DIPARTIMENTO DI MEDICINA VETERINARIA

CORSO DI DOTTORATO IN SCIENZE VETERINARIE

INDIRIZZO:

CICLO: XXXII

Coordinatore del Corso Prof.ssa Fiammetta Berlinguer

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Anno Accademico 2018-19

UNIVERSITÀ DEGLI STUDI DI SASSARI

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Chapter 1-Introduction

1.1 Genus Listeria

The genus Listeria includes short, Gram positive, facultative anaerobe, non-sporulating rods. They are capable of growing at temperatures between 0 and 45 °C, with motility at 20-30 °C and optimal growth between 30 and 37 °C (Halter *et al.,*2013). *Listeria* is also able to growth at pH between 4.4 and 9.4 and in presence of high salt concentration, until 13-14% wt/vol. In addition to the ability to grow in very variable conditions, *Listeria* also has great adaptability to environmental changes, such as during food production

Like several other bacteria, *Listeria* participates in the adhesion process and biofilm formation that causes the persistence of microorganisms in food processing environments. Biofilm is a sessile form of microbial life with an initial phase of adhesion of bacteria to the surfaces and a secondary phase with the production of a polymeric substance issued in the external environment (De Oliveira *et al*. 2010). This process causes the formation of a matrix that is composed of proteins, nucleic acids and polysaccharides. The matrix, that keeps the cells together, contains water channels that are used to exchange nutrients and waste (Sauer *et al*., 2007).

Several factors can influence biofilm formation process. They can be intrinsic or environmental. Intrinsic factors such as genetic determinants and combinations of expression of different genes but also environmental factors like availability of nutrients, presence of chemicals, adhesion surface compounds and temperature conditions (Camargo *et al.,* 2016; Henderson *et al*., 2019).

The resident microflora can influence the presence of *Listeria* in a positive or a negative way and it is difficult to have a single-specie culture in the processing environment. For example, it is demonstrated an improvement in biofilm formation when *Listeria* stays in presence of some species of *Pseudomonas* (Sasahara and Zottola 1993) or *Flavobacterium* (Bremer *et al.,* 2001) the other hand, it was proved that the presence of lactobacilli or competitive microflora can decrease the level of contamination of *Listeria* spp.. The inhibition is implemented through three different mechanisms: the prevention of adhesion, the production of antimicrobial agents like acid lactic and bacteriocins, the competition for nutrients (Mariani *et al*., 2011).

The possibility to growth at different conditions, the large adaptability of the microorganism and the ability to create biofilms are the keys that make *Listeria monocytogenes* a problem in processed products even with very low initial concentrations. *Listeria* is able to reach dangerous levels during storage even if carried out under the appropriate conditions of refrigeration and with the correct application of good hygiene practises (Cava-Roda *et al*. 2012; Aprea *et al.* 2018).

The genus *Listeria* includes several species with validly published names: *L. aquatica*, *L. booriae*, *L. cornellensis*, *L. fleischmannii*, *L. floridensis*, *L. grandensis*, *L. grayi*, *L. innocua*, *L. ivanovii*, *L. marthii*, *L. monocytogenes*, *L. newyorkensis*, *L. riparia*, *L. rocourtiae*, *L. seeligeri*, *L. weihenstephanensis* and *L. welshimeri.* In this group only *L. ivanovii* and *L. monocytogenes* are at the moment identified as pathogens (Orsi & Wiedmann, 2016). Since 2009, thanks to continuously evolving sequencing techniques, the genus has grown constantly, only in the 2018 other three species were sequenced: *Listeria thailandensis, Listeria goaensis* and *Listeria costaricensis* (Doijad *et al*. 2018; Leclercq *et al*. 2019; Núñez-Montero *et al*. 2018). The species are divided into two groups: *Listeria sensu strictu* and *sensu lato*. The first one is formed by species able to

growth within higher vertebrate hosts: *Listeria monocytogenes* and the five species that are more related with it (*Listeria marthii*, *Listeria innocua*, *Listeria welshimeri*, *Listeria seeligeri,* and *Listeria ivanovii*). The second one includes the other eleven,*L. grayi* and 10 Listeria species described starting from 2009 (Chiara *et al.* 2015).

Listeria monocytogenes is characterized by a high heterogeneity. The species includes four evolutionary lineages (lineages I, II, III, IV), further divisible into 13 different serotypes: 1/2b, 3b, 4b, 4d, 4e and 7 (lineage I) 1/2a, 3a, 3c and 1/2c (lineage II); 4a, 4c and 4b (lineages III and IV). The serotypes are assembled into four PCR serogroups (1/2a, 1/2b, 1/2c and 4b). The serotype 4b and lineage I are most commonly isolated from clinical cases, serotypes 1/2b, 1/2a, and 1/2c and lineage II in foods (Maury *et al*. 2016).

1.2 Listeriosis

Listeria monocytogenes is responsible of a food-borne disease called listeriosis. After the ingestion, *L. monocytogenes* overcomes numerous difficulties such as the pH in the stomach that is lower than 2.5, the presence of the volatile fatty acids in the gut consequently to the fermentation of sugars, the immune defence mechanisms such as macrophage action. The evolution of the microorganism has led to the creation of a gene expression and suppression system in the presence of low pH that causes the induction or down-regulation of more than 50 different proteins and a modification of the bacterial membrane and its lipid layers in order to control the intracellular pH homeostasis. Examples of this proteins are: internalin, listeriolysin and phospholipases.

After the penetration of *Listeria monocytogenes* through the intestinal barrier, *L. monocytogenes* exploits the macrophages as carriers to move to the target organs and to multiply protected from external attacks causing different symptoms (Conte *et al*., 2002).

The symptoms vary depending on the person affected: in immunocompetent individuals can be an occasional febrile gastroenteritis, abortion in pregnant women, new-borns can manifest meningitis and in elderly and immunocompromised there is a fatal bacteraemia (Buchanan *et al*. 2017). Listeriosis represents an increasing concern. In EU there was an increasing trend of confirmed listeriosis cases in the period between 2013 and 2017 with 2480 human cases only in 2017. The fatality rate that year was 13.8% (EFSA 2018) but it can be 20 and 30% (Zhu *et al.*, 2017).

Most cases of listeriosis are associated with the consumption of contaminated food, primarily unpasteurized milk and dairy products, ready to eat meat products and seafood. In the last few years there have been large-scale outbreaks (Desai *et al.*, 2019). Due to the absence of an antimicrobic treatment between production and consumption, such as heating, ready-to-eat foods are responsible of most of the recalls, outbreaks and costs of analysis. related with the presence of *Listeria monocytogenes* (Jordan & McAauliffe, 2018)

With Regulation (EC) 178/2002, at article 35, a rapid alert system was established in the European Community to monitor the health and nutritional risks of foods informing the Autority that analyses the data and provides the Commission and the Member States any information required to perform risk analysis. There were 3616 notifications due to the presence, suspect and outbreaks of Listeria monocytogenes reported by RASFF- the Rapid Alert System for Food and Feed in 2018 (European Commission 2018). In the same year, 28 member states of the European Union reported 2,549 invasive human cases of listeriosis that were confirmed by authorities. These

included 14 food-borne listeriosis outbreaks involving 158 human and 46 multicountry outbreaks. The fatality rate was 15.6% confirming that Listeriosis is one of the most serious food-borne that European Union put under surveillance (EFSA, 2019).

1.3 Protective cultures

Different strategies have been developed to control bacteria that cause foodborne disease like *Listeria monocytogenes.* It is possible to apply different approaches: physical methods such as pasteurization, radiation, ultrasound, UV, pulsed light, electricity, cold plasma; chemical methods with the addition of substances like benzoates, sorbates, nitrites and sulphites that are preservatives; the biological method that can be represented by the use of natural products with an antimicrobial activity or natural antagonists like bacteriophages or Lactic acid bacteria (LAB) (Yi *et al.* 2020).

Consumers are asking more and more for natural products without chemical additives like preservatives and antimicrobials. For this reason, research is trying to find more natural solutions like LABs, microorganisms capable to ferment carbohydrates obtaining lactic acid. LABs are classified as GRAS (Generally Recognized As Safe) and due to their ability to produce numerous compounds, like bacteriocins, useful to preserve foods from other microorganisms that can cause food spoilage, they are attracting significant attention in food processing and preservation (Muhammad *et al*. 2019).

The bacteria included into the group of the LABs are extremely heterogeneous and belong to different genera and species included in the order of *Lactobacillales*. Most of them are Gram-positive, catalase negative, not able to sporulate, reduce nitrate nor metabolize lactate. Over time they have adapted to numerous environmental conditions,

for this reason they can be anaerobic, microaerophilic aerobic or facultative anaerobic (Sharma *et al*. 2020) (Sánchez-Juanes *et al.,* 2020).

LABs can carry out their antimicrobial action through numerous mechanisms such as the production of lactic acid that, in the non-dissociated form, is able to pass the cytoplasmic membrane. When it is in the target cell, lactic acid is dissociated with the consequent acidification of the cytoplasm, the inhibition of the functions of the cell and the cancellation of the membrane potential. In addition, LABs compete for nutrients, they have the ability to decrease the levels of pH and to product substances with bactericidal or bacteriostatic effect (Delboni & Yang 2017).

Bacterial protein synthesis apparatus can synthetize low molecular weight peptides with antimicrobic activity. These peptides can be classified into narrow spectrum bacteriocins and broad spectrum bacteriocins if they are inhibitory against bacteria belonging from the same species or not respectively. Due to the great heterogeneity, the great amount of available information about their ribosomal synthesis and the possibilities for bio-medical and food applications, different classification of bacteriocins are continuously developed (Juturu & Wu 2018).

The utilization of LABs able to product bacteriocins to preserve food is preferred to the direct use of bacteriocins for several reasons that include legal and economic aspects. At the moment it is possible to find in the market only nisin and pediocin. Their isolation and purification is expensive and it is mandatory to declare them in the label, but not if it is used the bacteriocin producer LAB (Settier-Ramírez *et al*. 2020).

Bacteriocins act primarily against Gram positive pathogens like *L. monocytogenes* and *S. aureus* but, when the outer membrane is damaged, are active also against Gram negative. It is possible to add antimicrobial-producing LABs or directly the bacteriocins in the manufacture of dairy products, contributing to an improvement in the quality and

in the safety of the products by the introduction of an additional hurdle. If starter cultures are used, it is not necessary to obtain an approval or to declare their use in the labels. For this reason, producers prefer this solution (Arqués *et al*. 2015).

Bacteriocins are produced mainly during the exponential growth phase, but stress factors that result into less favorable growth conditions, such as low temperatures, low growth rates and presence of competing microflora, are apparently able to stimulate bacteriocins production. The explanation could lie in the fact that slow growth requires less energy, so there is more energy available for the polymerization of building blocks and the control of bacteriocin gene expression (De Vuyst *et al.*, 1996).

Actually nisin and pediocin are the most utilized but several other bacteriocins like subtilin, lichenicidin, cinnamycin, actagardine, epidermin, lacticin, carnobacteriocin, piscicolin, divergicin, mutacin, mundticin, mesenterocin, enterocin, sakacin, leucocin, curvacin, enterocin, lysostaphin, duramycin, brevinin, ruminococcin, curvaticin, and columbicin are known. The mechanism of action consists, probably, in the formation of ion selective pores with dissipation of the proton motive force and the depletion of intracellular ATC causing the permeabilization of the bacteria cell, the leakage of intracellular substrates and, sometimes, cell death. This activity is nonspecific and the bacteriocins producers are protected by a suite of protective proteins from the bacteriocin activity. Over time also pathogens and spoilage bacteria are showing some strategies to resist from cell permeabilization and pore formation (Kumariya *et al*. 2019).

1.4 High-Pressure Process

In the food industry the challenge is to eliminate the food-borne diseases preserving the flavor, color, taste and form of foods, improving food processing methods.

One of the technologies traditionally used is pasteurization that is really effective but, if this heat treatment is able to inactive pathogens and spoilage microorganisms, sometimes causes alterations in product quality and texture, destruction of colors and vitamins and off-flavor generation (Wang *et al*., 2016).

New non-thermal technologies have been developed. They can be physical treatments such as magnetic or electrical fields, ionization, light pulses, high hydrostatic pressures, ultrasound or chemical or biological agents like bacteriocins and enzymes. Only some of these technologies have been approved, others are still being studied to evaluate efficacy and convenience of commercial application (Trujillo *et al.*, 2000).

HPP is an innovative technology alternative to the heat treatment that subjects the product to very high pressures, up to 500-600 MPa. The treatment is efficient at low temperatures and after a short period of time, permitting to preserve the sensorial characteristics of the food and to extend its shelf life. There are numerous studies reporting the possibility to use the HPP technology to control spoilage and pathogenic microbes in fresh food (Evert-Arriagada *et al*., 2018). The system works thanks of a high hydrostatic pressure vessel, a pressure generating pump that intensifies the pressure and the yoke that ensure that the vessel is properly sealed. The effect of the treatment is instantaneous and uniform and independent of the equipment and the mass/time selected. The geometry and size of the product do not affect the results. Attention must be paid to the quality of the packaging material to prevent the pressure from breaking seal integrity (Mújica-Paz *et al*., 2011).

Moreover, HPP treatment is less expensive because it is needed a lower quantity of energy, the processing time is shorter and reduces the manipulations with less possibilities of post-production contaminations of the products (Martínez-Rodríguez *et al.* 2012).

The application of pressures between 400 and 800 MPa for a time up to 10 minutes is enough to achieve a reduction for pathogenic and spoilage vegetative microorganisms over 5 logs (Van Impe *et al*. 2018). Indeed, it is demonstrated that pressures levels between 400 and 600 MPa for several minutes are enough to inactivate vegetative cells. It is, however, necessary to underline that the application of excessively high pressures or the use of too long time, can cause alterations in the products such as oxidation of the lipids or changings in texture and that, from an economic point of view, the risk is to deteriorate the machinery (Stratakos *et al*., 2016).

The effectiveness depends on physicochemical properties of the treated product. There are three key factors that determine the success of the high pressures: process, product and target microorganisms. The inactivation of the cells can be consequent to an injury or the killing. Sometimes it is possible to have a sublethal injury that can be confused with a lethal one. In that cases the consequence can be the recovery or the death of the cells. The damages that high pressures can cause are: damages to cell membrane, inactivation of key enzymes modulating growth of cells and oxidative damages to cell membrane constituents (peptidoglycan layers) (Van Impe *et al*. 2018). At the same time, the principal quality characteristics of the products such as freshness, nutritional value, and sensory properties, are preserved. This because the treatment is able only to break the noncovalent bonds, so, compounds characterized by low-molecular weight like amino acids, vitamins, flavour molecules, remain unchanged (Martínez-Rodríguez *et al.* 2012).

When the process starts the temperature of the food sample rises because of the adiabatic heating allowing to reach the target temperature necessary for the process (Balasubramaniam *et al.,*2015). With the increasing of the sample temperature there is also a reduction of the volume of water as consequence of the restoration of the intrinsic equilibrium of the sample after the interruption of intermolecular forces in accordance

with the principle by Le Chatelier. In cheeses, the restoration of the equilibrium lead to changes in the microbiological, physicochemical, rheological and sensory characteristics. In these products there is a difference in the quantity and in the space distribution of water molecules, the structure is more compact and there are changes also in the enzyme interaction, the mineral balance and in the protein conformation. In particular, the primary structure is preserved, but there is denaturation of the secondary structures and changings in the tertiary structures (Tomasula *et al.*, 2014).

When the target process temperature and pressure are reached, the holding time starts, and the product stays at isothermal and isobaric conditions. The holding time is usually shorter than 10 minutes to avoid effects on the quality of the product, but also for economic reasons (Balasubramaniam *et al.,*2015).

In products like fresh oysters, processed meats, guacamole, fruit juice and fresh cheeses this technology is usually applied without highlight alteration of sensorial characteristics. Several studies on fresh cheeses demonstrated the efficacy of the treatment against Listeria monocytogenes with reductions up to 5 log cfu/g and more (Batty *et al.*, 2019).

Chapter 2-Thesis project

Listeriosis is a severe foodborne disease caused by *Listeria monocytogenes* characterized by a high fatality rate (till 30%) (Zhu *et al*. 2017). It is often linked with the consumption of ready to eat food (Jordan & McAuliffe 2018) and it was recognised as the cause of several large-scale outbreaks in the last few years (Desai *et al*., 2019).

Dairy products are often associated with outbreaks of listeriosis even if heat treatments, like the pasteurization of the milk, are applied. The heating is effective against *Listeria*, but often the problem is a recontamination of the product during manipulations after it, helped by the presence of the microorganism in biofilms and niches in the production plant (Cava-Roda *et al*. 2012).

Dairy products made with sheep milk are a very important part of the economy in Sardinia (Italy) and Ricotta salata, made with the whey obtained after the production of the cheese, is exported, like other products, to countries in Europe and also in USA. Lately, Ricotta salata was recalled due to the presence of *Listeria monocytogenes* and because it was recognized as the source of some outbreaks. The most serious outbreaks involving Ricotta salata in the last few years dates back to 2012 in 14 states of the USA with 22 cases, 20 hospitalizations and 4 deaths. The same cutter was used to cut Ricotta cheese and other cheeses causing a cross contamination, all the products were exported to USA.

The aim of this thesis is to evaluate not-heating methods to control the contamination of Ricotta salata cheese, in particular, the use of protective cultures and High-Pressure Process.

In the third chapter the maximum growth rate of *Listeria monocytogenes* in Ricotta salata cheese is calculated and the growth curve resulted in lab have been

compared with the one obtained utilizing a predictive software opensource. To perform the study, industrial Ricotta cheese produced by a Sardinian dairy plant, was inoculated with a known concentration of *Listeria monocytogenes*, vacuum packed and analysed every 48 hours for the enumeration of *L. monocytogenes* following the international standard ISO11290-2 2017.

The fourth chapter is about the characterization of a selection of protective cultures performed to evaluate their anti-listerial activity *in-vitro*. A pool of 4 resident strains of *Listeria monocytogenes* isolated from products and surfaces from Sardinian dairy plants plus one reference strain (NCTC 10788) has been used during a well diffusion assay. The commercial protective cultures, identified following the label indications, were used at different concentration, washed or not and at different temperatures.

In the fifth chapter there is the challenge test performed to confirm or not *in vivo* the results obtained *in vitro*. Ricotta salata samples were prepared inoculating them with *Listeria monocytogenes* plus one of the selected protective cultures or spraying only the pathogen, obtaining 3 groups of samples with one more group of blanc samples. The aim was to evaluate the behaviour of *Listeria* on Ricotta salata cheese with or without the protective cultures and compare it to find the more effective.

The sixth chapter is about the challenge test performed to evaluate the effectiveness of an HPP treatment of 500 MPa for 10 minutes. The Ricotta salata has been inoculated with to different concentrations of *Listeria*: 2 log to simulate a natural contamination and 5 log to evaluate the performance of the treatment.

The final chapter, the seventh, is a final discussion about the project and the results obtained.

Chapter 3 - *L. monocytogenes* Maximum growth rate (μ_{max}) in Ricotta Salata cheese in thermal abuse condition (8°C)

3.1 Introduction

Listeriosis is a serious foodborne disease caused by *Listeria monocytogenes* which is implicated in sporadic cases, disease outbreaks and product recall. Hence, there is a worldwide interest for what is now considered one of the major foodborne pathogens. *L. monocytogenes* is associated with two different forms of disease, non-invasive and invasive listeriosis. Non-invasive listeriosis is a milder form of disease known as febrile listerial gastroenteritis. Symptoms, including fever, watery diarrhoea, nausea, headache, pains in joints and muscles are self-limiting and generally occur in healthy individuals as a consequence of the ingestion of high doses of listeria. The invasive form is a more severe form of listeriosis affecting high-risk groups of the population, which include pregnant women, new-borns, elderly and immunocompromised individuals. The symptoms vary largely from miscarriage, stillbirth, premature delivery in pregnant women to meningitis and fatal bacteraemia in other individuals (Buchanan *et al*. 2017). Despite the low incidence, *Listeria monocytogenes* is characterized by a hospitalization rate >90% and a fatality rate of 25.9% (de Noordhout et al. 2014). In the last two decades, an increase of large-scale outbreaks has been recorded (Desai *et al.* 2019). In the EU, an increasing trend of confirmed listeriosis cases has been confirmed in the period between 2013 and 2017, with 2480 human cases only in 2017 (EFSA 2018).

Cases of listeriosis are associated with the consumption of contaminated food, primarily unpasteurized milk and dairy products, ready to eat meat products and seafood. Despite the fact that in many recent cases foods not previously implicated in human listeriosis were implicated (Desai *et al.* 2019), ready-to-eat foods (i.e. meat and meat

products, fish and fish products, and milk and milk products) continue to remain the main categories of foods linked with listeriosis (EFSA 2018).

In the period between March and October 2012, a listeriosis outbreak occurred in the USA involving 14 states, with 22 hospitalization and four deaths. The outbreak investigation linked the source of listeriosis with a ricotta salata cheese imported from Italy and produced in Sardinia (Acciari *et al*. 2016). The *L. monocytogenes* strain associated with the outbreak was a serotype 1/2a with an uncommon Pulsed Field Gel Electrophoresis profile in the USA. Before the severe outbreak, several recalls of ricotta salata due to *L. monocytogenes* contamination have been reported over the years (Spanu *et al.* 2013; 2015a). Ricotta salata is a traditional whey cheese produced using the whey remaining from the production of cheeses made with sheep milk. The whey is filtered, pre-heated at 60-65°C using a plate heat exchanger and transferred into large open kettles where it is heated until it reaches 85°C. After 15 minutes coagulation of the whey proteins starts, and the clots are collected with ladles and transferred in moulds lined with cheesecloths. The clots in the moulds are pressed for 24 hours at room temperature to enhance the drainage of residual whey. The Ricotta is then transferred to brine at 10 °C for 6 hours and then stored into a cold room at 1-2°C until they are vacuum packed using thermoforming vacuum films and stored at refrigeration temperature until sold. Alternatively, salt can be added to the whey before heating or the ricotta cheese can be dry salted (Casti *et al.,* 2016). The Regulation (EC) n. 2073/2005 provides the definition of foods that are not able to support the growth of *Listeria monocytogenes:* products with pH ≤4.4 or water activity (a_w) ≤0.92 and products with pH ≤5 and a_w ≤0.94. The same regulation clarifies that, throughout their shelf life, the level of contamination must not exceed 100 cfu/g. The studies that the Food Business Operator (FBO), responsible for food safety, should conduct to determine the shelf life of a product with respect to *L.*

monocytogenes, in particular ready to eat (RTE) foods able to support the growth of *L. monocytogenes*. In addition to physico-chemical analysis and the consultation of scientific literature, the FBO can use predictive mathematical modelling and tests to estimate the ability of the microorganism to grow or survive in the product during the shelf-life. The European Union Reference Laboratory (EURL) for *Listeria monocytogenes* prepared a guidance document "EURL *Lm* Technical Guidance Document for conducting shelf-life studies on *Listeria monocytogenes* in ready-to-eat foods". The document specifies how to implement and perform challenge test to assess the growth potential and to assess maximum growth rate of *L. monocytogenes* in food. The growth potential is the difference between the $\log_{10}$ cfu/g at the end and the $\log_{10}$ cfu/g at the beginning of the experiment. If the growth potential is >0.5 log cfu/g, the product is a RTE food able to support the growth of *L. monocytogenes,* if it is <0.5 log cfu/g, the product is not able to support the growth. Spanu *et al*., (2012) calculated that the growth potential of *L. monocytogenes* in ricotta salata cheese, stored for six months and analyzed after 6 hours and 2, 4 and six months, can be up to 6.9 log cfu/g. Considering the growth potential and the mean values of, pH and a_w, ricotta salata is a ready to eat food that supports the growth of *Listeria monocytogenes.*

The maximum growth rate (μ_{max}) is a laboratory study that measures the rate of growth of *L. monocytogenes* in contaminated food. It is calculated at a specific temperature from the growth curve obtained during the exponential growth phase of the strains used, plotting the natural logarithm of cell number against the time. The slope of the line obtained is the μ_{max}. If the growth potential is useful to classify the different products with respect to the ability to support growth, the maximum growth rate facilitates calculation of the maximum number of cells that can be present if the limit of 100 cfu/g is not to be exceeded during the shelf life of the food. It is possible to use predictive

software to estimate the μ_{max}, but considering the intrinsic properties of the food and the possible interaction with the extrinsic properties such as temperature, gas atmosphere, moisture that can influence the results, the data obtained in the laboratory on the food matrix allow a more accurate determination of the μ_{max}. In a laboratory made semi-soft cheese, Schvartzman *et al* (2011) showed that in 40% of cases where growth was predicted with Combase, no growth occurred in laboratory experiments. No information is available on the μ_{max} of *L. monocytogenes* in ricotta salata cheese. The objective of the present study was to assess the μ_{max} of *L. monocytogenes* in ricotta salata wedges at 8°C.

3.2 Materials and Methods

Ricotta salata samples

A total of 36 vacuum packed ricotta salata wedges were supplied from a local industrial cheese-making plant and used to evaluate the μ_{max} of *Listeria monocytogenes*. In order to account for variability of intrinsic properties (pH and a_w) among production batches, ricotta samples were randomly selected among each batch (12 samples from each batch). Ricotta wedges were transported and stored at refrigeration temperature (4±2°C) until their use for the experiment. Samples were labelled according to their use immediately after their arrival at the laboratory (within 24 hours after packaging). Challenge Units (CU) were defined as ricotta wedges artificially inoculated with *L. monocytogenes*; blank samples (BS) were defined as ricotta salata wedges not inoculated and used to evaluate the physico-chemical properties and confirm the absence of *L. monocytogenes* before the experiment.

Artificial inoculation

The experiment was designed according to the Technical guidance document for conducting shelf-life studies on *L. monocytogenes* in ready-to-eat foods prepared by the EURL for *L. monocytogenes* (Beaufort *et al.* 2014), except that a cocktail of 5 *L. monocytogenes* strains was used. The cocktail consisted of one was the reference strain NCTN 10887 (serotype 1/2a) and 4 wild-type strains selected from the Department of Veterinary Medicine of Sassari collection, previously isolated from sheep milk cheese-making plants and stored at -80°C. The strains were selected in order to be representative of the 4 major serovars associated with human illness (1/2a, 1/2b, 1/2c, 4b) (*Table 3-1*). The inoculum was prepared according to Spanu *et al* (2015b). Briefly, each strain was subcultured overnight on nonselective agar plates at 37 °C, then a single colony was picked and suspended in 10 ml of brain heart infusion (BHI) broth at 30°C in a shaking bath until the cells reached late exponential or the early stationary phase. After incubation, 1 mL of the suspension was transferred into 9 ml of BHI broth and incubated at 8°C for one week. Equal volumes of each of the 5 broth cultures were mixed into a sterile flask to obtain a "working culture". After a colony count, 2.5 ml of the appropriate dilution of the working solution, prepared in buffered saline solution (Sigma Aldrich, Missouri, USA), was evenly sprayed on the surface or ricotta salata wedges, in order to obtain a final a concentration of 50-100 cfu/g ricotta rind. After the inoculation, the samples were held for 5 minutes at room temperature to facilitate the absorption of the inoculum. Ricotta salata wedges were then individually vacuum packed and stored at 8°C.

Experimental design and analysis

The challenge studies were conducted in three independent trials, one for each ricotta salata batch. After the voluntary inoculation, the ricotta salata samples were stored at 8°C until analysis, which were performed at different testing times (T). The times were

defined as time zero (T_0), which was 6 hours after the inoculation, T_{48}, T_{96}, T_{144}, T_{192} and T_{240} which were, respectively, 48, 96, 14, 19 and 240 hours after inoculation. The number of samples, analysis and sampling times are summarised in *Table 3-2.*

For microbiological analysis, 25 g of ricotta were scratched under sterile conditions from the rind (2-5 mm depth) of each wedge and diluted into 225 ml of Buffered Peptone Water (Sigma-Aldrich, Missouri, USA). Enumeration of aerobic mesophilic bacteria was conducted according to ISO 4833-1 2013 while detection and enumeration of *L. monocytogenes* was conducted according to ISO11290-1/2 2017.

To account for possible interaction in *L. monocytogenes* survival and growth, blank samples were analysed for the determination of intrinsic properties and total bacterial counts. The pH and a_W were measured using pH meter GLP22 (Crison Instruments SA, Barcelona, Spain) and water activity meter Aqualab series 3 (Decagon, Pullman, WA, USA), respectively.

Growth rate of L. monocytogenes

The counts ($\log_{10}$ cfu/g) of *L. monocytogenes* obtained at the different sampling times were used to generate the growth curve of *L. monocytogenes* in ricotta salata wedges stored under mild abuse temperature (+8 °C). For each of the three curves obtained, one from each ricotta salata batch, the μ_{max} was estimated by non-linear regression using ComBase DMFit web edition (ComBase 2015). A growth curve generated using predictive model curves in the ComBase interface (imputing the following information: initial level of contamination, pH, a_W and storage temperature of ricotta salata wedges) was compared with the growth curve obtained with experimental data.

3.3 Results

Intrisic properties and total bacterial counts

The mean pH values of the ricotta salata batches ranged between 6.12±0.22 and 6.22±0.13 while mean a_W values ranged between 0.971±0.002and 0.970±0.004, respectively at T_0 and at T_{240}. The values of pH and a_W for each batch are given in *Table 3-3*. At T_0 the total viable count at 30°C was 6.71±1.23 cfu/g while at T_{240} it was 8.40±0.42.

Growth rate of L. monocytogenes

In blank samples, *L. monocytogenes* was always below the detection limit of 10 cfu/g. The level *L. monocytogenes* after voluntary contamination of ricotta salata wedges at T_0 was on average 1.80±0.52 cfu/g. During storage at 8°C for 240 hours the pathogen reached level as high as 6.33±0.43. The counts by batch for each sampling time are reported in *Table 3-3*. The μ_{max} was calculated independently from the growth curve obtained from each batch. The growth curve, the initial level of contamination, the duration of the lag phase and the μ_{max} for each batch are reported in Figure 1.

The average μ_{max} (calculated using DMFit) was 0.020±0.002 log cfu/h (0.48 log cfu/day) with a lag phase of 21.83±25.41 h. The predictive model was generated on an initial level of contamination of 1.8 log cfu/g, pH of 6.2, a_W of 0.970 and a storage temperature of 8°C. The growth rate obtained using the predictive model implemented on *ComBase* predictor was 0.023 log cfu/h. There was no significant difference ($p > 0.05$) between the value obtained from the predictive model and the value obtained with the experimental data (Figure 2).

3.4 Discussion

Among the dairy products produced in Sardinia from sheep milk, ricotta salata represents the highest risk with regard to listeriosis. Although there is a heating step involved in the manufacturing process, the process also includes phases that expose the product to contamination originating from the production environment. Phases downstream of the lethal treatment (i.e. whey heating) are the most critical for product contamination (Casti *et al.,* 2016). In fact, in salting, cooling and product washing areas conditions such as humidity, salinity and cold temperature, favourable to survival and growth of *L. monocytogenes* in the processing environment exist (Ibba *et al*. 2013; Spanu *et al.,* 2015a). The formulation of ricotta salata (pH of 6.3-6.7 and a_W 0.950-0.970) makes of this cheese a substrate favourable to the growth of *L. monocytogenes* (Casti *et al.,* 2016; Spanu et al. 2013). Previous investigations aiming to assess the growth potential of *L. monocytogenes* inoculated voluntarily onto ricotta salata cheese, demonstrated the ability of the organism to reach levels as high as about 7 log cfu/g, potentially threatening to human health (Spanu *et al.* 2012). However, the growth rate or lag phase were not calculated. In the frame of Regulation 2073/2005, food business operators should demonstrate that the product will not exceed 100 cfu/g during the entire shelf-life of the product under the foreseen storage and handling conditions. However, while the growth potential allows classification of whether or not a food supports the growth of *L. monocytogenes*, it does not allow estimation of the concentration of *L. monocytogenes* at a given day. This can be obtained, if the initial concentration is known, by assessing the maximum growth rate. Growth prediction can be obtained using predictive microbiology models implemented by several available software programmes such as ComBase

Predictor. Predictive models can be used by industry to minimize the length and cost process of microbial testing. However, predictive microbiological models do not replace laboratory analysis. The main limitation of predictive models is that they are derived from growth obtained in laboratory media, so they do not account for several environmental conditions and product formulations affecting microbial behaviour (Spanu *et al.* 2014). As a consequence, the models usually predict faster growth rates than are observed on the food, hence being overly conservative (Schvartzman *et al*., 2011; Fakruddin *et al.,* 2011). Therefore, it is necessary validate the models with specific laboratory experiment conducted on foods. The present study was specifically designed to assess the maximum growth rate of *L. monocytogenes* on Ricotta salata cheese wedges under thermal abuse conditions. The calculated growth rate was 0.020 log/hours. The results obtained with the voluntary inoculation of ricotta salata wedges were comparable with prediction of 0.023 estimated using the ComBase Predictor software. These results indicate that in the case of ricotta salata wedges, given the growth rate at 8°C, predictive models can be used to predict the maximum growth rate at another temperature. On a practical basis, the maximum growth rate significance stands with the definition of the shelf-life by the FBO. Such rate of growth corresponds to a 0.48 log cfu/g/day increase in *L. monocytogenes* counts, which implies that 5 days of storage are needed to exceed microbiological criteria of < 100 cfu/g defined by the European Regulation. The present study supports a previous investigation aimed at evaluating the growth potential of *Listeria monocytogenes* and demonstrating that ricotta salata represents a serious concern for consumers health (Spanu *et al.* 2012). It is essential for the food industry to ensure the safety of product placed on the market; therefore, specific control measures should be introduced in the production process to mitigate the risk of *L. monocytogenes* in ricotta salata. A strategy that has been proven effective in reducing the level of *L. monocytogenes* superficial contamination in ricotta

salata is the adoption of a hot water bath post-lethality treatment in the packed product. Such treatments were validated to reduce the level of contamination by up to 6 log cfu/g (Spanu *et al*. 2013; 2015c). Since the effect of the treatment is toward superficial contamination originating from the processing environment and no effect has been validated on the inner paste, it is vital to implement good hygienic practices during all phases of ricotta production

3.5 Tables and figures

Table 3-1 L. monocytogenes strains used for voluntary contamination of ricotta salata wedges.

Type of strain	Origin	Serogroup
Wild	Food (*ricotta salata*)	1/2a
Wild	Food (*ricotta salata*)	4b
Wild	Food (*Pecorino Romano*)	1/2b
Wild	Food environment (*floor drain*)	
Reference	Mammal, chinchilla l	1/2a

Table 3-2 Number of ricotta salata wheels and analysis performed at each sampling time.

		Sampling time (hours)						
Analysis	Test units	T_0	T_{48}	T_{96}	T_{144}	T_{192}	T_{240}	Total
Detection and enumeration of *L. monocytogenes*	CU[1]	6	6	6	6	6	6	36
Detection and enumeration of *L. monocytogenes*, aerobic mesophilic bacteria and intrinsic properties (pH and a_W)	BL[2]	6	-	-	-	-	6	12
Total		12	6	6	6	6	12	48

[1]Challenge Units: ricotta wedges inoculated voluntarily with *L. monocytogenes*; [2]blank samples: ricotta salata wedges not inoculated.

Table 3-3 Intrinsic properties of control samples after 6 h (T0) and 240 h (T240) of refrigerated storage

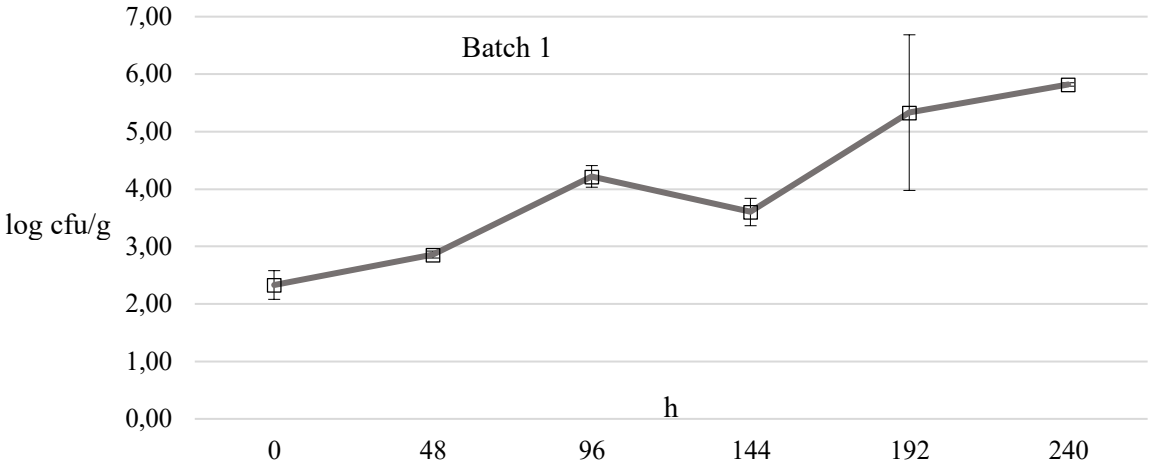
Analysis	T_0	T_{240}	T_0	T_{240}	T_0	T_{240}
pH	6.19±0.11	6.31±0.11	6.13±0.14	6.28±0.04	6.03±0.42	6.07±0.08
a_W	0.971±0.001	0.971±0.005	0.970±0.002	0.967±0.002	0.973±0.001	0.973±0.001

Table 3-4 Enumeration of L. monocytogenes (mean±sd log cfu/g) by batch during storage at 8°C for 240 hours.

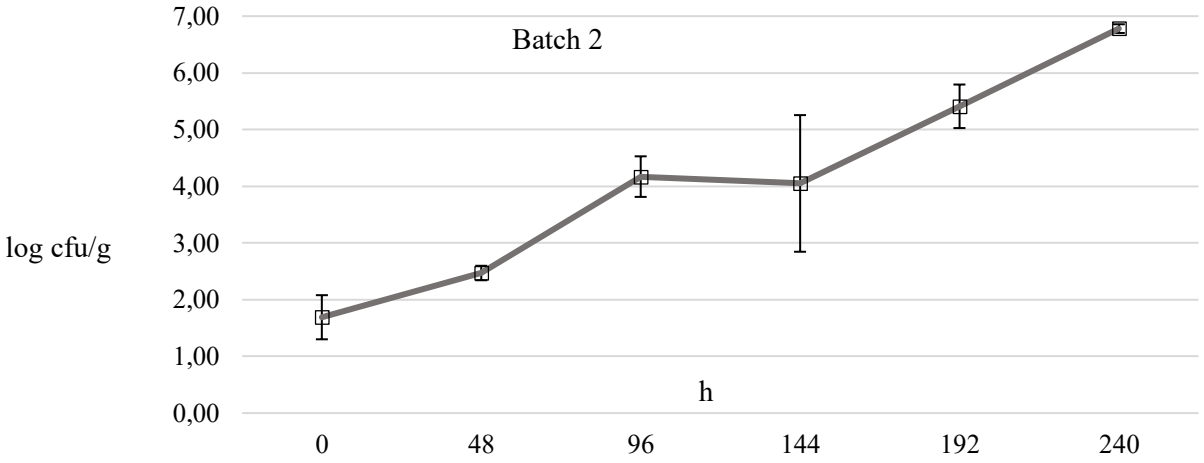
Analysis	Batch	T_0	T_{48}	T_{96}	T_{144}	T_{192}	T_{240}
Enumeration *L. monocytogenes*	1	2.33±0.35	2.86±0.08	4.22±0.27	3.60±0.34	5.33±1.91	5.82±0.05
	2	1.69±0.55	2.47±0.18	4.17±0.51	4.05±1.70	5.41±0.54	6.78±0.11
	3	1.39±0.13	1.85±0.78	2.60±0.08	4.08±0.01	5.36±0.10	6.37±0.01
	mean	1.80±0.52	2.39±0.58	3.66±0.87	3.91±0.81	5.37±0.89	6.33±0.43

T_0 is the day of voluntary contamination while T_{48}, T_{96}, T_{144}, T_{192} and T_{240} are respectively 48, 96, 144, 192 and 240 hours of storage.

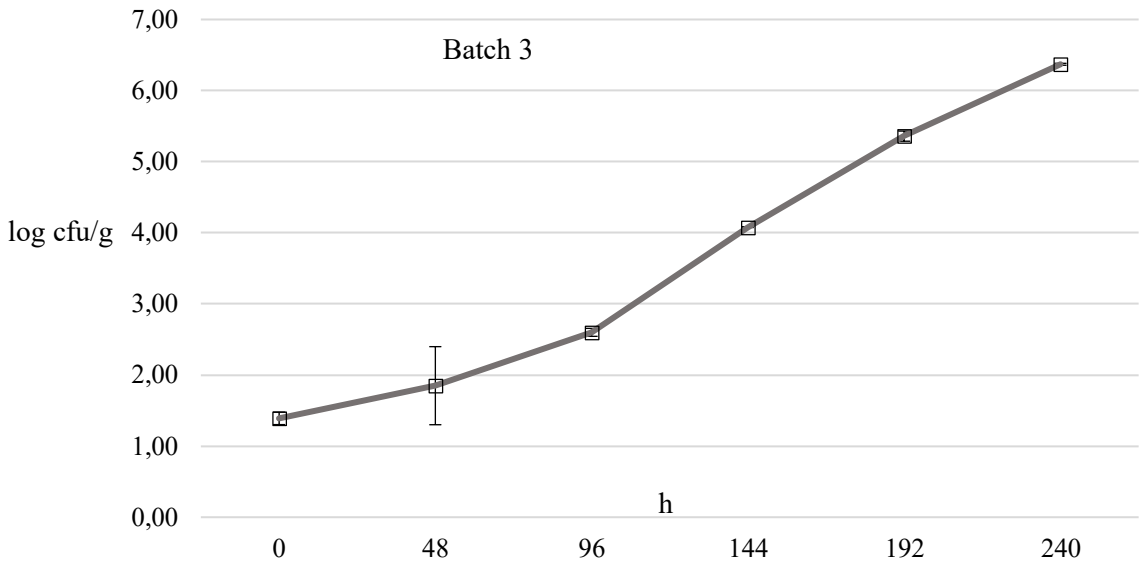
Figure 3-1 Growth curves of obtained plotting L. monocytogenes counts after voluntary contamination on 3 different batches of ricotta salata wedges.



initial value (cfu/g)	growth rate (μ_{max})
2.327 ± 0.565	0.0148 ± 0.00473

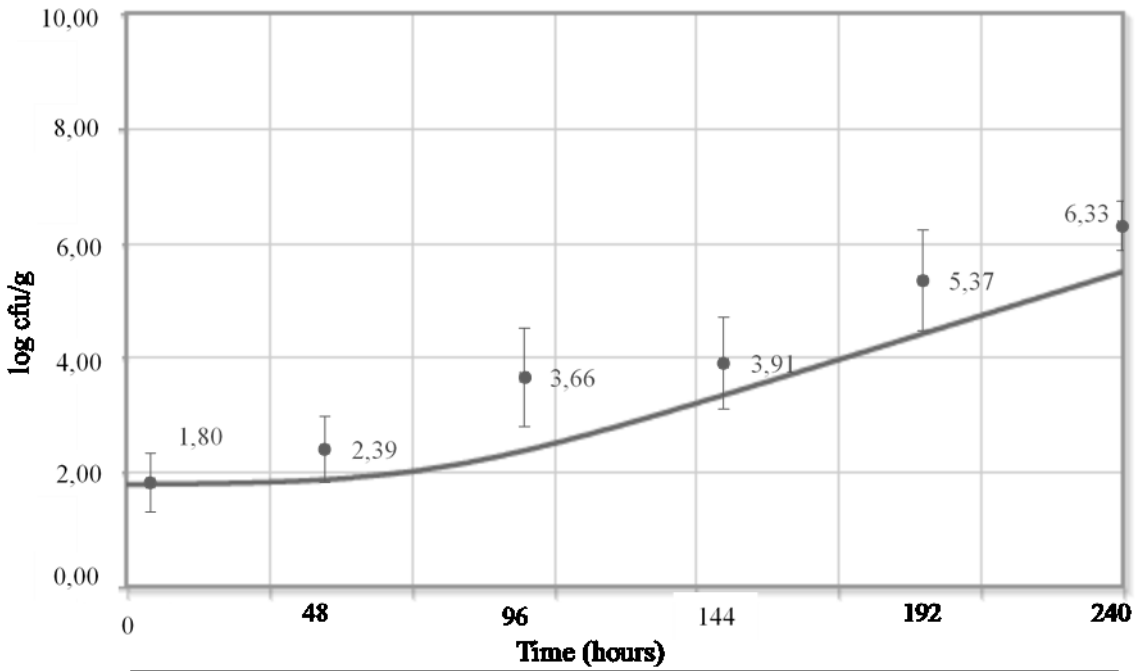


initial value (cfu/g)	growth rate (μ_{max})
1.678 ± 0.533	0.0206 ± 0.00417



initial value (cfu/g)	growth rate (μ_{max})
1.406 ± 0.194	0.0262 ± 0.0018

Figure 3-2- Growth curve of L. monocytogenes in ricotta salata wedges stored at 8°C for 240 h.



Initial level (cfu/g)	Temp (°C)	pH	aw	max growth rate (log cfu/h)	Dbl. time (hours)	Lag time (hours)	MPD (log cfu/g)
1.80	8	6.2	0.970	0.023	13.364	73.64	8.52

MPD = maximum population density; Dbl. Time = doubling time of the bacterium. Values in the graphs indicate counts of *L. monocytogenes* obtained from experimental data. ____ Indicates the growth curve estimated by ComBase Predictor

Chapter 4-Characterisation of protective cultures activity against *L. monocytogenes*

4.1 Introduction

Lactic Acid Bacteria (LAB) are part of a very heterogeneous group of bacteria members of different genera and species. They belong from different families included in the order *Lactobacillales*. Most of them are Gram-positive, catalase negative, non-spore forming, they do not reduce nitrate neither utilize lactate. They can be anaerobic, microaerophilic, aerobic or facultative anaerobes. These differences are caused by their adaptation to different environments, foods and surfaces od adhesion. A large part of them is able to explicate a positive effect for human health (Sharma *et al*. 2020) (Sánchez-Juanes *et al.,* 2020).

LABs are one of the strategies implemented by the food industry to contrast food spoilage microorganisms and pathogens, in particular *Listeria monocytogenes* with a clean-label biopreservation method. The idea is to have a competitive exclusion modulated by protective cultures able to control or inactivate undesirable microorganisms. These results are obtained competing for nutrients and sites of adhesion and also with the production of antimicrobial substances (Hossain *et al.* 2020). In general they can explicate their antimicrobial activity in several different way, in addition to the competition for nutrients and the sites of adhesion, they can product lactic acid that is able to pass the cytoplasmic membrane when it is in the non-dissociated form and, once in the cytoplasm, is dissociated. This reaction causes the lowering of the pH inside the intracellular environment with the inhibition of the activity of the cell and the annulment of the membrane potential. Furthermore, some LABs are able to synthetize substances

called bacteriocins that have bactericidal or bacteriostatic activity (Delboni & Yang 2017). The use of the bacteriocins-producer LABs is preferred to the direct use of bacteriocins. The protective cultures are less expensive because isolation and purification of the bacteriocins is not necessary. In addiction it is mandatory to declare the use of bacteriocins in the label and there are only two bacteriocins aviable in the market at the moment (Settier-Ramírez *et al*. 2020). Instead, the utilization of bacteriocin-producer protective cultures, is possible without an authorization.

Bacteriocins are more effective against Gram positive pathogens like *L. monocytogenes* and *S. aureus* but, in presence of damages of outer membrane, they can be used also against Gram negative (Arqués et al. 2015).

Choosing the protective cultures suitable for this study, was necessary to pay attention to the several metabolic activities carried out by LABs and not only the production of lactic acid. In fact they are able to lead physical and sensorial changes that include modifications in flavour, shelf life, volume, colour, astringency. They are responsible of the formation of some functional compounds like aglycones, terpenes or bioactive peptides and free aminoacids that are substrates for the formation of aldehydes, biogenic amines, esters and sulphur compounds (Sharma *et al*. 2020). For this reason, it was important to find protective cultures not able to change the sensorial characteristics of Ricotta salata cheese.

We obtained 5 protective cultures suitable to be used on ricotta salata cheese against *Listeria* from 3 commercial supplier. One of them, *Pediococci acidilactici*, was not specific for dairy product, but all the labelled characteristics were suitable for use in this study.

4.2 Materials and Methods

The 5 commercial protective cultures suitable for this phase of the study were selected on the basis of the labelled characteristics which were: the ability to grow at temperatures of 8°C, the resistance to NaCl concentration of 3% and anaerobiosis. Moreover, the cultures were not lactic acid producers to preserve the sensorial characteristics of the product. Using these parameters, the commercial cultures selected were: *Pediococcus acidilactici* (A)*, Lactobacillus plantarum* (B)*, Carnobacterium spp* (C)*, Lactobacillus sakei* and *Carnobacterium spp* (D)*, Lactobacillus plantarum* produced by another producer (E). To evaluate the efficacy of the protective cultures against *Listeria monocytogenes* was followed a modified protocol of the well diffusion assay written by Lewus et al 1991. The test was performed in triplicate using a petri dish per each strain and protective culture.

The strains were grown until the maximum concentration and then diluted to the desired concentration. BHI with agar at 1% was inoculated with a single strain of *Listeria monocytogenes* at the concentration of 10^6 log cfu/ml. 10 ml of the media were poured into petri dishes and left solidified under a laminar flow hood. Sterile tips were used to create 5 wells of 6 mm of diameter per each petri dish. One well was intended for negative control and was filled with sterile buffered saline solution, the other four for the protective cultures at different concentrations. To evaluate the performances simulating the use during the challenge test and in the dairy plant, it was decided to resuspend the protective cultures just before the filling of the wells. The count of 0.1 g of lyophilized protective cultures into 0.1l of buffered saline solution are shown in the *Table 4-1.*

To exclude the interaction with eventual excipients the test was performed with the cultures as they are and after washing passages. To rinse the protective cultures, 2 ml of the suspension at the desired concentration were centrifuged 3 times at 10000 rpm for 3 minutes. The supernatant was thrown away and the pellet was resuspended with 2 ml of buffered saline solution.

Every well was filled with 20µl of different samples:

1) buffered saline solution as it was as negative control,
2) the protective culture at the concentration of 10^8 log cfu/ml
3) the protective culture at the concentration of 10^8 log cfu/ml rinsed
4) the protective culture at the concentration of 10^7 log cfu/ml
5) the protective culture at the concentration of 10^7 log cfu/ml rinsed.

The petri dishes were left in the incubator at 30°C and controlled after 24 and 48 hours measuring the diameter of the inhibition halo in millimetres. The experiment was repeated with lower concentration (10^6 and 10^5 log cfu/ml) that were the working concentration declared by the producers. The four different concentrations *in vitro* were used to determine the ideal concentration *in vivo*.

The selection was made considering those cultures had achieved the best results in absolute terms and, at the same time, also their general trends.

4.3 Results

The negative controls were always negative. All the protective cultures showed their anti-listerial activity during the well diffusion assay but not always or

homogenously. The protective culture E showed a higher sensibility to external factors like oxygen and light and was effective only in the short time after opening the package even if in controlled storage conditions. At the lower concentrations, log 5 and log 6, the protective culture B did not show a halo of inhibition, while the protective culture A was mildly positive only in 3 out of 16. For this reason, it was decided to carry out the experiment only at the concentrations of log 7 and log 8.

The *Table 4-2* shows the results of the well diffusion assay with the higher concentrations.

On the base of these results a selection was made to find the two protective cultures suitable for the use during a challenge test against *Listeria monocytogenes* on ricotta salata cheese.

According to these data, there were 3 protective crops to choose from: A (*Pediococcus acidilactici*), C (*Carnobacterium spp.*) and E (*Lactobacillus plantarum*). In fact, protective culture B was the one with the worst results. There was no activity at log 7, with or without the washing passage, and at log 8 after washing. At log 8, when an activity was evident, it was lower than the other cultures and the halo was weak. The protective culture D was inactive at log 8 after the washing passage and at log 7 most of the times, also without the washing passage. When present, in the 33% of the samples, the positivity was always extremely low, and the halo was not well defined.

All the remaining cultures had shown good activity, but it was decided to use the C and E. Protective culture A reached very good results, but, the diameter of the halo produced by C and E, in some repetitions, was 14 millimetres that was the largest value registered during the experiment. The highest value for culture A was 12 millimetres.

4.4 Discussion

The aim of this phase of the project was to find the protective culture with the stronger effect *in vitro* and this step was necessary because Ricotta salata cheese does not own a specific natural microflora.

We obtained 5 protective cultures suitable to be used on ricotta salata cheese against *Listeria* from 3 commercial supplier. One of them, *Pediococci acidilactici*, was not dedicated to dairy products, but all the labelled characteristics were suitable for use in this study. In the market there are not protective cultures specific for ricotta salata cheese and it was considered better to examine as many options as possible even if only at an early stage of the experiment.

It is interesting to underline the behaviour of the two strains of *Lactobacillus plantarum*. It can depend on the strains utilized, in that case were not indicated by the producers, but also on the excipients.

4.5 Tables

Table 4-1-Counts of 0.1 g of powder into 0.1l of physiological solution. mean ± standard deviation. log 10 cfu/g.

Protective culture	*Mean ± standard deviation*
A	7,48±0,01
B	7,31±0,01
C	8,65±0,15
D	8,17±0,04
E	7,73±0,18

Table 4-2- Well diffusion assay, the diameters of the halo of inhibition, mensuration after 24 and 36 hours. PC=protective culture w=washed Average ± standard deviation (mm)

	24 hours					36 hours				
PC	NC	log8	log7	log8 w	log7 w	NC	log8	log7	log8 w	log7
A	0	11±1.22	6.33±0.52	9±0.93	6±0	0	11.22±1.09	8.43±1.81	9.67±0.82	6±0
B	0	9.67±1.03	0	0	0	0	8.88±1.36	0	0	0
C	0	11±1.58	6±0	6.56±0.73	6±0	0	11.22±1.20	6±0	6.44±0.73	0
D	0	11±1.22	6±0	6±0	0	0	11.44±1.59	6±0	6±0	0
E	0	11.44±1.74	7±1	9.67±0.82	0	0	11.89±1.36	7.20±0.45	9.67±1.37	0

Chapter 5 - Inhibition of *L. monocytogenes* in Ricotta Salata by protective cultures

5.1 Introduction

Listeria monocytogenes control strategies

Listeria monocytogenes is characterized by the ability to grow at different conditions comprising a wide range of temperatures (between 0 and 45°C), pH (between 4.4 and 9.4) and salt concentration (until 13-14 wt/vol %). It is also fundamental to its persistence the rapid adaptability to changing environmental conditions and the capability to form biofilm (Henderson *et al*. 2019).

Different strategies have been developed to control bacteria that cause foodborne disease like *Listeria monocytogenes.* It is possible to apply different approaches: physical methods such as pasteurization, radiation, ultrasound, UV, pulsed light, electricity, cold plasma; chemical methods with the addition of substances like benzoates, sorbates, nitrites and sulphites that are preservatives; the biological method that can be represented by the use of natural products with an antimicrobial activity or natural antagonists like bacteriophages or Lactic acid bacteria (LAB) (Yi *et al.* 2020). Some of these strategies are used as post-lethality treatments, mostly in ready to eat foods that are characterized by the absence of further treatments able to reduce or control *Listeria monocytogenes* before the consumption (Spanu *et al.,* 2015c).

Actually, in some Sardinian dairy plant, a post-lethality treatment is performed immerging the vacuum-packed ricotta salata cheese in hot water baths (85°C) for 90 minutes and chilling the immediately product till reaching 4±2°C. This treatment permits to eliminate contaminations with a concentration of *Listeria monocytogenes* about 6 log (Spanu *et al*. 2013).

Ricotta salata cheese and protective cultures

Because of the high temperatures reached during the production, the Ricotta salata cheese is not characterized by a typical microflora of its own, only some spores are able to survive, and subsequent contaminations are possible due to manipulations after the heating. Shelf life varies, on the base of the decision of the Food Business Operators, from 3 weeks to 6 months. The intrinsic characteristics, the technology and the shelf life, are the reasons way pathogens and spoilage microorganisms are able to reach high concentrations on Ricotta salata cheese (Casti et al., 2016). The utilization of commercial protective cultures can provide artificially the protection that other foods own naturally. While, to preserve fresh ricotta cheese, C*arnobacterium spp.* was used to prevent *Pseudomonas spp.* successfully (Spanu *et al*. 2018), nevertheless, in literature there are not examples of the use of protective culture on Ricotta salata cheese. Considering the LABs can explicate their protective activity through different mechanisms, it is necessary to make the selection on the base of the characteristics of the product. Some of them are able to produce lactic acid or other metabolites or lower the pH and, for this reason, are not suitable to be used on Ricotta salata cheese. Others compete for nutrients with unwanted microorganisms or produce bacteriostatic or bactericidal products (Delboni & Yang 2017). Bacteriocins are more effective against Gram positive, like *L. monocytogenes* but are active also against Gram negative if there are damages in the external membrane of the cells (Arqués *et al*. 2015).

5.2 Materials and Methods

Ricotta salata samples

The challenge test was performed on 180 wedges of vacuum-packed ricotta salata cheese belonging from 3 different production batches produced into an industrial Sardinian dairy plant in May 2018. The analyses were performed in 3 separate trials.

The wedges were stored at refrigeration temperature (4°C) until their utilization for the experiment. The samples, heat treated at 90°C for 20 minutes to eliminate natural contaminations and were divided into 4 groups: Blanc samples (B); Positive Controls (PC), inoculated with a pool of *Listeria monocytogenes* strains and the Challenge Units inoculated with a pool of *Listeria monocytogenes* strains and one of the two protective culture (CU1 and CU2). The samples have been analysed 6 hours after the inoculation (T_0) and it was decided to repeat the analysis 30, 60, 75 days after the inoculation (T_{30}, $T_{60}T_{75}$, respectively).

Protective cultures

To evaluate the possibility to use protective cultures to limit and control the growth of *Listeria monocytogenes* on Ricotta salata cheese, two different commercial cultures were selected on the base of the results of in vitro tests.

Both the cultures are characterized by specific properties that make them able to growth at the needed conditions:

- temperatures of 8°C during the storage;
- resistance to NaCl concentration of 3%;
- anaerobiosis.

Furthermore, to preserve the sensorial characteristics of the product, the cultures were not lactic acid producers.

The cultures were tested with others with the same characteristics utilizing the well diffusion assay, following the protocol by Lewus and Montville (1991). The tests were performed using the cultures at different concentrations (10^5, 10^6, 10^7 and 10^8 log

cfu/g) with or without an additional passage to wash the cells. The results of the different cultures were compared to choose the two with the best performances at test conditions. The following protective cultures were selected:

1) *Lactobacillus plantarum* (LP-C)
2) *Carnobacterium* spp. (CS-C)

The cultures lyophilized and stored at -18°C, have been resuspended in buffered saline water (Sigma-Aldrich, Missouri USA) to obtain the concentration of 10^8 log cfu/ml in the solution and 10^6 log/g on the surface of the product as indicated by the manufacturer in the label. 2.5 ml of the suspension has been sprayed on the surface of the samples, the product has been massaged to permit the adhesion of the cultures and, after the time necessary to let the products dry, the wedges have been packed under vacuum conditions and stored at 8°C.

Choice of the strains

To simulate a natural contamination, a pool of 4 strains of *Listeria monocytogenes* was created using strains from the collection at the Department of Veterinary Medicine of Sassari. The strains were isolated from dairy plants, in particular from Ricotta cheese and surfaces related to it. Moreover, the reference strain NCTC 10778 has been added to the others. Each strain was representative of one of the major serogroups of *Listeria monocytogenes*: 1/2a, 1/2b, 1/2c, 4b. All the strains were stored at -80°C in Brain Heart Infusion (BHI) with 10% glycerol.

Preparation of the inoculum

A subculture of each strain was incubated at 37°C overnight in Brain Heart Infusion (BHI) with 1.5% agar petri dishes. One colony from each subculture was transferred into a tube containing 10 ml of BHI and left 18 hours into a shaking bath at 30°C till reaching the early stationary phase. To adapt the strains at the storage conditions,

the suspensions were diluted 10/1 using BHI and incubated at 8°C for one week. To obtain the final inoculum, the mixture of the suspensions was diluted using buffered saline solution (Sigma-Aldrich, Missouri, USA) till reaching the final concentration. To control the inoculum level the suspension was diluted and enumerated on ALOA petri dishes. The final concentration was calculated to achieve a concentration lower than 10^2 log cfu/g of ricotta salata cheese.

Experimental design and analysis

The samples have been analysed at T_0, 6 hours after the inoculation, and at T_{30}, T_{60} and T_{75} after 30, 60 and 75 days respectively. To wait 6 hours is necessary to permit the adaptation of the microorganism to the new environment, but it is not enough to start the growth and distort the results. At each sampling time the pH and the a_W of all the groups of samples were measured to evaluate the eventual influence of the presence or the absence of the inoculum. In addition to chemical and physical analyses, microbiological analyses were performed to evaluate the behaviour of *Listeria monocytogene*s (ISO11290-2:2017) and of the total bacterial count (ISO 4833-1 2013). The enumeration of *Bacillus cereus* was performed using BACARA (bioMérieux-Lyon, France) following an alternative ISO16140:2016 validated method (1ml of 1:10 eluted sample was spreaded and left for 24 hours at 30°C) and the count of the *Enterobacteriaceae* eventually present was made according to ISO 21528-2:2017. De Man, Ragosa and Sharpe (MRS) agar (Oxoid, Basingstoke-UK) was used to enumerate LABs with a particular attention to colonies with an aspect attributable to *Lactobacillus plantarum* following ISO 15214 1998. Modified MRS was used to enumerate *Carnobacterium spp.,* usually in MRS there is acetate that causes an underestimation of this family of bacteria because of the inhibition of the growth. For the experiment MRS was modified omitting acetate and raising the value of the pH till 8.5 to improve the

selectivity of the media. For microbiological analysis, 25 g of ricotta were scratched under sterile conditions from the rind (2-5 mm depth) of each wedge and diluted into 225 ml of Buffered Peptone Water (Sigma-Aldrich, Missouri, USA).

5.3 Results

Intrisic properties

The average of the pH of the wedges analysed was 6.19±0.22 while the mean a_W value was 0.969±0.003. The values of pH and a_W for each batch are given in *Table 5-1.*

Microbiological profile

In all the samples except 2 the *Enterobacteriaceae* and in all the wedges yeasts and moulds, were always under the detection limit of the method in all the sampling times. Low positivity for *Bacillus cereus* was found in 2 samples of the first batch at T_0 and T_{30}, and in 3 samples of the third batch at T_0. In the second batch the level was always under the detection limit of the method at all the sampling times. The *Table 5-2* shows the Total Bacterial Count at T_0.

At the end of the experiment the differences were evident only for the Total Bacterial Count, but not for the other parameters (*B. cereus*, *Enterobacteriaceae* and yeasts and moulds), as shown in the *Table5-3* that indicates the values obtained for the Total Bacterial Count at the end of the experiment (T_{75}).

Listeria monocytogenes and protective cultures

Listeria monocytogenes was always under the detection limit of cfu/g in all the blanc samples at all the sampling times. At T_0 the count confirmed that the concentration of the inoculum was the desired one with an inoculum level always lower than 10^2 $\log_{10}$ cfu/g in all the different samples of the three batches as showed in the *Table 5-4.*

Listeria monocytogenes at T_{75} has achieved levels of 8.40±0.67 $\log_{10}$ cfu/g. The *Table 5-5* shows the individual data by sample.

The media utilised for the enumeration of *Lactobacillus plantarum* and *Carnobacterium spp.*, MRS and modified MRS, are not selective enough to permit to discriminate irrefutably the colonies or to limit the growth of background microorganisms. For this reason, it was not possible to enumerate precisely the protective cultures on the product, but only the concentration of the inoculum.

5.4 Discussion

The results obtained during the challenge test showed that the initial level of inoculation of *Listeria monocytogenes* was the desired one. To have less than 2 $\log_{10}$ cfu/g was fundamental to simulate a natural contamination. To examine the wedges at T_0 six hours after the preparation of the samples allowed the microorganisms to adapt to the new environmental conditions avoiding underestimations. At the same time this pause is not enough to permit the bacteria multiplication causing an overestimation.

Despite the good results obtained during the *in vitro* tests, the antilisterial activity of the protective cultures *in vivo*, has not been effective enough to contrast the growth of the pathogen. The causes of this can be manifold. First or all ricotta cheese is characterized by a varied bacterial background that can interfere with adhesion and multiplication of the protective culture. This interference may be due to the ability to alter the surrounding environment through the production of substances, even only the normal metabolites produced by bacteria. Furthermore, to simulate the use inside an industrial dairy plant, the protective cultures were suspended in a sterile, buffered saline solution at the moment of the utilization. In addiction *Listeria* is able to growth at temperature of

refrigeration (Halter *et al.,*2013) and at 8°C it is possible that they are less difficult to replicate than protective cultures, for which this value represents the minimum limit.

For these reasons it was not possible, for the culture, to start growing fairly tight to start early the production of bacteriocins, closely related to multiplication. Moreover, a delay in the adhesion and multiplication, limit the inhibition due to the competition for space and nutrients that are part of the mechanisms used to control unwanted microflora.

5.5 Tables

Table 5-1-pH and water activity for each batch at T_0, T_{30} and T_{75}. Average ± standard deviation. log cfu/g.

Analysis	Batch								
	1			2			3		
	T_0	T_{30}	T_{75}	T_0	T_{30}	T_{75}	T_0	T_{30}	T_{75}
pH	6.21±0.09	6.02±0.36	6.11±0.12	6.22±0.11	6.28±0.10	6.225±0.33	6.31±0.25	6.08±0.11	6.10±0.28
a_W	0.97±0.001	0.968±0.003	0.970±0.002	0.97±0.002	0.967±0.002	0.969±0.002	0.97±0.002	0.968±0.003	0.969±0.003

Table 5-2- Total Bacterial Count at T_0. Average ± Standard log cfu/g..

	Blanch samples	Listeria	Listeria+LP-C	Listeria+CS-C
$B1T_0$	5.34 ± 1.14	5.52 ± 1.44	7.00 ± 0.39	7.33 ±0.40
$B2T_0$	6.62± 0.56	6.35 ± 0.43	6.62 ± 0.33	7.00 ±0.40
$B3T_0$	5.34± 0.45	6.45 ± 0.72	7.08 ± 0.42	6.89 ±0.22

Table 5-3-Total Bacterial Count at T_{75}, Average ± standard deviation. log 10 cfu/g.

	Blanch samples	Listeria	Listeria+LP-C	Listeria+CS-C
$B1T_{75}$	9,04±0,25	9,39±0,10	9,14±0,33	9,37±0,07
$B2T_{75}$	6,45±0,24	8,80±0,01	8,55±0,18	8,92±0,23
$B3T_{75}$	7.89±0.25	9.18±0.09	8.90±0,35	8.74±0,07

Table 5-4- Listeria monocytogenes inoculum level. Average ± Standard deviation log cfu/g.

	Blanch samples	Listeria	Listeria+LP-C	Listeria+CS-C
$B1T_0$	(0/9)	1.33 ± 0.31	1.42 ± 0.10	1.16 ±0.28
$B2T_0$	(0/9)	1.10 ± 0.17	1.10 ± 0.17	1.20 ± 0.17
$B3T_0$	(0/9)	1.4 ± 0.46	1.87 ± 0.38	1.39 ± 0.12

Table 5-5- Listeria monocytogenes final level. Average ± Standard deviation log cfu/g.

	Blanch samples	Listeria	Listeria+LP-C	Listeria+CS-C
$B1T_{75}$	(0/9)	6,83±1,87	8,58±0,28	8,63±0,42
$B2T_{75}$	(0/9)	8,85±0,11	8,38±0,62	8,6±0,68
$B2T_{75}$	(0/9)	9.25±0.06	8.24±0.40	8.23±0.86

Chapter 6- High Hydrostatic Pressures treatment to inhibit *L. monocytogenes* in Ricotta Salata cheese

6.1 Introduction

High Hydrostatic Pressures (HHP) treatment

Several studies have been performed to evaluate preservation technologies alternative to heat treatments. They can be chemical or biological agents, such as bacteriocins, protective cultures and lytic enzymes or physical agents such as magnetic or electrical field, ionization, light pulses, ultrasounds and high hydrostatic pressures. Some of these strategies have been already applied, other are still studied to evaluate the commercial application (Trujillo *et al*., 2000). This technology is commonly used to treat several products including meat products, vegetable, fruit juice and cheese. In these products there are not significant differences in the sensorial characteristics between the product with or without the treatment (Batty *et al*., 2019). The use of HPP on packaged cheese is a way to control the growth of *Listeria monocytogenes* and to extend the shelf life in cold storage conditions (Tomasula *et al*., 2014).

In general, it is demonstrated that a treatment performed with pressures between 400 and 600 MPa for several minutes, is enough to inactivate vegetative microorganisms. However, it is important to limit the time of application and the pressures of the treatment because of two reasons: sometimes, it can cause alterations such as lipid oxidation and texture changes and, from a commercial point of view, to minimize the equipment wear. Therefore, it is necessary to find a balance between effectiveness and secondary effects utilizing the shorter time and the lower pressure possible (Stratakos *et al.*, 2016).

Several studies demonstrated the efficacy of the treatment against *Listeria monocytogenes* in different cheeses. The parameters selected and the results obtained were various for example in queso fresco a treatment of 600MPa for 5 minutes lead a reduction of 4.6 log cfu/g and in fresh goat cheese was achieved a reduction above 5 log cfu/g using 550 MPa or 450 MPa for 5 or 10 minutes respectively. The same values were obtained applying 500 MPa for 5 and 20 minutes on a model washed-curd cheese matrix (Batty *et al*., 2019). The application of different combination of time and pressure reduced on Turkish white cheese resulted in a reduction of *L. monocytogenes* of 3.1 log cfu/g at 300 MPa for 5-10 minutes and 4.4-4.9 log cfu/g at 600 MPa for 10-10 minutes (Akdemir *et al*., 2008).

Ricotta salata cheese

A Sardinian dairy plant produced the ricotta salata cheese used during this study. Ricotta salata is a traditional whey cheese produced in Sardinia using the whey obtained from the production of cheeses with ewe milk, mainly Pecorino Romano, an Italian PDO (Protected Designation of Origin). The technology of production makes this particular quality of ricotta suitable for the application of HPP and resistant at the treatment without modifications in shape and consistence unlike the ricotta gentile (a not salted quality that is creamy and too soft). The greater consistency is obtained maintaining the whey at temperatures above 85°C at least for 30 minutes during the coagulation of the whey proteins and leaving the mass obtained under a press for 24 hours. The blend is, in this phase, inside moulds coated by cheese cloths to permit the leakage of the remaining whey (Casti *et al.,* 2016).

6.2 Materials and Methods

Ricotta salata samples

To evaluate the effectiveness of the HPP treatment, 108 wedges of vacuum-packed ricotta salata cheese weighing 250g per each, have been made by a Sardinian industrial manufacturer in April 2019. The samples belonged from three different production batches and the experiment was carried out in 3 separated trials. The wedges of ricotta salata were sent to the IRTA-Institute of Agrifood Research and Technology a research centre in Spain with the industrial equipment and the microbiology laboratory to perform the treatment and host the inoculation and the analyses of positive controls and of the samples at T_1. The next steps have been performed in the laboratories of inspection of food of animal origin at the department of veterinary medicine in Sassari. The shipments and the storage were made at refrigeration temperature (4±2°C).

The samples were divided into 4 groups:

- Positive Samples + (PS+), 9 wedges of ricotta salata inoculated with high concentration inoculum;
- Positive Samples - (PS-), 9 wedges inoculated with low concentration inoculum;
- Challenge Units + (CU+), 45 wedges of ricotta salata inoculated with high concentration inoculum and HPP treated;
- Challenge Units - (CU-), 45 wedges inoculated with low concentration inoculum and HPP treated.

In addition to the experimental samples, 18 wedges of ricotta salata cheese have been analysed to assess the absence of natural contaminations.

The ricotta salata cheese was stored at 4±2°C during all the study.

Preparation of the inoculum

Five strains of *Listeria monocytogenes* were selected from the collection of the Department of Veterinary Medicine, four wild strains plus one reference strain, representative of all the main serogroups as showed in *Table 3.1*, the strains were singularly subcultured and incubated at 37°C overnight in Brain Heart Infusion (BHI) with 1.5% agar petri dishes.

One colony from each subculture was transferred into a tube containing 10 ml of BHI and left 18 hours into a shaking bath at 30°C till reaching the early stationary phase. To adapt the strains at the storage conditions, the suspensions were diluted 10/1 using BHI and incubated at 4°C for one week. To obtain the final inoculum, the mixture of the suspensions was diluted using buffered saline solution (Sigma-Aldrich, Missouri, USA) till reaching the final concentration. To control the inoculum level the suspension was diluted and enumerated on ALOA petri dishes. The samples have been inoculated with a mix of 5 different *Listeria monocytogenes* strains at 2 concentrations: 2 and 5 log10 cfu/g. The low level of contamination has been chosen to simulate a natural contamination; the high level was necessary to estimate the reduction of the contamination. 90 wedges were treated with HPP, the other 18, nine with low level and 9 with high level, were analysed 6 hours after the inoculation to evaluate the initial level of the inoculum. It was decided to wait 6 hours to give the strains the possibility to adapt to new conditions but not enough to start multiplication. 2.5 ml of the suspensions (10% of the weight) were spreaded on the surface of the products in a laminar flow cabinet. Every sample was massaged to permit the adsorption of the liquid and the adhesion of the bacteria and then singularly vacuum packed.

Experimental design and analysis

At all the sampling times, all the samples were analysed to evaluate the modification of the microbiologic profile of the ricotta salata cheese wedges after the HPP treatment. The analysis have been performed at different times: PS+ and PS- have been analysed at T_0, 6 hours after the inoculation while CU+ and CU- have been analysed at T_1, T_{14}, T_{21}, T_{30} and T_{60} respectively 1, 14, 21, 30 and 60 days after the inoculation and the treatment. The parameters considered are: *Listeria monocytogenes*, analysis for the detection and enumeration, according to ISO11290-1 2017 and ISO11290-2:2017 respectively; the evaluation of the Total Bacterial Count at 30°C according to ISO 4833-1 2013; the enumeration of the mesophilic lactic acid bacteria following the standard ISO 15214 1998; the count of the *Enterobacteriaceae* eventually present was made according to ISO 21528-2:2017. To perform the microbiological analysis, 25 g of ricotta were scratched under sterile conditions from the rind (2-5 mm depth) of each sample and diluted with 225 ml of Fraser Broth Base (Sigma-Aldrich, Missouri, USA).

HPP treatment

The ricotta salata wedges, after inoculation, have been singularly vacuum packed. To prevent leakages and cross contaminations the wedges have been packed three by three into a secondary bag. Other bags have been used to pack together two secondary bags per each. In addiction another bag was added. A total of four bags per each sample were used to pack the ricotta salata cheese.

The treatment applied on the samples was 500 MPa at 10°C for 300 seconds.

6.4 Results

Microbiological profile

Bacillus cereus was under the detection limit in all the samples at every sampling time.

The Total Bacterial Count was reduced immediately after the treatment, when there has been a reduction of about 3 log 10 cfu/g. Indeed, as showed in the *Table 6-1* the initial level about 8 log cfu/g has been reduced by the HPP treatment up to 6 log cfu/g with an increase up to 9 log cfu/g after 21 days and stability in the following sampling times until the end of the experiment.

The enumeration of lactic acid bacteria followed the same trend of the Total Bacterial Count as showed in the *Table 6-2* with an initial average count of 8.08±0.27, an average count of 4.43±0.42 at T_1 immediately after the treatment until reaching an average count of 9.30±0.13 at T_{60}.

The trend followed by the *Enterobacteriaceae* was different, as shown in *Table 6-3*. Indeed, it is possible to highlight that, with an initial level of *Enterobacteriaceae* about 7 log cfu/g, 24 hours after the treatment, only one sample out of 9 was positive. At the end of the experiment, no sample has reached the initial level.

The concentration of *Listeria monocytogenes* obtained with the inoculation was determined analysing the Positive Samples 6 hours after it. The qualitative analysis of these samples was not performed. The *Table 6-4* shows the results of the analysis for the enumeration of *Listeria monocytogenes* at T0.

With regards to the CU- samples, the analysis at T_1 showed that in only one sample was possible to enumerate *Listeria monocytogenes*, but, at the qualitative analysis, the samples were all positive except one. In the quantitative analysis at T_{14}, only one sample was above the detection limit of the method while, at the qualitative analysis, all the samples were positive. Even at T_{30} it was possible to enumerate *Listeria monocytogenes* only in one sample, but the qualitative analysis at the same sample time

was positive only for five samples out of nine. At the last sample time, T_{60}, as many as three samples out of nine were positive and *Listeria monocytogenes* resulted present at the qualitative analysis of six samples out of nine.

If instead we go to consider the CU+ samples, it is possible to note a decrease up to values of 1.57±0.21 log cfu/g and all samples positive for both quantitative and qualitative analysis at T_1. At T_{14} the enumeration at the quantitative analysis was 3.31±1.17 but four samples out of nine were negative and three of them were negative also at the qualitative analysis. After one week, at T_{21}, the average was 3.24±062 and three samples out of nine were negative for both the analysis: enumeration and presence. The concentration increased further at T_{30} with an average of 4.36±0.03, but only three samples out of nine were positive at the quantitative analysis. While, At the qualitative analysis, six samples out of nine were positive. At the end of the experiment, T_{60}, the average was 3.10±0.45 with six samples out of nine positive and only one negative sample out of nine at the qualitative analysis. The *Table 6-5* shows the evolution of *Listeria monocytogenes* after the treatment while the *Table 6-6* sums up the positive samples at the qualitative analysis.

6.5 Discussion

24 hours after the treatment a reduction of the Total Bacterial Count is evident. The difference is reduced at T_{14} and at T_{21} the initial level is exceeded in all the batches. That indicates that the microflora on the surface of the product was affected by the treatment but, after a recovery period, the replication started again until the achievement of a concentration about 9 log cfu/g. The same happened with the other parameters with

the exception of *Enterobacteriaceae* that remained at a lower level than the initial one, till the end of the experiment.

The initial enumeration of *Listeria monocytogenes* in the low-level samples was the desired one, while, in the high-level samples, it was slightly lower but enough to achieve the objectives.

Considering the results of the quantitative analysis of the high-level samples the results showed that there was a reduction of 3 log and that the microorganism was affected enough to replicate slower than usual, reaching a level of 3.10±045 log after 60 days. That is a very low increase considering the rapidity of replication of *Listeria monocytogenes* on Ricotta salata cheese, even if under refrigerated storage condition.

Also the results obtained enumerating the low level samples were good, but not enough to guarantee the safety of the product especially when evaluated together with the qualitative analysis. It is clear that the treatment was able to cause a lethal damage on part of the microorganisms, but there was another part that suffered sublethal damage. The risk is the recovery of the cells with the replication restart up to dangerous levels of contamination.

Taking into account the fact that the pathogen was affected by the treatment, it would be desirable investigate this possibility with different combinations of time and pressures and for the entire shelf life.

6.6 Tables

Table 6-1 Total Bacterial Count at T_0, T_1, T_{14}, T_{21}, T_{30} and T_{60} (average ± standard deviation log cfu/g). At T0 the samples are not treated.

	Batch	T_0	T_1	T_{14}	T_{21}	T_{30}	T_{60}
Low level	1	8.16 ±0.27	5.93±0.29	8.41±0.55	*nd*	9.64±0.49	9.38±0.14
	2	8.54±0.18	5.97±0.16	8.20±0.70	*nd*	9.47±0.12	9.47±0.10
	3	8.34±0.09	6.15±0.50	8.37±0.41	*nd*	9.26±0.24	9.43±0.02
High level	1	8.04±0.27	6.04±0.39	7.67±0.48	9.16±0.11	9.57±0.11	9.45±0.12
	2	8.59±0.17	6.33±0.35	8.73±0.17	9.06±0.23	9.18±0.20	9.29±0.09
	3	8.54±0.14	6.24±0.13	8.88±0.12	9.32±0.09	9.28±0.21	9.22±0.03

Table 6-2-Lactic Acid Bacteria at T_0, T_1, T_{14}, T_{21}, T_{30} and T_{60} (average ± standard deviation log cfu/g). At T_0 the samples are not treated.

	Batch	T_0	T_1	T_{14}	T_{21}	T_{30}	T_{60}
Low level	1	8.52±0.24	4.20±0.37	8.21±0.06	*nd*	9.19±0.16	9.27±0.23
	2	8.19±0.19	4.73±0.85	-	*nd*	9.24±0.14	9.33±0.22
	3	8.49±0.28	5.40±0.68	7.56±0	*nd*	8.80±0.19	9.29±0.08
High level	1	7.47±0.55	4.51±0.15	7.85±0.19	8.74±0.41	8.03±0.74	9.40±0.11
	2	8.42±0.31	5.56±0.31	-	9.20±0.13	8.38±0.54	0.29±0.06
	3	8.38±0.04	5.15±0.16	-	9.30±0.15	7.98±0.42	9.24±0.07

Table 6-3- Enterobacteriaceae at T_0, T_1, T_{14}, T_{21}, T_{30} and T_{60} (average ± standard deviation log cfu/g). At T_0 the samples are not treated. In brackets the number of positive samples per each batch

	Batch	T_0	T_1	T_{14}	T_{21}	T_{30}	T_{60}
Low level	1	6.84±0.58	-	2.17±0	*nd*	4.98±0.59	5.84
	2	6.69±0.17	-	4.59±0.31	*nd*	4.98±0.78	5.09±0.80
	3	6.88±0.23	-	2.97±0.21	*nd*	4.96±0.24	4.66±0.20
High level	1	6.73±0.25	-	3.17±1.33	-	3 (1/3)	-
	2	7.15±0.17	-	3.35±0.80	4.03±0.41	4.29±0.76	4.70±1.10
	3	6.65±0.33	1.54±0.34	3.61±0.40	4.83±0.28	5.02±0.53	3.83±1.44

Table 6-4- Listeria monocytogenes inoculum level (average ± standard deviation log cfu/g).

Batch	Low level	High level
1	2.21 ± 0.23	4.27 ± 0.45
2	2.33 ± 0.26	4.16 ± 0.09
3	2.30 ± 0.39	4.34 ± 0.14

Table 6-5 Listeria monocytogenes at T1, T14, T21, T30, T60. Average ± standard deviation log cfu/g. In brackets the number of positive samples per each batch

	Batch	T_1	T_{14}	T_{21}	T_{30}	T_{60}
Low	1	1.30 (1/3)	(0/3)		(0/3)	2 (1/3)
level	2	(0/3)	3±0 1/9		2.48 (1/3)	(0/3)
	3	(0/3)	(0/3)		(0/3)	2.69±0.30 (2/3)
High	1	1.80±0.04	3.19±0.32	3.87±0.51	4.72±0.03 (1/3)	4.65±0.40
level	2	1.39±0.36	3.42±2.01	3.55±0.72 (2/3)	(0/3)	2.3 (1/3)
	3	1.52±0.24	(0/3)	2.30±0 (1/3)	3.99 (1/3)	2.34±0.49 (2/3)

Table 6-6 Positive samples at qualitative analysis for Listeria monocytogenes. Three samples per batch.

	Batch	T_1	T_{14}	T_{21}	T_{30}	T_{60}
Low level	1	(3/3)	(3/3)	nd	(2/3)	(2/3)
	2	(3/3)	(3/3)	nd	(2/3)	(2/3)
	3	(2/3)	(3/3)	nd	(1/3)	(2/3)
High level	1	(3/3)	(3/3)	(3/3)	(3/3)	(3/3)
	2	(3/3)	(2/3)	(2/3)	(0/3)	(3/3)
	3	(3/3)	(1/3)	(1/3)	(3/3)	(2/3)

Chapter 7-General discussion

This study investigated possible solutions suitable to control *Listeria monocytogenes* in ricotta salata cheese, considering technologies that are commonly used to preserve other foods, never applied on this product. In the last few years, ricotta salata cheese was subject to recals due to listeriosis outbreaks or, recurrently, for unacceptable levels of L. monocytogenes contamination. This made necessary to provide research programs aiming to give a more comprehensive knowledge about the interaction between the microorganism and this product. *L. monocytogenes* is able to survive under extreme environmental conditions thanks to its capability to form biofilm and giving also persistent contaminations. The food contact and non-food contact surface represent the source of the contamination in food processing facilities, frequently occurring in post process steps (e.g. salting, ripening, product maintenance and portioning). The use of high temperatures forms during the production inactivate bacterial vegetative cells that eventually contaminate the milk whey, but this phase is followed by a sequence of manipulations that can lead to post-process contamination (Casti *et al.*, 2016b; Spanu *et al.,* 2013). The under vacuum packaging of ricotta salata cheese, that can reduce the growth of the aerobic microrganisms, does not prevent the *L. monocytogenes* growth.

The possibility to replicate at refrigeration temperatures, permits the microorganism to reach infectious doses, in foods that may support its growth, even with low level of initial contamination (Barría *et al.,* 2020). For this reason, the curve of growth was built, the maximum growth rate under conditions of thermal abuse was calculated and the use of a predictive software was validated. The results obtained in this study demonstrated that, at 8°C, the maximum growth rate is 0.020±0.002 log cfu/h (0.48 log

cfu/day) confirming that ricotta salata cheese represents the ideal environment for Listeria not only because of its intrinsic characteristics (pH of 6.3-6.7 and a_W 0.950-0.970).

The curve and the growth rate permitted to evaluate the interaction between Listeria monocytogenes and ricotta salata cheese without treatments.

In literature it is possible to find the evidences that the use of protective cultures on dairy products leads to uneven results. The use of bacteriocins producer bacteria is preferred to the direct use of bacteriocins because of the food legislation and because, alone, they cannot guarantee protection against contamination of dairy products (Silva *et al.,* 2018). It is necessary to underline that ricotta salata cheese is a whey cheese, the coagulation of the whey proteins is due to heating and the technology does not include a phase of acidification ((Casti *et al.,* 2016). As the process does not involved a fast and huge microbial growth, the protective cultures should be applied only on the final products, affecting the possibility for these microorganisms to reach easily high levels in the product. The protective cultures growth should occur during the post-process steps, in under vacuum and refrigerated environments, that limit the choice of microbial species that can be used only to that ones that can grow also at low temperature in anaerobiosis.

Another limiting factor that should be considered is represented by the sensorial characteristics of the product. As non-lactic acid bacteria growth is not provided during the production, resulting in pH ranging between 5.7-6.4, the protective cultures to be used should be limited to species with low ability to produce lactic acid from lactose. Despite the good results obtained during the tests *in vitro*, where a halo of inhibition showed clearly the anti-listerial activity, the challenge test demonstrated that the protective cultures used for this project were not able to contrast the growth of *Listeria monocytogenes* on ricotta salata cheese. The sampling times did not permit to understand if there was an initial inhibition and a subsequent recovery or if the *Listeria*

monocytogenes cells grew so fast that the protective cultures were not able to adapt themselves to the new environmental conditions. A slow growth can limit the protective activity, not only because the microorganism produces bacteriocins during active replication, but also because the inhibition is also due to indirect mechanisms such as competition for nutrients and space of adhesion. It is desirable to investigate if it is possible, like on other dairy products, the utilization of protective cultures in association with other hurdle technologies able to work in synergy.

The last part of the project involved the HPP technology, a treatment already used on several foods, included dairy products (Batty et al 2019). The ricotta salata wedges were suitable for these treatment because of the texture, that is hard enough to resist to high pressures, and the packaging technology because usually ricotta salata is vacuum packed into plastic film bags. Obviously, the plastic bags have to be suitable for the utilization with high pressures.

Considering only the enumeration results, *Listeria monocytogenes* was highly affected by the pressures at 500 MPa for 5 minutes. The treatment was able to reduce the concentration of the pathogen and the reduction was enough to limit it, in most of the samples, under 100 cfu/g. Unfortunately, there was at least a positive sample at every sampling time at the quantitative analyses and most of ricotta wedges was positive at the qualitative analyses. The ricotta salata has got a very long shelf-life, sometimes even 6 months, depending on the producers. This period can be enough to permit the recovery of the damaged cells and the beginning of the multiplication that, as showed in the first part of this project, can be extremely rapid, until reaching 6 log in 10 days. It could be interesting to indagate the behaviour of *Listeria monocytogenes* on ricotta salata cheeses using other combinations of time and pression, but always taking into consideration that

to strong or to long treatments can alter the sensorial characteristics of ricotta and can cause wear of machinery that is not convenient from a commercial point of view.

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