. The "δ" is defined as 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 "δ" depends on many factors, the most important are the choice of the strain to inoculate, the food intrinsic properties (pH, content in NaCl, a_W, nutritional characteristics of the food and associated microflora) and extrinsic properties (temperature and gas atmosphere of incubation). This study can be applied, as the EC Regulation 2073/2005, to foods that support the growth of *Listeria monocytogenes* other than those intended for infants and for special medical purposes., Foods where "δ" is greater than 0.5 $\log_{10}$ cfu/g are considered foods that support the growth of *Listeria monocytogenes*, instead the foods in which "δ" is less than 0.5 $\log_{10}$ cfu/g do not support the growth of *L. monocytogenes*.

Maximun growth rate (μ_{max})

Microbiological challenge tests assessing maximum growth rate (μ_{max}) is a study that measures in a fixed time the rate of growth of *L. monocytogenes* in an artificially contaminated food, stored under foreseeable conditions at fixed temperature. The data of the exponential growth of the microorganism are related with the growth time and is

obtained a straight-line. The straight-line slope indicates the maximum growth rate (μ_{max}) of the bacterium. The results obtained with a study of the maximum growth rate, depends on the used strain, on the intrinsic properties of food and on extrinsic properties of incubation (temperature, gas). The determination of the μ_{max} allow the determination of *L. monocytogenes* concentration at a given day when the initial concentration is known or the determination of the maximum concentration of *L. monocytogenes* in the product at production which ensure that the limit of 100 cfu/g will not be exceeded at the end of the shelf life.

Validation of a post lethality treatment

The Challenge test can be used also to validate post-lethality treatments in order to test the efficacy of a process able to inactivate *L. monocytogenes* in a food. Such treatments are applied to the finished product after it has already undergone to killing step (i.e. lethal treatment). The treatments are applied on the packaged, or immediately before the aseptic packaging, to eliminate or reduce the level of post-process contamination. The post-lethality treatments to inactivate *Listeria monocytogenes* are treatments in which the packaged food is subjected to high temperatures, high pressure or radiation. In order to validate a post-lethality treatment able to determine a reduction of *Listeria monocytogenes* it is necessary to artificially inoculate the food product with high concentrations of the microorganism (eg. 10^6-10^7cfu/g). These concentrations of inoculum, even if not very representative of the real contamination levels, allow to

quantify the inactivation potential of the studied treatment. In order to design a When conducting Challenge test aimed to validate the *L. monocytogenes* inactivation it is necessary to take into account several factors.

First of all the appropriate level of artificial contamination should be selected. If too low values (<10 cfu/g) are chosen, this could lead to some difficulties in the bacteria count, to false negative results and therefore in the underestimation of the growth potential of *L. monocytogenes*. This could give the wrong conclusion that the product is safe, representing a threaten for human health. On the contrary, too high values of inoculum (> 10,000 cfu/g) could overgrowth the effect of the applied killing step and as result to take the unnecessary decision to shorten the shelf-life of the product. The contamination level depend on the kind of the study. The "Technical Guidance Document on shelf-life studies for *Listeria monocytogenes* in ready-to-eat foods" suggests a contamination level within the 100 cfu/g (Beaufort et al., 2008); other organizations suggest an inoculum level of about 10^2-10^3 cfu/g (FDA, 2001). Some author suggest that in order to validate a post-lethality treatment are necessary inoculum levels of about 10^6-10^7 cfu/g (Scott *et al.*, 2005).

The selection of the strains used for the inoculum is another important factor. It is generally suggested to use a mixture of several strains (from three to five) in order to take into account to the variability between different serotypes. Care must be taken to the physiological state of bacterial cells (living cells and in log phase or early stationary

phase) and their adaptability to the product (eg. acidity, refrigeration temperatures).

Finally, the method used to contaminate the product should be as similar as possible to

the natural contamination.

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CHAPTER 2

Thesis Project

Thesis Project

As consequence of the high temperatures applied to the whey during curd coagulation (above 80 °C), Ricotta salata is characterized by the presence of poor typical microflora, with the only exception of the possible survival of *B. cereus* spores (De Santis *et al.,* 2008; Spanu *et al.*, 2016). The absence of a competitive microflora, the intrinsic characteristic of the product (pH, a_w an nutrients) and the particular open system production make Ricotta salata a product extremely favorable to the growth of pathogenic and spoilage microorganisms.

The safety of Ricotta salata relies in the strict application of hygienic procedures (GMP and GHP) during the production process, with special regard to the steps including manual manipulation of the product (i.e. from molding to packaging).

Another key element in the control of microbiological hazards is the respect of the cold chain, as thermal abuse may favor the development of pathogenic and spoilage microorganisms at levels that can impair the organoleptic characteristics of the product and the consumer's health.

Among the microorganisms frequently recovered from the processing environments of Sardinian cheese making plants, *L. monocytogenes* represents the greatest concern for human health. In fact, Ricotta salata supports the growth of *Listeria monocytogenes* and it is frequently isolated from the surface of the product (3.7%) but rarely, from the paste (De Santis *et al.*, 2005).

The packaging of ricotta salata depends on the final use of the product, being ricotta salata wheels vacuum packed as a whole in shrinking bags if intended to be consumed grated, for mixing with other cheeses or as an ingredient, or cut into wedges before packaging if consumed plain.

Whereas the *Listeria monocytogenes* contamination of the product are prevalent on the rind and occur principally at the end of the production process (post-production contaminations), we resolved to developed a post-lethality treatment for the surface of vacuum packed Ricotta salata designed to control the contamination of *Listeria monocytogenes*.

The application of water bath heat treatment in vacuum packed ricotta salata is a possible strategy to control *L. monocytogenes* superficial contamination.

The general aim of the thesis was to individuate the best time – temperature combination in order to inactivate the *L. monocytogenes* contamination on the rind of vacuum packaged Ricotta salata cheese wheels and wedges.

The Challenge test was the method used for the validation of post-lethality thermal treatment. With this purpose the challenge test method was first studied and subsequently the inactivation studies on Ricotta salata cheese were developed.

The thesis is based on three scientific papers already published in international journals.

The first contribution of the thesis (Chapter 3) is a review published in the Italian journal of Food safety 2014, volume 3:4518 pages 231 – 237, entitled "Microbiological challenge testing for *Listeria monocytogenes* in ready-to-eat food: A practical approach" where the candidate is a coauthor. The present is a review of the existing literature on the challenge test and it describes the methodology for implementing such laboratory studies. All the main aspects for the conduction of *L. monocytogenes* microbiological challenge test were considered, from the selection of the strains, preparation and choice of the inoculum level and method of contamination, to the experimental design and data interpretation. The objective of the work is to provide an exhaustive and practical guideline for laboratories that want to implement *L. monocytogenes* challenge testing on ready to eat foods. The methodology described was used in the planning and conduction of the experiments illustrated in Chapter 4 and Chapter 5.

The second contribution to the thesis (Chapter 4) was published in the scientific journal Food Control 2015, volume 50 pages 740 – 747, entitled "Comparison of post-lethality thermal treatment conditions on the reduction of *Listeria monocytogenes* and sensory properties of vacuum packed ricotta salata cheese" where the candidate is a coauthor. The objective of the present study was to select a heat treatment able to inactivate *L. monocytogenes* count of at least 5 log in Ricotta salata. Nine temperature time combinations, 75 °C, 85 °C and 90 °C applied for 15 min, 25 min and 40 min each were tested in ricotta wheels artificially contaminated with a mixture of 5 *L.*

monocytogenes strains. Inactivation was assessed respectively one day and 30 days after heat treatment. The efficacy of treatments was evaluated based on the reduction in *L. monocytogenes* counts, on the impact on sensory properties and on the cost of the treatment. Two out of nine treatment combinations were effective in reducing *L. monocytogenes* contamination level of 5 log. Regarding the sensory properties no significant difference were observed after the heat treatments.

The third contribution to the thesis (Chapter 5) was published in Journal of Food Science 2015 volume 80 pages M1549 – 1556, entitled "Inactivation of *Listeria monocytogenes* using Water Bath Heat Treatment in Vacuum Packed Ricotta salata Cheese Wedges". In the present study 9 different time temperature combinations, 75, 85, and 90 °C applied for 10, 20, and 30 min each, were tested on artificially contaminated Ricotta salata cheese wedges. The extent of the lethal effect on *L. monocytogenes* was assessed 1 and 30 days after the application of the hot water bath treatment. Five of 9 combinations, 75 °C for 30 min, 85 °C for 20, and 30 min, and 90°C for 20 and 30 min, demonstrated to meet the process criteria of at least 5 log reduction. No significant difference was observed in sensory properties after the heat treatments.

CHAPTER 3

Microbiological challenge testing for *Listeria monocytogenes* in ready to eat foods: a practical approach.

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The contribution of the doctoral candidate to the review in this chapter mainly concerned the bibliographic research. The candidate contributed also in manuscript writing and editing of publication. The candidate presented this work to the conference AIVI in Bologna 10-12 September 2014.

Microbiological challenge testing for *Listeria monocytogenes* in ready to eat foods: a practical approach.

Abstract

Food business operators are primary responsible for the safety of the food they place on the market. The definition and validation of the product shelf-life is an essential part for ensuring microbiological safety of the food and health of consumers. In the frame of the Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs, food business operators shall conduct shelf-life studies in order to assure that their food do not exceed the food safety criteria throughout the defined shelf-life. In particular this is required for ready to eat foods that support the growth of *Listeria monocytogenes*. Among the other studies, food business operator can rely on the conclusion drawn by microbiological challenge test. A microbiological challenge test consists in the artificial contamination of a food with a pathogen microorganism and is aimed to simulate its behavior during processing and distribution under the foreseen storage and handling conditions. A number of documents published by international health authorities and research institutions describe how to conduct challenge studies. The authors review the existing literature and described the methodology for implementing such laboratory studies. All the main aspects for the conduction of *L. monocytogenes* microbiological challenge test were considered, from the selection of the strains, preparation and choice of the inoculum level and method of contamination, to the experimental design and data interpretation. The

objective of the present document is to provide an exhaustive and practical guideline for laboratories that want to implement *L. monocytogenes* challenge testing on ready to eat foods.

Introduction

Regulation (EC) No 852/2004 (European Commission, 2004) on the hygiene of foodstuffs states that the primary responsibility for food safety rests with the food business operators (FBOs), which are legally responsible for the determination of the date of minimum durability of the foodstuffs they place on the market. According to Regulation (EC) No 2073/2005 (European Commission, 2005) on microbiological criteria for foodstuffs, FBOs shall ensure the compliance of their products with the limits set by the Regulation until the end of the shelf-life. When defining the product shelf-life FBOs should base their decision on scientific evidences. In particular, for ready to eat foods that support the growth of *Listeria monocytogenes*, the Regulation describes a series of studies that may be conducted. Among the indicated shelf-life studies are the challenge studies. Although the Regulation indicates the opportunity of conducting such studies, it does not describe how to perform them. Two main guidance documents have been published describing the methodology to conduct shelf-life studies for *L. monocytogenes* in ready to eat foods. The first, directed to laboratories, is a technical guidance document on shelf life studies for *L. monocytogenes* in ready to eat food, prepared by the EU Community Reference Laboratory (CRL) for *L. monocytogenes*

(Beaufort et al., 2014). The second document, intended for FBOs, is the Guidance document on *L. monocytogenes* shelf-life studies for ready to eat foods, under Regulation (EC) No. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs (EC/DG SANCO, 2008). There are a number of existing available documents published by health authorities at international scale supporting the implementation of challenge testing as a control measure of L. monocytogenes in ready to eat foods (USFDA, 2008; Chilled Food Association, 2010; NZFSA, 2011; Health Canada, 2012; FSIS, 2014). A challenge study consists in the artificial contamination of the food with the target microorganism under controlled experimental conditions. These studies are intended to determine whether a RTE food is able to support the growth of *L. monocytogenes* or not during the designated shelf-life. A further application of challenge studies is to validate the efficacy of lethality treatment applied to ready to eat foods intended to reduce or eliminate the pathogen (Scott et al., 2005). Many factors should be taken into account in designing, conducting and interpreting the results of a challenge study. Some of them are related to the laboratory conditions while other are related to product formulation, manufacturing process, packaging, conditions during distribution and consumption. Neglect all these aspects could lead to flawed conclusions and invalidate the study. Therefore, the aim of the present document is to review the existing available documents and to provide FBOs, research laboratories and official control authorities with a practical guide to design and perform challenge studies for *L. monocytogenes* in ready to eat foods.

Materials and methods

Laboratories performing challenge studies should be aware that for handling *L .monocytogenes* a biosafety level 2 is required and expert microbiologists are needed.

Several factors must be considered when conducting a challenge study. A brief description of all the main aspects follows.

Selection of *L. monocytogenes* strains

It is usually recommended to use a pool of at least three to five different strains so that differences in growth and survival among strains are taken into account. The inoculum should include strains isolated from the processing environment or from outbreaks associated with the food being tested (Scott et al., 2005). The serotypes most frequently involved in human listeriosis (1/2a, 1/2b and 4b) should be part of the inoculum. Strains obtained from international culture collections, i.e. American Type Culture Collection (ATCC) or National Collection of Type Cultures (NCTC) can also be used. However, wild type strains are more likely to adapt and growth on the food matrix as compared to reference strains (Guyer and Jemmi, 1991; Skalina, 2010; Spanu et al., 2012; Spanu et al., 2013). Alternatively to the use of pathogens, surrogate microorganism can be used (i.e. Listeria innocua) when the study is to be conducted into a processing facility. These microorganisms have similar characteristics with the target microorganism, except for the pathogenicity. Although it is reasonable to assume that surrogates have similar behaviour, they should be tested to demonstrate similar growth and resistance as compared with *L.*

monocytogenes and for possible interaction with food formulation and background microflora (Scott et al., 2005). After the selection of the strains a genetic characterization should be conducted in order to determine if the strains recovered from the challenged samples are the same that were inoculated. This characterization could be performed with several methods such as Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Pulsed Field Gel Electrophoresis (PFGE), DNA microarrays and gene sequencing.

Preparation of the inoculum

The procedure for the preparation of the inoculum should always start from strains stored at -80°C with glycerol, avoiding subculturing of strains for more than five passages (AOAC, 2006). Streak strains onto non selective agar medium (e.g., trypticase soy agar, TSA or brain heart infusion agar, BHIA) and incubate for 24 h at 37°C. Pick a single pure colony and transfer into tubes containing non selective nutrient broth (trypticase soy broth, TSB or brain heart infusion broth, BHI) and incubate at 37°C for a time sufficient for the strains to reach the same physiological state (late exponential phase or early stationary phase). Overnight incubation up to 36 h, depending on the use of static or shaken incubation, is usually appropriate to obtain cells in stationary phase (ca. 1 x 109 cells/mL). Prepare a second subculture in broth medium and incubate at refrigeration temperature for the time sufficient to reach the late exponential phase or early stationary phase. Preliminary test should be performed to determine the incubation time and

enumeration confirmed by colony counting on agar plates. This phase is essential to adapt the strains when the challenge is conducted in RTE refrigerated foods. This may require also adaptation to pH, water activity (aW) or other hostile conditions (e.g. NaCl concentration, preservatives) characteristic of the tested food. The preparation of each of the strains to be mixed in the inoculum should be performed separately. Once adapted, mix individual cultures in equal volume to obtain a working stock solution, which will be used, after appropriate dilution, for the contamination of the product.

Inoculum level

Prepare adequate serial dilutions in phosphate buffered saline (PBS) or sterile saline solution (0.85% NaCl) to obtain the desired level of contamination.The level of the inoculum to be used depends on the objective of the study. If we were to determine the growth of *L. monocytogenes* or the stability of a product formulation, it is usually recommended to obtain a final concentration between 102-103 cfu/g of product (USFDA, 2001; Uyttendaele et al., 2004; Scott et al., 2005; Beaufort et al., 2014; NACMCF, 2010; Augustin et al., 2011). Although natural contamination of most foods in generally lower, this level allows enumeration of *L. monocytogenes*. In some circumstances lower levels can also be used but in this case the detection limit of the enumeration method should be increased, by using duplicate plates (e.g. 2mL of the suspension onto 6 plates) or by using the most probable number (MPN) method (NACMCF, 2010; Corry et al., 2010). Failure to enumerate *L. monocytogenes* could lead to the incorrect conclusion that the product is

safe. On the other hand, if too high of an inoculum level is used, the microorganisms may overcome the ability of the product formulation to inhibit *L. monocytogenes* growth. Instead, the validation of a lethal treatment requires higher inoculum levels. This depends on the extent of reduction we desire to validate. Inoculum levels of approximately 105-107 cfu/g of product are generally suggested (USFDA, 2001; Scott et al., 2005; NACMCF, 2010). According to the Listeria Rule issued by the Food Safety and Inspection Service (FSIS) the validation of a post-lethality treatment should demonstrate at least 1-log reduction of *L. monocytogenes* to be considered effective (FSIS, 2003), but higher levels of inactivation can be demonstrated according to circumstances. The Codex Alimentarius Commission (2004) defined the effect on the frequency and/or concentration in a food that must be achieved by the application of one or more control measure to provide or contribute to a food safety objective (FSO) or adequate level of protection (ALOP), as the performance criterion (PC). Different public health risk for *L. monocytogenes* are accepted by international health authorities which lead to a "zero tolerance" policy in ready to eat meat products recommended by USDA, while the SCVPH of EC (2005) considered 102 cfu/g a FSO at the time of consumption valid to provide an ALOP. When selecting the appropriate PC, FBOs should consider the type of food, the possible survival, growth and recontamination during the product shelf-life.

Experimental design

A number of factors should be taken into account when designing challenge studies. The duration of the study should be at least equal to the shelf-life of the product and analysis performed at least the day the product is inoculated ("day 0") and at the end of the shelf ("day end"). A sufficient number of intermediate sampling intervals (at least 4-5) should be set over time. Ideally, an additional time should be considered (1.5 times the shelf-life), to simulate the eventuality the product is consumed beyond its assigned durability (Scott et al., 2005). At each interval a minimum of 3 inoculated test units should be analyzed. Increasing the number of the units tested at each analysis point will increase the confidence of the study. Along with inoculated test units, a number of control units should be analyzed at each sampling interval. Controls are represented by uninoculated units ("blank samples") used for detecting the level of natural contamination with *L. monocytogenes,* the background microflora and physical-chemical characteristics of the product. The determination of the background microflora is essential in order to evaluate possible interaction that may affect the growth of *L. monocytogenes*. The physical-chemical properties of the product (i.e. aw, moisture, salt level, pH, preservatives levels, gas concentrations in Modified Atmosphere Packaging, etc.) should be monitored through the shelf life to account for factors that may affect the growth or inactivation rate of *L. monocytogenes*. As far as sample size, duplicate or triplicate sample units should be tested at each interval point. The study should be ideally repeated in three independent trials

using three different batches of the same product so that product variation is considered (Scott et al., 2005; Beaufort et al., 2014).

Inoculation method

The inoculation procedure should be performed in such a way that product formulation is not changed. Therefore, the inoculum volume should not exceed 1% of the product weight or volume. For products that are packaged in MAP the inoculation procedure shall ensure that the gas composition is similar to what is expected in the uninoculated products (Beaufort et al., 2014).

Contamination should be as close as possible to natural contamination. Liquid product can be directly inoculated with an appropriate volume of the mixed culture at the desired concentration, while solid products can be sprayed, dipped, spreaded or mixed with the inoculum. A holding period after the inoculation is needed to allow the inoculum to attach to the product (Health Canada, 2012). The level of contamination should be confirmed by testing control positive unit after the inoculation.

Food product storing conditions

After the artificial contamination and for the entire duration of the study the products should be packaged under the same condition as intended for marketing (under vacuum, modified atmosphere, etc.). The temperature should mimic the foreseen conditions of the product during storage and distribution (e.g. refrigerated). Although out of the control of food industries, poor consumers handling of the product during dispatch, storage and

domestic usage should be taken into account. Therefore, temperature abuse in the distribution chain could also be simulated by incubating the food at temperatures above the refrigeration temperature. The time and the temperature used should be justified by detailed information: the 75th percentile of the observation for the country where the stage of cold chain is located (Beaufort et al., 2014).

Samples analysis

The detection and enumeration of *L. monocytogenes* should be conducted according to standard methods (ISO 11290-1, 1996; ISO 11290-2, 1998) as stated in the Regulation No. 2073/2005. When the study is aimed to evaluate the efficacy of a lethality step, it is required to use an enrichment method in order to detect *L. monocytogenes* that may be no longer recovered with the enumeration method. Inoculated units should be analyzed at day 0, day end and at all intermediate points for the enumeration of *L. monocytogenes*. Non-inoculated units should also be analyzed at the same intervals by the detection method. When assessing the lethality of a killing steps (e.g. heath treatment, high pressure), detection and enumeration method should be conducted on treated units after (the same day) the delivery of the lethal treatment. The lethal treatment may not kill all Listeria cells that may survive in the product. Such injured cells may repair themselves and recover their ability to growth, being as dangerous as uninjured cells. However, sublethally injured cells may not be cultured on selective media due to the presence of antibiotics, organic dyes and other selective agents. Therefore, testing foods after heat

treatment requires techniques that enable to detect sublethally injured cells. Although the use of non selective media on one hand allows the recovery of damaged cells, on the other hand cannot differentiate the target microorganism from background microflora. The Thin Agar Layer (TAL) method is a method which consists in the overlay of a nonselective agar medium onto agar plates containing a selective medium that combines the ability to enumerate and to differentiate heat injured cells (Kang and Fung, 1999; Wu and Fung, 2001). Determination of the relevant physical-chemical characteristic (pH, aw, salt content, preservatives concentration) that can affect the inactivation or growth of Listeria monocytgenes should be evaluated over product shelf-life. To take into account the variability of product formulation, when performing challenge study the formulation that is the most permissive for Listeria growth should be tested. Standard methods should be used when conducting the analysis. Gas composition should be monitored in product that are MAP packed to check if their concentration is stable throughout the entire shelf-life. In table 1 is reported an example of experimental design with the test units, sampling point and analysis to be conducted when performing a challenge study. Testing for background microflora in control units gives important indication of their effect on the shelf-life of the product. The presence of starter (i.e. lactic acid bacteria) can compete with *L. monocytogenes* limiting its growth, while other contaminant microorganisms can spoil the product before *L. monocytogenes* could grow to risk levels.

Results

Assessing growth potential

The growth potential (δ) is defined as 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 (Beaufort et al., 2014). The $\log_{10}$ cfu/g at day 0 and the $\log_{10}$ cfu/g at day end are obtained taking the median of the $\log_{10}$ cfu/g concentration among the test units at the beginning and at the end of the study, respectively. Their difference is computed independently for each batch. The maximum difference between these values is the growth potential. A food is considered able to support the growth of *L. monocytogenes* if the δ is higher than 0.5 $\log_{10}$ cfu/g, while it is assumed that the food is not able to support the growth if the δ is lower than 0.5 $\log_{10}$ cfu/g. In table 2 is reported a selection of studies assessing the growth potential of L. monocytogens obtained in different ready to eat food after artificial contamination.

Assessing lethality

In this case it is not always necessary to analyze inoculated units at each sampling point. Since the objective is to validate the lethality of a process it is necessary to examine the product at the start of the trial, after the process (treatment) and at the end of its designated shelf-life (end-point determination). To determine if the process is capable of deliver the required level of lethality against *L. monocytogenes* (performance standard or D), the difference between the level of $\log_{10}$ cfu/g after the inoculum and at the end-point is computed. As for the determination of the growth potential, the log reduction should be

calculated independently for each batch. To account for a margin of safety the lowest log reduction obtained should be compared with the highest expected contamination. The result obtained can be expressed as log reduction of the target microorganism and the performance standard D is the number of log reduction. FSIS require for a post-lethality treatment to be validated to demonstrate at least 1-log reduction (i.e. a 90% reduction of the pathogen), while a reduction on 5 log is considered a full lethality treatment (FSIS, 2014). If microbiological challenge testing fails to demonstrate the predetermined level of D, the study is not invalid, a lower D can still be validated. In table 3 is reported a selection of studies assessing the effectiveness of thermal pasteurization, irradiation and high-pressure decontamination technologies applied on different ready to eat food artificially contaminated with *L. monocytogenes*.

Discussion and Conclusions

Microbiological challenge tests are a tool aimed to simulate the behavior of pathogens or spoilage microorganisms on a food during processing and distribution under the foreseen storage and handling conditions. They consist in laboratory based study in which the food is artificially contaminated with a known initial concentration of the target microorganism. Microbiological challenge study can be used to determine whether or not a food supports the growth of pathogenic microorganism or as performance criterion of a process intended to deliver a lethal effect. It is worth conducting challenge studies on ready to eat food when their formulation does not guarantee to prevent the growth of the

microorganism during the designated shelf-life. The increased demand of minimally processed ready to eat food poses a special attention in the definition of their shelf- life. In fact, these foods are generally characterized by mild heat treatment, minimal preservatives concentration in the formulation and storage at refrigeration temperatures. This may be inadequate to kill or to prevent the growth of an important pathogenic microorganism such as *L. monocytogenes* (Peck, 2006). Furthermore, the risk of listeriosis associated with ready to eat food is increased by the always more extended shelf-life required by the market, giving the opportunity to *L. monocytogenes* of growing to levels exceeding the limit set by health authorities. The definition of the durability of ready to eat foods should be based on studies aimed to assess the ability of *L. monocytogenes* to growth or to survive in the product under the foreseen storage condition for the entire shelf-life. Durability studies, assessing the growth of *L. monocytogenes* in naturally contaminated foods, can also be conducted. Although more realistic, the drawbacks of durability studies are that the interpretation of the results is complicated by the probability of testing contaminated food samples (which depends by the prevalence of the contamination), the low level and the uneven distribution of the initial contamination. As an example of the relationship existing between prevalence of microbial contamination of a food and the potential for recovery, the number of test units needed to detect one or more positive per lot with 95% confidence level is 4 when the prevalence of contamination is 100%, while it increases to 299 when the prevalence is

1% (Midura and Bryant, 2001). On the other hand challenge studies allow knowing the initial contamination level and need lower samples units to draw conclusions. However, the results are valid only for the food and for the particular conditions tested. If any significant change occurs in the product formulation or in the process, the study should be repeated. Challenge studies needs the support of an expert food microbiologist and should not be performed in the microbiology laboratory of the food processing plant. A well designed challenge study can be of a great support for food business operator in validating lethal treatments or product formulation aimed to control survival or growth of *L. monocytogenes* for the entire shelf-life. A proper definition of the fate of *L. monocytogenes* through the processing, distribution and successive handling of ready to eat foods is essential in order to comply with all applicable legislative and regulatory requirements. When validating the effect of an antimicrobial agent or process it should be pointed that these strategies are aimed control *L. monocytogenes* contamination in post-lethality exposed products (e.g. slicing, curing, packaging and other unit operations conducted after the lethality step). Therefore they should not be considered as an alternative to the implementation of proper sanitation and preventive hygienic measures.

Figures and Tables

Table 1. Experimental design indicating the type of analysis, the testing time and the relative minimum number of test units to perform per batch.

Analysis	Test units	testing time				
		T_0	T_1	T_2	T_n	T_{end}
Detection and enumeration of *L. monocytogenes*	IU[1]	3	3	3	3	3
	NC[2]	3	3	3	3	3
	BS[3]	3	-	-	-	-
Background microflora	NC[2]	3	3	3	3	3
	BS[3]	3	3	3	3	3
physical-chemical characteristic	NC[2]	3	3	3	3	3
	BS[3]	3	3	3	3	3

IU1: units inoculated with *Listeria monocytogenes*; NC2: negative control, inoculated with sterile physiological water; BS3: uninoculated blank samples.

Table 2. Examples of studies aimed to assess the growth potential in different ready to eat food artificially contaminated with *Listeria monocytogenes*

type of food	Ready to eat food	pH	aw	incubation temperature	time	growth log₁₀ cfu/g	reference
meat products	cooked ham slices	6.2	0.975	7°C	5 days	2.0	Uyttendaele et al., 2004
	sliced deli meat	6.1-6.3		7°C	35 days	7.0	Beumer et al., 1996
dairy products	ricotta salata cheese	5.8-6.3	0.940-0.950	4°C-6°C	60 days	3.05-4.87	Spanu et al., 2012
fishery products	smoked salmon	5.8-6.3	0.93-0.96	4°C-10°C	30 days	2.5-4.5	Guyer & Jemmi, 1991
	salmon preparations		0.997	4°C-8°C	7 days	1.3-6.42	Midelet-Bourdin et al. , 2010
produce and salads	ready-to-eat vegetables	6.2-7.2		7°C-15°C	6 days	0.21-3.34	Sant'Ana et al., 2012
	green leafy vegetables			7°C-10°C		0.5-1.5	Carlin & Nguyen-The, 1994
	garlic cheese salad	5.5		3°C-7°C	2 days	0.44-0.99	Skalina, 2010
	smoked ham salad	5.0-5.1		3°C-7°C	2 days	0.26-1.11	Skalina, 2010
	shrimp–tomato salad	5.5		3°C-7°C	2 days	0.48-0.64	Skalina, 2010

Table 3 part 1. Examples of studies aimed to assess the efficacy of different post-lethality treatment on *Listeria monocytogenes* counts applied in artificially contaminated ready to eat foods.

type of product	Ready to eat food	type of treatment	paramether	Performance standard $D*$	reference
meat products	sliced deli meat	Hot water bath	65 °C for 10''- 5'	3.0-4.8 log$_{10}$	McCormick et al., 2003; Selby et al., 2006; Mangalassary et al., 2008
	sliced deli meat		85 °C for 10''	>6.0 log$_{10}$	McCormick et al., 2003
	deli meat		90.6-96.1°C for 2'-10'	2.0-4.0 log$_{10}$	Muriana et al., 2002
dairy products	cooked turkey breast		96°C for 50 min	7 log$_{10}$	Murphy et al., 2003a
	ricotta salata cheese		90°C for 90 min	6 log$_{10}$	Spanu et al., 2013
meat products	fully cooked frankfurters	Steam pasteurization	100°C for 1.5''	3.0 log$_{10}$	Murphy et al., 2005a; Murphy et al., 2006
	fully cooked bologna logs		100°C for 2.5'	2.0 log$_{10}$	Murphy et al., 2005b
	Fully cooked chicken leg quarters		96 °C for 22 min	7 log$_{10}$	Murphy et al., 2003b
meat products	RTE deli meat	electron beam irradiation	1.0-2.5 kGy	2.0-3.0 log$_{10}$	Foong et al., 2004; Concepción Cabeza et al., 2007
fishery products	cold-smoked salmon		1.0-1.5 kGy	2.5-3.0 log$_{10}$	Su et al., 2004; Medina et al., 2009
meat products	Frankfurters	Gamma irradiation	0.49-2.6 kGy	1.0-5.0 log$_{10}$	Sommers and Thayer, 2000; Knight et al., 2007
	RTE deli meat		0.52-2.5 kGy	1.0-5.0 log$_{10}$	Zhu et al., 2005; Jin et al., 2009
	cured ham		0.75-0.90 kGy	>2.0 log$_{10}$	Fu et al., 1995
dairy products	Feta cheese		2.5 kGy	3.0 log$_{10}$	Konteles et al., 2009
fishery products	seafood salad		0.7 kGy	>2.0 log$_{10}$	Foley et al., 2005

Table 3 part 2.

	Ready to eat food	type of treatment	paramether	Performance standard D*	reference
produce	chopped romaine lettuce	High pressure processing	0.56 kGy	2.6-2.9 $\log_{10}$	Mintier et al., 2006
meat products	frankfurters		300 MPa	1.0 $\log_{10}$	Lucore et al., 2000
	RTE deli meat		400-450 MPa for 10' at 12-17 °C	1.1-3.4 $\log_{10}$	Morales et al., 2006; Marcos et al., 2008
	dry-cured ham		600 MPa for 5' at 15 °C	3.85 $\log_{10}$	Hereu et al., 2012
	RTE deli meat		600-700 MPa for 3-10' at 10-31 °C	3.0-3.5 $\log_{10}$	Hayman et al., 2004; Jofré et al., 2008; Jofré et al., 2009
dairy products	Gorgonzola cheese		400 MPa for 90'' at 20 °C	2 $\log 10$	López-Pedemonte et al., 2007
			500 MPa for 110'' at 5-20°C	5-$\log_{10}$	López-Pedemonte et al., 2007
	cheese		600 MPa for 10' or 700 MPa for 5'	2.0 $\log 10$	Carminati et al., 2004
fishery products	cold-smoked salmon		450 MPa for 10' at 12°C	3 $\log_{10}$	Medina et al., 2009

*$\log_{10}$ reduction after the application of the treatment.

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Tesi di Dottorato in Scienze Veterinarie – Ciclo XXIX - Indirizzo: Produzione, Qualità e Sicurezza Alimentare - Università degli Studi di Sassari

CHAPTER 4

Comparison of post-lethality thermal treatment conditions on the reduction of *Listeria monocytogenes* and sensory properties of vacuum packed ricotta salata cheese.

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The contribution of the doctoral candidate to the paper presented in this chapter concerned several aspects of the experiment, from samples collection, preparation of inoculum, experimental inoculation, microbiological analysis and data statistical interpretation. The contribution of the candidate was also in the writing and editing of the publication.

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Carlo Pala – "Development of a post-lethality treatment to reduce the risk of *Listeria monocytogenes* contamination in ricotta salata cheese"
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Comparison of post-lethality thermal treatment conditions on the reduction of *Listeria monocytogenes* and sensory properties of vacuum packed ricotta salata cheese

Abstract

Ricotta salata is a whey protein cheese produced in Sardinia that in the last decades has been linked to several recalls and in 2012 to a severe human listeriosis outbreak. Contamination of ricotta salata with *L. monocytogenes* mainly occurs during post-process handling and generally origins from the processing environment. The application of water bath heat treatment in vacuum packed ricotta salata is a possible strategy to control *L. monocytogenes* superficial contamination. The objective of the present study was to select a heat treatment able to inactivate *L. monocytogenes* count of at least 5 log. Nine temperature time combinations, 75 °C, 85 °C and 90 °C applied for 15 min, 25 min and 40 min each were tested in ricotta wheels artificially contaminated with a mixture of 5 *L. monocytogenes* strains. Inactivation was assessed respectively one day and 30 days after heat treatment. The efficacy of treatments was evaluated based on the reduction in *L. monocytogenes* counts, on the impact on sensory properties and on the cost of the treatment. Two out of nine treatment combinations, i.e. 85 °C for 40 min and 90 °C for 40 min, were effective in reducing *L. monocytogenes* contamination level of 5 log. No significant difference was observed in sensory properties after the heat treatments.

Therefore both combinations are eligible to conduct a successive study aimed to extend the shelf-life of ricotta salata up to several months.

Introduction

Ricotta salata is a traditional whey protein cheese obtained in Sardinia (Italy) through the heat coagulation of the whey remaining after the production of sheep's milk cheeses. The main phases of production technology of ricotta salata are described as follows. Traditionally is manufactured using the whey remaining after the production of hard sheep's milk cheese, usually Pecorino Romano PDO (protected denomination of origin), which is stored in a silo at 45 °C until use. The whey is filtered and preheated at 60-70 °C using a plate heat exchanger. The whey is then transferred in large open kettles with approximately 1,200-1,500 liters capacity, added with 1% by weight of sodium chloride and heated to temperature above 80 °C for 30 minutes. As a result of heating, curd start floating on the top of liquid, this is collected using perforated scoops and transferred into plastic molds. The so called ricotta "*Toscanella*" is formed into cylindrical shapes and pressed to enhance drainage. The curd is salted either by dry-salting (5% w/v) or by brine-salting and dried for about 10 days in cold rooms at 10-12 °C. The manufacturing process result in cheese wheels weighing approximately 3 kg with a pH of 6.1-6.9, a_W of 0.940-0.970, moisture of 50-60% (< 50% if intended for grating), fat of 28-33% and protein of 14-23% (Spanu, Scarano, Spanu, Penna, Virdis, & De Santis, 2012; Spanu, Spanu, Pala, Virdis, Scarano, & De Santis, 2013). The final product is individually packed in vacuum

bags and stored at refrigeration temperature with a set shelf-life which differs from three weeks up to several months, depending on the food business operators. Packaging of ricotta salata depends on the final use of the product, being ricotta salata wheels vacuum packed as a whole in shrinking bags if intended to be consumed grated, for mixing with other cheeses or as an ingredient, or cut into wedges before packaging if consumed plain. No preservatives are used for shelf life extension. In recent years contamination of ricotta salata with *Listeria monocytogenes* leaded voluntary recalls by international companies importing the product from Sardinia. In 2008 the European Commission documented a case of *L. monocytogenes* infection associated with the consumption of ricotta salata cheese (RASFF, 2008). The most recent and severe episode occurred in the USA where a multistate outbreak of listeriosis linked to ricotta salata imported from Italy caused 20 hospitalization and 4 deaths (CDC, 2012). Heat treatments, such as thermization and pasteurization, applied to milk during cheese making and to whey during ricotta production inactivate *Listeria* cells to levels of approximately 3 to 6 $\log_{10}$ cfu (Buazzi, Johnson, & Marth, 1992; Casadei, Esteves de Matos, Harrison, & Gaze, 1998; ICMSF, 1996; Villani, Pepe, Mauriello, Moschetti, Sannino, & Coppola, 1996). Contamination of whey cheeses with *L. monocytogenes* origins from the processing environment and is localized almost exclusively on the rind, with a reported prevalence in ricotta salata of approximately 20% (Pintado & Malcata, 2000; Lioliou, Litopoulou-Tzanetaki, Tzanetakis, & Robinson, 2001; Ibba, Cossu, Spanu, Virdis, Spanu, Scarano, & De Santis,

2013; Spanu, Scarano, Ibba, Spanu & De Santis, 2015). The intrinsic properties of ricotta

salata support the growth of *L. monocytogenes,* once onto the product, to level as high as

$7.0 \log_{10}$ cfu g^{-1} of rind, potentially harmful to human health (Spanu, Scarano, Spanu,

Penna, Virdis, & De Santis, 2012). Ricotta salata produced in Sardinia is mainly exported

in North America and in other European countries. However, international health

authorities accept different health risk for *L. monocytogenes*, leading to an absence in 25

g recommended by FDA and 10^2 cfu g^{-1} criteria at the time of consumption set by

European Commission (EC) Regulation No. 2073/2005. Even with a strict application of

good hygienic practices during production, superficial contamination of ricotta salata

could not be totally avoided, but only reduced (Tompkin, Scott, Bernard, Sveum, &

Gombas, 1999). Therefore, the application of alternative control strategies should be

applied if the product is exposed to environmental contamination after the lethality

treatment (e.g., cooking) and before packaging (FSIS, 2014). In order to reduce *L.*

monocytogenes contamination in ready to eat food, a number of post-package

decontamination methods have been proposed, such as thermal pasteurization, irradiation

and high-pressure. The efficacy of these decontamination technologies in different ready

to eat products have been reviewed (Zhu, Du, Cordray, & Ahn, 2005). The final choice

of the treatment to apply in ready to eat food stays on the food business operator based

on scientific evidences on the efficacy, but is certainly cost-oriented. Heat post-lethality

treatments (i.e. hot water bath and steam pasteurization) are widely used in the food

industry due to their effectiveness in reducing the load of pathogenic microorganisms (Arnoldi, 2002; Orta-Ramirez & Smith, 2002). The effectiveness of a thermal treatment is influenced by several factors such as temperature-time ratio, food composition, size and weight of the product and microorganism characteristics (Doyle, Mazzotta, Wang, Wiseman, & Scott, 2001; Ray, 2004; Sofos, 2002; Yen, Sofos, & Schmidt, 1991). Hence, the validation of a post-lethality treatment should be designed around the product, taking into account the formulation, packaging and the expected storage and use conditions. Therefore, results obtained on a specific product cannot be extended on another product, even if similar. Previous research demonstrated the efficacy of the immersion of vacuum packed ricotta salata wheels in water bath at 85 °C for 90 min in reducing *L. monocytogenes* counts of 6 $\log_{10}$ cfu g^{-1} of rind (Spanu, Spanu, Pala, Virdis, Scarano, & De Santis, 2013). However, the effect on sensory characteristics of such treatment was not investigated. Any technological interventions that negatively affect the sensory quality of a product become useless for a commercial purpose. For this reason sensory evaluation play a non negligible role in this type of investigation.

The objective of the present study was to compare 9 different temperature-time conditions for the superficial treatment of whole ricotta salata wheels. The efficacy will be evaluated taking into account the extent of reduction and survival of artificially inoculated *L. monocytogenes* and the impact on sensory properties. The results will be used to select

the temperature-time ratio to perform a further study aimed to extend the shelf-life of ricotta salata up to 180 days.

Materials and methods

Ricotta salata samples

A total of 465 vacuum packed ricotta salata wheels were provided by a local cheese-making plant using sheep milk. Samples were randomly selected from 3 different batches (155 ricotta wheels for each batch) and stored in a cold room at 4±2 °C until the experiment was performed. Immediately after their arrival samples were labeled according to their use for the experiment. Experimental Units (EUs) were defined ricotta salata wheels artificially contaminated with *L. monocytogenes* and successively submitted to heat treatment. Positive Controls (PCs) were defined ricotta salata wheels artificially contaminated with *L. monocytogenes*. Blank Samples (BLs) were defined the units not inoculated and used to evaluate the level of natural contamination of ricotta salata with *L. monocytogenes*. Sensory Units (SEs) were defined the not inoculated samples used to evaluate sensory properties either after heat treatment (SEt) or as control with no treatment (SEc). Composition Units (CUs) were defined not inoculated samples used for the determination of intrinsic properties (pH and a_W) and composition (moisture, fat and proteins) after heat treatments.

Artificial inoculation

The Technical Guidance document prepared by the EU Community Reference Laboratory (CRL) for *L. monocytogenes* (Beaufort, Cornu, Bergis, Lardeux, & Lombard, 2014) was used for the experiment designing. A mixture of 5 *L. monocytogenes* strains was used to artificially contaminate EUs and PCs ricotta salata wheels. Of the strains that composed the inoculum one was the reference strain ATTC 19111 (serovar 1/2a) obtained from American Type Culture Collection (Manassas, VA, USA), while the other four were wild-type strains (respectively serotypes 1/2a, 1/2b, 1/2c and 4b), previously recovered from the cheese-making plant environments or from ricotta salata. The wild-type strains were selected in order to be representative of the main serotypes associated with foodborne listeriosis. All the strains were stored at -80 °C in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, UK) with glycerol (15% v/v). The inoculum level was aimed to demonstrate a reduction in *L. monocytogenes* level, or Performance Criterion (PC), of 5 $\log_{10}$ cfu g^{-1} of rind, considered to suffice to attain a Food Safety Objective (FSO) of 10^2 cfu g^{-1} throughout the entire storage period under refrigeration. Previous experiments were conducted to standardize the preparation of inoculum according to the indications contained in the Guidelines for conducting *Listeria monocytogenes* challenge testing of foods (Scott, Swanson, Frier, Pruett jr., Sveum, Hall, Smoot, & Brown, 2005). In order to prepare cells in the same physiological state (late exponential or early stationary phase) each strain was separately inoculated into tubes containing BHI broth and cultured

overnight at 30 °C in a shaking water bath (100 rev min^{-1}). To adapt cultures at refrigeration temperatures, cells were then subcultured into 10 mL of BHI and incubated at 4±2 °C for approximately 15 days. A "mixed working culture" was obtained by transferring equal volumes of each individual culture into a sterile flask. The concentration was adjusted to ca. 10^7 cfu mL^{-1} using sterile saline solution (0.85% NaCl). Plate count on Trypticase Soy Agar (TSA, Biolife, Milan, Italy) was used to confirm concentrations. The whole surface of ricotta salata wheels was evenly sprayed with 2 mL of *L. monocytogenes* mixed culture using an atomizer. A holding period of 15 min at room temperature was allowed to inoculated samples in order to let the suspension attach, after which ricotta salata were individually vacuum packed in shrink bags (Criovac Cook-In HT-3000, Sealedair Ltd., St Neots, UK) and stored at refrigeration temperature until further use.

Heat treatment and experimental design

The experiment was conducted in three independent trials, one for each batch, conducted one month apart.

Heat treatment was performed by immersion of vacuum packed ricotta salata wheels in hot water bath. Nine different temperature- time conditions were tested: 75 °C, 85 °C and 90 °C applied for 15 min, 25 min and 40 min each. The number and the types of ricotta salata samples used for each treatment condition are reported in table 1. Immediately after the heat treatment ricotta salata wheels were immersed in a tank

containing iced water for approximately 2 hours and then stored at 4±2 °C until analysis.

The analysis points or testing times (T) were: the day of inoculum and heat treatment, defined as T_0; 24 hours after heat treatment, defined as T_1 and 30 days after heat treatment defined as T_{30}. T_1 was performed the day subsequent the heat treatment to avoid false negative caused by the presence of sub-lethally injured *L. monocytogenes* cells that may survive the heat treatment but are not immediately culturable. The PCs were analysed at T_0, 6 hours after inoculation to assess if the level of contamination was effectively 10^5 $\log_{10}$ cfu g^{-1}. The BLs were also examined at T_0, to account for eventual natural contamination of ricotta salata with *L. monocytogenes*. The EUs and CUs were submitted to heat treatment and analysed at T_1 and T_{30}. Part of SE units were treated (SEt) and part, used as negative controls (SEc), were no treated (Table 1).

The sampling plan with sample units, testing times and related analysis is summarized in table 2. The effective temperature obtained on ricotta salata surface during each heat treatment was monitored using an additional ricotta salata wheel where a data logger (KT 20T, Kimo, Montpon Ménestérol, France) was placed 1.5 cm below the surface and the temperature recorder during the treatment.

Microbiological analysis

Detection and enumeration of *L. monocytogenes* (ISO 11290-1:1996/Amd 1:2004; ISO 11290-2:1998/Amd 1:2004) and enumeration of aerobic mesophilic bacteria (ISO 4833:2003) were conducted on 25 g of ricotta salata aseptically collected cutting the rind

up to 2 cm. In order to detect the presence of sublethally injured cells that may survive in the product but may not be cultured on selective media, on heat treated samples the enumeration of *L. monocytogenes* was also conducted using the Thin Agar Layer (TAL) method. The TAL method consists in the overlay of a nonselective agar medium onto agar plates containing a selective medium that combines the ability to enumerate and to differentiate heat injured cells (Kang and Fung, 1999; Wu and Fung, 2001). From each positive sample, 5 suspected colonies of *L. monocytogenes* were submitted to phenotypic identification. Multiplex PCR was carried out to confirm identification and to separate the major serovars (1/2a, 1/2b, 1/2c and 4b) into distinct serogroups (Doumith, Buchrieser, Glaser, Jacquet, & Martin, 2004). The *prs* gene, specific for *Listeria* spp. was used as internal amplification control. A selection of the strains recovered from EUs at T_{30} was submitted to pulsed-field gel electrophoresis (PFGE) to confirm that the strains recovered were the same that were inoculated. From each of the 3 replicate were selected up to five strains for each temperature-time combination. In order to capture as much variability as possible a preliminary screening of isolates to submit to PFGE was conducted based on the serogroups. PFGE was carried out using the protocol proposed by Graves & Swaminathan (2001). The obtained restriction profiles were analysed by visual examination to distinguish inoculated strains among each other and from strains originating by natural contamination.

Physico-chemical properties and composition

Intrinsic properties and chemical composition of ricotta salata were determined to account for possible interaction with *L. monocytogenes* survival and growth. PH and a_w were measured using pH meter GLP22 (Crison Instruments SA, Barcelona, Spain) and water activity meter Aqualab 4TE (Decagon, Pullman, WA, USA), respectively. Near infrared transmittance (NIT) compositional analyzer (FOSS, Eden Prairie, MN, USA) was used for the analysis of fat, moisture, protein and total solids. Differences in intrinsic properties and composition ($\bar{x} \pm$SD) of ricotta salata cheese between the different temperature-time combinations used for the heat treatment and over time (T_1 and T_{30}) were compared using Fisher's least significant difference (LSD) test. Statistical analysis was performed with Statgraphics Centurion XVI software (StatPoint Technologies, Warrenton, VA, USA).

Sensory analysis

The "Difference from control test" was applied to highlight sensory differences between heat treated samples (SEt) and the negative control (SEc). This test is very helpful to determine difference between one or more samples against the control and, if the difference is significant, to measure its size (Meilgaard, Civille, & Carr, 1999). On the other hand this test can cause a sensory fatigue when many samples have to be taken into account because, during each session, the control sample as reference and as blind sample must be served. In order to avoid the sensory fatigue only five out nine temperature-time combination were evaluated: 75 °C and 90 °C treated for 15 min and 40 min, and 85 °C

treated for 25 min. Thirty judges (14 females and 16 males, aged 25-50 years) specialized in dairy products, previously selected for their sensitivity and after attending a course of 60 hours in sensory analysis (ISO 8586-1: 1993), evaluated the samples against an untreated control on a numerical category scale (0 = no difference and 9 = very large difference). Ricotta salata samples were kept at 4-6 °C until sensory assessments. Before analysis the ricotta samples were portioned extracting two opposing slices. The slices were further portioned into parallelepiped pieces (5 x 1.5 x 1.5 cm) and served, at room temperature, in odorless plastic containers marked with a random three-digit number (Meilgaard, Civille, & Carr, 1999). Judges were also provided by a tray containing an unsalted cracker and a glass of water. The evaluation was carried out in a randomized and balanced order (Macfie, Bratchell, Greenhoff, & Vallis, 1989). Statistical analysis was performed with Statgraphics Centurion XVI software (StatPoint Technologies) by the one-way ANOVA (factor: samples) and the Fisher's LSD.

Validation of heat treatment

The experiment was conducted in three independent trials for each of the 9 temperature-time combinations. Samples used in each trial belonged to three different production batches (batch A, B and C). Analyses were conducted at two different sampling times (T_1 and T_{30}). For each heat treatment combination and sampling time were analyzed three samples. To account for a margin of safety, the effectiveness of heat treatment, i.e. performance standard (Δ) was considered in the worst conditions, i.e. the minimum level

of $\log_{10}$ cfu g^{-1} reduction in *L. monocytogenes* counts. Reduction obtained as consequence of heat treatments was calculated independently for each batch, by computing the minimum difference between the concentration ($\log_{10}$ cfu g^{-1}) before the treatment (T_0) and after the treatment (T_1 and T_{30}) observed in the triplicate samples.

Statistical analysis

Mean mesophilic bacteria counts ($\log_{10}$ cfu g^{-1}), intrinsic properties ($\bar{x} \pm SD$) and composition ($\% \pm SD$) between the different temperature-time combinations at T_1 and T_{30} were compared using Fisher's least significant difference (LSD) test. All statistical analyses were performed with Statgraphics Centurion XVI software (StatPoint Technologies, Warrenton, VA, USA).

Results

L. monocytogenes contamination and background microflora

Natural contamination of ricotta salata rind with *L. monocytogenes* occurred in 6 out of 18 BLs (30.0%) all originating from the first batch. Enumeration of *L. monocytogenes* was possible in five BLs, showing a level of contamination of 2.68±0.51 $\log_{10}$ cfu g^{-1} ($\bar{x}$ ±SD). The mean level of artificial contamination expressed as $\log_{10}$ cfu g^{-1} ($\bar{x} \pm SD$) obtained on the rind of PCs units at T_0 was 4.82±0.43, 5.5±0.04 and 5.36±0.09 in the first, second and third replicate, respectively. Enumeration of aerobic mesophilic bacteria was conducted on 18 BLs, 9 PCs and 162 EUs. In BLs aerobic mesophilic population ($\bar{x} \pm SD$ $\log_{10}$ cfu g^{-1}) was 7.41±0.47, 7.45±1.04, 7.83± 1.00 while in PCs was 7.21±0.31,

7.78±0.68 and 8.83±0.11 in the first, second and third batch respectively. The mean $\log_{10}$ cfu g^{-1} reduction in total bacterial counts observed at T_1 ranged between ca.1.0 and 3.0 for ricotta treated at 75 °C, 4.0 and 5.5 for treatment at 85 °C and from 5 to 6 for 90 °C. After 30 days of refrigerated storage the microbial population increased of less than 1.0 $\log_{10}$ cfu g^{-1} in ricotta salata treated at 75 °C and of ca. 1.0 and 2.0 $\log_{10}$ cfu g^{-1} in samples treated respectively at 85 °C and 90 °C. Pair-wise comparison of aerobic mesophilic bacteria counts between ricotta salata samples submitted to the 9 treatment combinations and between samples analysed at T_1 and T_{30} are reported in table 3.

Inactivation and survival of L. monocytogenes

The lethal effect was evaluated on 9 EUs (3 for each replicate) for each temperature-time combination at T_1 and T_{30}. The minimum differences in *L. monocytogenes* counts between T_1 and T_0 (Δ_1) and T_{30} and T_0 (Δ_{30}) for each of the 9 treatments are reported in table 4. At T_1 three out of nine combinations, i.e. 85 °C for 40 min, 90 °C for 25 min and 90 °C for 40 min, were effective either with the enumeration and detection methods. However, the 90 °C for 25 min combination showed the survival of *L. monocytogenes* after storage at 4 °C for 30 days. The complete description of the effect of each treatment at T_1 and T_{30} is reported in table 5 and figure 1.

Overall, 334 strains were confirmed as *L. monocytogenes* by molecular identification. Twenty-seven strains (90.0%) isolated from BLs were serogroup 1/2a, while 3 (10.0%) were serogroup 1/2c. Of the 49 strains isolated from PCs, 17 (34.7%) were serogroup

1/2a, 15 (30.6%) serogroup 1/2b, 11 (22.4%) serogroup 1/2c and 6 (12.2%) serogroup 4b. From EUs at T_1 were isolated 95 strains which were grouped as follows: 32 (33.7%) serogroup 1/2a, 12 (12.6%) serogroup 1/2b, 16 (16.8%) serogroup 1/2c and 35 (36.8%) serogroup 4b. From EUs at T_{30} were isolated 160 strains which were grouped as follows: 52 (32.5%) serogroup 1/2a, 23 (14.4%) serogroup 1/2b, 41 (25.6%) serogroup 1/2c and 44 (27.5%) serogroup 4b. Of *L. monocytogenes* recovered from EUs at T_{30} were submitted to PFGE 19, 13 and 30 strains from batch A, B and C respectively. Strains recovered showed the same PFGE profile of the inoculated strains and belonged exclusively to the wild type: 18 (29.0%) were 1/2a, 12 (19.4%) were 1/2b, 13 (21.0%) were 1/2c and 19 (30.6%) were 4b.

Temperature monitoring

The initial temperature of ricotta salata rind before the immersion in hot water was 6.0 ±0.8 °C. Figure 2 shows the temperature profile recorded on the rind of ricotta salata during water bath heat treatments for the three temperatures.

Sensory features

The results of sensory analysis (average values ±SD) are shown in table 7. The blind control allowed estimating the *placebo* effect, produced by asking to find a difference when in fact no differences exist. Statistic inferences are estimates by comparing the samples and the blind control. All the heat treated samples are significantly different from the untreated one (blind control) with the exception of that treated at lowest temperature-

time combination (75 °C for 15 min). However the difference size goes from 1.3 to 1.8 points that converted in the verbal scale correspond to "slight/moderate difference". The heat treated samples were not different between each other, meaning that heating from 75 to 90 °C for a time ranging between 15 and 40 min do not significantly change the sensory characteristics of ricotta salata.

Ricotta salata composition

Physico-chemical characteristics were determined on untreated samples (18 BLs) and heat treated samples (162 CUs). Intrinsic properties values ($\bar{x}$±SD) for untreated samples were 6.42±0.09 for pH and 0.963±0.01 for a_w. Composition values (%±SD) were respectively of 55.35±2.09 for moisture, 21.75±2.42 for fat, 14.55±1.37 for proteins and 4.56±1.38 for salt. Differences in intrinsic properties and composition between the 9 treatment combinations at T_1 and T_{30} are reported in table 6. PH and a_w were always within limits for *L. monocytogenes* growth and no significant difference was observed between values of ricotta salata submitted to heat treatment with different temperature-time conditions (P >0.05).

Discussion

In the last decades ricotta salata has been associated with several recalls due to *L. monocytogenes* contamination and more recently even with foodborne listeriosis outbreaks. Contamination of ricotta salata with *L. monocytogenes* mainly origins from food processing environment and is localized almost exclusively on the rind (Pilo,

Marongiu, Corgiolu, Virdis, Scarano, & De Santis, 2007). Whole ricotta salata wheels are generally intended to be consumed grated including the rind. Post-process control strategies are needed in order to comply with international health authorities limits. Hot water bath treatment in vacuum packed ricotta salata has been previously evaluated, demonstrating to be an effective and economic method to inactivate surface contamination of ricotta salata cheese (Spanu, Spanu, Pala, Virdis, Scarano, & De Santis, 2013). However, optimization of the process was needed in order to account for the level of reduction in *L. monocytogenes* counts ($\log_{10}$ cfu g-^{1}), changes in sensory properties and cost of the treatment. In the present study was compared the listericidal effect of nine temperature-time combinations for the treatment of artificially contaminated whole ricotta salata wheels. The recovery of *L. monocytogenes* from not inoculated blank samples, confirms that natural contamination of ricotta salata is not a rare finding. The prevalence of contamination of ricotta salata produced in Sardinia is estimated around 20% (Ibba, Cossu, Spanu, Virdis, Spanu, Scarano, & De Santis, 2013; Spanu, Scarano, Ibba, Spanu & De Santis, 2015). This level can result, when ricotta is stored at refrigeration temperatures for up to two months, in concentration of the pathogens of approximately 10^{7} $\log_{10}$ cfu g-^{1}, potentially harmful to human health (Spanu, Scarano, Spanu, Penna, Virdis, & De Santis, 2012). The present study was aimed to validate the temperature-time combinations able to reduce *L. monocytogenes* concentration of 5 log

cfu g^{-1}, considered sufficient to comply with the food safety objective of <100 cfu g^{-1} for the products placed on the market during their shelf-life (EC, 2005).

Enumeration of *L. monocytogenes* in positive control units confirmed that the desired level of contamination of 10^5 cfu g^{-1} was obtained. Out of nine temperature-time combinations only two, 85 °C for 40 min and 90 °C for 40 min, showed to be effective in reducing *L. monocytogenes* to undetectable levels either at T$_0$ and T$_{30}$. These combinations allowed to reach respectively 56.3±1.5 °C and 57.7± 1.4 °C on ricotta surface, effective in killing *L. monocytogenes*. On one hand, little or no efficacy was observed for treatment conducted at 75 °C, regardless of the time of application, while on the other hand no efficacy was demonstrated for treatment conducted for 15 min, regardless of the temperature used. Despite an initial inactivation of up to 5.0 log at T$_0$ as consequence of treatments performed at 75 °C (i.e. 25 and 40 min), *L. monocytogenes* was still culturable with the detection method. The survival and successive growth during storage at refrigeration temperature for 30 days resulted in counts as high as ca. 7 log. Failure of heat treatment at 75°C to inactivate *L. monocytogenes* could be explained with the difference between water temperature and the maximum temperature obtained on ricotta rind (47.5 °C). Treatment conducted at 85 °C for 25 min resulted in a temperature on ricotta rind of 49.8±1.5 °C which allowed *L. monocytogenes* survival to concentrations of up to 1 log at day zero. As consequence the microorganism grew during the successive storage to level as high as 5 log. Although *L. monocytogenes* was not countable with the

enumeration method after 30 days in samples treated at 90 °C for 25 minutes, it was still detectable with the qualitative method. No guarantee can be provided that the pathogen will not growth to levels potentially dangerous to human during ricotta salata shelf-life. The highest temperature detected on ricotta salata rind after 25 min was 52.5 °C for the treatment at 90 °C. This could explain the presence of heat injured cells, which recovered after the subsequent storage at refrigeration temperature for 30 days. Strains capable to survive and growth after heat treatment belonged to the wild type, suggesting that they are characterized by a greater resistance as compared with reference strains. As far as the gap between the temperature of water during treatments and the temperature recorded on the ricotta salata rind it should be noted that temperatures were detected 1.5 cm below the ricotta surface, which may underestimate the effective temperature reached on the interface between packaged ricotta and water. The heat transfer is a function of the thermal properties of foods, which depend, among other factors, by chemical composition and temperature. However, due to the complexity of heat transfer calculations, specific experiments should be conducted in order to define the specific thermal properties of ricotta salata. Changes in sensory properties of heat treated ricotta salata were observed with respect of untreated samples, but no differences were among treatments. This indicates the feasibility of using more protective treatments with no negative implication for ricotta salata sensory profile.

Conclusion

Contamination of Ricotta salata with *L. monocytogenes* can effectively be controlled by the application of water bath heat treatment applied after packaging of the product. Treatments performed at 85 °C for 40 min or 90 °C for 40 min can be effectively used to obtain a reduction of 5 log of the pathogen. No significant difference was observed in the sensory properties between the treatments. Although treatments at 85 °C for 40 min might gather food processors favors as compared to 90 °C for 40 min, the latter may provide a greater safety of the product when is stored for periods of time longer than 30 days. Treatments applied for 40 min, either at 85 °C and 90 °C, are eligible as combination of choice to be used in a further study to assess the efficacy as post-lethality treatment aimed to extend ricotta salata shelf-life.

Figures and Tables

Figure 1. Reduction in *L. monocytogenes* counts ($\log_{10}$ cfu g^{-1}) of artificially contaminated Ricotta salata wheels (T_0) analyzed 24 h (T_1) and 30 days (T_{30}) after water bath heat treatment with 9 temperature-time combinations.
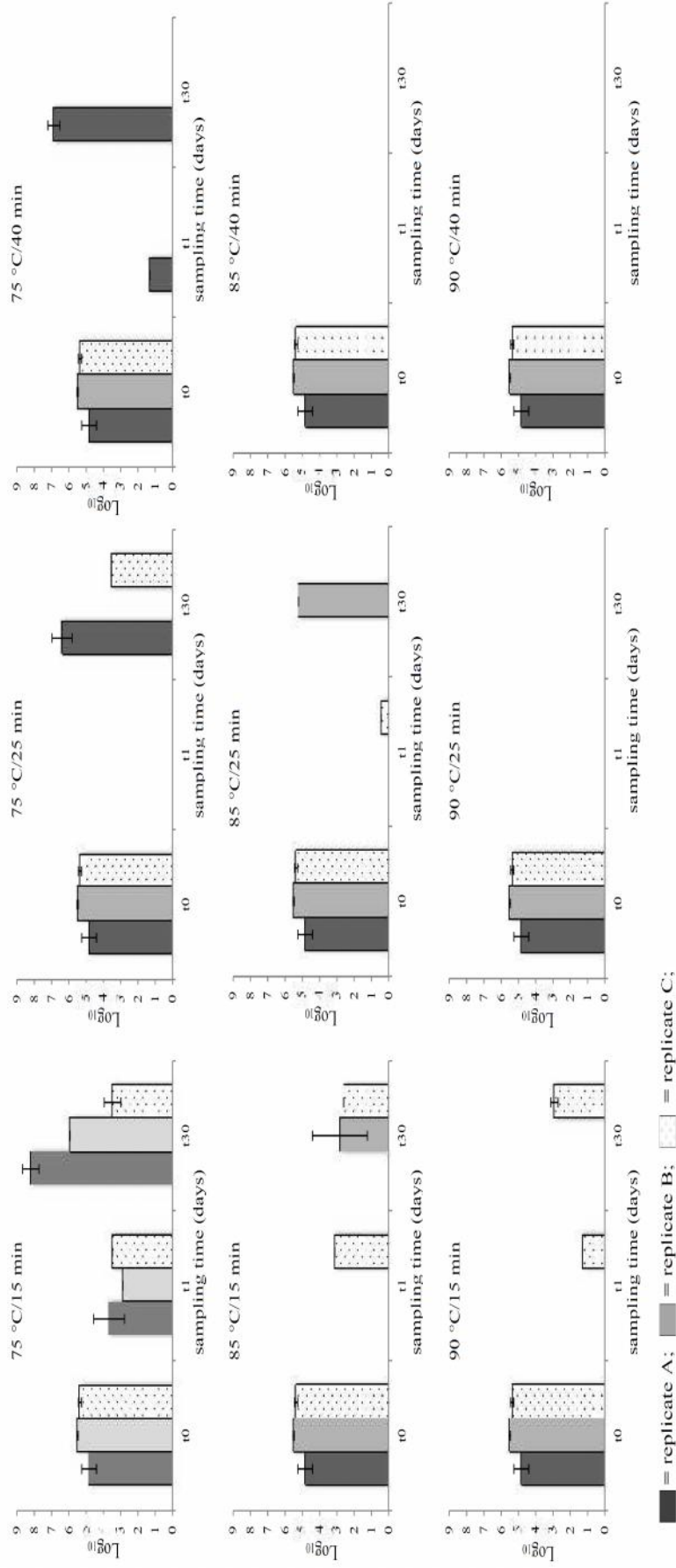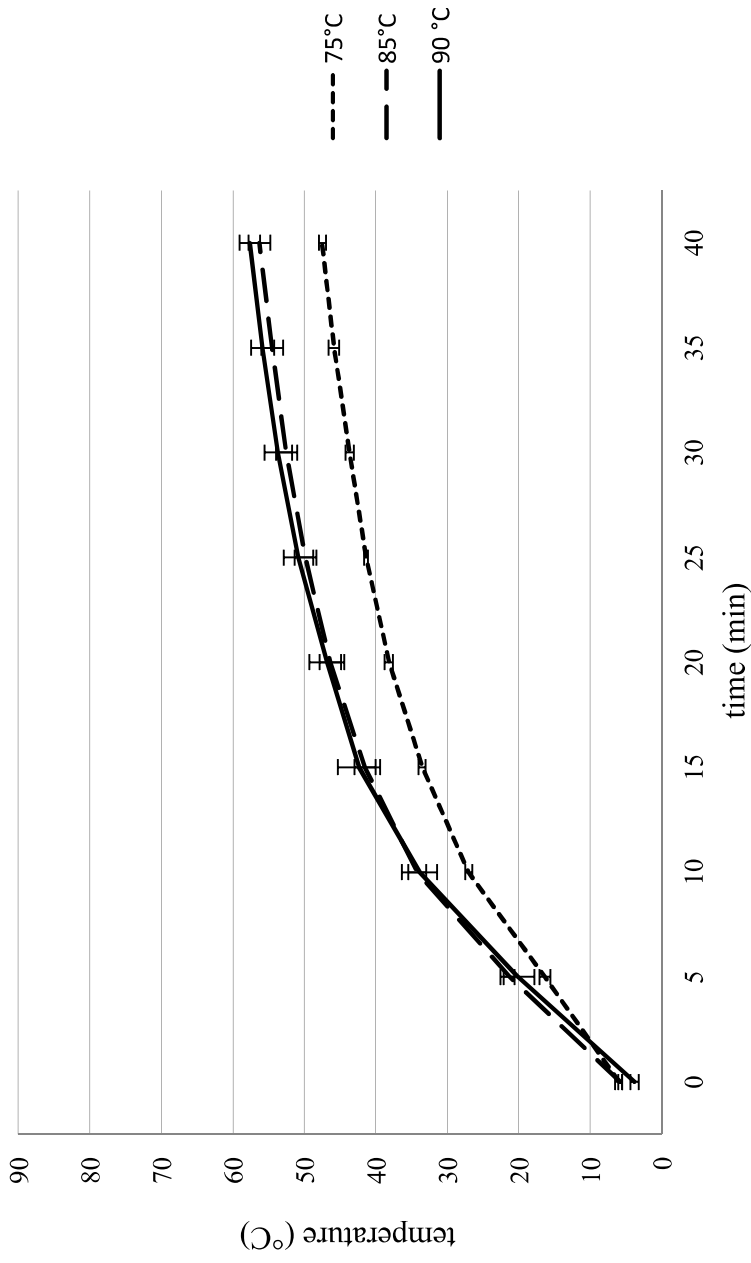
Figure 2. Temperatures recorded 1.5 cm below ricotta salata surface during water bath heat treatment

Each data point is the mean of temperatures recorder in the three replicates (batch A, B and C).

Table 1. Temperature-time combinations used for water bath heat treatment of ricotta salata

Temperature-time condition		Ricotta salata samples					
Temperature	Minutes	BL[1]	PC[2]	EUs[3]	CUs[4]	SEs[5]	Total
No treatment	-	18	9	-	-	42	69
75 °C	15	-		18	18	12	48
	25	-		18	18	3	39
	40	-		18	18	12	48
85 °C	15	-		18	18	3	39
	25	-		18	18	12	48
	40	-		18	18	3	39
90 °C	15	-		18	18	12	48
	25	-		18	18	3	39
	40	-		18	18	12	48
Total		18	9	162	162	114	465

[1]BLs (Blank Samples): not inoculated units; [2]PCs (Positive Controls): samples inoculated with *L. monocytogenes*; [3]EUs (Experimental Units): samples inoculated with *L. monocytogenes* and successively heat treated; [4]CUs (Composition Units): heat treated units for physico-chemical analysis; [5]SEs (Sensory Units): samples used to assess the effect of heat treatment on sensory properties.

Table 2. Number of ricotta salata wheels and analysis performed at each sampling time.

Analysis	Test units	T_0^a	T_1^b	T_{30}^c	Total
			Sampling time		
Detection and enumeration of *L. monocytogenes* and aerobic mesophilic bacteria	BLs[1]	18	-	-	-
	PCs[2]	9	-	-	9
	EUs[3]	-	81	81	162
	CUs[4]	-	81	81	162
Intrinsic properties and composition	BLs[1]	18	-	-	-
	CUs[4]	-	81	81	162
Sensory analysis	SEs[5]				38

Superscript letters are referred to the time between inoculation and analysis: [a] = day of inoculum and heat treatment; [b] = 24 hours after heat treatment; [c] = 30 days after heat treatment. Superscript numbers are referred to test units: [1]BLs (Blank Samples): not inoculated units; [2]PCs (Positive Controls): samples inoculated with *L. monocytogenes*; [3]EUs (Experimental Units): samples inoculated with *L. monocytogenes* and successively heat treated; [4]CUs (Composition Units): heat treated units for physico-chemical analysis; [5]SEs (Sensory Units): samples used to assess the effect of heat treatment on sensory properties.

Table 3. Comparison of aerobic mesophilic bacteria counts ($\log_{10}$ cfu g^{-1}; $\bar{x} \pm$ SD) of heat treated ricotta salata with 9 different temperature-time combinations analyzed 24 h after the treatment (T_1) and after storage at refrigeration temperature for 30 days (T_{30}).

Treatment			Aerobic mesophilic bacteria		
Temperature	Minutes	+ve/n	T_1	+ve/n	T_{30}
75 °C	15	9/9	6.94 ± 0.62^A	9/9	7.06 ± 0.80^A
	25	9/9	5.69 ± 0.55^B	9/9	6.20 ± 1.36^A
	40	9/9	5.12 ± 0.42^B	7/9	6.50 ± 1.28^A
85 °C	15	9/9	3.90 ± 0.85^C	9/9	4.88 ± 0.88^B
	25	9/9	3.71 ± 0.94^C	5/9	4.58 ± 1.14^B
	40	9/9	2.45 ± 1.97^D	4/9	4.14 ± 1.78^{BC}
90 °C	15	9/9	2.02 ± 1.22^D	6/9	4.15 ± 0.82^{BC}
	25	9/9	1.68 ± 1.29^D	3/9	2.90 ± 0.78^C
	40	9/9	2.01 ± 1.12^D	3/9	3.67 ± 1.62^{BC}

Means in the same column on the same testing time (T_1 or T_{30}) with different capital letter are significantly different ($P<0.05$).

Table 4. *Listeria monocytogenes* reduction (Δ)[a] on ricotta salata rind after water bath heat treatment

Temperature	Batch	15 min		25 min		40 min	
		ΔT_1	ΔT_{30}	ΔT_1	ΔT_{30}	ΔT_1	ΔT_{30}
75 °C	A	-0.23	4.18	-4.54	2.26	-3.24	2.57
	B	-2.59	0.48	-5.45	-5.45	-5.45	-5.45
	C	-1.78	-1.36	-5.27	-1.73	-5.27	-5.27
85 °C	A	-4.54	-4.54	-4.54	-4.54	-4.54	-4.54
	B	-5.45	-1.51	-5.45	-0.22	-5.45	-5.45
	C	-2.15	-2.67	-3.97	-5.27	-5.27	-5.27
90 °C	A	-4.54	-4.54	-4.54	-4.54	-4.54	-4.54
	B	-5.45	-5.45	-5.45	-5.45	-5.45	-5.45
	C	-3.97	-2.13	-5.27	-5.27	-5.27	-5.27

[a]Values are the difference between concentration ($\log_{10}$ cfu g^{-1}) the day of artificial inoculation (T_0) and 24 hours (ΔT_1) and 30 days (ΔT_{30}) after treatment. For each batch and for each temperature-time combination values are the minimum difference between the initial contamination level and the maximum count after the treatment in the triplicate samples.

Table 5. Enumeration and detection of *L. monocytogenes* in ricotta salata artificially contaminated and heat treated with different temperature-time combinations and relative compliance with Regulation CE limits evaluated 24 h (T_1) and 30 days (T_{30}) after the heat treatment.

Treatment			*L.monocytogenes*					
			T_1			T_{30}		
			Enumeration		Detection in 25 g	Enumeration		Detection in 25 g
Temperature	Minutes	Batch	log₁₀ cfu/g	+ve/n	+ve /n	log₁₀ cfu/g	+ve/n	+ve /n
75 °C	15	A	3.67±0.89	2/3	3/3[1]	8.20±0.48[2]	3/3	3/3
		B	2.86±0.00	1/3	1/3[1]	5.93±0.00[2]	1/3	3/3
		C	3.49±0.00	1/3	3/3[1]	3.47±0.48[2]	3/3	3/3
	25	A	0.00±0.00	0/3	1/3[1]	6.39±0.58[2]	2/3	3/3
		B	0.00±0.00	0/3	1/3[1]	0.00±0.00[2]	0/3	0/3
		C	0.00±0.00	0/3	1/3[1]	3.54±0.00[2]	1/3	2/3
	40	A	1.30±0.00	2/3	3/3[1]	6.87±0.34[2]	3/3	3/3
		B	0.00±0.00	0/3	0/3	0.00±0.00	0/3	0/3
		C	0.00±0.00	0/3	0/3	0.00±0.00	0/3	0/3
85 °C	15	A	0.00±0.00	0/3	0/3	0.00±0.00	0/3	1/3
		B	0.00±0.00	0/3	0/3	2.82±1.58[2]	2/3	2/3
		C	3.12±0.00	1/3	2/3[1]	2.60±0.00[2]	1/3	3/3
	25	A	0.00±0.00	0/3	0/3	0.00±0.00	0/3	0/3
		B	0.00±0.00	0/3	0/3	5.23±0.00[2]	1/3	1/3
		C	1.30±0.00	1/3	1/3[1]	0.00±0.00	0/3	1/3
	40	A	0.00±0.00	0/3	0/3	0.00±0.00	0/3	0/3
		B	0.00±0.00	0/3	0/3	0.00±0.00	0/3	0/3
		C	0.0±0.00	0/3	0/3	0.00±0.00	0/3	0/3
90 °C	15	A	0.00±0.00	0/3	0/3	0.00±0.00	0/3	2/3
		B	0.00±0.00	0/3	0/3	0.00±0.00	0/3	1/3
		C	1.30±0.00	1/3	3/3[1]	2.92±0.22[2]	2/3	2/3
	25	A	0.00±0.00	0/3	0/3	0.00±0.00	0/3	0/3
		B	0.00±0.00	0/3	0/3	0.00±0.00	0/3	2/3
		C	0.00±0.00	0/3	0/3	0.00±0.00	0/3	0/3
	40	A	0.00±0.00	0/3	0/3	0.00±0.00	0/3	0/3
		B	0.00±0.00	0/3	0/3	0.00±0.00	0/3	0/3
		C	0.00±0.00	0/3	0/3	0.00±0.00	0/3	0/3

Compliance are intended as follows: [1]not compliant with the Regulation CE 2073/2005 detection limits before the food has left the immediate control of the food business operator (T_1); [2]not compliant with the Regulation CE 2073/2005 enumeration limits for the products placed on the market during their shelf-life (T_{30}).

Table 6. Intrinsic properties ($\bar{x}\pm$SD) and composition (%±SD) of ricotta salata submitted to 9 different heat treatment combinations and analyzed 24 h (T_1) and 30 days (T_{30}) after storage at refrigeration temperature.

Treatment		pH		a_W		Moisture %		Fat %		Proteins %		NaCl %	
Temperature	Min	T_1	T_{30}	T_1	T_{30}	T_1	T_{30}	T_1	T_{30}	T_1	T_{30}	T_1	T_{30}
75 °C	15	6.41±0.12[A]	6.32±0.06[A]	0.959±0.01[A]	0.952±0.01[A]	54.96±1.44[A]	54.59±1.67[A]	20.97±1.88[A]	20.87±2.29[A]	15.19±3.38[AB]	14.75±1.35[AB]	5.02±0.84[A]	5.50±1.33[A]
	25	6.42±0.11[A]	6.36±0.05[A]	0.954±0.01[A]	0.951±0.01[A]	54.90±2.08[A]	54.94±1.36[A]	21.73±2.01[AB]	22.70±3.01[A]	15.56±2.63[B]	14.05±1.09[A]	5.04±0.72[A]	5.57±0.79[A]
	40	6.41±0.13[A]	6.35±0.07[A]	0.950±0.01[A]	0.654±0.01[A]	55.09±2.23[A]	53.53±1.93[A]	20.90±3.11[A]	22.71±2.32[A]	14.44±1.29[ABC]	14.56±0.93[AB]	5.18±0.30[A]	5.20±0.74[AB]
85 °C	15	6.41±0.12[A]	6.36±0.09[A]	0.952±0.01[A]	0.953±0.01[A]	54.53±2.04[A]	54.17±2.16[A]	21.31±2.26[AB]	21.65±2.52[A]	14.20±2.32[ABC]	15.01±1.54[AB]	5.26±1.12[A]	4.98±0.95[AB]
	25	6.41±0.12[A]	6.33±0.08[A]	0.956±0.01[A]	0.956±0.01[A]	55.40±2.03[A]	54.07±2.74[A]	21.23±1.73[AB]	22.29±3.13[A]	13.97±1.04[ABC]	15.37±1.12[AB]	5.24±0.84[A]	4.63±0.59[B]
	40	6.38±0.10[A]	6.33±0.11[A]	0.953±0.01[A]	0.956±0.01[A]	54.72±1.81[A]	53.91±2.12[A]	21.65±2.21[AB]	22.48±2.09[A]	14.95±1.78[ABC]	14.94±0.97[AB]	5.16±0.76[A]	5.09±0.76[AB]
90 °C	15	6.40±0.11[A]	6.33±0.09[A]	0.953±0.01[A]	0.954±0.01[A]	54.30±1.64[A]	53.61±1.62[A]	23.07±0.88[B]	22.75±2.15[A]	13.33±0.56[C]	14.48±1.50[AB]	5.36±0.66[A]	5.18±0.44[AB]
	25	6.38±0.12[A]	6.35±0.15[A]	0.955±0.01[A]	0.954±0.01[A]	54.78±1.66[A]	54.24±2.24[A]	21.74±2.01[AB]	22.52±2.36[A]	14.38±2.00[ABC]	14.33±0.82[AB]	5.12±0.90[A]	5.32±0.79[AB]
	40	6.35±0.11[A]	6.35±0.12[A]	0.954±0.01[A]	0.955±0.01[A]	54.58±1.37[A]	54.22±2.02[A]	23.03±1.08[B]	21.80±2.26[A]	13.70±0.54[AC]	15.50±2.66[B]	5.19±0.87[A]	5.03±0.93[AB]

Each data point is the mean of three samples. For each parameter means in the same column on the same testing time (T_1 or T_{30}) with different capital letter are significantly different (*P*<0.05).

Table 7. Mean values and standard deviations of sensory differences among the control (SEc) and the samples heat treated (SEt).

Temperature-time condition	Differences
Blind samples	$2.3^{a}* \pm 1.5$
75 °C x 15 min	$3.4^{ab} \pm 2.3$
75 °C x 40 min	$3.8^{b} \pm 2.0$
85 °C x 25 min	$4.1^{b} \pm 2.4$
90 °C x 15 min	$3.6^{b} \pm 2.3$
90 °C x 40 min	$3.9^{b} \pm 2.5$

Mean values with different superscript letters are significantly different among samples.* ($P \leq 0.05$).

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CHAPTER 5

Inactivation of *Listeria monocytogenes* using Water Bath Heat Treatment in Vacuum Packed Ricotta Salata Cheese Wedges

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The contribution of the doctoral candidate to the paper presented in this chapter concerned several aspects of the experiment, from samples collection, preparation of inoculum, experimental inoculation, microbiological analysis and data statistical interpretation. The contribution of the candidate was also in the writing and editing of the publication.

Inactivation of *Listeria monocytogenes* using Water Bath Heat Treatment in Vacuum Packed Ricotta Salata Cheese Wedges

Abstract

Ricotta salata cheese is frequently contaminated on the surface with *L. monocytogenes.* Water bath heat treatment in vacuum packed whole ricotta salata cheese wheels demonstrated to be effective in inactivating *L. monocytogenes*. However the risk of cross contamination in ricotta salata wedges is increased during cheese cutting. Therefore, the effectiveness of heat treatment in ricotta salata wedges has to be demonstrated conducting a new validation study. In the present study nine different time temperature combinations, 75°C, 85°C and 90°C applied for 10 min, 20 min and 30 min each, were tested on artificially contaminated ricotta salata cheese wedges. The extent of the lethal effect on *L. monocytogenes* was assessed one day and 30 days after the application of the hot water bath treatment. Five out of nine combinations, 75°C for 30 min, 85°C for 20 and 30 min and 90°C for 20 and 30 min, demonstrated to meet the process criteria of at least 5 log reduction. Sensory analyses were also conducted in order to account for the potential impact on sensory features of ricotta salata wedges which showed no significant differences between treatments.

Practical Application

The present study allowed to select water bath heat treatments of vacuum packed ricotta salata wedges effective to reduce *L. monocytogenes* contamination. Such treatments can

be successfully applied by food business operator to meet compliance with microbiological criteria through the designated shelf-life.

Introduction

Ricotta salata is a whey protein cheese largely produced in Sardinia (Italy) using the whey remaining after the production of sheep's milk cheeses, mainly Pecorino Romano PDO (protected denomination of origin). After filtration and pre-heating at temperature of 60-70°C, the whey is heated in open kettles under gentle agitation for about 30 min to temperature of 85-90°C. As consequence of whey protein coagulation, the curd starts floating on the surface and then is scooped into perforated plastic hoops lined with cheesecloth. In order to increase drainage, the curd is pressed for approximately 24 hours. Ricotta is salted by dry salting or by brine-salting and dried in storage rooms under controlled temperature (10-12°C) and humidity for 10-15 days. The final moisture of the product depends on its intended use, usually 53-55% if for grating and between 55-60% if is to be consumed as such. Traditionally ricotta salata is a cylindrical shaped wheel (ricotta *Toscanella*) with an average weight of approximately 3 kg, pH of 6.1-6.9, a_W of 0.940-0.970, fat of 28-33%, protein of 14-23% and NaCl of 3-4% (Spanu and others 2012; Spanu and others 2013; Spanu and others 2015a). Ricotta salata wheels are individually packed in vacuum bags as a whole or after cutting into smaller wedges of ca. 200-300 g. Wedges are shaped as triangular prisms with height, width and length of ca. 3.5 cm, 8.5 cm and 12 cm, respectively. The shelf-life of the product differs from one

business operator to another, but it usually ranges between few weeks up to several months at refrigeration temperature. Despite the application of good hygiene practices together with the application of procedures based on the HACCP principles during manufacturing, recalls of ricotta salata produced in Sardinia due to *Listeria monocytogenes* contamination have been reported in recent years (RASFF 2008; CDC 2012). Raw milk and whey can be excluded as the origin of the contamination in consideration of heat treatments usually applied during cheese making (i.e. thermization and pasteurization) and whey protein coagulation (Buazzi and others 1992; Casadei and others 1998; ICMSF 1996; Villani and others 1996). *L. monocytogenes* contamination in ricotta salata is generally limited to the product surfaces and origins from the processing environment. Previous studies reported a prevalence of *L. monocytogenes* in ricotta salata rind of ca. 20% (Pintado and Malcata, 2000; Lioliou and others 2001; Ibba and others 2013; Spanu and others 2015b). Contamination of ricotta salata is rarely observed in the inner paste and is generally a consequence of flaws in the application of production hygiene procedures (Spanu and others 2015a). Cutting ricotta salata wheels into smaller portions and repacking for retail sale can expose the product to cross-contamination originating from utensils used to cut the cheese, thus increasing the risk of *L. monocytogenes* contamination. Previous investigation demonstrated a *L. monocytogenes* growth potential in contaminated ricotta salata wheels of ca. 5 $\log_{10}$ cfu g^{-1} on the rind during the first two months of storage at refrigeration temperature (Spanu and others

2012). This represents a serious concern in such product, since ricotta salata does not have an actual rind and it is generally assumed to be whole edible. There is evidence that food business operator cannot demonstrate compliance with microbiological food safety criteria of 10^2 cfu g^{-1} at the time of consumption set by European Commission (EC) Regulation No. 2073/2005 throughout the shelf-life. This issue is even more stringent considering that ricotta salata is largely exported in North America where the limit recommended by FDA is more restrictive, requiring the absence in 25 g.

Post-process decontamination procedures are widely used in ready to eat food industry to reduce the risk of *L. monocytogenes* contamination in packaged products (Zhu and others 2005). These include methods such as thermal pasteurization, irradiation and high-pressure referred to as post-lethality treatments since they are applied to the final product or sealed package of product in order to reduce or eliminate the level of pathogens resulting from contamination from post-lethality exposure (FSIS 2014; Spanu and others 2014). To describe the overall effect of a control measure on a specific hazard at a step it is used the term *performance criterion* (PC), which is defined as: the effect in frequency and/or concentration of a hazard in a food that must be achieved by the application of one or more control measures to provide or contributes to an food safety objectives or appropriate level of protection, as applicable (CAC, 2004).

Hot water bath treatments applied in whole ricotta salata cheese wheels have been previously proven to be effective in reducing *L. monocytogenes* contamination on the rind

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up to 5-6 $\log_{10}$ cfu g^{-1} (Spanu and others 2015a). However, a thermal treatment should take into account several factors such as temperature-time combination, microbiological profile, composition and product size and weight (Doyle and others 2001; Ray 2004; Sofos 2002; Yen and others 1991).

Effectiveness of the immersion of portioned vacuum packed ricotta salata in hot water bath needs to be validated with an ad hoc study.

The aim of the present study was to evaluate the efficacy of hot water bath treatment on the reduction of *L. monocytogenes* in artificially contaminated ricotta salata wedges. Nine different temperature-time combinations were compared in order to identify the process criteria required to deliver a 5 log reduction of *L. monocytogenes* concentration. The impact of post-lethality treatments on sensory properties of ricotta salata has been also evaluated. Inactivation of *L. monocytogenes* was assessed 24 hours and 30 days after the treatment. The suitable treatment combination will be selected to conduct a successive shelf -life study to assess compliance of treated ricotta salata wedges for the designated durability.

Materials and methods

Ricotta salata samples

A local cheese-making plant supplied 465 vacuum packed ricotta salata wedges, obtained from the whey remaining after the production of sheep milk cheeses. Ricotta salata samples belonged to 3 different production batches (155 from each batch). Ricotta salata

wedges were transported and stored at refrigeration temperature (4±2°C) until use for the experiment within 24 h. Part of ricotta salata wedges (inoculated units) were artificially contaminated with *L. monocytogenes* while another part (uninoculated units) was not contaminated. Inoculated units included ricotta salata samples submitted to heat treatment, or experimental units (EUs), used to assess the efficacy of the treatment and positive controls (PCs), used to check for the inoculum level. Uninoculated samples included blank samples (BLs), used to evaluate the level of natural contamination of ricotta salata with *L. monocytogenes*; sensory units (SEs) used to evaluate sensory properties after heat treatment; composition units (CUs) samples used for the determination of intrinsic properties (pH and a_W) and composition (moisture, fat and proteins). Detailed description of the number of ricotta salata wedges included in each of the sample types is showed in table 1.

Artificial contamination

The artificial contamination of EUs and PCs ricotta salata wedges was conducted according to the Technical Guidance document prepared by the EU Community Reference Laboratory (CRL) for *L. monocytogenes* (Beaufort and others 2014). In the preparation of the inoculum was used a mixture of 5 *L. monocytogenes* strains composed by the reference strain ATTC 19111 (serovar 1/2a) obtained from American Type Culture Collection (Manassas, VA, USA) and by other four wild-type strains (respectively serotypes 1/2a, 1/2b, 1/2c and 4b), previously recovered from the

cheese-making plant environments or from ricotta salata. Selection of strains and preparation of the inoculum have been previously described (Spanu and others 2015a). The target level of contamination was 5 $\log_{10}$ cfu g^{-1} of rind, which was obtained spraying ricotta salata wedges with 2 mL of inoculum at a concentration of 10^7 cfu mL^{-1}. The concentration of the inoculum was confirmed by plate count on Trypticase Soy Agar (TSA, Biolife, Milan, Italy). The whole surface of ricotta salata wedges was evenly sprayed using an atomizer. Before ricotta salata samples were individually vacuum packed in shrink bags (Criovac Cook-In HT-3000, Sealedair Ltd., St Neots, UK) a holding period of 15 min at room temperature was allowed to let the inoculum attach (Health Canada, 2012). Vacuum packed ricotta salata wedges were then stored in cold room at 4±2°C until further analyses. The inoculum was performed under a microbiological safety cabinet and the operator worn protection devices.

Heat treatment and testing times

Independent trials were conducted for each of the three production batches. The post-lethality treatment was performed by immersion of vacuum packed ricotta salata wedges in a stainless steel vat containing hot water. Nine different temperature-time conditions were tested: 75°C, 85°C and 90°C applied for 10 min, 20 min and 30 min each. Ricotta salata samples used for each treatment

condition are reported in table 1. Once treated, in order to firm up ricotta salata wedges, the samples were immersed for approximately 2 hours in iced water and then stored at

$4\pm2°C$ until analysis. The testing times (T) were: T_0 which was the day of the artificial contamination and heat treatment; T_1 and T_{30} which were respectively 24 hours and 30 days after heat treatment. At T_0 were analyzed PCs to assess if the level of contamination was effectively $10^5 \log_{10}$ cfu g^{-1} and BLs to check for eventual natural contamination of ricotta salata with *L. monocytogenes*. The EUs and CUs were submitted to heat treatment and analysed at T_1 and T_{30}. The SE units were either submitted to heat treatment (SEt) or used as negative controls (SEc). Types of ricotta salata samples, analysis performed and sampling times are summarized in Table 2. Temperatures obtained during treatments were monitored using datalogger (KT 20T, Kimo, Montpon Ménestérol, France) positioned on the surface of an additional ricotta salata sample. Loggers were button shaped stainless steel recorder with a diameter of ca. 17 mm and height < 6 mm which were inserted with their lower side 0.5 cm deep in the paste and their upper side in the interface between ricotta rind and packaging bag.

Microbiological analysis

Ricotta salata rind was aseptically collected cutting the wedges surfaces up to 2 cm in depth. Detection and enumeration of *L. monocytogenes* (ISO 11290-1:1996/Amd 1:2004; ISO 11290-2:1998/Amd 1:2004) and enumeration of aerobic mesophilic bacteria (ISO 4833:2003) were conducted in accordance with standard methods. The potential presence of sublethally injured cells that may survive the heat treatment but may not be cultured on selective media was investigated using the Thin Agar Layer (TAL) method (Kang &

Fung, 1999; Wu & Fung, 2001). This method combines the ability of nonselective agar media to growth injured cells and of selective media to differentiate microorganism. Preliminary identification of *L. monocytogenes* was confirmed by picking 5 suspected colonies from each positive sample which were submitted to phenotypic and molecular identification (Doumith and others 2004; De Santis and others 2007). To confirm that strains recovered at T_{30} were the same as the ones used in the inoculum, a selection (up to 5 strains from each temperature time combination) was submitted to pulsed-field gel electrophoresis (PFGE), according to the protocol proposed by Graves and Swaminathan (2001). The PCR serogroups were used as preliminary screening criteria of isolates to be submitted to PFGE. Comparison of restriction profiles allowed to differentiate between inoculated strains and strains originating by natural contamination.

Centesimal composition and intrinsic factors

Ricotta salata centesimal composition (fat, moisture, protein, total solids and sodium chloride) and intrinsic factors (pH and a_W) were determined to account for possible interaction with *L. monocytogenes* survival and growth. Compositional data were obtained by near infrared transmittance (NIT) using a compositional analyzer (FOSS, Eden Prairie, MN, USA), while pH and a_w were measured using pH meter GLP22 (Crison Instruments SA, Barcelona, Spain) and water activity meter Aqualab 4TE (Decagon, Pullman, WA, USA), respectively. Comparison of centesimal composition and intrinsic factors ($\bar{x} \pm$SD) of ricotta salata wedges submitted to heat treatment with the nine different

temperature-time combinations and over time (T_1 and T_{30}) was conducted using the Fisher's least significant difference (LSD) test, performed with Statgraphics Centurion XVI software (StatPoint Technologies, Warrenton, VA, USA).

Sensory analysis

The "Difference from control test" allowed to determine if a significant difference between the treated (SEt) samples and the negative control (SEc) exist and to estimate the size of this difference (Meilgaard and others 1999). In order to quantify the experimental noise (*placebo* effect), the control sample (SEc) must be served during each session both as reference and as blind sample. This cause a sensory fatigue especially if all of nine time-temperature combinations samples have to be taken into account, for this reason the sensory analysis was restricted to only five heat treatments corresponding to the lowest and highest time-temperature combinations (75°C and 90°C, 10 min and 30 min) and the central point (85°C treated for 20 min). All of these heat treatments were performed and evaluated in triplicate. Thirty assessors equally distributed between females and males, aged from 25 to 50 years were selected after attending a course of 60 hours in sensory analysis (ISO 8586-1: 1993) applied to dairy products. Assessors were asked to determine the difference between the control sample (SEc) and the blind coded test sample (SEt) on a numerical ten points scale (0 = no difference and 9 = very large difference). The Ricotta salata wedges were portioned into parallelepiped pieces (5 x 1.5 x 1.5 cm) and served in a randomized and balanced order (Macfie and others 1989), in odorless plastic containers

marked with a random three-digit number (Meilgaard and others 1999) at room temperature. Assessors were also provided with an unsalted cracker and a glass of water in order to rinse their mouth after each evaluation. Raw data (n=540) were analyzed by the two-way ANOVA ($P \leq$ 0.05) using the Statgraphics Centurion XVI software (StatPoint Technologies, Warrenton, VA, USA).

Validation of heat treatment

The effectiveness of each of the nine heat treatment combinations was evaluated computing the minimum difference between *L. monocytogenes* concentration ($\log_{10}$ cfu g^{-1}) before the treatment (T_0) and after the treatment (T_1 and T_{30}) and expressed as performance criterion or performance standard (Δ.) The Δ was computed independently for each of the three production batches and for each value was obtained from triplicate samples.

Statistical analysis

Comparison of Mean mesophilic bacteria counts ($\log_{10}$ cfu g^{-1}), intrinsic properties ($\bar{x}$ $\pm$SD) and centesimal composition (%$\pm$SD) between temperature-time combinations at T_1 and T_{30} was conducted using Fisher's least significant difference (LSD) test. All statistical analyses were performed with Statgraphics Centurion XVI software (StatPoint Technologies, Warrenton, VA, USA).

Results

L. monocytogenes contamination and background microflora

In all BLs natural contamination with *L. monocytogenes* was never observed. In artificially contaminated ricotta salata wedges (PCs) *L. monocytogenes* mean count expressed as $\log_{10}$ cfu g^{-1} ($\bar{x}\pm SD$) was 5.24±0.11, 5.4±0.05 and 5.03±0.4 in the first, second and third batch, respectively. Aerobic mesophilic bacteria enumeration ($\bar{x}\pm SD$ $\log_{10}$ cfu g^{-1}) in BLs was 4.86±0.31, 6.44±0.46, 6.53± 0.41 while in PCs was 5.67±0.19, 7.12±0.26 and 6.35±0.62 in the first, second and third batch respectively. At T_0 the reduction in mean $\log_{10}$ cfu g^{-1} total bacterial counts changed according to temperature, ranging between ca.1.5 and 3.0 for ricotta treated at 75°C and up to 5.0 for treatment at 85°C and 90°C, while bacterial counts at T_{30} was a function of the time of heat treatment, with an average increase of 2.5-3.0 observed only in ricotta samples treated for 10 min. Pair-wise comparison of aerobic mesophilic bacteria counts between temperature-time combinations at T_1 and T_{30} is reported in table 3.

Post lethality treatment

The fate of *L. monocytogenes* (inactivation, survival or growth) was assessed for each temperature-time combination at T_1 and T_{30}. Despite treatment applied for 10 min showed a certain degree of reduction of *L. monocytogenes* concentration at T_0 (between ca. 1 log at 75°C and 5 log at 90°C), in the successive 30 days of refrigerated storage *L. monocytogenes* concentration increased to levels as high as the initial contamination or

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even higher. After the water bath treatment applied for 30 min with all three different temperatures, *L. monocytogenes* was not recovered with the detection method, suggesting a full efficacy of treatments (figure 1). The treatments for 20 min were not effective only for the lowest temperature tested. The performance standard or Δ of each of the nine treatment combinations is reported in table 4.

L. monocytogenes strains characterization

Overall, 217 strains were confirmed as *L. monocytogenes* by molecular identification, 45 from PCs and 172 from EUs, respectively. Of the strains recovered from PCs, 11 belonged to serogroup 1/2a, 12 to serogroup 1/2b, 3 to serogroup 1/2c and 19 to serogroup 4b. Of the strains recovered from EUs 68 (39.5%) were serogroup 1/2a, 48 (27.9%) serogroup 1/2b, 32 (18.6%) serogroup 1/2c and 24 (13.9%) serogroup 4b. Comparison of PFGE profiles of the 48 *L. monocytogenes* strains selected from EUs at T_{30} showed identical profiles with strains used to contaminate the ricotta salata wedges. None of the strains showed similar profile with the reference strain ATCC 19111.

Temperature monitoring

The temperature recorded on ricotta salata rind before hot water bath treatment was 3.7±0.5°C. The graph in figure 2 shows the evolution of the temperature on ricotta salata rind over time for each of the three water temperature used during treatments.

Ricotta salata composition

Intrinsic properties values ($\bar{x}\pm$SD) determined on the 18 BLs were 6.40$\pm$0.16 for pH and 0.977$\pm$0.01 for a_w, while composition values (%$\pm$SD) were respectively of 53.92$\pm$3.29 for moisture, 21.50$\pm$3.75 for fat, 16.50$\pm$1.52 for proteins and 3.42$\pm$0.28 for NaCl. Composition analysis was also conducted on 162 treated samples (CUs) and differences in intrinsic properties and composition between the 9 treatment combinations at T_1 and T_{30} are reported in table 5. Ricotta salata wedges pH and a_w showed no significant difference with respect to the heat treatment ($P>0.05$) and were always above the lower limits for *L. monocytogenes* growth.

Sensory features

Difference from the control test performed by ANOVA showed no significant difference ($P=0.45$) (treatment effect) between the control sample (SEc) and the test sample (SEt) (table 6). Mean and standard deviation of the control, provided as blind samples (blind control), are statistically comparable with the means and the standards deviation of the heat treated samples, indicating that the heat treatment do not produce an effect greater than the experimental noise. Since all of the five heat treated samples, the lowest and highest time-temperature combinations (75°C and 90°C, 10 min and 30 min) and the central point (85°C treated for 20 min), are not significant different, it is reasonable to extend the same result on the four time-temperature combinations included in this field and not evaluated by the sensory analysis: (85°C for 10 min and 30 min, 75°C and 90°C

for 20 min). Moreover the ANOVA shows that also the factor "replicate" is not significant (P=0.84) (table 6) validating both the heat treatments and the sensory analysis.

Discussion

Contamination of ricotta salata with *L. monocytogenes* is mainly a post process contamination originating from the processing environment and is generally limited to the rind (Pilo and others 2007). This represents a serious concern since ricotta salata wedges are consumed as such and the rind is edible. Ricotta salata production includes pressing of the curd which could favour deepening of the microorganism in the first centimeters of the paste. In addition the risk of cross contamination in ricotta salata wedges is increased by transfer of the microorganism through blades during mechanical cutting. Despite the contamination of ricotta salata with *L. monocytogenes* is not a rare finding, the observed prevalence could range from 0.0% to as high as 20.0-30% (Spanu and others 2015b), in the present study was never detected from blank samples. The processing environment represents the main contamination route. Therefore, the reason for this wide variability of contamination between premises is largely due to difference in the implementation of good hygienic and good manufacturing practices by the processor. A cheese making plant of proven history in implementing and maintaining hygienic procedures provided ricotta salata samples used in the present study. In addition, in order to avoid as much as possible the occurrence of natural contamination, the production of ricotta salata batches intended to be used in the present study where

monitored, to guarantee that all the necessary measure where put in place to limit contamination of the product. However, in many circumstances the prevention of environmental contamination is almost impossible to avoid by the mere use of hygienic measures. Therefore, it is essential for food business operator to implement strategies to control the contamination in order to comply with microbiological limits for *L. monocytogenes* in RTE foods. A number of post-lethality treatments have been proposed, among which hot water bath of vacuum packed ricotta salata demonstrated to be effective (Spanu and others 2013; Spanu and others 2015a). The present study evaluated the listericidal effect of nine different water bath temperature-time combinations in vacuum packed ricotta salata wedges. The performance criterion of the treatment was to reduce *L. monocytogenes* concentration of 5 log cfu g^{-1} from ricotta salata surface. In order to account for the potential presence of sublethally injured cells, which during this period may recover their ability to growth, the fate of *L. monocytogenes* was evaluated also after 30 days of refrigerated storage. Efficacy of treatments was conditioned by exposition time rather than temperature. None of the treatments applied for 10 min resulted effective, while all temperatures inactivated *L. monocytogenes* when the treatment was prolonged up to 30 minutes. Intermediate situation was observed for the 20 minutes treatments which were effective at 85°C and 90°C. Although *L. monocytogenes* was inactivated to some extent (ca. 4 log) by treatments at 85°C and 90°C for 10 minutes, the cells surviving to the heath treatment increased in the subsequent storage to levels as high as 5 log.

Temperatures registered by dataloggers on ricotta salata surface explain the effect on *L. monocytogenes* inactivation. Treatments conducted at 75°C only when applied for 30 min allowed to obtain temperature on the product > 70°C which were maintained for 10 min, while after the 10 min and 20 min treatments the maximum temperature registered were respectively 65.5°C and 70°C. Although the treatment at 85°C for 10 min reached 72.6°C, it stayed above 70°C on ricotta surface only for 2 min, justifying the survival of *L. monocytogenes*. Treatments at 85°C for 20 min and 30 min guaranteed on ricotta surface temperature above 70°C for 12 min and 23 min, respectively. Similar behavior was observed for treatment at 90°C for 10 min which reached 76.3°C on the product surface but temperature above 70°C were maintained only for 4 min. The 90°C for 20 min and 30 min hesitated in maximum temperature of 84.6°C and 88.1°C on ricotta rind which were above 70°C for 14 min and 24 min respectively. Strains capable to survive and growth after heat treatment belonged to the wild type, suggesting that they are characterized by a greater resistance or a better adaptation to the substrate as compared with reference strains. However it should be considered that the actual temperature recorded by dataloggers is the balance between hot water bath temperature at the interface product-packaging and cold product temperature on the lower face of the datalogger. Despite differences in treatment efficacy, the sensory analysis reveals that the heat treated samples (SEt) are not significant different from the untreated control samples (SEc),

indicating that any heat treatments between 75°C for 10 min and 90°C for 30 min does not affect the sensory properties of ricotta salata wedges.

Conclusion

Superficial contamination of ricotta salata rind with *L. monocytogenes* is difficult to avoid even with strict production hygiene procedures. Compliance with microbiological limits could be obtained with the application of control strategies aimed to reduce the contaminations originating in the post-processing environment. Post-lethality treatments such as hot water bath applied on packaged ricotta salata are effective and feasible treatment to kill *L. monocytogenes* from ricotta salata surface. Eligible treatments in ricotta salata wedges are 75°C for 30 min, 85°C for 20 and 30 min and 90°C for 20 and 30 min. These treatments should not be intended as an alternative measure to good manufacturing and good hygiene practices but rather an additional tool to obtain compliance with microbiological criteria.

Figures and Tables

Figure 1. Inactivation of L. monocytogenes ($\log_{10}$ cfu g-1) in artificially contaminated ricotta salata wedges analyzed 24 h (T1) and 30 days (T30) after water bath heat treatment with 9 temperature time combinations.
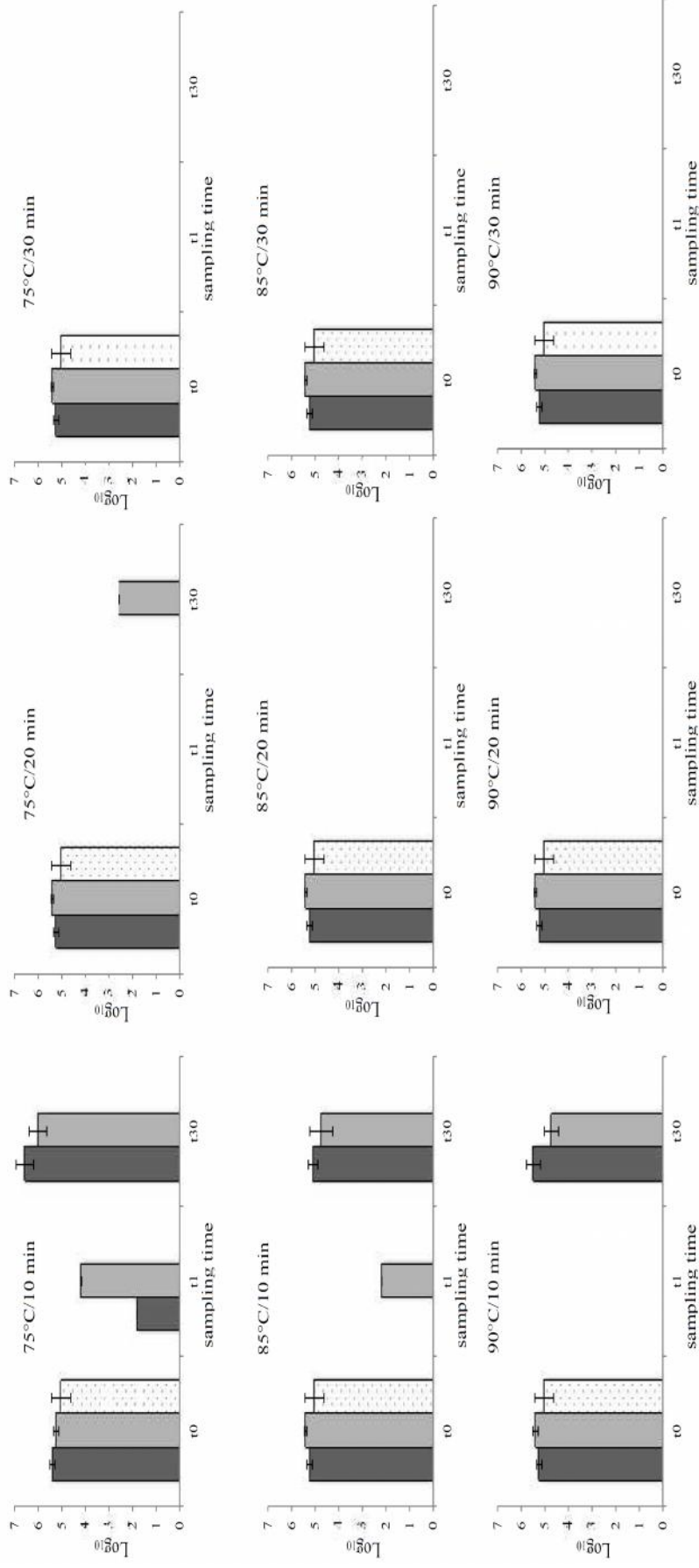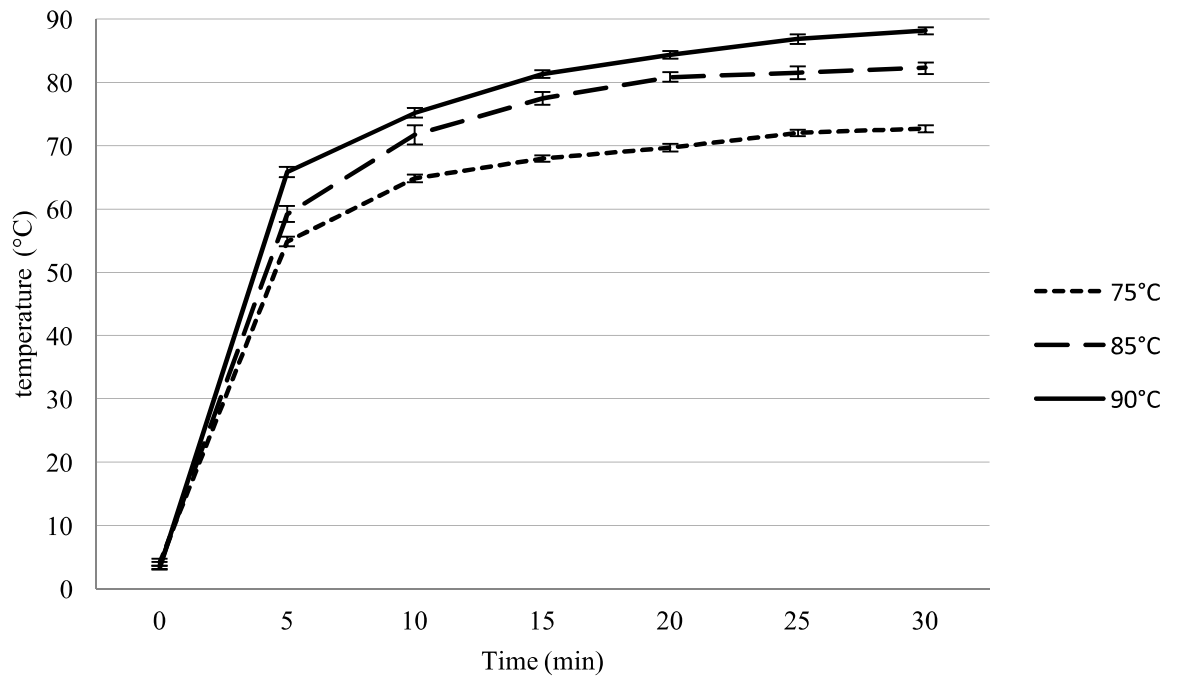
Figure 2. Temperatures recorded during water bath heat treatment on the interface ricotta salata rind packaging bag surface.

Each data point is the mean of temperatures recorder in the three replicates (batch A, B and C).

Table 1. Types of treatment and ricotta salata wedges samples used in the hot water bath validation study.

Temperature-time condition		Ricotta salata samples					
Temperature	Minutes	BL[1]	PC[2]	EUs[3]	CUs[4]	SEs[5]	Total
No treatment	-	18	9	-	-	42	69
75°C	10	-		18	18	12	48
	20	-		18	18	3	39
	30	-		18	18	12	48
85°C	10	-		18	18	3	39
	20	-		18	18	12	48
	30	-		18	18	3	39
90°C	10	-		18	18	12	48
	20	-		18	18	3	39
	30	-		18	18	12	48
Total		18	9	162	162	114	465

[1]BLs (Blank Samples): not inoculated units; [2]PCs (Positive Controls): samples inoculated with *L. monocytogenes*; [3]EUs (Experimental Units): samples inoculated with *L. monocytogenes* and successively heat treated; [4]CUs (Composition Units): heat treated units for physico-chemical analysis; [5]SEs (Sensory Units): samples used to assess the effect of heat treatment on sensory properties.

Table 2. Types of ricotta salata samples, analysis and testing times.

Analysis	Test units	Sampling time T_0^a	T_1^b	T_{30}^c	Total
Detection and enumeration of *L. monocytogenes* and aerobic mesophilic bacteria	BLs[1]	18	-	-	18
	PCs[2]	9	-	-	9
	EUs[3]	-	81	81	162
	CUs[4]	-	81	81	162
Intrinsic properties and composition	BLs[1]	18	-	-	-
	CUs[4]	-	81	81	162
Sensory analysis	SEs[5]				38

Superscript letters are referred to the time between inoculation and analysis: [a] = day of inoculum and heat treatment; [b] = 24 hours after heat treatment; [c] = 30 days after heat treatment. Superscript numbers are referred to test units: [1]BLs (Blank Samples): not inoculated units; [2]PCs (Positive Controls): samples inoculated with *L. monocytogenes*; [3]EUs (Experimental Units): samples inoculated with *L. monocytogenes* and successively heat treated; [4]CUs (Composition Units): heat treated units for physico-chemical analysis; [5]SEs (Sensory Units): samples used to assess the effect of heat treatment on sensory properties.

Table 3. Comparison of aerobic mesophilic bacteria counts ($\log_{10}$ cfu g^{-1}; $\bar{x} \pm$ SD) of heat treated ricotta salata wedges with 9 different temperature-time combinations analyzed 24 h after the treatment (T_1) and after storage at refrigeration temperature for 30 days (T_{30}).

Treatment			Aerobic mesophilic bacteria		
Temperature	Minutes	+ve/n	T_1	+ve/n	T_{30}
75°C	10	8/9	3.89 ± 0.90^A	9/9	6.64 ± 0.27^A
	20	5/9	3.14 ± 1.08^A	7/9	3.01 ± 0.38^C
	30	2/9	3.41 ± 0.58^{AB}	1/9	1.48 ± 0.0^C
85°C	10	6/9	3.04 ± 1.14^{ABC}	9/9	5.51 ± 1.18^B
	20	3/9	1.10 ± 0.17^D	5/9	1.91 ± 0.75^C
	30	2/9	1.15 ± 0.21^D	2/9	1.67 ± 0.95^C
90°C	10	7/9	1.98 ± 0.93^{BCD}	8/9	5.08 ± 1.55^C
	20	1/9	1.00 ± 0.0^{CD}	1/9	1.48 ± 0.0^C
	30	1/9	1.00 ± 0.0^{CD}	1/9	1.00 ± 0.0^C

Means in the same column on the same testing time (T_1 or T_{30}) with different superscript letter are significantly different ($P \leq 0.05$). +ve/n are the number of samples where aerobic mesophilic bacteria could be enumerated.

Table 4. Enumeration, detection and reduction (Δ)a of *Listeria monocytogenes* on the surface of ricotta salata wedges artificially contaminated and heat treated in hot water bath with different temperature-time combinations.

Treatment			*L.monocytogenes*							
			T$_1$				T$_{30}$			
			Enumeration		Δ	Detection in 25 g	Enumeration		Δ	Detection in 25 g
Temperature	Minutes	Batch	+ve/n	log$_{10}$ cfu/g	T$_1$-T$_0$	+ve/n	+ve/n	log$_{10}$ cfu/g	T$_{30}$-T$_0$	+ve/n
75 °C	10	A	1/3	4.16±0.00	-0.99	3/3	3/3	6.01±0.37	1.28	3/3
		B	2/3	1.82±1.16	-2.73	3/3	3/3	6.56±0.47	1.73	3/3
		C	0/3	ND	-4.76	3/3	3/3	6.01±0.38	1.63	3/3
	20	A	0/3	ND	-5.15	0/3	3/3	ND	-5.15	0/3
		B	0/3	ND	-5.36	0/3	1/3	2.57±0.00	2.79	1/3
		C	0/3	ND	-4.76	0/3	0/3	ND	-4.76	0/3
	30	A	0/3	ND	-5.15	0/3	0/3	ND	-5.15	0/3
		B	0/3	ND	-5.36	0/3	0/3	ND	-5.36	0/3
		C	0/3	ND	-4.76	0/3	0/3	ND	-4.76	0/3
85 °C	10	A	0/3	ND	-5.15	2/3	2/3	5.09±0.20	0.08	2/3
		B	1/3	2.18±0.00	-3.18	3/3	3/3	4.74±0.49	-0.29	3/3
		C	0/3	ND	-4.76	0/3	0/3	ND	-0.25	0/3
	20	A	0/3	ND	-5.15	0/3	0/3	ND	-5.15	0/3
		B	0/3	ND	-5.36	0/3	0/3	ND	-5.36	0/3
		C	0/3	ND	-4.76	0/3	0/3	ND	-4.76	0/3
	30	A	0/3	ND	-5.15	0/3	0/3	ND	-5.15	0/3
		B	0/3	ND	-5.36	0/3	0/3	ND	-5.36	0/3
		C	0/3	ND	-4.76	0/3	0/3	ND	-4.76	0/3
90 °C	10	A	0/3	ND	-5.15	3/3	3/3	5.48±0.30	0.67	3/3
		B	0/3	ND	-5.36	1/3	2/3	4.72±1.83	0.65	2/3
		C	0/3	ND	-4.76	2/3	3/3	5.58±0.07	0.88	3/3
	20	A	0/3	ND	-5.15	0/3	0/3	ND	-5.15	0/3
		B	0/3	ND	-5.36	0/3	0/3	ND	-5.36	0/3
		C	0/3	ND	-4.76	0/3	0/3	ND	-4.76	0/3
	30	A	0/3	ND	-5.15	0/3	0/3	ND	-5.15	0/3
		B	0/3	ND	-5.36	0/3	0/3	ND	-5.36	0/3
		C	0/3	ND	-4.76	0/3	0/3	ND	-4.76	0/3

aValues are the difference between concentration (log$_{10}$ cfu g^{-1}) the day of artificial inoculation (T$_0$) and 24 hours (ΔT$_1$) and 30 days (ΔT$_{30}$) after treatment. For each batch and for each temperature-time combination values are the minimum difference between the initial contamination level and the maximum count after the treatment in the triplicate samples. +ve/n are the number of samples where *L. monocytogens* could be enumerated. ND are the samples where *L. monocytogenes* was below the detection limit.

Table 5. Intrinsic properties ($\bar{x}$±SD) and composition (%±SD) of ricotta salata submitted to 9 different heat treatment combinations and analyzed 24 h (T1) and 30 days (T30) after storage at refrigeration temperature.

Treatment		pH		a_W		Moisture %		Fat %		Proteins %		NaCl %	
Temperature	Min	T1	T30	T1	T30	T1	T30	T1	T30	T1	T30	T1	T30
75°C	10	6.36±0.13[a]	6.30±0.12[a]	0.975±0.001[a]	0.976±0.000[a]	52.94±2.58[a]	52.69±3.33[a]	20.49±2.20[a]	22.88±4.16[a]	18.00±1.74[a]	16.30±1.56[a]	3.30±0.52[a]	3.36±0.26[ab]
	20	6.33±0.10[a]	6.27±0.14[a]	0.976±0.000[a]	0.977±0.001[a]	53.50±2.91[a]	54.85±3.15[a]	21.45±4.08[a]	20.67±3.58[a]	17.16±1.35[a]	16.75±1.62[ab]	3.23±0.36[a]	3.46±0.22[abc]
	30	6.38±0.10[a]	6.23±0.17[a]	0.977±0.000[ab]	0.977±0.000[a]	53.04±2.84[a]	54.05±2.64[a]	20.96±2.28[a]	22.23±3.16[a]	18.13±1.50[a]	16.20±1.28[a]	3.25±0.15[a]	3.34±0.26[ab]
85°C	10	6.35±0.08[a]	6.24±0.13[a]	0.978±0.000[ab]	0.976±0.000[a]	53.21±3.57[a]	54.41±2.86[a]	20.92±2.65[a]	20.62±2.23[a]	17.85±1.72[a]	16.62±1.12[ab]	3.31±0.26[a]	3.56±0.38[bc]
	20	6.36±0.11[a]	6.27±0.07[a]	0.979±0.000[ab]	0.975±0.000[a]	54.67±2.07[a]	53.43±4.10[a]	20.15±2.08[a]	20.61±2.11[a]	17.29±1.50[a]	18.19±2.25[b]	3.31±0.23[a]	3.52±0.22[a]
	30	6.39±0.09[a]	6.28±0.08[a]	0.979±0.000[ab]	0.980±0.001[a]	54.13±3.65[a]	55.31±3.63[a]	20.42±2.05[a]	20.41±1.96[a]	18.04±2.17[a]	17.15±2.19[ab]	3.22±0.26[a]	3.28±0.18[a]
90°C	10	6.40±0.11[a]	6.26±0.13[a]	0.981±0.001[b]	0.978±0.001[a]	53.27±3.42[a]	55.02±3.98[a]	22.33±3.89[a]	20.51±3.37[a]	16.99±1.77[a]	16.82±1.45[ab]	3.24±0.27[a]	3.43±0.28[abc]
	20	6.39±0.11[a]	6.25±0.11[a]	0.981±0.001[b]	0.977±0.001[a]	53.65±4.02[a]	54.02±2.80[a]	21.00±3.00[a]	21.28±2.38[a]	17.91±1.97[a]	17.44±1.71[ab]	3.35±0.35[a]	3.61±0.27[c]
	30	6.34±0.09[a]	6.24±0.08[a]	0.981±0.001[b]	0.978±0.001[a]	54.92±2.84[a]	54.73±4.02[a]	20.84±2.93[a]	21.53±3.74[a]	16.88±1.97[a]	16.97±1.61[ab]	3.41±0.27[a]	3.42±0.20[abc]

Each data point is the mean of three samples. For each parameter means in the same column on the same testing time (T1 or T30) with different superscript letter are significantly different (P≤ 0.05).

Table 6. Means and Standard deviation of sensory results

Treatment	Differences from the control		
Blind control	3.0 [a]	±	2.1
75°C x 10 min	2.9 [a]	±	2.0
75°C x 30 min	3.3 [a]	±	2.2
85°C x 20 min	3.5 [a]	±	2.3
90°C x 10 min	3.1 [a]	±	2.1
90°C x 30 min	3.1 [a]	±	1.9

Mean values with different superscript letters are significantly different among samples.* ($P \leq 0.05$).

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CHAPTER 6

General Conclusion

General Conclusions

A recent listeriosis outbreak linked to the consumption of Ricotta salata occurred in the USA and the numerous products recalled in several Member States of the European Union and in other countries over the last decades, highlighted the necessity to develop strategies aimed to control *Listeria monocytogenes* contamination in Ricotta salata cheese.

Ricotta salata contaminations origins mainly from the processing environment and are frequently limited to the rind of the product. Ricotta salata, as other ready to eat products, is consumed as it is or grated (rind included), without any further processing. It is generally accepted that contamination of Ricotta salata is mainly a post process contamination originating from the processing environment. The stages successive to curd coagulation such as moulding, pressing, salting, storage and portioning are the most critical for product contamination. The prevalence of *L. monocytogenes* contamination on the rind of Ricotta salata is ca. 20% (Spanu et al., 2015). The physic-chemical composition of Ricotta salata and in particular the pH and a_W, are favourable to *L. monocytogenes* growth. During the prolonged refrigerated storage, in the absence of a competitive microflora, *Listeria monocytogenes* that can reach a concentration as high as 10^7 log $_{10}$ ufc/g^{-1} (Spanu *et al.,* 2012).

In order to comply with most restrictive safety standards required by the international market (i.e. absence of listeria in 25 g), it is necessary for the dairy industry

the use of systems that ensure the inactivation of *Listeria monocytogenes* at the end of the production process. Zhu *et al.*, (2005) described the post-process treatment such as pasteurization, irradiation and high pressure for the inactivation of *L. monocytogenes* in RTE foods. Inactivation treatment must be specifically designed and validated for each product. The physic-chemical and microbiological characteristics of the product that may affect the efficacy of the treatment should be taken into account. Additionally, the influence on the organoleptic properties, feasibility and cost of the treatment should be considered.

The feasibility of using water bath heat treatment to reduce the risk of contamination with *L. monocytogenes* in Ricotta salata rind has already been proven under experimental conditions (Spanu et al., 2013). Such treatment conducted at 85 °C for 90 min was effective in reducing *Listeria monocytogenes* concentration of ca. 10^6 cfu/g. However, it is necessary to establish the most effective time-temperature combination in order to contain the costs of the treatment and to guarantee the preservation of organoleptic and sensory characteristics of the product. Therefore, the experiment described in the present thesis were aimed to validate which time temperature combination was both able to inactivate a 5 log cfu/g^{-1} concentration of *L. monocytogenes* from the rind of Ricotta and suitable at industrial level.

There is the need to select the appropriate treatment taking into account the type of product. In particular, ricotta salata can be placed on the market as the whole wheel or

after cutting into wedges. For this reason, two independent experiment were conducted, one for ricotta salata wheels (Chapter 4) and ricotta salata wedges (Chapter 5). The purpose of the treatment were to demonstrate the compliance of ricotta with microbiological criteria laid down by Regulation EC 2073/2005.

In Ricotta salata wheels two out of nine treatment combinations, i.e. 85°C for 40 min and 90 °C for 40 min, were effective in reducing *L. monocytogenes* contamination level of 5 log. In Ricotta salata wedges five out of 9 combinations, 75 °C for 30 min, 85 °C for 20, and 30 min, and 90°C for 20 and 30 min, demonstrated to meet the process criteria of at least 5 log reduction.

As far as the results obtained on the sensory features of ricotta salata, it is necessary to distinguish between whole wheels and wedges. "Treated" Ricotta salata wheels showed a significant difference when compared to untreated ricotta. This indicates that the water bath heat treatment had an impact on the sensory properties of ricotta salata. It is worth to note that sensory analysis were principally conducted in order to highlight sensory differences among the heat treatments, rather than with untreated samples. Provided that the heat treatments are necessary under a food safety prospective, the objective was to select among the treatments effective to control *L. monocytogenes* which one had the less impact on the sensory properties. Only the mildest treatment was comparable with untreated samples, however with detriment of the safety of the product. In addition, the difference from control test is designed to detect a difference, but no further indication is

given on the sensorial attributes (i.e. which one is better). Therefore, it can be concluded that no sensory differences were observed among the different time-temperature combinations. Unlike whole ricotta salata wheels, for ricotta salata wedges no differences has been observed in sensory properties between heat treated and untreated samples. This could be explained considering the smaller size and weight of ricotta wedges which allowed the use of milder time-temperature combinations as compared to the whole wheels. In particular, the time ricotta samples were exposed to heat was up to 20 minutes shorter in the wedges, reasons for these differences. For both products it is necessary a deeper investigation of the sensory properties with a specific descriptive profile study aimed to assess the impact of heat treatment on sensory feature of ricotta salata.

The recovery of *L. monocytogenes* in the inner paste of treated samples leads to two additional consideration of post-lethality treatment. The first is that such treatment are aimed to control the level of contamination only on the product surface (i.e. the rind that in ricotta salata is edible). The temperature detected one cm in depth from the product rind showed a ΔT of ca. 40 °C, being ineffective in killing Listeria cells. This evidence justify the use of listericidal heat treatment to control superficial contamination of the product. The second consideration is relative to the need to follow strict hygiene procedures during manufacturing of Ricotta salata. Since the product undergoes several manipulation during the production process, it is particularly exposed to secondary contamination originating from processing environment (i.e. personnel, utensils and

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several food contact and non-food contact surfaces). If good manufacturing and good hygienic practices are not implemented, during the production process, a possible contamination could occur in the inner paste. As an example, filling moulds with cold ricotta curd recovered from drainage tables at the end of the production shift, could favour the contamination of the inner paste with *L. monocytogens*. At this stage listeria cells within the curd mass are protected from the thermal treatment and can survive and multiply during the refrigerated storage. This could represent a serious concern for consumers' health.

We can conclude that it is possible to suggest the use of water bath heat treatment as a strategy to control the superficial contamination of *Listeria monocytogenes* in vacuum-packed ricotta salata. However, it is necessary to stress that this post-lethality treatments are intended to be used as additional (and not alternative) step in the mitigation of the risk of listeriosis associated to the consumption of ricotta salata.

Microbiological challenge testing for *Listeria monocytogenes* in ready-to-eat food: a practical approach

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