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# *Listeria monocytogenes* in milk and cheese: studies of prevalence, behaviour and growth modelling during cheesemaking and shelf life

Ph. D. thesis

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At Luca

If you really want to do something, you will find a way.
If you don't, you will find an excuse.
(African proverb)

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# 1. INTRODUCTION

## 1.1. *Listeria monocytogenes*

### 1.1.1. Basic informations

*Listeria* are Gram-positive, facultative anaerobic, non-spore-forming, rod-shaped bacteria with a low G+C content. The genus consists of six species i.e. *Listeria monocytogenes*, *Listeria ivanovii*, *Listeria seeligeri*, *Listeria innocua*, *Listeria welshimeri* and *Listeria grayi*, of which only *L. monocytogenes* is the primary human pathogen although there have been rare reports of illnesses caused by *L. seeligeri, L . ivanovii* and *L. innocua* (Perrin et al., 2003; Gasanov et al., 2005). *Listeria* is a small (0.5-2 µm x 0.5 µm)bacillus, isolated or arranged in small chains, motile at 20-25°C. It is aerobic and facultatively anaerobic, catalase-positive except for a few rare strains, oxidase negative and hydrolyses esculin. *Listeria* ferments many carbohydrates without producing gas. Strains of *L. monocytogenes* species are always D-xylose negative and produce lecithinase. They are generally β-haemolytic and L-rhamnose positive. This species is divided into 13 serovars based on somatic and flagellar antigens. Since 2005, these serovars have been replaced by 5 genoserogroups determined by PCR: IIa (serovars 1/2a and 3a), IIb (serovars 1/2b and 3b), IIc (serovars 1/2c and 3c), IVb (serovars 4b, 4d and 4e) and L (other serovars). Of these, IVb followed by IIa and IIb are the genoserogroups most frequently implicated in human cases (EURL, 2014).

*L. monocytogenes* is an opportunistic intracellular pathogen that has become an important cause of human foodborne infections worldwide (Liu, 2006). Foodborne listeriosis, caused by the pathogen *L. monocytogenes*, is a relatively rare but serious disease with high fatality rates (20–30%) compared with other foodborne microbial pathogens, such as *Salmonella*. (FAO/WHO, 2005). While *L. monocytogenes* causes a relatively mild gastroenteritis in healthy adults, the illness can be severe in susceptible individuals. Basically, *L. monocytogenes* most often affects those with a severe underlying disease or condition (e.g. immunosuppression, HIV/AIDS, chronic conditions such as cirrhosis that impair the immune system); pregnant women;

unborn or newly delivered infants; the elderly. Symptoms range from flu-like illness to severe complications including meningitis, septicaemia, spontaneous abortion or listeriosis of the newborn (FAO/WHO, 2005). The *Listeria* species are tolerant to extreme conditions such as low pH, low temperature and high salt conditions (Sleator et al., 2003; Liu et al., 2005). *L. monocytogenes* is a psychrotrophic bacteria which is able to grow at -1.5°C, and thus may grow well at refrigeration temperatures. The microorganism has the ability to persist in food-processing areas and equipment (EURL, 2014). Therefore, it can be found in a variety of environments, including soil, sewage, silage, water, effluents and foods. With globalization and increased consumption of manufactured ready-to-eat foods throughout the world, it is hardly surprising that *L. monocytogenes* has become recognized as an important opportunistic human foodborne pathogen.

### 1.1.2. Legislations

#### *1.1.2.1 EU Regulation*

Ready To Eat (RTE) products are of particular concern for contamination with *L. monocytogenes* because they may support the growth of the pathogen during refrigerated storage. In addition, since RTE products can be consumed without further cooking, there is a greater possibility of the occurrence of foodborne illness from these products if they become contaminated. Lethality treatments, such as cooking meat and poultry products, generally eliminate *L. monocytogenes*; however, RTE products can be re-contaminated after the lethality treatment by exposure to the environment during peeling, slicing, repackaging, and other processing steps. By controlling sanitation in the post-lethality processing environment or implementing interventions in their products, establishments can ensure that their RTE products do not become contaminated with *L. monocytogene* (FSIS, 2014).

In 2005, the European Commission (EC) defined a food safety criteria limit of 100 colonies forming units (CFU)/g for Ready To Eat foods "unable to support the growth of *L. monocytogenes*" (EC, 2005). The EC regulation also states that as necessary, Food Business Operators (FBO) shall conduct studies to evaluate the growth of *L. monocytogenes* that may be present in the product, during its shelf life

under reasonably foresee able conditions of storage, distribution, and use. Annex II of the regulation text suggests different approaches to classify the RTE product:

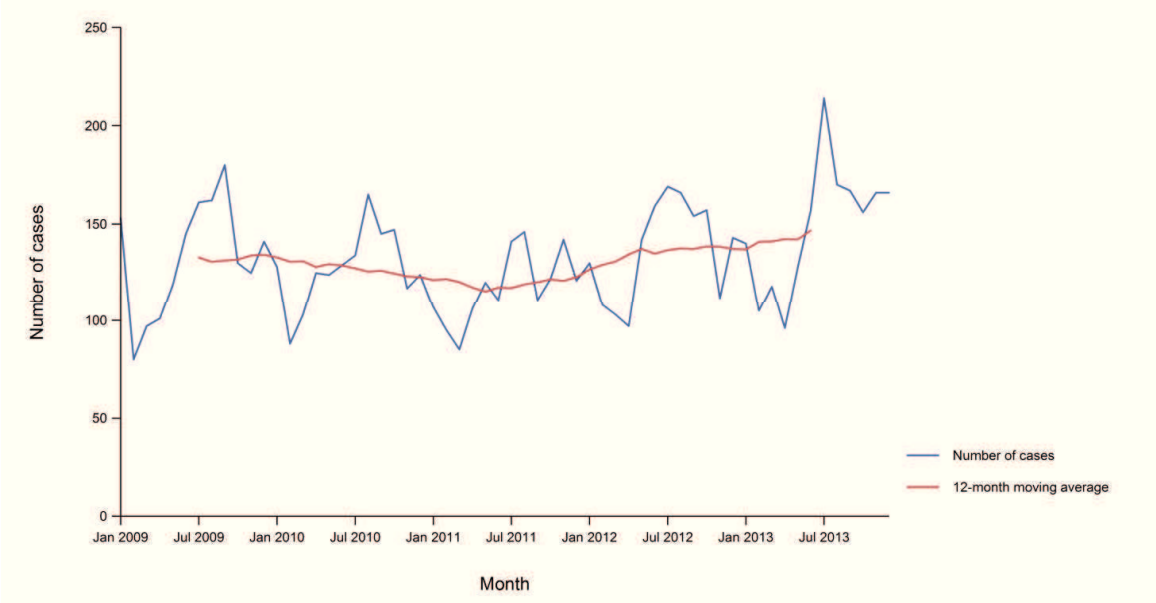
a) the first approach regards the analysis of physical-chemical properties, such as pH, aw, salt content, concentration of preservatives and type of packaging system, taking into account the storage and processing conditions, the possibilities for contamination and the planned shelf life. Accordingly, the products "with pH ≤ 4.4 or aw ≤ 0.92, those with pH ≤ 5.0 and aw ≤ 0.94 and those with a shelf life of less than five days" are automatically included in the category of RTE foods that are unable to support the growth of L. *monocytogenes*.
b) the second approach is based on the available scientific literature and research data regarding the growth and survival characteristics of the pathogens of concern. Within such approach, the use of predictive mathematical modelling is also allowed, with the growth of pathogen being assessed as a function of relevant critical factors.
c) as third approach, the so-called challenge tests have been addressed, where the ability of appropriately inoculated organisms to grow or survive in the product under different storage conditions are investigated (EC, 2005).

## 1.2. *L. monocytogenes* in food

### 1.2.1. ESFA Report

As reported by EFSA (2015), in 2013, 27 European Membre States (MS) reported 1,763 confirmed human cases of listeriosis. The EU notification rate was 0.44 cases per 100,000 population which was an 8.6 % increase compared with 2012. The vast majority of cases were reported to be domestically acquired. A seasonal pattern was observed in the listeriosis cases reported in the EU in the period 2009-2013, with large summer peaks and smaller winter peaks (**Fig. 1**). Fifteen MS provided information on hospitalisation for all, or the majority, of their cases (which represented 42.1 % of all confirmed cases reported in the EU) in 2013. On average, 99.1 % of the cases were hospitalised. This is the highest proportion of hospitalised cases of all zoonoses under the EU surveillance and reflects the focus of the EU surveillance on severe, systemic listeriosis infections.

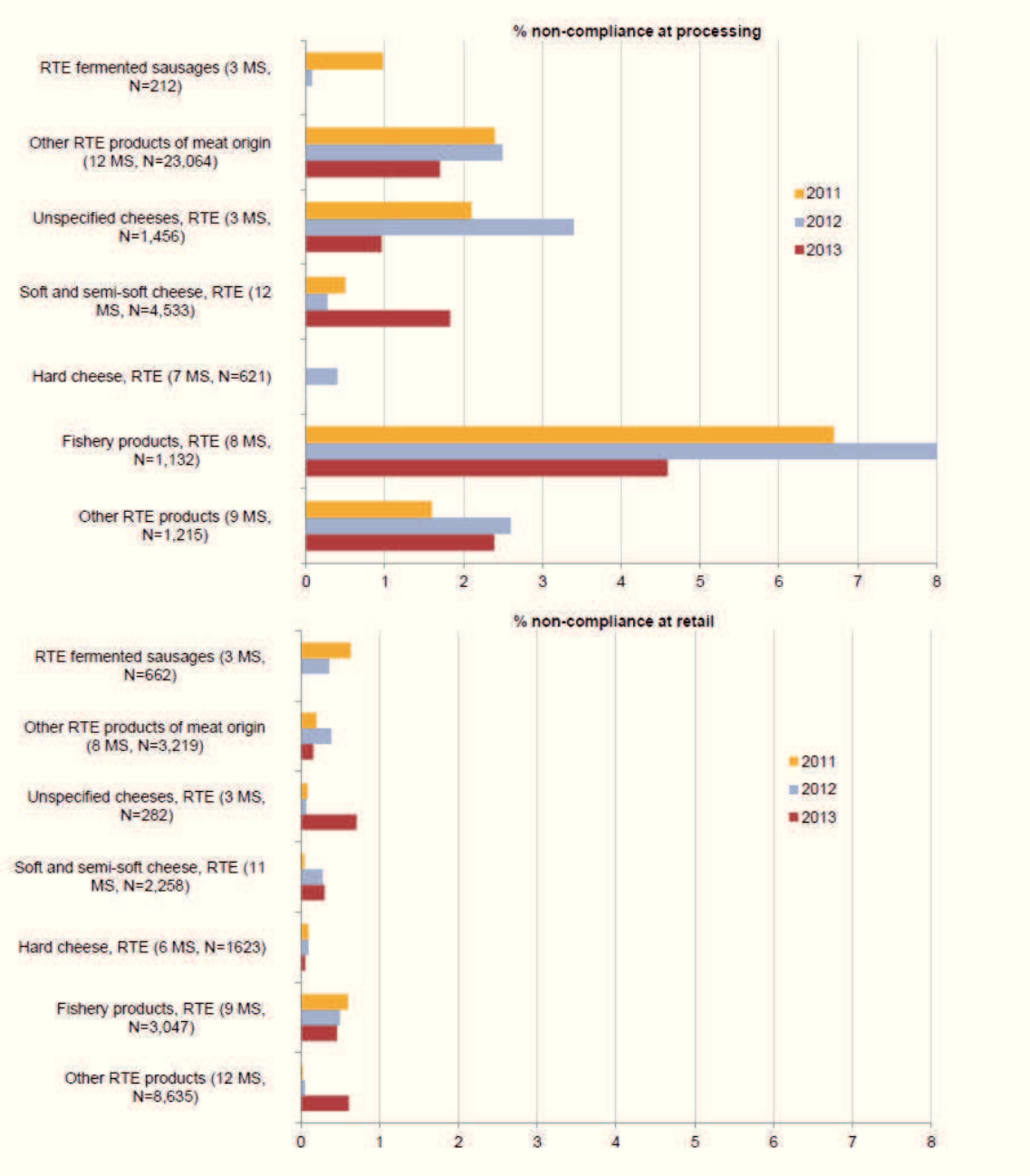
Figure 1. Trend in reported confirmed cases of human listeriosis in the EU/EEA, 2009-2013 (EFSA, 2015).




For RTE products on the market, very low percentages (< 1 %) were generally found to not comply with the criterion of ≤ 100 CFU/g. However, higher levels of non-compliance (primarily presence in 25 g) were reported in samples of RTE products at the processing stage, ranging from none to 4.6 % of single samples.

As in previous years, all samples of RTE food intended for infants and for medical purposes were compliant with the *L. monocytogenes* criteria both at processing (one MS) and at retail (four MS). All RTE milk samples collected at either processing (11 MS) or retail (seven MS) were also compliant. As observed in the past two years, the food category with the highest levels of non-compliance at processing was RTE fishery products (4.6 % of single samples and 19.9 % of batches), mainly in smoked fish (**Fig. 2**).

Figure 2. Proportion of single samples at processing and retail non-compliant with EU *L. monocytogenes* criteria, 2011-2013 (EFSA, 2015).



Among samples from RTE products of meat origin, other than fermented sausages, low levels of non-compliance were observed at processing (1.7 % of single samples and 2.8 % of batches), where non-compliance was reported from 11 MS. At retail, very low levels of non-compliance were reported (0.2 % of single samples and 0.1 % of batches), with a few non-compliant products reported by three MS. In the case of fermented sausages, all tested products were found to meet the *L. monocytogenes* criterion (no levels exceeding 100 CFU/g) at both processing and retail.

For soft and semi-soft cheeses, low levels of non-compliance were observed in investigations at processing (1.8 % of single samples and 0.3 % of batches). Non-compliance primarily occurred in soft and semi-soft cheeses made from raw or low heat-treated cow's milk. At retail, the levels of non-compliance were very low (0.3 % of single samples and 0.4 % of batches), and the few non-compliant products were reported from three MS. Low levels of non-compliance were also observed in unspecified cheeses at processing (1 % of single samples) and at retail (0.7 %).

Hard cheeses are assumed not to support the growth of *L. monocytogenes*. All tested units complied with the criteria of levels not exceeding 100 CFU/g at processing and retail, except for one single sample of hard cheese made from pasteurised cow's milk sampled at retail.

Among samples of unspecified cheeses, low levels of non-compliance were observed at processing (1.0 % of single samples) and at retail (0.7 %). However, at retail, the level of *L. monocytogenes* non-compliance observed in unspecified cheese was the highest of all the RTE foods at the same sampling stage.

### 1.2.2. RASFF: Food and Feed Safety Alerts

The EU has one of the highest food safety standards in the world – largely thanks to the solid set of EU legislation in place, which ensures that food is safe for consumers. A key tool to ensure the cross-border follow of information to swiftly react when risks to public health are detected in the food chain is RASFF – the Rapid Alert System for Food and Feed.

Created in 1979, RASFF enables information to be shared efficiently between its members (EU-28 national food safety authorities, Commission, EFSA, ESA, Norway, Liechtenstein, Iceland and Switzerland) and provides a round-the-clock service to ensure that urgent notifications are sent, received and responded to collectively and

efficiently. Thanks to RASFF, many food safety risks had been averted before they could have been harmful to European consumers. Vital information exchanged through RASFF can lead to products being recalled from the market. A robust system, which has matured over the years, RASFF continues to show its value to ensure food safety in the EU and beyond. The RASFF annual report 2015 gives a profound insight into the activity of the RASFF giving detailed figures for the types of notifications, products, hazards and countries that have been reported through the RASFF in 2015, including important developments in what is reported in the system as well as about the system itself. The RASFF portal features an interactive searchable online RASFF database. It gives public access to summary information about the most recently transmitted RASFF notifications as well as search for information on any notification issued in the past.

Since June 2014, consumers have had the use of a new tool, the "RASFF consumers' portal", which provides latest information on food recalls and public health warnings in all EU countries. (European Commission, 2010).

As reported by the RASFF annual report for 2015 (RASFF, 2015), the number of notifications for *L. monocytogenes* stayed at the same - high level as in 2014. Reporting Listeria in smoked salmon is still frequent, mostly processed in Poland (20) and mainly notified by Italy; the issue mentioned in the 2014 RASFF annual report about a dispute over shelf life studies is continuing. Other product categories often reported for *L. monocytogenes* are cheeses mostly from France (18, most often reported to be made from raw milk) and from Italy (6, Gorgonzola).

### 1.2.3. *L. monocytogenes* in milk and cheeses

The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks, reported that the majority of strong evidence food-borne outbreaks were associated with foodstuffs of animal origin including dairy product. In 2013, 2.14% of strong-evidence food-borne outbreaks (18 on 839 outbreaks) were attributed to the consumption of cheese and dairy products (11 and 7 outbreaks respectively) in Europe (EFSA/ECDC, 2015). From 1982 to 2010, sixty-four reported human cases and outbreaks in Europe, the United States and Canada related to the consumption of dairy products were referred in a non-exhaustive list by Verraes et al. (2015). The presence of foodborne pathogens in raw

milk and bulk tank milk has been widely reported (D'Amico & Donnelly, 2010; De Reu et al., 2004; Hassan et al., 2000; Jayarao & Henning, 2001; Kousta et al., 2010). The prevalence of pathogens in milk is influenced by numerous factors such as farm size, number of animals on the farm, hygienic conditions, farm management practices, variation in sampling and types of samples evaluated, differences in detection methodologies used, geographical location, and season. However, in spite of the variation, all of these surveys clearly demonstrated that milk can be a major source of foodborne pathogens of human health significance (Oliver et al., 2005). In this contest, *L. monocytogenes*, responsible of listeriosis, represents one of the most serious food safety concerns. It has been isolated from many foods (Gianfranceschi et al., 2003), among which milk and cheeses (Bernini et al., 2013; Manfreda et al., 2005; Pintado et al., 2005) and it has been involved in numerous outbreaks occurring after consumption of contaminated milk and milk products throughout the world (Donnelly, 2001; Lyytikainen et al., 2000). In particular, dairy products contaminated with *L. monocytogenes* have been implicated at almost the half of the reported listeriosis outbreaks in Europe (Lundén et al., 2004). The pathogen is widespread in nature and lives naturally in plants and soil environments. Its ability to survive and grow over a wide range of environmental conditions, including refrigeration temperatures, high salt concentration and low pH, makes it a potential hazard in foods (Ryser, 2007). Moreover, *L. monocytogenes* into food processing plants results in reservoirs that are difficult to eradicate: this is the case of biofilms that are a constant issue in food processing environments (Oliver et al., 2005). So, in addition to the risk associated to direct consumption, the introduction of raw contaminated milk into dairy processing plants represents a risk to human health if milk is used unpasteurized for cheese making or in case of cross contamination (Kousta et al., 2010).

The survival and growth of *L. monocytogenes* in a dairy environment depends on the manufacturing, ripening and storage conditions used for the cheeses, even when the cheese is stored at refrigeration temperatures (Pintado et al., 2005). In this regard, research into soft-ripened and blue-veined cheeses like Gogonzola cheese is an important field (Carminati et al., 2004; Lomonaco et al., 2009; Manfreda et al., 2005). Gorgonzola, one of the most important Italian cheeses, awarded with the “Protected Denomination of Origin” (PDO) from the European Commission (EU Regulation,

1996), represents an optimal food substrate for the growth of *L. monocytogenes* (Lomonaco et al., 2012). Gorgonzola is a blue-veined, mould ripened cheese, made with pasteurised cow's milk and characterised by a final pH ranging from 4.5 to 6.5 and a sodium chloride content of 2.3-7%. During ripening, biochemical changes due to the proteolytic activity of *Penicillium roqueforti*, that develops as an internal blue-green mould, cause an increase in pH, influencing the microflora's evolution and favouring possible contamination by ubiquitous dairy microorganisms and food-borne pathogens, among which *L. monocytogenes* has raised particular concern (Mucchetti & Neviani, 2006). In 2006 Gianfranceschi et al. (2006) described the first case of listeriosis associated with the consumption of contaminated Gorgonzola cheese in Italy. Although the pasteurization of milk inactivates the pathogen, it can enter the product after contact with the environment during ripening (Cocolin et al., 2009). Contamination during processing principally depends on the ripening and storing conditions of the cheeses, and together with the characteristics of the *L. monocytogenes* and of the product, these conditions can make pathogen survival and multiplication possible (Ryser, 2007). This contamination, if present, consists of a small number of cells and is limited to the rind (Carminati et al., 2004; GOLIS Project, 2004), which has been declared not edible by the Consortium for the Protection of Gorgonzola Cheese. However, *L. monocytogenes* can still be a threat to consumer health because contamination can be transferred to the internal paste during slicing. In fact, it is know how the bacterial transfer from surfaces to food or food to food, called “cross-contamination”, can occurs via hands or hands contact surfaces as well as equipment like cutting boards or knifes. Different studies on pathogen cross-contamination of foods have been documented (Cogan et al., 1999; Wachtel et al., 2003) and, in general, it is estimated that cross-contamination is involved in 39% of food-borne outbreaks (Evans et al., 1998).

## 1.3. Predictive microbiology in food

### 1.3.1. Basic concepts

#### *1.3.1.1 Traditional methods*

Microbiological challenge testing has been and continues to be a useful tool for determining the ability of a food to support the growth of spoilage organisms or pathogens (US FDA, 2009).

There are several types of challenge studies that deal with validation of food safety processing procedures, product storage conditions, and shelf life. Food safety–related challenge studies differ according to the objective of the study, such as a pathogen growth inhibition study or a pathogen inactivation study or a combination of the two, and depend on the type of product, production process, and hazard analysis of the product. Food safety–related challenge studies include the following:

a) Pathogen growth inhibition studies. These studies are used to evaluate the ability of a particular food product formulation with a specific type of processing and packaging to inhibit the growth of certain bacterial pathogens when held under specific storage conditions (time and temperature).
b) Pathogen inactivation studies. These studies are used to evaluate the ability of a particular food product formulation, a specific food manufacturing practice, or their combination to cause the inactivation of certain bacterial pathogens. These studies may also be impacted by food storage and packaging conditions and must account for these variables.
c) Combined growth and inactivation studies. Combined studies may be used to evaluate the ability of a particular food or process to inactivate certain bacterial pathogens and to inhibit the growth of certain other pathogenic bacteria or to achieve a level of inactivation followed by inhibition of the growth of survivors or contaminants introduced after processing (NACMCF, 2010).

In predictive microbiology, it is needed to evaluate the behavior of a particular strain (or a cocktail of different strains) to subsequently calculate kinetic parameters. Microbiological challenge tests also play an important role in the

validation of processes that are intended to deliver some degree of lethality against a target organism or group of target organisms (for example, a 5 log reduction of *Escherichia coli* O157:H7 for fermented meats). Selection of microorganisms to use in challenge testing and/or modeling depends on the knowledge gained through commercial experience and/or on epidemiological data that indicate that the food under consideration or similar foods may be hazardous because of pathogen growth. An appropriately designed microbiological challenge test will validate that a specific process is in compliance with the predetermined performance standard. The design, implementation, and assessment of microbiological challenge studies form a complex task that depends on factors related to how the product is formulated, manufactured, packaged, distributed, prepared, and consumed. Failure to account for specific product and environmental factors in the design of the test could result in flawed conclusions. Microbiological challenge studies can be used in specific cases for the determination of the potential shelf life of certain refrigerated or ambient-stored foods. The determination of whether challenge studies are appropriate or useful must consider such factors as the likelihood of the product to support growth of spoilage organisms or pathogens or include knowledge of the previous history of the product. When conducting a microbiological challenge study, a number of factors must be considered:

a) the selection of appropriate pathogens or surrogates,
b) the inoculation level,
c) the method of inoculation,
d) the duration of the study,
e) formulation factors and storage conditions,
f) sample analyses.

These are described next.

a) Selection of appropriate strains or surrogates

The ideal organisms for challenge testing are those that have been previously isolated from similar formulations. Additionally, pathogens from known foodborne outbreaks should be included to ensure the formulation is robust enough to inhibit those organisms as well. For certain applications, surrogate microorganisms may be used in challenge studies in place of specific pathogens. For example, introducing pathogens into a processing facility is not feasible; therefore, it is desirable to use

surrogate microorganisms in those cases. An ideal surrogate is a strain of the target pathogen that retains all other characteristics except its virulence. In any case, it is important to incubate the microorganisms in standardized conditions, preferably similar to those encountered in the food.

b) Inoculation level

The inoculum level used in the microbiological challenge study depends on whether the objective of the study is to determine product stability and shelf life or to validate a step in the process designed to reduce microbial numbers. Typically, an inoculum level between $10^2$ and $10^3$ colony-forming units (cfu)/g of product is used to ascertain the microbiological stability of a formulation. If the inoculum level is too low, microorganisms could not grow in the food product because of the increased lag phase, so that one can assume in certain cases that food formulation assures food safety when it is not low. In contrast, at high inoculum levels, microbial growth could be overestimated. For studying lethality processes, higher levels of microorganisms are needed (generally more than $10^6$ cfu/g).

c) Method of inoculation

The method of inoculation is another extremely important consideration when conducting a microbiological challenge study. Every effort must be made not to change the critical parameters of the product formulation undergoing challenge. A variety of inoculation methods can be used depending upon the type of product being challenged. In aqueous liquid matrices such as sauces and gravies with high aw (>0.96), the challenge inoculum may be directly inoculated into the product with mixing, using a minimal amount of sterile water or buffer as a carrier. Use of a diluent adjusted to the approximate aw of the product using the humectant present in the food minimizes the potential for erroneous results in intermediate aw foods. In studies where moisture level is one of the experimental variables, the inoculum may be suspended in the water or liquid used to adjust the moisture level of the formulation. Products or components with aw < 0.92 may be inoculated using the atomizer method with a minimal volume of carrier water or buffer. Again, the product should always be checked to ensure that the final product aw or moisture level has not been changed. A short post-inoculation drying period may be needed for some products before final packaging. A minimum volume of sample should be inoculated so that a minimum of three replicates per sampling time is available

throughout the challenge study. In some cases, such as in certain revalidation studies and for uninoculated control samples, fewer replicates may be used.

d) Duration of the study

The microbiological challenge test should be conducted for at least the whole shelf life period of the food product. Some regulatory agencies recommend extending the duration of the study a margin beyond the desired shelf life because it is important to determine what would happen if users held the product beyond its intended shelf life and then consumed it. The frequency of analysis depends on the environmental conditions under which the food is subjected. It may be desirable to test more frequently (for example, daily or multiple times per day) early in the challenge study (that is, for the first few days or week) and then reduce the frequency of testing to longer intervals.

e) Formulation factors and storage conditions

When evaluating a formulation, it is important to understand the range of key factors that control its microbiological stability. It is, therefore, important to test each key variable singly or in combination in the formulation under worstcase conditions. Experimental temperature should be similar to real processing, distribution, and sale conditions. In a last step, the use of temperature shift might be recommended, such as storing the food product at one specific temperature for a portion of its shelf life, after which time the product may be subjected to elevated temperatures.

f) Sample analysis

In challenge tests, it is recommended to analyze at least three replicates per analytical point, although more replicates would be needed when requiring more accuracy in the results. The culture media to be used will depend on the type of microorganism to be controlled, but if the food product contains high concentration levels of competitive flora, it would be better to use selective media. Similarly, if the targeted microorganism is a toxin producer, toxin concentration should be measured during the study period. In parallel, control samples (uninoculated) may be analyzed in the same way as inoculated samples to evaluate the effect of the food flora on the analytical period of time (Perez-Rodriguez & Valero, 2013; NAMFC, 2010).

#### 1.3.1.2 *Predictive models*

The ability to predict events has always been in the centre of human intellectual activity, let it be religion or science. Inferring predictions from observations has been one of the first practical results of science, and a triumph of logic by which those inferences were made. It was the ancient Greeks who gave logic a strict, formal framework, which (hand-in-hand with the development of mathematical methods and the rigorous language by which they were described) triggered the rise of an art and craft what is called today "Mathematical modelling". When this is used to describe microbial responses to food-related environments, we call it "Predictive (food) microbiology".

Predictive microbiology is based on the premises that a) bacterial responses (i.e. their growth, survival and death) to environmental are reproducible, therefore can be described by kinetic equations; and b) their stochastic variation can be characterized in a similarly consistent way by probability distributions. Therefore, based on previous observations, it is possible to predict the behaviour of foodborne organisms under given environmental conditions (Ross and McMeekin, 1994), and to estimate the accuracy of those predictions. It follows directly that predictive microbiology must be based on two pillars: structured, systematically organized databases and mathematical models. The two are closely interconnected, helping each other's development and validation. Besides, in order to make the results available for various stakeholders, it is essential that they are accessible (primarily on the web); easy to manage and understand. Web-based software tools are essential means of knowledge translation in the interest of food safety management (Baranyi & Cosciani-Cunico, 2013).

It is a goal of food microbiologists to know in advance the behavior of microorganisms in foods under foreseeable conditions. To do so, an exhaustive control of physicochemical factors that could influence microbial growth is needed (such as temperature, pH, aw, salt), as well as a deep knowledge about the biological characteristics of the target microorganism(s). The premises behind the scientific basis of predictive microbiology are that microbial responses in foods are in certain way reproducible against several extrinsic and intrinsic environmental factors (Ross et al., 2000). This behavior can be translated into diverse mathematical models that

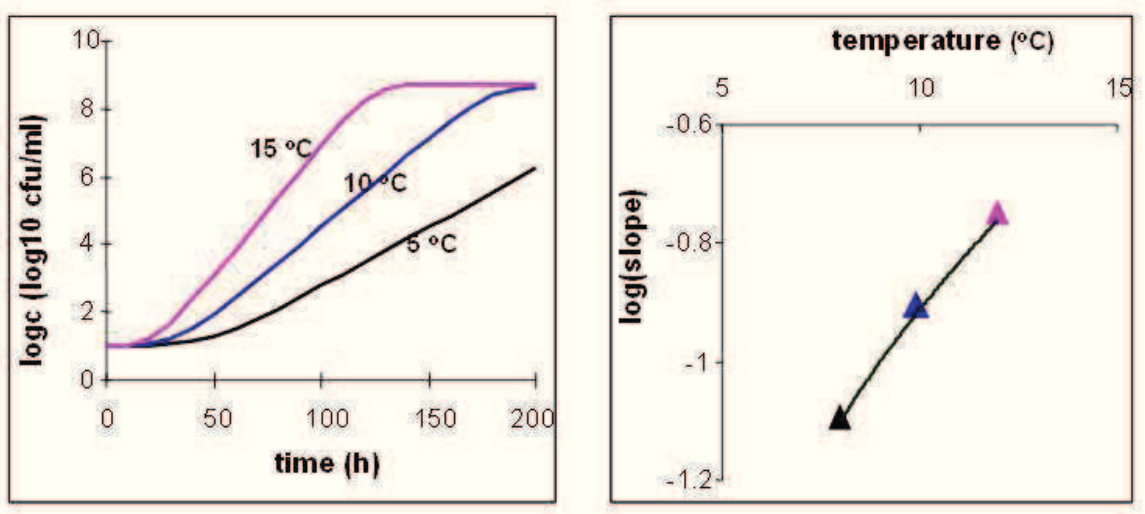
estimate microbial growth/inactivation/toxin production/probability of growth, etc. This emerging area was redefined as modeling of microbial responses in foods. McKellar and Lu (2004) presented a detailed review of the predictive models published so far.

With the aim of performing comparative studies, several authors suggested different classifications of predictive models based on their final purpose, the type of microorganism to be studied, and their impact on food spoilage or food safety. Basically, predictive models are split up into three groups: survival/inactivation models, boundary (growth/no growth) models, and growth models. Basing on their development, models can be classified as follows:

a) Primary models: aim to describe the kinetics of a process with as few parameters as possible while being able to accurately define the growth and inactivation phases. They are represented as the increase (or decrease) in population density against time (**Fig. 3**).
b) Secondary models: describe the effect of environmental conditions (i.e., physicochemical and biological factors) on the values of the parameters of a primary model (**Fig. 3**).
c) Tertiary models: based on computer software programs that provide an interface between the underlying mathematics and the user, allowing model inputs to be entered and estimates to be observed through simplified graphical outputs.

Whiting & Buchanan (1994a) called the foregoing integrated software-based models 'tertiary models.' They defined tertiary-level models as personal computer software packages that use the pertinent information from primary- and secondary level models to generate desired graphs, predictions, and comparisons. Primary-level models describe the change in microbial numbers over time, and secondary-level models indicate how the features of primary models change with respect to one or more environmental factors such as pH, temperature, and aw.

Figure 3. Sigmoid primary model for bacterial growth in batch. The exponential growth (appearing as linear on log-scale) is preceded by the lag period and followed by the stationary phase. A frequently used approach is to model the logarithm of the slopes as a function of the environment, called the secondary model. In the above case, the environment is characterized by one variable only, the temperature (Baranyi & Cosciani-Cunico,2013).




### 1.3.1.3 *Developing Predictive Models: Fitting Methods*

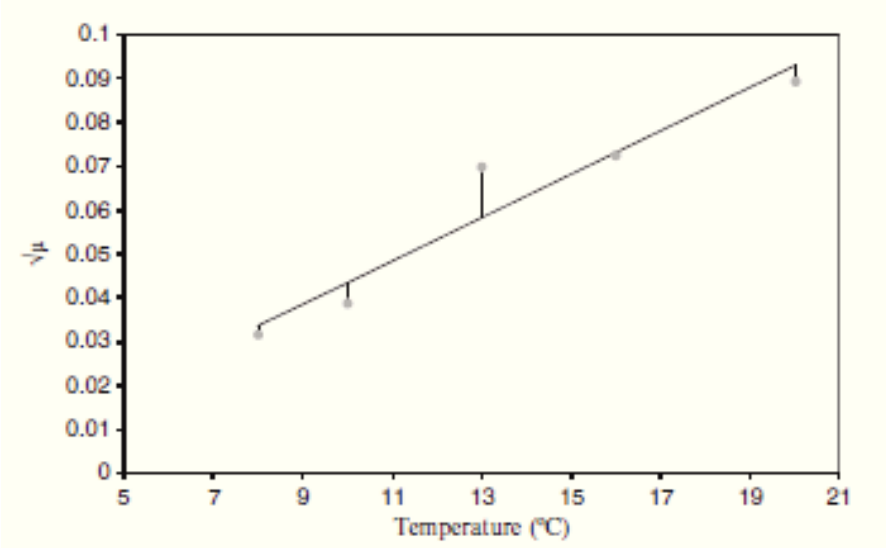
As reported by Baranyi & Cosciani-Cunico (2013), mathematical modelling is based on simplifications. It is important to appreciate that modelling is not simply compiling a set of equations. Modelling, in some way, is the art of omitting the unnecessary. It cannot be an automated process, because the extent and nature of those omissions depend on the purpose of the modelling.

As mentioned, when the need for applying mathematical models to a scientific field arises, a straightforward first step is to borrow terminologies and methods/models of fellow sciences. This is what happened when the linear death kinetics were introduced for the survival of *Clostridium botulinum* spores during heat treatment. The model was reminiscent of those used to describe biochemical reactions and population dynamics in adverse environments. It omitted many details, such as the food matrix, or simply the fact that it is not easy to interpret the log-linear decrease when only a few cells are left. However, the model served well in circumstances, when computational tools and data were much less abundant than today.

In predictive microbiology, most models are devoid of biological basis, and therefore are built on the basis of an empirical approach. Hence, mathematical models are derived by searching for the mathematical function(s) that adequately fit observed

values. The growth data (concentration vs. time) are used to derive primary models whereas kinetic parameters from primary models, that is, lag time, maximum growth rate, and maximum population density, are used to obtain secondary models. In both cases, the selection of the mathematical function(s) to be fitted should be based on a reasonable concordance between the theoretical basis and characteristics of the model and the observed behavior of the variable. To illustrate this, a simplified example based on growth data of *E. coli* O157:H7 in culture broth measured over time at different incubation temperatures between 5°C and 25°C was used. In this case, we focus on the secondary model. If the estimated values of $\sqrt{\mu}$ for the growth data are plotted against the corresponding incubation temperatures, a roughly straight-line relationship may be observed between both variables as evidenced by the data points represented in **Fig. 4**. Thus, predictive microbiology practitioners should look for mathematical functions featured by a straight-line pattern. In this case, based on available secondary models one finds that the square root model could be applied to fit the observed growth for the suboptimal temperature range (Ratkowsky 1983).

Figure 4. A Ratkowsky's model (straight line) for suboptimal temperatures fitted to square root transformed maximum growth rate values. Vertical lines between data points and the straight line represent the residuals of the fitted Ratkowsky's model (Perez-Rodriguez & Valero, 2013).




Regression methods are applied to estimate the function's parameters that best describe the observations. The regression method most widely used to fit mathematical functions to data is the least squares method (LS). This method

consists of deriving the parameter's values that minimize the sum of the squares of the differences between observed values and those predicted by the fitted model, which are so-called residuals. The residuals represent the distances between data points and the best-fit model and are visualized as vertical lines, as shown in **Fig. 4**. When the data present unequal variance, parameter estimates may improve using weighted least squares (WLS). The principle here is to assign to each observation a weight that reflects the error of the measurement. In general, the weight assigned to the observation, will be a function of the variance of this observation. Despite its popularity and versatility, LSM has its problems. Probably, the most important drawback of LSM is its high sensitivity to outliers (i.e., extreme observations) (Abdi, 2007).

#### *1.3.1.4 Model validation*

Validation is an essential step in the modeling process. Models cannot be applied if a validation process is not previously accomplished, which typically consists of confirming the predictions experimentally by using any quantitative method (Dym 2004). In predictive microbiology, experimental analysis of growth in food is the basis of model validation (challenge tests): experimental growth data (i.e., observations) are compared with the model predictions (Gíbson et al., 1988; Sutherland & Bayliss, 1994). Although validation should be performed on food, in many cases, because of the economic cost derived from challenge tests, data from scientific literature or artificial media are also used for validation. When validation is performed with data sets taken from the same experimental conditions as those used to elaborate the model, validation is the so-called internal validation and aims at determining if the model can sufficiently describe the experimental data. Some authors have carried out internal validation studies obtaining good results (Garcìa-Gimeno et al., 2002; Zurera et al., 2004). The external validation is based on the comparison between predictions and independent data sets, that is, either observations obtained from challenge test (Whiting & Buchanan 1994b; te Giffel & Zwietering 1999; Ross et al., 2000) or data taken from scientific literature (Fernandez et al., 1997; McClure et al.,1997).

## 1.3.2. Predictive microbiology as useful tool

### *1.3.2.1 A tool for improving food safety and quality*

As reported by Perez-Rodriguez & Valero (2013), despite the improvement in food technology and processing, currently microbiological hazards are associated with some food commodities; thus, their evaluation, control, reduction, and/or elimination are important for different commissions, governments, and organisms related to public health. As previously stated, predictive microbiology is based on the use of mathematical models to estimate microbial behaviour in foods. For food industries, application of this knowledge could be of great interest for assuring food safety and quality. During the past several years there has been substantial advance in both the concepts and methods used in predictive microbiology. Coupled with 'userfriendly' software and the development of expert systems, these models are providing powerful new tools for rapidly estimating the effects of formulation and storage factors on the microbiological relations in foods. Thus, predictive microbiology is understood as a scientific-based tool covering an integrated approach that improves food safety and quality. Predictive models can be effectively applied throughout the whole food chain, from raw material acquisition to end products. The utility of predictive microbiology will be further enhanced when is recognized as an effective rapid method (McMeekin et al., 2002).

Traditionally, food operators are confident, based on limited information, about their process or product. Indeed, scarcity in available resources and facilities to develop rapid and cost-effective techniques together with the increasing demand of safer and more stable food products from consumers have decreased the investment in research and development sources. Despite the earlier development of predictive microbiology in the science field, in the 1980s it was accorded more awareness because the outbreaks occurring with traditional food-borne pathogens, such as *Salmonella* spp. in eggs or *L. monocytogenes*, which can grow at refrigeration temperatures.

From that time on, national governments and food authorities have prioritized the use of food research for improving food safety. Some of the potential applications of predictive microbiology are summarized below.

Hazard Analysis and Critical Control Points (HACCP)

• Preliminary hazard analysis

• Identification and establishment of critical control points

• Corrective measures

• Evaluation of variables interaction

Risk Assessment and Risk Management

• Estimation of microbial population dynamics along the food chain

• Exposure assessment toward a specific pathogen

• Design of scientifically based management strategies to assure food safety

Shelf life studies

• Growth prediction of spoilage or pathogenic microorganisms in foods

Innovation and development of a new product

• Evaluation of the impact of microbial spoilage in a food product

• Effect of processing on food quality and safety

• Evaluation of the effect of other additional factors throughout the food chain

Hygienic measures and temperature integration

• Evaluation of the consequences of chill chain application on microbial spoilage

• Optimization of thermal and nonthermal inactivation processes

Education

• Education of both scientific and nonscientific staff

• Implementation and training of computing-based decision systems

Experimental design

• Estimation of the number of samples to be prepared

• Definition of intervals within each factor to be analyzed

Other current applications of predictive microbiology in an industrial context are wide but can be summarized into three groups (Membré & Lambert, 2008):

• Development of new products: developing alternative product formulations by the assessment of growth of spoilage and pathogenic microorganisms; this provides the definition of safer storage conditions, thus increasing shelf life;

• Operational support: supporting food safety decisions that need to be made when implementing or running a food manufacturing operation; also, setting critical

control points (CCPs) in HACCP, assessing impact of process deviations on microbiological safety and quality of food products;

• Incident support: estimating the impact on consumer safety or product quality in case of problems with products on the market.

For food industries, besides economical issues, the desire to produce safe foods using strategies is based on understanding sources and magnitudes of hazards. These are the main reasons why HACCP and risk assessment are taking part in any decision-making process (McMeekin & Ross, 2002). Among their main principles, they must assess human exposure to pathogens in foods. Clearly, this information is rarely available. Predictive microbiology models are commonly used to quantify the human exposure of bacteria through the ingestion of foods. To translate these concepts into food safety levels, risk-based metrics are used as a systematic approach to food safety based on hazard identification and control. These metrics (i.e., Performance Objectives and Food Safety Objectives) identify and evaluate key steps in the food production chain, which have the greatest effect on risk associated with hazards; it is often applied subjectively (Stringer, 2005).

Thus, predictive microbiology gives improved, quantitative insight into the food properties that are considered of importance to the safety and quality of foods (McMeekin et al., 1993a; Zwietering et al., 1992). Specifically, published studies of potential applications in meat (McDonald & Sun, 1999; Sumner & Krist, 2002; Pin et al., 2011) and fish industries (Koutsoumanis & Nychas, 2000; Dalgaard et al., 2002; Ross et al., 2003) are described.

Determination of shelf life is one of the most promising applications of predictive microbiology to food industries, rendering a reliable and economic tool for obtaining rapid estimations (Shimoni & Labuza, 2000; Castillejo-Rodríguez et al., 2002; Mataragas et al., 2006); this is also related to the applicability of time-temperature indicators for monitoring of spoilage (Vaikousi et al., 2009). In this context, the development and application of structured quality and safety assurance systems based on prevention through monitoring, recording, and controlling of critical parameters during the entire life cycle of the products, seem to be a prerequisite. More recent applications are focused on the use of predictive modeling to reduce the impact of climate change and seasonal variations on the safety and quality of foods

(Janevska et al., 2010). This goal can be reached starting with supply chain data to recalculate periodically changes in model parameters used for prediction of risk levels or shelf life, for example, the probability for contamination of the product with certain pathogens, growth rate, or initial count of spoilage microorganisms.

Application of appropriate statistical analysis would identify significant variations in the trends in terms of decreased safety or shelf life of the product, which would require further attention and corrective actions. The development of a predictive model comprises different stages, depending on the model type, application, or time/resources to be committed. Some relevant questions are often arisen before the construction of a new or modified predictive model. As literature sources contain a large quantity of developed models, much information is already gained by what one has really to analyze if carrying out additional experimental trials to create new models regarding a particular hazard/food combination.
Despite each particular situation, general questions (Q) could be formulated:
Q1: Are data and/or models sufficiently available in the literature?
Q2: Will the predictive model significantly improve current knowledge in the field?
Q3: Are available laboratory resources sufficient to perform all analyses in controlled conditions?
Q4: Do the authors have 'a priori' knowledge about the main environmental conditions affecting microbial growth/survival of the studied hazard(s) and food(s)?
Q5: According to these conditions, is it possible to develop a full-factorial design?
Q6: Is the mathematical model comprehensive and representative of the observed behaviour of the studied hazard(s)?
Q7: Could the model be validated with additional measurements or external data?
Q8: Could the model be effectively applied for food industries or authorities under a given set of conditions?

#### *1.3.2.2 Database and free-software-tool: some examples*

One of the main objectives pursued in predictive microbiology is quantification of microbial responses in foods. However, the complexity of the food environment has been recognized, which thus makes it difficult to quantify or even to categorize some of its features and their potential effects on microbial population dynamics or the

ability to recover a target organism from a food sample. An additional difficulty is that, with the background information on the environment and with currently available techniques to measure microbial responses, both variability and uncertainty may be large (Ratkowsky, 2004), which can produce wider confidence limits and a reduction in the accuracy of microbial estimates. It should be noted that predictive models themselves constitute a simplification of the real biological responses of microorganisms affected by different combinations of physicochemical and biological environmental factors. When these factors are identified, the same simplification is carried out as when a process is characterized by some mathematical variables. Predictive microbiology software programs are based on databases and mathematical models. Behind predictive software programs are the raw data upon which the models are built. The relationship between mathematical models and data bases provides the fast comparison of large amounts of data under a standardized and harmonized recording format.

A data base is a large collection of data organized in a specific form for rapid search and retrieval. Creation of data bases in predictive microbiology can provide a solution to inaccuracy in measurements, which can be compensated by increasing their number, to variability in responses and to data exchange between different research and academic institutions. The largest and most used database, called ComBase (Combined, or Common, i.e., joint, Database of microbial responses to food environments) (**Fig. 5**) was launched at the Fourth International Conference on Predictive Modeling in Foods, Quimper, France, June 2003. Its technical details can be read in Baranyi & Tamplin (2004) and on the website (www.combase.cc).

Figure 5 Example of a record in ComBase.


This database was developed in the Institute of Food Research, Norwich, UK (IFR), to pool available predictive microbiology data. Soon, the leaders of FSA and USDA-ARS agreed that incorporating all their data in this common data base would be mutually beneficial. The European Commission also embraced the idea, and now the original Food MicroModel and PMP datasets have been supplemented with additional data submitted by supporting institutes, universities, and companies, mainly from Europe. Furthermore, data have also been compiled from the scientific literature and a continuous updating process is carried out. Accessibility and application of resources of data involves thousands of researchers, risk assessors, legislative officers, food manufacturers, and their laboratory managers at no expense. Users can compare observations with independent predictions gained from other software packages or with external data. If ComBase is accepted internationally as the benchmark, the number of sources generating different views on risk can be decreased (McMeekin et al., 2006).

Nowadays, the great development of computational sciences and software engineering have enabled obtaining user-friendly software that, based on specific

mathematical algorithms, are able to fit many types of mathematical function to observations including qualitative and quantitative data. In predictive microbiology, observations usually correspond to either counts describing microbial growth and inactivation or kinetic parameter values obtained under different environmental conditions. In such cases, different fitting procedures can be used depending on the type of model and variable considered. Linear and nonlinear regression methods are applied to data on the basis of the type of mathematical function to be fitted. Most software incorporates both types of fitting procedure and, in some cases, specific developments and algorithms are implanted to optimize fitting procedure. The Excel adding DMfit is a free software application for predictive microbiology modeling developed by the Computational Biology group at Institute of Food Research (Norwich, UK). The application is a Microsoft Excel Add-In to fit log counts versus time data, providing kinetic parameters such as growth/death rate and lag time/shoulder. The available primary models are the reparameterized Gompertz equation (Zwietering et al., 1990) and the Baranyi model (Baranyi et al., 1995), including a modification for fitting optical density data. Furthermore, the application includes a module to fit secondary models encompassing the gamma model, Ratkwosky model, and polynomial models. The latest version of Dmfit can be downloaded at the Combase website http://www.combase.cc/. In addition, there is an on-line version, which can be executed in the Internet, including some basic features taken from the Excel macro. This version is also available through the combase's webpage. GInaFiT is a freeware add-in for Microsoft Excel aimed to fit several inactivation models (Geeraerd et al., 2005) to experimental data provided by users in an Excel spreadsheet. This application has been developed by the chemical and biochemical process technology and control group (Biotec) at the University of Leuven. The application includes a collection of the most representative inactivation models highlighting the log-linear model (e.g., Bigelow model), and the Weibull model and its adaptations. The software can be downloaded on-line at http://cit.kuleuven.be/biotec/downloads.php and once installed, executed in the Microsoft environment. Similar to this application, an Excel macro developed by Prof. M. Peleg of the Department of Food Science at University of Massachusetts enables fitting the Weibull model to inactivation data. The macro files can be downloaded at a specific website containing other similar macros concerning

predictive microbiology developed by Prof. Peleg's research group (http://people.umass.edu/~aew2000/GrowthAndSurvival/GrowthAndSurvival.html).

### 1.3.3. Predictive models in Quantitative Risk Assesment

The process of conducting a Microbial Risk Assessment is a structured, systematic approach to integrate and evaluate information from diverse sources concerning the origin and fate of pathogens along the food chain and to determine the magnitude of public health risks. In addition, predictive microbiology can help risk assessors and risk managers to make decisions concerning risk mitigation in food products because it is possible to know the microbial behavior in a medium, or in a food through different mathematical models, as a function of certain intrinsic or extrinsic environmental factors. FAO/WHO (Food Agriculture Organization/World Health Organization) taking the lead from WTO in 1995, introduced, through meetings of experts, the concept of risk analysis as a systematized and 'rational' scheme in which the development of food standards on national and international scale is based (FAO/WHO, 1995).

This approach also could be followed by food industries and food companies, even though in this case its development is mainly intended to improve the Hazard Analysis and Critical Control Points (HACCP) programs and to assess, from the bio-sanitary point of view, new designs and novel products (van Gerwen & Gorris, 2004; Voysey, 2000). This food safety management approach has been completed and developed through the inclusion of other concepts such as the Food Safety Objective (ICMSF, 2002). The use of predictive modeling will help in choosing the most appropriate levels of factors to be used to meet target management measures. The main achievement within a HACCP system lies on setting quantitative levels, which can be transmitted to governments and national authorities to improve food safety. The Risk Analysis, according to the FAO/WHO (1995), consists of three components (**Fig. 6**): Risk Assessment, Risk Management, and Risk Communication.

Figure 6. Interaction between the elements of risk analysis (FAO/WHO, 2006).


Risk assessment is 'the qualitative and/or quantitative evaluation of the nature of the adverse effects associated with biological, chemical, and physical agents which may be present in food' (FAO/WHO, 1995). Risk assessment is structured in four steps: Hazard Identification, Hazard Characterization, Exposure Assessment, and Risk Characterization. The document entitled Principles and Guidelines for the Conduct of Microbiological Risk Assessment (Alinorm 99/13A) provided specific definitions for the four steps of Microbiological Risk Assessment:

1. Hazard Identification. 'The identification of biological agents capable of causing adverse health effects and which may be present in a particular food or group of foods'.

2. Hazard Characterization. 'The qualitative and/or quantitative evaluation of the nature of the adverse health associated with the hazard'.

3. Exposure Assessment. 'The qualitative and/or quantitative evaluation of the likely intake of a biological agent via food, as well as exposure from other sources if relevant'.

4. Risk Characterization. 'The process of determining the qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known or potential adverse health effects in a given population based on Hazard Identification, Hazard Characterization and Exposure Assessment'.

Microbial Risk Assessment (MRA) can be defined as a scientific approach intended to estimate the microbial risk associated with food-borne pathogens (Codex Alimentarius Commission 1999). Lammerding & Paoli (1997) pointed out that MRA assesses the impact of changes and trends in the food supply chain; thus, MRA should be able to predict how active or passive changes during processing, distribution, and consumption of foods affect public health. By taking a quantitative approach (i.e., using numerical information), MRA yields more accurate estimations reducing misinterpretation or bias when risk managers use this information. In general, Quantitative MRA (QMRA) is preferred to qualitative MRA, when necessary data and quantitative information are available (Lammerding & Fazil, 2000). QMRA aims to quantitatively describe the effect of food processes, from farm to fork, on microbial risk. To this end, microbial prevalence (e.g., percent of contaminated servings) and concentration (e.g., log cfu/g) along the food chain should be quantified. However, some limitation arises because of the lack of knowledge or information, for example, during pasteurization when pathogen levels are reduced to undetectable levels at which microbiological analysis is not effective or also at the moment of consumption where performing a quantitative analysis is not feasible in a practical sense (Lammerding & Fazil, 2000). Thus, predictive models should be incorporated into QMRA to estimate the quantitative effect of determined steps or stages along the food chain for which no data are available or data collection is difficult. Perhaps the work that best summarizes this approach is one of the first published works approaching QMRA and predictive microbiology in a strict sense, which was carried out by Cassin et al. (1998). Of the four components constituting MRA methodology, two components require an important contribution of predictive models, that is, hazard characterization and exposure assessment. Hazard characterization is mainly based on applying dose–response models. This type of model establishes a mathematical relationship between ingested dose of the food-borne pathogen and host response in terms of probability of infection, illness, or death (Buchanan et al., 2000). Information from outbreaks, epidemiological studies (Strachan et al., 2005), and experimentation with animal and humans (studies in vivo) are used to derive dose–response models, although important limitations are associated with this kind of studies because of ethical issues, scarcity of information, natural variability, etc.

In quantitative exposure assessment, predictive models are intended to describe prevalence and concentration changes at different stages along the food chain (Klapwijk et al., 2000). In other words, the final goal of an exposure assessment study is to know the exposure level to a pathogenic microorganism in terms of prevalence and concentration at the moment of consumption. In this case, predictive microbiology can provide suitable models to better describe different bacterial processes in food-related environments, such as bacterial growth and inactivation (e.g., pasteurization), bacterial transfer (e.g., cross-contamination during handling of foods), and growth probability under determined storage and preservation conditions. Many of the models can be now used in a more applicable context to determine the risk associated with certain food(s) and pathogenic microorganism(s). Although important efforts have been made to agree on a standardized methodology (van Gerwen et al., 2000; FAO/WHO, 2008; Bassett et al., 2012), the application of predictive models in QMRA studies is still a complex task, a blend of science and art when information is scarce or data are not conclusive.

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# 2. AIM OF THE THESIS

Among food-borne pathogens, *L. monocytogenes* represents one of the most serious food safety concerns. In particular, dairy products are often a source of this infection. During 2007 and 2009 in Italy there was an increase of notifications of listeriosis with the most cases reported in the Centre-North of Italy. This is probably attributable both to a real increase of listeriosis in Italy and to surveillance implementation. However, statistically significant increasing trends in listeriosis notification rates from 2005 to 2009 were noted in Italy as elsewhere in Europe (EFSA/ECDC, 2011).

In the present research studies, raw milk, raw milk cheeses and Gorgonzola cheese (frequently contaminated dairy products) were considered like to:

a) monitor the prevalence of *L. monocytogenes*: the collected data may be a useful tool for the quantitative risk assessment study for human listeriosis linked to the consumption of raw milk and cheese made from raw milk or Gorgonzola cheese in Italy;
b) study the behaviour of *L. monocytogenes* in cheeses as a function of the environmental condition, when contaminated at different time points (in milk, during the cheesemaking, during the ripening or the storage) to investigate the effect of physicochemical and microbiological changes on the behaviour of *L. monocytogenes* during the manufacture process. These informations may increase knowledge concerning the impact of the process on the survival of pathogenic microorganisms.
c) develop useful tools to predict the behaviour of the pathogen as a function of the environmental conditions (temperature, pH, aw, lactic acid bacteria competition etc.): these may be considered valuable tools to support the monitoring surveys carried out by officers of the Regional Veterinary Authority, especially when the products (milk or cheeses) are found positive to the presence of *L. monocytogenes*. In this case, knowing the product storage conditions (storage temperature profile and duration of shelf life) allows to predict the growth of *L. monocytogenes*, as to assess the safety of the product before it reaches the final consumer.

# 3. SURVEY OF PREVALENCE AND SEASONAL VARIABILITY OF *Listeria monocytogenes* IN RAW COW MILK FROM NORTHERN ITALY

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# Survey of prevalence and seasonal variability of *Listeria monocytogenes* in raw cow milk from Northern Italy

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ABSTRACT

*Listeria monocytogenes* is an important food-borne pathogen causing meningitis, meningo-encephalitis and abortion. Both sporadic and epidemic human listeriosis cases are associated with the consumption of contaminated foods. To assess the potential risk to consumer health, the presence of *L. monocytogenes* was investigated using qualitative and quantitative methods in raw milk (bulk tank milk and milk for vending machine) collected from 2010 to 2013 in Northern Italy (Lombardy and Emilia-Romagna regions). Overall, *L. monocytogenes* was detected in 145 on 8716 of raw milk samples, with a prevalence of 1.66% (95% C.I. 1.4%–1.7%). The prevalence ranged from 0.52% (95% C.I. 0.3%–0.9%) in 2012 to 2.7% (95% C.I. 2.0%–3.8%) in 2013, but no trend of increase was observed in four-years of investigation. The pathogen was detected from 2.2% (95% C.I. 1.9%–2.6%) of bulk tank milk and from 0.5% (95% C.I. 0.3%–0.8%) of milk for vending machine. A significative difference ($p < 0.05$) of the prevalence data was observed between data collected in two different regions of Northern Italy with an higher prevalence in Lombardy. In addition to the geographical area, the *L. monocytogenes* presence was influenced also by the seasonal period of collection samples, with peaks in spring and autumn. These results confirm the raw milk can be a source of foodborne illness outbreaks if consumed without sanitizing treatments, but the low prevalence and the low contamination levels (more than 80% of the contaminated samples contained <10 cfu $ml^{-1}$ of *L. monocytogenes*) proving the hygienic quality of the milk produced in Northern Italy.



## 1. Introduction

The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks, reported that the majority of strong evidence food-borne outbreaks were associated with foodstuffs of animal origin including dairy product. In 2013, 2.14% of strong-evidence food-borne outbreaks (18 on 839 outbreaks) were attributed to the consumption of cheese and dairy products (11 and 7 outbreaks respectively) in Europe (EFSA/ECDC, 2015). From 1982 to 2010, sixty-four reported human cases and outbreaks in Europe, the United States and Canada related to the consumption of dairy products were reported in a non-exhaustive list by Verraes et al. (2015). The presence of foodborne pathogens in raw milk and bulk tank milk has been widely reported (D'Amico and Donnelly, 2010; De Reu, Grijspeerdt, & Herman, 2004; Gaya, Saralegui, Medina, & Nunez, 1996; Hassan, Mohammed, McDonough, & Gonzalez, 2000; Jayarao & Henning, 2001; Kousta, Mataragas, Skandamis, & Drosinos, 2010; Moshtaghi & Mohamadpour, 2007; Van Kessel, Karns, Gorski, McCluskey, & Perdue, 2004; Waak, Tham, & Danielsson-Tham, 2002). The prevalence of pathogens in milk is influenced by numerous factors such as farm size, number of animals on the farm, hygienic conditions, farm management practices, variation in sampling and types of samples evaluated, differences in detection methodologies used, geographical location, and season. However, in spite of the variation, all of these surveys clearly demonstrated that milk can be a major source of foodborne pathogens of human health significance (Oliver, Jayarao, & Almeida, 2005).

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In this contest, *Listeria monocytogenes*, responsible of listeriosis, represents one of the most serious food safety concerns. It has been isolated from many foods (Gianfranceschi, Gattuso, Tartaro, & Aureli, 2003), among which milk and cheeses (Bernini et al., 2013; Dalmasso & Jordan, 2014; Manfreda, De Cesare, Stella, Cozzi, & Cantoni, 2005; Pintado, Oliveira, Pampulha, & Ferriera, 2005; Torres-Vitela et al., 2012) and it has been involved in numerous outbreaks occurring after consumption of contaminated milk and milk products throughout the world (Donnelly, 2001; Linnan et al., 1988; Lundén, Tolvanen, & Korkeala, 2004; Lyytikainen et al., 2000). In particular, dairy products contaminated with *L. monocytogenes* have been implicated at almost the half of the reported listeriosis outbreaks in Europe (Lundén et al., 2004). The pathogen is widespread in nature and lives naturally in plants and soil environments. Its ability to survive and grow over a wide range of environmental conditions, including refrigeration temperatures, high salt concentration and low pH, makes it a potential hazard in foods (Ryser, 2007). Moreover, *L. monocytogenes* into food processing plants results in reservoirs that are difficult to eradicate: this is the case of biofilms that are a constant issue in food processing environments (Oliver et al., 2005). So, in addition to the risk associated to direct consumption, the introduction of raw contaminated milk into dairy processing plants represents a risk to human health if milk is used unpasteurized for cheese making or in case of cross contamination (Kousta et al., 2010).

Considering the threat represented by the pathogen in raw milk, a survey was conducted from January 2010 to September 2013, involving a large number of samples collected in different geographical area in the North of Italy. This research aimed to give a considerable overview of *L. monocytogenes* presence in raw cow milk, intended both for cheese making and for direct consumption, by evaluating the prevalence of the pathogen at farm level also in relation to the seasonality.

## 2. Materials and methods

### 2.1. Samples collection

A total of 8716 raw cow milk samples were collected from January 2010 to September 2013 in 942 farms located in Lombardy and Emilia-Romagna regions, Northern Italy. All the samples, consisting in 5897 samples of bulk tank milk intended for cheese making and 2819 samples of milk intended for sale in automatic vending machines, were collected into sterile containers, kept below 4 °C during transportation and analyzed within 2 h after receipt. Samples were collected in the frame of Food Business Operator's self-control programs or in the frame of monitoring surveys officers of the Regional Veterinary Authority.

### 2.2. Detection and enumeration of L. monocytogenes

All samples were tested for the presence of *L. monocytogenes* on 25 ml of raw cow milk by means of qualitative methods.

The samples collected from 2010 to 2011 were examined qualitatively according to ISO 11290-1 (ISO, 1996). The samples collected from 2012 to 2013 were examined by a biomolecular method (real-time PCR) (Biorad AFNOR BRD 07/10-04/05) (AFNOR, 2004) to detect *L. monocytogenes* DNA. Samples testing positive were retested under a microbiological protocol according to ISO 11290-1 (ISO, 1996). Typical colonies ($n = 5$) presumed to be *Listeria* spp. were streaked from Agar Listeria acc. to Ottaviani & Agosti (ALOA) (Biolife Italiana, Teramo, Italy) supplemented with ALOA enrichment-selective supplements (Biolife Italiana) onto Tryptone Soya Yeast Extract Agar, TSYEA (Oxoid, Basingstoke, UK) and plates were incubated at 37 °C for 24 h. By following the Gram's staining, catalase reaction and tumbling motility were performed using the pure cultures obtained from TSYEA. The isolates resulted positive to phenotypic tests were inoculated on 5% sheep blood agar (Oxoid) to determine the Beta haemolytic reaction. For following confirmation, carbohydrate utilization and CAMP tests were performed.

On samples found to be positive, the enumeration of *L. monocytogenes* was carried out according to the method described by ISO 11290-2 (ISO, 1998) to evaluate the prevalence of contamination level.

### 2.3. Data analyses

The prevalence of *L. monocytogenes* in raw milk, calculated as proportion between positive samples on total sample, was expressed in percentage values. Statistical analysis was performed by Epi tools (http://epitools.ausvet.com.au): the confidence intervals (C.I.) of proportions were calculated with using the binomial exact method and the statistical significance of differences between proportions was evaluated by Chi-square ($\chi^2$) test.

## 3. Results and discussion

From 2010 to 2013, 8716 raw milk samples, intended both for cheese making and for vending machine, were collected in Northen Italy. Samples were taken from local farms within self-control sampling programs and by the official veterinarians within state surveillance programs and were investigated for the presence of *L. monocytogenes*.

The results are summarized in Table 1. The prevalence values in raw milk ranged from 0.52% (95% C.I. 0.3%–0.9%) in 2012 to 2.73% (95% C.I. 2.0%–3.8%) in 2013, but no trend of increase was observed in four-year investigation. Overall, *L. monocytogenes* was detected in 145 raw milk samples out of 8716, with a prevalence of 1.66% (95% C.I. 1.4%–2.0%). This result is mainly due ($p > 0.05$) to bulk tank milk contamination rather than to raw milk intended for vending machine. In fact, concerning bulk tank milk, *L. monocytogenes* was found in 2.22% (95% C.I. 1.9%–2.6%) of samples (131/5897) in four years of survey (Table 2). These findings were in agreement with those reported in several studies carried out internationally and recently published, in which the prevalence of *L. monocytogenes* in raw milk has ranged from 'not detected' to 7.1%. In particular, the pathogen was "not detected" in Norway in 2011 (Jakobsen, Heggebø, Sunde, & Skjervheim, 2011), and to the extent of 0.68% in New Zeland in 2012 (Hill, Smythe, Lindsay, & Shepherd, 2012), 2.12% in Turkey in 2006 (Aygun & Pehlivanlar, 2006), 2.61% in Algeria in 2007 (Hamdi, Naïm, Martin, & Jacquet, 2007), 5.5% in Finnish in 2013 (Ruusunen et al., 2013), 6.1% in North-West Spain in 2007 (Vilar, Yus, Sanjuán, Diéguez, & Rodríguez-Otero, 2007) and 7.1% in USA in 2011 (Van Kessel, Karns, Lombard, & Kopral, 2011). Previously, in 2005, the prevalence of *L. monocytogenes* in bulk tank milk has been reported to range from 1 to 12% (Oliver et al., 2005), therefore, a reduction of contamination samples seems to have been monitored in the last years. Anyway, the above data collected in different world areas underline the wide variability of the

**Table 1**
Detection and prevalence of *L. monocytogenes* in raw milk collected in Northern Italy according to the year of sampling.

| Year | Samples | Positive for *L. monocytogenes* (%) |
|---|---|---|
| 2010 | 1728 | 20 (1.16%) |
| 2011 | 3150 | 76 (2.41%) |
| 2012 | 2519 | 13 (0.52%) |
| 2013 | 1319 | 36 (2.73%) |
| Total | 8716 | 145 (1.66%) |

**Table 2**
Detection and prevalence of *L. monocytogenes* in different categories of raw milk collected in Northern Italy according to the year of sampling.

| Year | Bulk tank milk | | Raw milk for vending machine | |
|---|---|---|---|---|
| | Samples | Positive for *L. monocytogenes* (%) | Samples | Positive for *L. monocytogenes* (%) |
| 2010 | 1176 | 20 (1.70%) | 552 | 0 |
| 2011 | 2639 | 68 (2.58%) | 511 | 8 (1.57%) |
| 2012 | 1317 | 9 (0.68%) | 1202 | 4 (0.33%) |
| 2013 | 765 | 34 (4.44%) | 554 | 2 (0.36%) |
| Total | 5897 | 131 (2.22%) | 2819 | 14 (0.50%) |

*L. monocytogenes* prevalence, which can be due to several factors, as geographical area, size farm, types of housings for the cattle. As further confirmation of this variability, the overall data we collected in this study show that even in the same geographical area there may be a different prevalence of *L. monocytogenes*. In Lombardy region, the pathogen was detected in 96 (2.6%; 95% C.I. 2.1%–3.1%) of 3721 samples analyzed and the prevalence was statistically highest ($p < 0.05$) compared to that found in Emilia-Romagna region, where *L. monocytogenes* was detected in 26 (1.2%; 95% C.I. 0.8%–1.8%) of 2176 milk samples (data not shown). The different prevalence values can be due to the different treatment of the cattle usually practiced in the two Italian regions. In Lombardy, the most of the cattle is fed with silages, the housing of cattle is indoors and these practices, when combined with poor hygiene on the farm, may contribute to contamination of milk, in agreement with Husu, Seppänen, Sivelä, and Rauramaa (1990) and Sanaa, Poutrel, Menard, and Serieys (1993). In contrast with this, many researchers have identified the raw milk as a source of *L. monocytogenes*, but environmental and fecal contamination during the transportation and storage of milk have also been reported (Frece, Markov, Cvek, Kolarec, & Delas, 2010). Moreover, *L. monocytogenes* may also directly contaminate milk from animals with mastitis (Hird & Genigeorgis, 1990).

A low prevalence of *L. monocytogenes* was observed over the four years survey in raw milk samples indented for vending machine (Table 2). The pathogen was detected in 0.50% (95% C.I. 0.03%–0.8%) of samples (14/2819), but the prevalence ranged from 0% (95% C.I. 0%–0.7%) in 2010 to 1.57% (95% C.I. 0.7%–3.1%) in 2011. A similar result was obtained by Bianchi et al. (2013) who detected the *L. monocytogenes* in 1.6% of the milk samples for vending machine during the monitoring survey from 2009 to 2011 in another Italian region (Piedmont region, near Lombardy).

*L. monocytogenes* is widely extended throughout the environment (Fenlon, Wilson, & Donachie, 1996). This pathogen has the ability to survive in stress conditions and it is able to grow at low temperatures in several food types as cooked meat (Daminelli et al., 2014), vegetables (Sant'Ana, Barbosa, Destro, Landgraf, & Franco, 2012), cold-smoked salmon (Beaufort et al., 2007), milk and cheese (Schvartzman, Belessi, Butler, Skandamis, & Jordan, 2010). To prevent the growth of the pathogen in raw milk, in Italy, where the sale and the distribution of unpacked raw milk via automatic self-service vending machines was authorized since 2007, the product must be maintained at constant temperature between 0 °C and 4 °C, and the customers are instructed to boil the milk before consumption. Even if the most important aspect remains the hygienic quality of the product, these practices can contribute to improve the safety of the raw milk, as shown the low prevalence of *L. monocytogenes* reported in the present study.

To evaluate the seasonal influence on the *L. monocytogenes* presence in bulk tank milk, in Table 3 were reported the prevalence data for each year (from 2010 to 2013), broken down by each month. In four years, only in January on a total of 188 samples analyzed, milk samples positive for *L. monocytogenes* have never been found. Considering the seasonal variability, the *L. monocytogenes* prevalence was statistically lower ($p < 0.05$) during the winter season, with a prevalence of 0.8% (95% C.I. 0.3%–1.7%), in contrast with the spring prevalence of 3.04% (95% C.I. 2.3%–3.9%), the summer prevalence of 1.91% (95% C.I. 1.4%–2.6%) and the autumn prevalence of 2.33% (95% C.I. 1.6%–3.3%) (Table 3). Previous studies on *Listeria* spp. prevalence in raw milk reported some evidence of seasonal variation. Atil, Ertas, and Ozbey (2011) in eastern Turkey observed a high prevalence in spring and winter; in France, Meyer-Broseta, Diot, Bastian, Rivière, and Cerf (2003) reported peaks in winter. Ryser (1999) reported that seasonal variations in *Listeria* prevalence may be related to silage feeding, with higher prevalence in months when silage is fed to animals. In fact, *L. monocytogenes* could be present in silage, in which the pathogen can multiply if the silage has been inadequately fermented (pH above 5.0 to 5.5) (Husu, 1990). Seasonal differences in *L. monocytogenes* prevalence were observed in our monitoring, but

**Table 3**
Seasonal detection and prevalence of *L. monocytogenes* in bulk tank milk collected in Northern Italy according to the year of sampling.

| Season | Month | Sample positive/sample analyzed (%) | | | | Total for month | Total for season |
|---|---|---|---|---|---|---|---|
| | | 2010 | 2011 | 2012 | 2013 | | |
| Winter | December | 0/58 (0%) | 2/106 (1.89%) | 0/45 (0%) | nc | 2/209 (0.96%) | 6/746 (0.8%) |
| | January | 0/47 (0%) | 0/49 (0%) | 0/70 (0%) | 0/22 (0%) | 0/188 (0%) | |
| | February | 0/108 (0%) | 1/51 (1.96%) | 3/158 (1.9%) | 0/32 (0%) | 4/349 (1.15%) | |
| Spring | March | 2/174 (1.15%) | 7/233 (3%) | 0/215 (0%) | 21/212 (9.91%) | 30/834 (3.6%) | 56/1844 (3.04%) |
| | April | 0/74 (0%) | 8/225 (3.56%) | 0/79 (0%) | 12/167 (7.19%) | 20/545 (3.67%) | |
| | May | 1/112 (0.89%) | 5/218 (2.29%) | 0/76 (0%) | 0/59 (0%) | 6/465 (1.29%) | |
| Summer | June | 4/84 (4.76%) | 5/297 (1.68%) | 0/80 (0%) | 0/73 (0%) | 9/534 (1.69%) | 37/1934 (1.91%) |
| | July | 2/116 (1.72%) | 8/463 (1.73%) | 5/212 (2.36%) | 1/148 (0.68%) | 16/939 (1.7%) | |
| | August | 2/129 (1.55%) | 10/121 (8.26%) | 0/162 (0%) | 0/49 (0%) | 12/461 (2.6%) | |
| Autumn | September | 6/119 (5.04%) | 12/485 (2.47%) | 1/154 (0.65%) | 0/3 (0%) | 19/761 (2.5%) | 32/1373 (2.33%) |
| | October | 3/97 (3.09%) | 5/273 (1.83%) | 0/35 (0%) | nc | 8/405 (1.98%) | |
| | November | 0/58 (0%) | 5/118 (4.24%) | 0/31 (0%) | nc | 5/207 (2.42%) | |

nc: Data not collected.

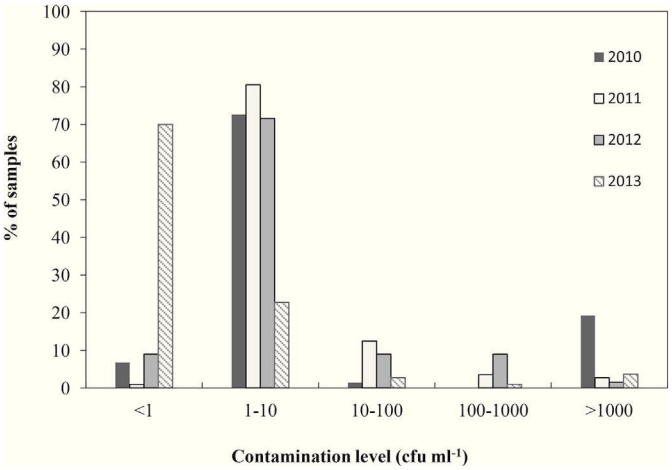


**Fig. 1.** Counts of *L. monocytogenes* in bulk tank milk according the year of sampling, expressed as percentages of samples containing colony numbers ranging from <1 to >1000 cfu $ml^{-1}$.

more studies are needed to determine the validity and the causes of these differences. In fact, in the present study, the reduced number of samples found positive to *L. monocytogenes* in the winter season may be due to the lower number of samples analyzed (746 samples against over 1000 samples analyzed in each of the other seasons), or to other variables such as the type of cattle feed (fresh grass or silage), the hygienic conditions of breeding or the presence of cattle infected with mastitis.

The contamination levels of *L. monocytogenes* in bulk tank milk samples are reported in Fig. 1 as percentage of samples with pathogen concentration variable between <1 and >1000 cfu $ml^{-1}$. Overall, more than 80% of the contaminated samples contained <10 cfu $ml^{-1}$ of *L. monocytogenes*. This trend is reflected from 2010 to 2012, while in 2013 the majority of the positive samples contained <1 cfu $ml^{-1}$ of the pathogen, as shown in Fig. 1. It is difficult to compare the results from this study with those conducted in other countries, because most studies express the results only qualitatively. However, a study by Meyer-Broseta et al. (2003), shows that even in France, the level of contamination of bulk tank milk was very low, generally less than 1 cfu $ml^{-1}$ of the *L. monocytogenes*.

## 4. Conclusions

During 2007–2009 in Italy there was an increase of notifications of listeriosis with the most cases are reported in the Centre-North of Italy. This is probably attributable both to a real increase of listeriosis in Italy and to surveillance implementation (Pontello et al., 2012). However, statistically significant increasing trends in listeriosis notification rates from 2005 to 2009 were noted in Italy as elsewhere in Europe (EFSA/ECDC, 2011). In the present study the raw milk was found positive to the presence of *L. monocytogenes*, confirming the milk as potential source of the food borne disease, but the low prevalence and the reduced level of pathogen concentration, when present, can highlight the hygienic quality of the milk produced in Northern Italy. Furthermore, the collected data in the present study can be a useful tool for the quantitative risk assessment study for human listeriosis linked to the consumption of raw milk and cheese made from raw milk in Italy. Furthermore, the collected data in the present study can be a useful tool for the quantitative risk assessment study for human listeriosis linked to the consumption of raw milk and cheese made from raw milk in Italy.

## Disclosure

Authors declare that no conflict of interests exists.

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# 4. BEHAVIOUR OF *Listeria monocytogene* AND *Escherichia coli* O157:H7 DURING THE CHEESE MAKING OF TRADITIONAL RAW-MILK CHEESES FROM ITALIAN ALPS

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# Behaviour of *Listeria monocytogenes* and *Escherichia coli* O157:H7 during the cheese making of traditional raw-milk cheeses from Italian Alps

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## Abstract

The behaviour of *Listeria monocytogenes* and *Escherichia coli* O157:H7 was studied during the manufacture and ripening of two traditional Italian Alps cheeses. Each cheese type was manufactured in a pilot plan from raw cow milk (without the addition of starter cultures) artificially inoculated with *L. monocytogenes* and *E. coli* O157:H7 to a final concentration of about 4 log CFU/mL. The pathogens were enumerated throughout the cheese making and ripening processes to study their behaviour. When the milk was inoculated with 4 Log CFU/mL, the pathogens counts increased in the first time during the manufacturing process and then remained constant, until the end of ripening, or decreased significantly. Results indicate that the environment and nature of food borne pathogens affected the concentration of the bacteria during the manufacturing and ripening process. Thus, the presence of low cells numbers of *L. monocytogenes* and *E. coli* O157:H7 in milk destined for the production of raw milk cheeses characterized by a cooking of the curd less than 48°C can constitute a hazard for the consumer.

## Introduction

The food business operators (FBOs) have to check the hygiene of their production following the European Commission (EC) Regulation No. 2073/2005 (European Commission, 2005). In the online database, Rapid Alert System for Food and Feed (RASFF), created by the EC, it is published that, in the last ten years, 55 alerts were regarding the presence of *Listeria monocytogenes* and verocytotoxin *Escherichia coli* (VTEC) in raw milk cheeses, mainly produced in France (RASFF, 2007). In fact, among dairy products, the raw milk cheese, characterized by the cooking of the curd at temperature less than 48°C, are known to be the most frequently contaminated (CDSC, 2000; Conedera *et al*., 2004; Bielaszewska *et al*., 1997; EFSA, 2013; Farrokh *et al*., 2013) and it is documented that contaminated raw milk cheeses, with short ripening time (less than 60 days) could generate severe outbreak (Health Canada, 2013).

Many regional cheeses throughout Europe are manufactured from unpasteurized milk, and there is growing concern that fresh cheeses, made by raw milk, could be contaminated by food pathogens (Vernozy-Rozand *et al.,* 2005). Traditional, raw milk cheeses, obtained by the cooking of the curd at temperature less than 48°C, are produced in Alps area, and while more data are available for the French cheeses (Miszczycha *et al*., 2013) few is known about the behaviour of food pathogens during the cheese making of Italian raw milk cheeses produced in Alps area.

The cheese manufacturing process affects strongly the eco-system in which the food pathogen could be present. The cheese making temperature, the pH and $a_w$ reduction, the presence of indigenous bacterial population, are all variables that can modify the behaviour of undesirable bacteria (Buchanan *et al*., 1993). For this reason, many cheese making processes are registered in the Minister of Health web site on quality and safety of Italian food product (www.ars-alimentaria.it).

The purpose of this work was to study the behaviour of *L. monocytogenes* and *E. coli* O157:H7 in two cheeses produced in Alps area, by challenge test performed in a pilot plan at the Veterinary Public Health Institute of Lombardy and Emilia Romagna, Brescia, Italy.

## Materials and Methods

### Raw milk

A total of 700 L of raw cow milk were collected at different time during the summer season in the Alps in Northern Italy. Milk was collected from the bulk ripening tank and maintained refrigerated at 4±0.5°C for transportation to the pilot plan and processed immediately.

### Bacterial cultures

Two multi-strain cocktails of *L. monocytogenes* and *E. coli* O157:H7 were used in this experiment. *L. monocytogenes* ATCC® 19115 and two wild strains (isolated from cheeses; BVR; www.ibvr.org) and *E. coli* O157:H7 ATCC®35150 and two wild strains (isolated from milk; BVR; www.ibvr.org) were used in the challenge test. The bacterial cultures were prepared in agreement with Dalzini *et al*. (2015). Raw milk was separately inoculated with two multi-strain cocktails, with the ratio of 1:100 v/v, in order to obtain an initial milk contamination of about 4 Log CFU/g (contaminated cheese). For the production of not contaminated cheeses the milk was inoculated with sterile peptone water (PW) (CONDA, Madrid, Spain) at the same ratio.

### Cheese preparation

Two different cheeses were manufactured in pilot plan. Both types of cheeses were done following specifications of producers (www.ars-alimentaria.it). The manufacturing process were summarized in Table 1 (for cheese A) and in Table 2 (for cheese B). In order to produce cheese A (short ripened cheese), a total of 150 L of raw cow milk was used. During the process, no heat treatment was applied to the curd. Cheeses were ripened on wooden boards at 4-5°C for 60 days with turning over every 1-3 days. A total of 30 cheeses (1 kg each) were obtained: 10 cheeses contaminated with *L. monocytogenes*, 10 cheeses contaminated with *E. coli* and 10 non contaminated cheeses. In order to produce cheese B (long ripening cheese), a total of 150 L were used. The curd was cooked at 45°C for 15 min and then moulded into 80 by 300 mm cylindrical wooden moulds. Cheeses were ripened at 12°C for 4 months. Five cheeses, 8 kg each, were obtained.

### Bacterial and physico-chemical analysis

While the milk was not diluted, the solid

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samples were transferred in a sample bags (NEOMED, Milan, Italy) and homogenized 1:3 w/v in sterile PW for 3 min in a Stomacher 400 blender (Seward Medical, London, UK). Serial 10 fold dilutions of control samples test material were prepared in sterile PW. In contaminated samples, the enumeration of *E. coli* O157:H7 was performed in agreement with Vernozy-Rozand *et al*. (2005) and Coscian-Cunico *et al*. (2014), while the *L. monocytogenes* enumeration was carried out according to the standard method ISO 11290-2 (ISO, 1998a). To verify the natural contamination of raw milk, at time zero, the enumeration of pathogens was also investigated in not contaminated samples.

In not contaminated samples, the enumeration of lactic acid bacteria (LAB) was performed according to the standard methods ISO 15214 (ISO, 1998b). All analyses were carried out in milk, curd after the extraction, and in cheese at different sampling times during the ripening step.

The physical-chemical analyses were carried out in not contaminated sample. The pH values (Hanna Instrument, Woonsocket, RI, USA), and the water activity ($a_w$) (Decagon Devices, Inc., Pullman, WA, USA) were measured.

## Statistical analysis

For the short ripened cheese, the average and standard deviations of *L. monocytogenes* and *E. coli* O157:H7 microbial counts were determined from the average of three samples at each sampling time, while the average and standard deviations of LAB microbial counts, as well for the physical-chemical values, were determined from the average of two samples at each sampling time.

For the long ripened cheese, the average and standard deviations of *L. monocytogenes* and *E. coli* O157:H7 microbial counts were determined from the average of two samples at each sampling time, while LAB microbial counts, as well for the physical-chemical values, were determined from a single sample at each sampling time.

Analysis of variance (ANOVA) was carried out. The significance was statistically analysed by Student *t*-test at a 95% confidence interval ($P<0.05$) using R statistical software version 2.7.0 (R Development Core Team, 2008).

**Table 1. Conditions of manufacturing process of short ripened cheese (cheese A), made from raw milk, from the cheese making to the end of ripening time.**

| Manufacturing step | Duration (hours) | Temperature (°C) |
|---|---|---|
| Addition of cheese rennet* | 0.5 (0.02) | 32 |
| Acidification and coagulation | 0.75 (0.03) | 22-18 |
| Cutting coagulum into 0.5 cm cubes and sineresis | 1 (0.04) | 22-18 |
| Moulding° | 0.5 (0.02) | 22-18 |
| Draining and overturning | 24 (1) | 22-18 |
| Manual salting | 0.5 (0.02) | 22-18 |
| Draining and inversion | 48 (2) | 22-18 |
| Ripening | 1368-1440 (57-60) | 5-6 |

*1:10000 mL/mL; °40 by 200 mm quadratic wooden moulds. Values in parenthesis represent days.

**Table 2. Conditions of manufacturing process of long ripened cheese (cheese B) made from raw milk, from the cheese making to the end of shelf life.**

| Manufacturing step | Duration (hours) | Temperature (°C) |
|---|---|---|
| Addition of cheese rennet* | 0.5 (0.02) | 36 |
| Acidification and coagulation | 0.5 (0.02) | 21 |
| Cutting of the coagulum in 4 cm cubes and then in grains of rice | 1 (0.04) | 21 |
| Cooking of the curd, sineresis | 0.25 (0.01) | 45-48 |
| Moulding° | 0.5 (0.02) | 38 |
| Pressing of the curd | 3 (0.125) | 21 |
| Draining and overturning | 36 (1.5) | 20-18 |
| Manual salting | 192 (8) | 12 |
| Ripening | 1488 (120) | 12 |

*1:10000 mL/mL; °80 by 300 mm cylindrical wooden moulds. Values in parenthesis represent days.

**Table 3. $p_H$, aw values and lactic acid bacteria concentration in milk, curd and cheeses during manufacturing and ripening.**

| Cheese type | Test material | pH | $a_w$ | LAB (Log CFU/mL, CFU/g) |
|---|---|---|---|---|
| Cheese A | Milk | 6.75±0.03 | ND | 4.66±0.01 |
| | Curd | 6.5±0.08 | 0.967±0 | 8.38±0.05 |
| | Cheese (1)° | 5.56±0.3 | 0.981±0.002 | 9.22±0.11 |
| | Cheese (15)° | 5.24±0.09 | 0.983±0.01 | 9.42±0.08 |
| | Cheese (30)° | 5.39±0.06 | 0.971±0.006 | 9.2±0.08 |
| | Cheese (60)° | 5.4±0.15 | 0.947±0.007 | 8.93±0.25 |
| Cheese B | Milk | 6.73 | ND | 4.07 |
| | Curd | 6.68 | 0.991 | 6.81 |
| | Cheese (1)° | 6.03 | 0.994 | 8.57 |
| | Cheese (3)° | 5.12 | 0.993 | 8.77 |
| | Cheese (7)° | 5.24 | 0.989 | 8.99 |
| | Cheese (10)° | 5.22 | 0.997 | 9.07 |
| | Cheese (20)° | 5.11 | 0.968 | 8.96 |
| | Cheese (30)° | 5.11 | 0.96 | 8.83 |
| | Cheese (60)° | 5.02 | 0.95 | 8.77 |
| | Cheese (90)° | 5.04 | 0.956 | 8.65 |
| | Cheese (120)° | 5.12 | 0.951 | 8.67 |

LAB, lactic acid bacteria; ND, not determined; CFU, colony forming unit. °Day of ripening. Values are represented as mean±standard deviation of two replicates samples for the short ripened cheese (A); while as a single value for long ripened cheese (B). Values in parenthesis represent days.

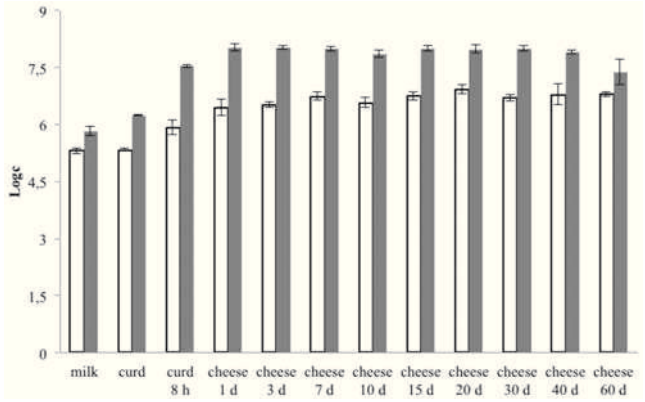


**Figure 1. Logc concentration of *Listeria monocytogenes* (white bars) and *Escherichia coli* O157:H7 (grey bars), throughout the cheese making and ripening of short ripened cheese. Values are obtained from the average and standard deviations of three samples at each sampling time.**

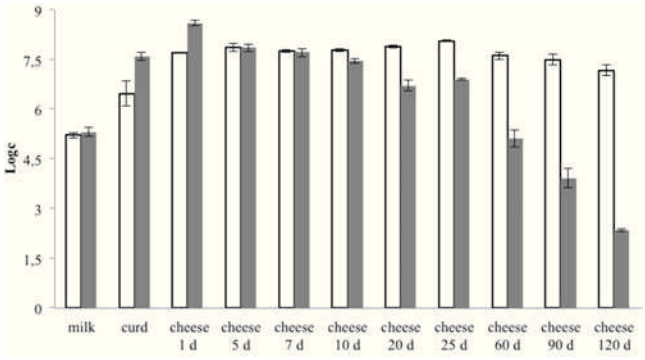


**Figure 2. Logc concentration of *Listeria monocytogenes* (white bars) and *Escherichia coli* O157:H7 (grey bars), throughout the cheese making and ripening of long ripened cheese. Values are obtained from the average and standard deviations of two samples at each sampling time.**

## Results

Microbiological analysis in not contaminated milk revealed the absence (<0.47 log CFU/mL) of *E. coli* O157 and *L. monocytogenes* (data not shown). The LAB concentration and the physical-chemical properties of milk, curds and cheeses measured on not contaminated cheeses, were shown in Table 3. In the cheese with a short ripening time (cheese A) the pH decreased during the coagulation step, reaching pH 5.56±0.3 at the end of the draining, and remained almost stable until the end of ripening (pH 5.4±0.15). In the cheese with a long ripening time (cheese B) the pH reached, after 3 days, the pH 5.12 and remained constantly low until the end of the ripening. The $a_w$ decreased gradually during the ripening until it reached almost 0.95 in both cheeses. Analyses of raw milk indicated that, before the cheese making, the LAB concentration was 4.66 and 4.07 Log CFU/mL respectively in cheese A and B. During the process, LAB concentration increased up to 9.22 and 8.57 Log CFU/g in both cheeses and remained relatively stable during the ripening step. Different *E. coli* O157:H7 and *L. monocytogenes* behaviour were observed during both manufacturing processes. In both contaminated cheese types, the pathogen concentrations increased significantly during the first hours of the manufacturing process (Figures 1 and 2). This can be due to the concentration of milk proteins in the curd (Miszczycha *et al.*, 2013). While *L. monocytogenes* concentration remained stable during the ripening time in both cheeses, the concentration of *E. coli* O157:H7 remained stable during the ripening time of short ripening cheese, but decreased significantly (P>0.05, more than 6 Log CFU/g) in long ripening cheese (Figures 1 and 2).

## Discussion

In the present study we observed as the manufacturing process of raw milk cheese can affect the behaviour of two important food pathogens such as *L. monocytogenes* and *E. coli* O157:H7 in different ways. In fact, the cheese-making process and the physical chemical variables of the two cheeses are different and this seems to affect more the behaviour of the Gram negative *E. coli* than the Gram positive *L. monocytogenes* (El-Ziney *et al.*, 1998). In particular, the *L. monocytogenes* concentration remained stable during the type manufacturing process of both cheeses and showed a low variability during the ripening step. The pathogen reached the apparent stationary phase at the beginning of ripening time and remained constant. This can be explained considering that the amount of organic acid, produced by the lactic acid bacteria, inhibited the growth of *L. monocytogenes* (Le Marc *et al.*, 2002; Mellefont *et al.*, 2008; Cornu *et al.*, 2011). During the short ripened cheese type process, even the *E. coli* O157:H7 concentration did not change, as previously reported in a *Formaggella* produced in the northwest area of Lombardy region (Cosciani-Cunico *et al.*, 2014). Conversely, in the long ripened cheese, the pathogen concentration decreased significantly, which can be due to the lower pH of cheese during the ripening step (under 5.3 value) in agreement with Buchanan (1993). The same result was previously observed in a French goat cheese made with raw milk in which *E. coli* O157:H7 concentration decreased gradually during the ripening phase (Vernozy-Rozand *et al.,* 2005).

## Conclusions

The present study confirms the influence of the cheese-manufacturing environment on the *L. monocytogenes* and *E. coli* O157:H7 growths, in particular during the first days of the process as reported by others authors in different cheese types (Ryser and Marth, 1987; Ramsaran *et al.*, 1998; www.combase.cc). Results indicate that *L. monocytogenes* and *E. coli* O157:H7 can survive the manufacturing process. Thus, the presence of low numbers of these pathogens in milk, destined for the production of raw milk cheeses characterized by a short ripening time, can constitute a threat to the consumer. Even *L. monocytogens* did not decrease during the ripening of the long ripening time cheese, considered in this study, while *E. coli* O157:H7 decreased significantly. Knowing the intrinsic end extrinsic variables of these two traditional Italian Alps cheeses, observed data reported in this study could be used to validate dynamic predictive mathematical model published in the literature (Baranyi and Roberts, 1995).

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# 5. MODELLING THE BEHAVIOUR OF *Listeria monocytogenes* DURING MAKING CHEESE FROM RAW MILK

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**Modelling the behaviour of *Listeria monocytogenes* during making cheese from raw milk**

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**Abstract**

The presence of *Listeria monocytogenes*, even if at low levels, in raw milk used for to produce raw milk cheeses represents a safety issue. The present study shows the inhibitory effect of starter culture bacteria at different levels on the growth of *L. monocytogenes* thanks to the "Jameson effect". The experimental data ware used to validate a model developed in this study to describe the behavior of *Listeria* as a function of the temperature and the lactic acid bacteria growth. The predicted data were in good agreement with observations in cheese, showing a discrepancy between 2.18% and 4.64%. This model may be a very useful tool to support the monitoring surveys carried out by officers of the Regional Veterinary Authority. In fact, knowing the manufacturing conditions allows to predict the growth of *L. monocytogenes*, as to assess the safety of the product before it reaches the final consumer.

## 1. Introduction

In 2013, a total of 12 *Listeria* outbreaks were reported by the European Member States due to different food categories like 'Cheese', 'Meat and meat products', 'Pig meat and products thereof', 'Vegetables and juices and other products thereof (mixed salad)'. Raw milk may be a source of foodborne illness outbreaks, specially due to *L. monocytogenes* pathogen, if consumed without sanitizing treatments. *L. monocytogenes* was often detected in samples of soft and semi-soft cheeses made from raw or low heat-treated milk than in samples of cheeses made from pasteurised milk (EFSA, 2015; Verraes et al., 2015). In Northern Italy the prevalence of *L. monocytogenes* was very low, about of 1.66% (95% C.I. 1.4%e1.7%) (Dalzini et al., 2016), but the raw milk is often used to produce a large variability of soft or semi-

soft cheese, therefore is useful to know the behaviour of the pathogen throughout the cheesemaking. In fact, numerous studies shown that the survival or growth of *L. monocytogenes* during the manufacture of cheese depends on several factors like on technological parameters during cheesemaking, ripening and storage, on nature and activity of starter culture, on the rate of fermentation and on the temperature (Morgan et al., 2011; Pitt et al., 1999). As reported by Xanthiakos et al. (2005) traditional approaches to milk safety that rely heavily on regulatory inspection and sampling regimes cannot sufficiently guarantee consumer protection because: (i) they are time-consuming, (ii) 100% sampling and inspection is financially and logistically impossible and (iii) temperature abuse in the chill-chain, a major cause of safety problems, is not being dealt with. An alternative approach to traditional methods is to rely on quantitative microbiology (McMeekin et al. 1997). To model *L. monocytogenes* behavior, in this study we used an approach based on the description of the "Jameson effect" i.e. the simultaneous stop of the growth of all microorganisms when the dominant bacterial population reaches its stationary phase. As reported by Le Marc et al. (2009), this approach is also empirical but unlike the previous one does not require the estimation of additional parameters. The "Jameson effect" was first defined in enrichment broths (Jameson, 1962) and was also observed in various fish and meat products (Coleman et al., 2003; Ross et al., 2000;). Giménez & Dalgaard (2004) successfully applied a model based on the description of the "Jameson effect" to describe the inhibitory effect of LAB on the growth of *L. monocytogenes* in salmon. The hypothesis underlying this effect is that the microorganisms are similarly inhibited by the production of the same by-product, or by the depletion of the same nutrient. It can be hypothesized, however, that in some situations one or several

species are more sensitive than the dominant flora to end-products and/or to the depletion of some limiting substrates and inhibited earlier.

The aims of this study were (i) to study the influence of different inoculums levels of starter culture on the behavour of *L. monocytogenes* during the manufacturing of an Italian soft cheese made with raw milk and (ii) to develop of a predictive model to describe this effect, as well as the growth of LAB as a function of temperature, using data available in literature (www.combase.cc).

## 2. Materials and methods

### *2.1. Behaviour of L. monocytogenes during the manufacturing of raw milk cheese made with different inoculums levels of starter culture*

#### *2.1.1. Milk contamination and cheesemaking*

In order to study the behaviour of *L. monocytogenes* during the cheesemaking and the ripening of soft raw milk cheese as a function of different starter culture concentrations, a total of 300 l of raw cow milk was provided by local farms and transported to IZSLER's laboratory in refrigerate condition (4°C). *L. monocytogenes* registered strain ATCC® 19115™; were used to contaminate the milk and the inoculum was prepared following what indicated by Dalzini et al. (2015). As starter culture was used a multi-strain cocktail of *Lactococcus lactis* ATCC® 11454™ and *L. lactis* CRA 26 (from the collection of CRA-FLC of Lodi-Italy) (Carminati et al., 1989). Milk was inoculated with starter culture at different inoculums levels and cheese rennet, and incubated at 21 °C for the acidification and coagulation steps. After 40 minutes, the coagulum was cut into 4 cm cubes and let stand for 60 min to induce syneresis. The curd was transferred into square moulds, and let stand at 22°C for 48

h, turning the moulds every 2h. Finally, the cheeses were dry-salting and ripened for 20 days in climatic chamber at 12°C with 85% Relative Humidity (RH).

*2.1.2. Experimental design:*

From 300 L of milk was prepared 3 type of cheese with different inoculums levels:

Cheese A: 40 L of milk was inoculated with 1% v/v of *L. monocytogenes* and with the multi-strain cocktail of *L. lactis* in order to give a final concentration of 2 log cfu $mL^{-1}$ for each bacterial culture (Contaminated cheese); another batch (40 L) of milk was inoculated only with the multi-strain cocktail of *L. lactis* (2 log cfu $mL^{-1}$) (Control cheese).

Cheese B: 40 L of milk was inoculated with 1% v/v of *L. monocytogenes* (2 log cfu $mL^{-1}$) and with the multi-strain cocktail of *L. lactis* (6 log cfu $mL^{-1}$) (Contaminated cheese); another batch (40 L) of milk was inoculated only with the multi-strain cocktail of *L. lactis* (6 log cfu $ml^{-1}$) (Control cheese).

Cheese C: 40 L of milk was inoculated with 1% v/v of *L. monocytogenes* (2 log cfu $mL^{-1}$) and with the multi-strain cocktail of *L.s lactis* (8 log cfu $mL^{-1}$) (Contaminated cheese); another batch (40 L) of milk was inoculated only with the multi-strain cocktail of *L. lactis* (8 log cfu $mL^{-1}$) (Control cheese).

*2.1.3. Microbiological and physicochemical analyses*

The microbiological analyses on milk were performed through direct plate count. Subsequently, in the case of curd and cheese, 25 g of contaminated and control samples were first transferred separately into plastic one-chamber filter stomacher bags (NEOMED, Milano, Italy) and then homogenized 1:3 (w:v) in sterile Peptone Water (PW, CONDA, Madrid, Spain) for 3 min using a Stomacher 400 blender

(Seward Medical, London, UK). Decimal dilutions in sterile PW were then prepared. For the enumeration of *L. monocytogenes* in contaminated cheeses, appropriate dilutions were surface-plated onto duplicate plates of differential-chromogenic ALOA agar (Microbiol Diagnostici, Cagliari, Italy) (ISO, 1998). Mesophilic Lactic Acid Bacteria (LAB) were enumerated in control cheeses by pour plating 1 ml of appropriate dilution in de Man, Rogosa and Sharpe Agar (MRSA) (Microbiol Diagnostici, Cagliari, Italy). Typical colonies of each microorganism were counted after the incubation of plates at 37 °C for 24-48 h (ALOA) or 48-72 h (MRSA).

The physicochemical analyses were performed on 10 g of control samples examined for each sampling time. The pH was measured on 10 g of control samples using an HI 223 Calibration checkTM Microprocessor pH meter (Hanna Instrument, USA) equipped with a Gel-Glass electrode (Hamilton, Switzerland). Water activity ($a_w$) was measured at 25 °C with the $a_w$ recorder AquaLab, series 3, Model TE (Decagon Devices, Inc., Pullman, USA) in accordance with ISO/FDIS 21807 (ISO/FDIS, 2004). The time/temperature profile during cheesemaking/ripening/shelf life was monitored using electronic data loggers (cox tracer, Cox Technologies, Belmont N.C., USA).

Each analyses was performed on three replicate samples.

### *2.2. Model development*

#### *2.2.1. Modelling*

The mathematical model was developed using experimental data collected from ComBase database

(www.combase.cc) about *L. monocytogenes* grown on milk (raw, heated or sterile) with pH between 5.4 and 7 and temperature from 4°C and 37°C. Growth curves for

each temperature and strain were built separately by fitting data to the Baranyi model (Baranyi & Roberts, 1994) using DMFit version 2.1 Excel_add-in (www.ifr.ac.uk/safety/DMfit) to measure growth parameters [maximum specific growth rate ($\mu_{max}$), Lag time and maximum population ($y_{end}$)]. The data used for model development are shown in detail in **Table 1**. The specific growth rates obtained were modelled as a function of temperature using the model of Ratkowsky et al. (1982):

(Equation 1):

(1)

$$\sqrt{\mu} = b(T - T_0)$$

In this model, $\sqrt{\mu}$ is the square root of maximum specific growth rate, $b$ is the slope of the regression line, T is temperature and $T_0$ is a conceptual minimum temperature for microbial growth, where T

was given in °C.

The prediction of *Listeria* growth under dynamic temperature profile was based on the time-temperature profile during the cheesemaking of an Italian generic raw milk soft cheese, in conjunction with the square root model for the estimation of the 'momentary' $\mu_{max}$. Under dynamic temperature profile, models were solved numerically using the second order Runge-Kutta method.

*2.2.2. Model validation*

To validate the developed model in dynamics temperature conditions, the predictions of *L. monocytogenes* concentration were compared with measurements obtained from the cheesemaking of an Italian generic raw milk soft cheese made

with different inoculums levels of starter culture. Bias (B), accuracy factor (A) and discrepancy between model predictions, *pred*, and observations in foods, *obs*, were estimated as reported by (Baranyi et al., 1999) and briefly described below in Equations 2, 3 and 4:

(2) $$\text{Discrepancy (\%)} = (e^{A}\text{-}1)100$$

where:

(3) $$A = \sqrt{\frac{\sum_{i=1}^{n}(\text{Ln } pred - \text{Ln } obs)^2}{n}}$$

where A is the accuracy factor, n is the number of observations, and:

(4) $$B = \frac{\sum_{i=1}^{n} \text{Ln } pred - \text{Ln } obs}{n}$$

The accuracy factor is a measure of the average difference between observed and predicted values and the bias factor indicates a systematic over- or under-estimation of microbial growth. The bias factor values were calculated so that numbers higher than 1 always indicated that predicted growth was higher than observed growth. Graphic comparison of predicted and observed values were used to illustrate distribution of data and to evaluate model performance for different sub-sets of the dataset. Natural logarithms values were plotted as suggested by Pin et al. (2011). To take the inhibitory effect of the LAB concentration into account, we assumed that no cell division took place after LAB reached a concentration level (Jameson effect).

## 3. Results and discussion

As reported by Cornu et al. (2011), *L. monocytogenes* and lactic acid bacteria (LAB) are among the bacteria that are best adapted to grow at low temperatures. Both populations may then be in competition in foods. Competitive growth has been intensively investigated in environments such as cold-smoked salmon, and poultry and meat products including pork meat products, such as cooked ham (Amézquita & Brashears, 2002; Beaufort et al., 2007; Coleman et al., 2003; Delignette-Muller et al., 2006; Mellefont and Ross, 2007). Soft cheeses have been implicated in a number of sporadic cases of listeriosis (De Buyser et al., 2001) and many authors have shown that *L. monocytogenes* can survive in soft cheese during ripening (Morgan et al., 2001; Cosciani-Cunico et al., 2015). In this study was reported the behaviour of *L. monocytogenes* inoculated during the manufacturing of raw milk cheese made with different inoculums levels of starter culture. The results of microbiological and physicochemical analyses during the manufacturing of raw milk cheese made with different inoculums levels of starter culture were reported in Table 2.

Similar results were observed by Cosciani-Cunico et al., (2015) in soft raw milk cheese where LAB concentration increased up to 9.22 Log cfu $g^{-1}$ during the milk coagulation, the pH decreased reaching pH 5.56 ± 0.3 at the end of the draining, and remained almost stable until the end of ripening (pH 5.4 ± 0.15), and the *Listeria* concentration increased significantly during the first hours of the cheesemaking and then it remained stable during the ripening.

In this study the pathogen concentration did not exceed the maximum level of 8 cfu $g^{-1}$ but reached a maximum concentration of 5.18 ± 0.05log cfu $g^{-1}$ (at the end of the ripening in Cheese B) and then the growth was suppressed (Table 2). This was observed for each replicates and may be due to a non-specific competition for

nutrients by the pathogen and the indigenous microflora present at high concentration in the matrix. Similar competition was also observed by other authors between *L. monocytogenes* and the natural biofilm microflora of wooden shelves used for the cheese ripening, when the pathogen growth were completely suppressed when the natural microflora reaches their maximum concentration (Cosciani-Cunico et al., 2015; Guillier et al., 2008). This may be due to the Jameson effect described like a 'race' for nutrients between microbial species. In this 'race', both initial inoculum levels and growth parameters are important factors to determine which of the species are likely to overcome others (Mellefont et al., 2008). As reported by Le Marc et al. (2009), inhibition of pathogens by LAB is usually attributed to the production of weak acids, especially lactic acid. During the fermentation process, the lactic acid produced decreases the pH by the process of dissociation. Both decreasing pH and increasing undissociated lactic acid have inhibitory effects on any pathogenic bacteria possibly present. However other mechanisms may be also involved. The hurdle effect of complex microbiota on *L. monocytogenes* seems to be highly variable and remains unclear at this time. It is probably related to multiple factors such as competitive interactions and/or synergistic effects of antagonistic metabolites produced in situ, such as organic acids, volatile compounds and $H_2O_2$ (Irlinger & Mounier, 2009).

During last years, several mathematical models describing the effect of temperature on the kinetic parameters of *L. monocytogenes* have been published (Buchanan and Phillips 1990; Xanthiakos et al., 2005). Considering the relevance of the LAB on the behavior of *L. monocytogenes* in raw milk, in this study a mathematical model based on Jameson effect was developed.

The growth data of *L. monocytogenes* in milk were collected form ComBase web site (www.combase.cc) and from the IZSLER data collection. The log count were fitted to the primary model of Baranyi & Roberts (1994) in order to calculate the kinetic parameters maximum specific growth rate ($\mu_{max}$). Representative growth curves of the pathogen in milk naturally contaminated are shown in Figure 1. The $\mu_{max}$ was further modelled as a function of temperature using a square root-type model (Figure 2). The parameter of the model (slope) and the correlation coefficients for the regression equation were 0.0189 ($h^{-1}$) and 0.74 respectively. Similar results were reported by Xanthiakos et al. (2005) in their study, where the specific growth rates of *L. monocytogenes* in milk were modelled as a function of temperature using the square-root type model, obtaining a slope of 0.024 ($h^{-1}$). The calculated value for the theoretical minimum temperature of growth (Tmin) was -0.57°C. The model described in this study does not include a lag phase. As a consequence, the model may be biased and over-predict bacterial concentration in both static and changing conditions. As reported by Pin et al. (2011) modelling the dependence of the lag period on the environment requires experimental work to quantify the effect of the previous and current environment on the lag time. This information is required to predict the transition from lag to exponential growth phase or vice-versa under fluctuating environments. With the required set of experiments, predicting the duration of the lag period and the transitions from exponential to the lag phase and vice-versa under fluctuating environmental conditions can be achieved (Munoz-Cuevas et al., 2010). Graphic comparison of predicted and observed values were used to illustrate distribution of data and to evaluate model performance (Figure 3). Validation studies were necessary in order to evaluate the efficiency of the obtained predictive models before practical application. External validation was carried out

using independent data sets obtained in our experimental design, but not included in the development of models, as recommended by te-Giffel and Zwietering 1999. The growth data of *L. monocytogenes* during the manufacturing of raw milk cheese made with different inoculums levels of starter culture were used for external validation. To predict the growth of *L. monocytogenes* as a function of Jameson effect, the growth simulation was stopped when the starter cultures reach their maximum concentration (about at 8 log cfu $g^{-1}$).

Model predictions were compared with observations in foods (Table 3). A reported by Pin et al. (1999) values between 25 and 50% for the discrepancy between model predictions and observations have been reported as acceptable when validating other models; then, the data predicted were in good agreement with observations in cheese stored at dynamic temperature, showing a discrepancy between 2.18% and 4.64%. The bias factors ranged from 1.06 to 1.18 exceeding the value of 1. This indicated that predicted growth was higher than observed growth. In fact, the model was develop considering the growth rate of *L. monocytogenes* mainly in milk heat-treated, in which never competition with indigenous bacteria can slow down the pathogen growth. The comparison between predicted and observed growth of *L. monocytogenes* during the manufacturing of three type of soft cheese made with different inoculums levels of starter culture is shown in Figure 4 were is possible to observe experiments with good agreement between predictions and observations. The experimental data highlights that the growth of *L. monocytogenes* was inhibited when the LAB have reached a critical density.

In conclusion, the presence of low levels of *L. monocytogenes* in raw milk destined for the production of raw milk cheeses characterized by a short ripening time, can constitute a threat to the consumer. Using high levels of starter culture bacteria is

possible to inhibit the *Listeria* growth thanks to the competitions defined as Jameson effect. The develop of a simple mathematical model to describe the behavior of *Listeria* as a function of the LAB growth, may be a very useful tool to support to support the monitoring surveys carried out by officers of the Regional Veterinary Authority. In fact, knowing the manufacturing conditions (fermentation and ripening temperature profiles, LAB counts) allows to predict the growth of *L. monocytogenes*, so as to assess the safety of the product before it reaches the final consumer.

## Disclosure

Authors declare that no conflict of interests exists.


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**Table 1.** Experimental data collected from ComBase database (www.combase.cc) and IZSLER collection about *L. monocytogenes* grown in milk at different temperature and pH used to develop the predictive model.

| Product | n° curves | Temperature (°C) | pH |
|---|---|---|---|
| Chocolate milk | 5 | 4 - 35 | 6.6 |
| Fermented milk | 4 | 25 | 6.5 |
| Goat milk | 2 | 8 | 6.6 |
| Milk | 44 | 4 - 35 | 5.4 - 6-7 |
| Non fat milk solids | 2 | 10, 22 | 6.6 |
| Pasteurised milk | 9 | 13 - 35 | 5.24 - 6.42 |
| Reconstituted non-fat dry milk | 9 | 10 - 35 | 6.4 - 6.6 |
| Semi-skimmed milk | 7 | 4 - 30.4 | 6.6 - 6.8 |
| Skimmed milk | 33 | 4 - 22 | 6.6 - 6.7 |
| UHT milk | 45 | 0 - 37 | 6.5 - 6.7 |
| Whole milk | 38 | 3 - 37 | 6.6 - 6.8 |
| Naturally contaminated* | 5 | 12 - 37 | 6.5 |

* Data from IZSLER collection

**Table 2.** *L. monocytogenes* (Lm) and Mesophilic Lactic Acid Bacteria (LAB) log count, pH and $a_w$ values measured during the manufacturing of three type of soft cheese made with different inoculums levels of starter culture. The data represent the average values ± standard deviation of three samples for each cheese type.

| Parameters | Milk | Curd | Cheese | Cheese at different ripening times (days) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 4 | 5 | 7 | 12 | 15 | 20 |
| Cheese A | | | | | | | | | | | |
| Lm[a] | 2.49 ± 0.00 | 2.25 ± 0.00 | 2.59 ± 0.10 | 3.67 ± 0.08 | 4.61 ± 0.08 | 4.72 ± 0.13 | 4.92 ± 0.13 | 4.71 ± 0.13 | 4.78 ± 0.31 | 4.95 ± 0.22 | 4.87 ± 0.2 |
| LAB[a] | 2.55 ± 0.07 | 2.59 ± 0.07 | 4.37 ± 0.11 | 7.5 ± 0.08 | 8.87 ± 0.20 | 9.18 ± 0.03 | 9.16 ± 0.09 | 8.92 ± 0.1 | 9.48 ± 0.08 | 9.29 ± 0.03 | 9.62 ± 0.45 |
| pH | 6.75 ± 0.00 | 6.68 ± 0.01 | 6.72 ± 0.01 | 6.71 ± 0.01 | 6.24 ± 0.02 | 5.87 ± 0.12 | 5.38 ± 0.04 | 5.45 ± 0.08 | 5.31 ± 0.05 | 5.27 ± 0.07 | 5.33 ± 0.06 |
| aw | 0.999 ± 0.001 | 0.998 ± 0.001 | 0.998 ± 0.002 | 0.995 ± 0.002 | 0.99 ± 0.001 | 0.973 ± 0.008 | 0.966 ± 0.002 | 0.962 ± 0.002 | 0.961 ± 0.004 | 0.956 ± 0.004 | 0.950 ± 0.007 |
| Cheese B | | | | | | | | | | | |
| Lm[a] | 2.43 ± 0.07 | 2.5 ± 0.22 | 2.75 ± 0.09 | 4.51 ± 0.12 | 4.71 ± 0.13 | 4.67 ± 0.11 | 4.87 ± 0.04 | 4.75 ± 0.08 | 5.02 ± 0.1 | 5.1 ± 0.05 | 5.18 ± 0.05 |
| LAB[a] | 6.47 ± 0.01 | 6.26 ± 0.06 | 7.39 ± 0.34 | 8.34 ± 0.17 | 8.76 ± 0.03 | 8.83 ± 0.13 | 8.88 ± 0.05 | 8.81 ± 0.09 | 9.33 ± 0.12 | 9.22 ± 0.07 | 9.12 ± 0.07 |
| pH | 6.72 ± 0.00 | 6.66 ± 0.01 | 6.66 ± 0.01 | 6.33 ± 0.04 | 5.8 ± 0.06 | 5.51 ± 0.07 | 5.33 ± 0.09 | 5.22 ± 0.01 | 5.27 ± 0.06 | 5.22 ± 0.07 | 5.19 ± 0.09 |
| aw | 0.998 ± 0.001 | 0.998 ± 0.001 | 0.995 ± 0.002 | 0.997 ± 0.002 | 0.995 ± 0.001 | 0.972 ± 0.019 | 0.962 ± 0.018 | 0.965 ± 0.003 | 0.96 ± 0.01 | 0.955 ± 0.004 | 0.952 ± 0.005 |
| Cheese C | | | | | | | | | | | |
| Lm[a] | 1.3 ± 0.11 | 1.81 ± 0.07 | 1.33 ± 0.75 | 1.74 ± 0.07 | 1.26 ± 0.71 | 0.47 ± 0 | 1.64 ± 0.17 | 1.56 ± 0.02 | 1.86 ± 0.21 | 1.61 ± 0.22 | 1.64 ± 0.22 |
| LAB[a] | 8.10 ± 0.05 | 8.11 ± 0.04 | 8.58 ± 0.09 | 8.70 ± 0.13 | 8.91 ± 0.01 | 8.81 ± 0.12 | 8.77 ± 0.07 | 8.66 ± 0.03 | 9.00 ± 0.17 | 8.86 ± 0.08 | 8.75 ± 0.07 |
| pH | 6.65 ± 0.00 | 6.54 ± 0.01 | 6.31 ± 0.01 | 5.82 ± 0.03 | 5.4 ± 0.08 | 5.35 ± 0.02 | 5.25 ± 0.1 | 5.21 ± 0.02 | 5.29 ± 0.07 | 5.2 ± 0.02 | 5.32 ± 0.06 |
| aw | 0.997 ± 0.002 | 0.996 ± 0.001 | 0.995 ± 0.003 | 0.998 ± 0.001 | 0.989 ± 0.004 | 0.97 ± 0.006 | 0.945 ± 0.02 | 0.94 ± 0.023 | 0.934 ± 0.018 | 0.922 ± 0.014 | 0.931 ± 0.003 |

[a] Values are mean log cfu $g^{-1}$±standard deviation

[b] Means with different uppercase letters within a row are significantly different (HSD Tukey's test, $p<0.05$)

**Table 3.** Indices of model performance evaluated in this study: Accuracy factor (A), Bias factor (B) and Discrepancy percentage (D).

| Food | A | B | D (%) |
|---|---|---|---|
| Cheese A | 1.15 | 1.11 | 2.18 |
| Cheese B | 1.09 | 1.06 | 1.97 |
| Cheese C | 1.64 | 1.18 | 4.64 |

**Figure 2.** Relation between the square root specific growth rate ($\sqrt{\mu_{max}}$) of *L. monocytogenes* and the temperature in different milk type.

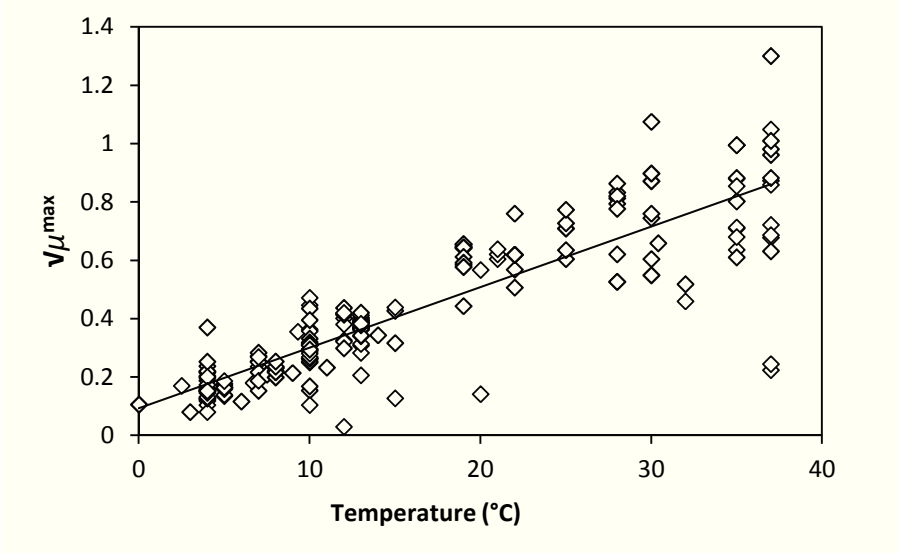


**Figure 3.** Comparison between predictions and observations used to fit the models.

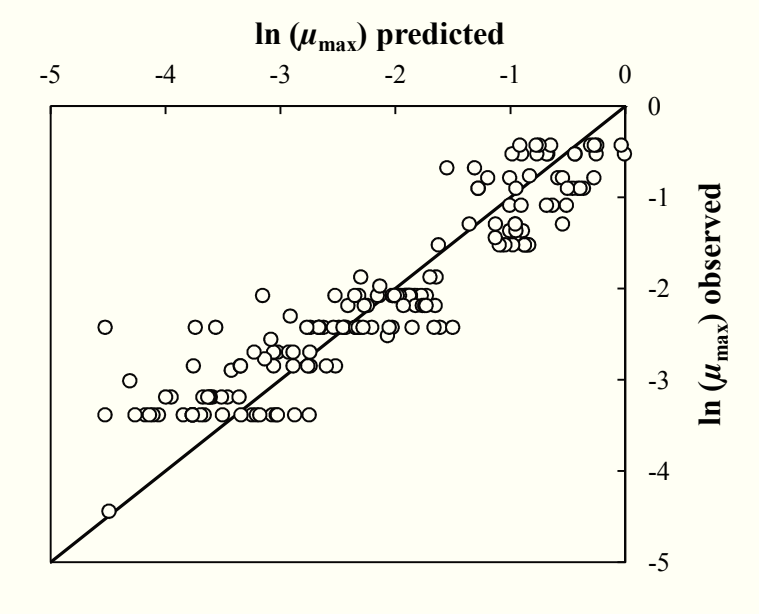
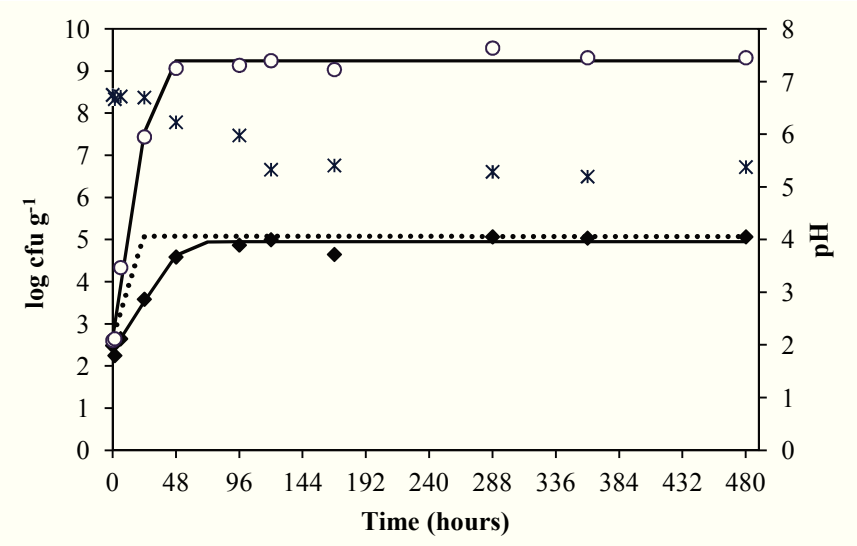
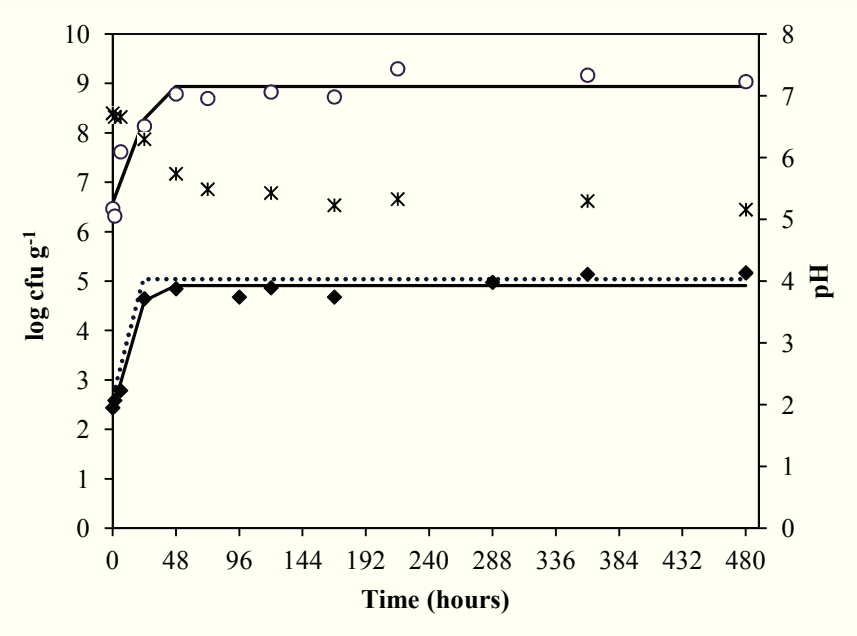


**Figure 4.** Comparison between predicted and observed concentrations of *L. monocytogenes* during the manufacturing of three type of soft cheese made with different inoculums levels of starter culture. The figure reported the data from a single replicate for each cheese type of the pH values (*), LAB (○) and *L. monocytogenes* (♦) log counts with their interpolation (solid line) (interpolations have a visual interest only). The *Listeria* prediction were reported as dashed line.

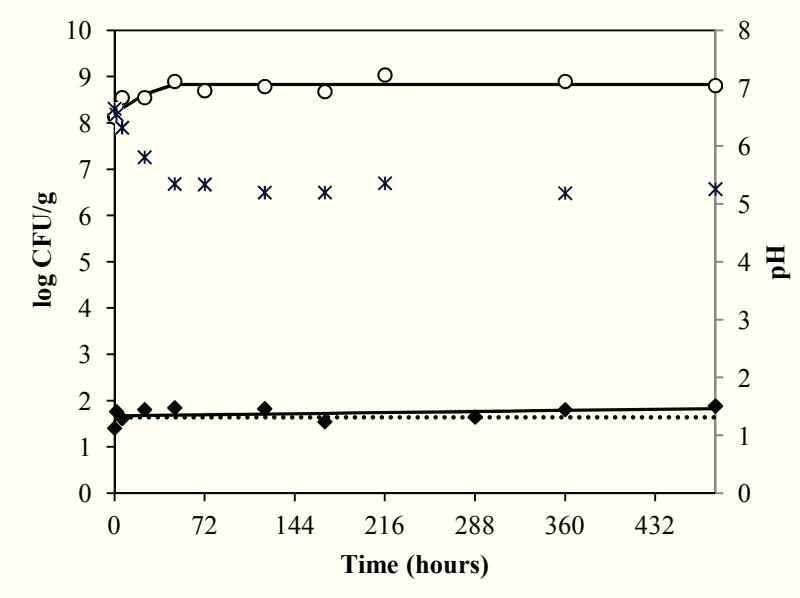
Cheese A




Cheese B



Cheese C



# 6. ANTI-*Listeria* ACTIVITY OF BIOACTIVE FOOD PACKAGING ON ARTIFICIALLY CONTAMINATED SLICED CHEESE

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# ANTI-LISTERIA ACTIVITY OF BIOACTIVE FOOD PACKAGING ON ARTIFICIALLY CONTAMINATED SLICED CHEESE

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## ABSTRACT

The aims of this study were to (1) study the optimal growth condition for two *Lactococcus lactis* strains to produce the anti-listeria compounds and (2) evaluate the effect of films activated with a twofold concentrated cell-free supernatant of *La. lactis* to inhibit *Listeria monocytogenes*. For the growth conditions, skimmed milk added to either Ml7 or MRS provided the highest levels of anti-listeria activity assayed (800 arbitrary unit per milliliter) for both *La. lactis* strains. A significant decrease of *Li. monocytogenes* counts was observed in cheese packed in films treated with an average decrease of 2.12 log colony-forming unit per gram after 15 days of storage.

## PRACTICAL APPLICATIONS

The antimicrobial film for food packaging can be used for the preservation of perishable foods such as the sliced cheese. This film developed in our study inhibits *Listeria monocytogenes* growth in sliced cheese, and does not have an adverse effect on the lactic microbiota, contributing to the preservation of the quality of the product. Thus, it could be a useful tool for the control of the development of pathogens in foods during storage of dairy products.

## INTRODUCTION

*Listeria monocytogenes* is a gram-positive pathogen, with the ability to adapt to a wide range of conditions such as refrigeration temperatures, acidic foods and high salt foods; characteristics that render its presence in food-processing environments undesirable although often inevitable (Tompkin 2002). In fact, it can be found in a wide variety of raw and processed foods such as dairy products, various meats, seafood and fresh produce (Lianou and Sofos 2007; Bernini *et al.* 2013), and it has been associated with several outbreaks of human listeriosis, sometimes involving large numbers of consumers (Aureli *et al.* 2000).

Antimicrobial packaging is an important method in controlling microbial growth on food surfaces, contributing to the promotion of microbial food safety by acting as a hurdle against the growth of food spoilage as well as foodborne pathogenic microorganisms (Dawson *et al.* 2003). Recently, increased research attention has been given to the use of bacteriocins in the so-called intelligent packaging whereby a packaging material is coated with an antimicrobial substance to inhibit, reduce or delay the growth of pathogenic or spoilage microorganisms upon contact with the food (Appendini and Hotchkiss 2002; Iseppi *et al.* 2008; Massani *et al.* 2013). Bacteriocin-activated plastic films for food packaging have been developed for the storage of liquid milk or dairy products (Mauriello *et al.* 2005), hamburgers (Mauriello *et al.* 2004), hot dogs (Franklin *et al.* 2004), frankfurters (Ercolini *et al.* 2006), cooked ham (Marcos *et al.* 2007), cold smoked salmon (Neetoo *et al.* 2008) and sliced cheese (Chollet *et al.* 2008). Intensive studies to elucidate the fundamental structural and functional properties of bacteriocins have been useful. However, applied research carried out with a view to determining the impact of food components and processing methods on the structure, solubility and activity of bacteriocins is of extreme importance when considering potential food applications (Deegan *et al.* 2006). There is an extensive background of earlier work

performed to optimize the antimicrobial compounds (as bacteriocins) in *Lactococcus lactis*, and many of these studies have described de Man Rogosa Sharpe (MRS) and Ml7 broths for optimal cellular growth and expression (Parente *et al*. 1994).

For these purposes, the aims of this study were to (1) investigate the anti-listeria properties of two *La. lactis* strains (registered and wild); (2) study the culture media and the growth conditions in which both strains show a maximum antimicrobial activity against *Li. monocytogenes*; and (3) evaluate the anti-listeria activity of polymeric films coated with *La. lactis* cell-free supernatant (CFS), during the storage of artificially contaminated sliced cheese. The ability of the coated films to leave the indigenous bacteria count unchanged on the cheese was also investigated in this study.

## MATERIALS AND METHODS

### Bacterial Strains and Culture Conditions

*Li. monocytogenes* ATCC 19115 serotype 4b was used as an indicator strain for all assays. The strain was daily cultivated (1% v/v of inoculum) in brain heart infusion broth (Oxoid, Milano, Italy) at 37C. *La. lactis* ATCC 11454 and *La. lactis* CRA 26 (from the collection of CRA-FLC of Lodi-Italy) (Carminati *et al*. 1989) were cultured using the following experimental media:

(1) MRS broth (Oxoid) and Ml7 broth (Oxoid) at their standard concentrations per the manufacturer's recommendations;

(2) MRS or Ml7 at 25% of their standard concentrations plus 25% of liquid skimmed milk (nonfat milk UHT; Centrale Del Latte Di Brescia S.P.A., Brescia, Italy) (25% MRS + 25% skimmed milk or 25% Ml7 + 25% skimmed milk).

To evaluate the production of anti-listeria compounds related to growth condition of *La. lactis*, the effects of culturing parameters, incubation time and number of transfers to fresh culture medium were studied to optimize the expression of antimicrobial compounds and their release into media. Prior to use in defined experimental media, 100 µL of each *La. lactis* stock culture was grown in either MRS or Ml7 ("preinoculum"; 50 mL of broth in 250 mL flasks) and incubated at 30C for 30 h in the shaker incubator (175 rpm). From the preinoculum, 5 mL of aliquots of the cell suspension was transferred into 50 mL of each experimental medium in 250 mL flasks and incubated for another 30 h at 30C. Cultures were transferred five times (30C for 3 h) using 5 mL of aliquots of broth culture for each new volume of the respective medium. After every 30 h of incubation period, 30 mL of cell suspensions was aseptically withdrawn from the flasks and tested for pH, microbial count and anti-listeria activity. Every type of media examined was performed in triplicate. The pH values were measured in 10 mL of suspension using a HI 223 calibration check microprocessor pH meter (Hanna Instrument, Padova, Italy) equipped with a gel glass electrode (Hamilton, Switzerland). For the enumeration of *La. lactis* (ATCC 11454 and CRA 26), decimal dilutions in physiological solution were prepared and the appropriate dilution was surface plated (100 µL) onto duplicate plate of MRS agar (Oxoid) and incubating at 30C for 48–72 h in aerobic condition.

The anti-listeria activity was examined using the agar well diffusion method: CFS of each strain were obtained by centrifugation at 10,000× *g* for 20 min at 4C (Jouan centrifuge CR422, Winchester, VA) and then adjusted to pH 6.0 by applying NaOH to exclude the inhibitory effect of organic acids, and heat treated for 10 min at 80C to destroy extracellular proteases and $H_2O_2$ (Biscola *et al*. 2013). Briefly, 20–30 µL of serial twofold dilutions of the CFS was added to wells of 5 mm in diameter cut in tryptic soy agar plates (TSA; Oxoid) previously inoculated with the indicator strain *Li. monocytogenes* ATCC 19115 (approximately$10^5$ colony-forming unit [cfu]/mL) and allowed to diffuse. The plates were incubated at 37C for 24 h and examined for zones of inhibition. The antimicrobial activity was expressed in arbitrary units per milliliter (AU/mL), and determined as the reciprocal of the highest dilution yielding a zone of growth inhibition on the indicator lawn (Rogers and Montville 1991).

### Production of Concentrated Cell-Free Supernatant

CFS from each culture of *La. lactis* (ATCC 11454 and CRA 26) in MRS plus skimmed milk, grown at 30C for 30 h, was collected, dialyzed against distilled water and twofold concentrated by ultrafiltration through 2,000 molecular weight cut-off exclusion membranes (Sartocon Slice; Sartorius AG, Goettingen, Germany). The inhibitory activity of concentrated cell-free supernatant (CCFS) was determined and quantified as AU/mL by agar well diffusion assay as described earlier.

### Activation of Plastic Films

A commercial low-density polyethylene film for food packaging was used in this study for the coating treatment with the CCFS from a culture of *La. lactis* (ATCC 11454 and CRA 26). Twenty-five microliter per square centimeter of CCFS was sprayed manually onto one side of the film. After coating, the film was dried by exposure to room temperature (22 ± 1C) in a biological safety cabinet.

### Anti-Listeria Activity of Activated Films

The antibacterial activity of the coated films against *Li. monocytogenes* ATCC 19115 was evaluated by two

different methods: (1) *in vitro*, on the agar plates; (2) *in vivo*, through the contact with artificially contaminated cheese samples. The cheese chosen for the experiment *in vivo* was a pasta filata cheese, made from whole pasteurized cow's milk and characterized by a firm paste.

In order to resemble a superficial development of listeria on the surface of food products and the antimicrobial effect of the developed films on superficial growth of listeria, a quantity of 0.1 mL of a suspension containing about $10^5$ cfu of *Li. monocytogenes* was spread plated on TSA agar plates, the active face of the coated films was located in contact with the agar surface and the plates were incubated at 37C for 24 h. After the incubation, the antimicrobial activity was revealed by the absence of growth of the indicator listeria in the part of the plate in contact with the coated films. Untreated films were used as controls.

The direct evaluation of the anti-listeria activity of coated films *in vivo* was determined in sliced semi-hard cheese contaminated on the surface with $10^7$ cfu/mL suspension of *Li. monocytogenes* ATCC 19115 in sterile physiological solution. The contaminated samples were packed in plastic trays and covered with *La. lactis* ATCC 11454 and *La. lactis* CRA 26 CCFS coated films. Contaminated samples packed with untreated films were used as controls. The samples were stored at 12C for 20 days. At specific time intervals (0, 1, 2, 3, 6, 8, 10, 13, 15, 18 and 20 days), the sliced cheese (about 15 g) were placed in sterile plastic bags, added with 135 mL of sterile physiological solution and homogenized for 1 min in Stomacher 400 blender (Seward Medical, London, UK). Serial 10-fold dilutions of the obtained suspensions were spread in duplicate on ALOA agar (Microbiol Diagnostici, Cagliari, Italy) for enumeration of artificially inoculated *Li. monocytogenes* and on MRS agar for enumeration of mesophilic lactic acid bacteria (LAB) to evaluate the antimicrobial effect of coated film on indigenous bacteria of cheese. Plates were incubated aerobically at 37C for 48–72 h. The experiments were carried out in triplicate.

### Statistical Analyses

All the experiments were repeated three times and the final results were calculated as the average of the replicates. The average data ± standard deviations were calculated with Excel program (Microsoft Corp.). In order to determine the statistical significance between bacterial counts in cheese packed with film untreated (control) and film coated with CCFS, the Tukey's test was performed at the 95% ($P < 0.05$) confidence interval with R statistical software version 2.7.0 (R Development Core Team 2008).

## RESULTS AND DISCUSSION

According to Kim *et al.* (1998) and Vessoni Penna *et al.* (2005), the successive transfer of cells into fresh media may provide conditions for greater bacteriocin production above the "ceiling concentration." For this purpose, in our study, five successive transfers in M17 and MRS culture media at 100% or at 25% of their standard concentrations plus milk were carried out for both *La. lactis* strains to evaluate the best conditions in which was expressed the activity against *Li. monocytogenes* ATCC 19115. The pH and the microbial counts of *La. lactis* ATCC 11454 and CRA 26 strains after five successive transfers in the different media are presented in Table 1. The CFS of *La. lactis* strains tested against *Li. monocytogenes* by the agar well diffusion assay showed an anti-listeria activity (expressed in AU/mL) with a range between 200 and 800 AU/mL (Table 1) even after the addition of NaOH and the heat treatment, which allowed the exclusion of the antimicrobial effect due to acids, peroxidase and $H_2O_2$. Thus, *La. lactis* CRA 26 was confirmed to be able to produce other anti-listeria compounds that could be bacteriocins, in agreement with Carminati *et al.* (1989).

In our study both strains showed an anti-listeria activity, but the highest levels assayed (800 AU/mL) were reached when the milk was added to Ml7 and MRS culture media. Vessoni Penna *et al.* (2005) studied the nisin expression related to the growth conditions of *La. lactis* subsp. *lactis* ATCC 11454 and the effects of culturing parameters (such as media components, incubation time and number of culture transfers to fresh medium) to optimize the expression of nisin and release into media. In accordance to their study, the skimmed milk added to either M17 or MRS provided the highest levels of anti-listeria activity. In M17 culture media with added milk, the highest antimicrobial activity was already observed after the first transfer to *La. lactis* ATCC 11454, and after the third transfer to CRA 26 strain (Table 1). Although in MRS culture media with added milk, the same activity was observed for both strains after the first transfer. This result may be due to a higher release of bacteriocins from cells into growth medium with acid pH. In fact, it is known that cells of bacteriocin-producing bacterial strains adsorb the bacteriocin molecules that they produce (Parente *et al.* 1994). But in culture media with milk, *La. lactis* cells ferment lactose to lactic acid that causes a decrease in the medium pH (Table 1) and then the release of bacteriocin, in agreement with Yang *et al.* (1992) who studied the influence of pH on bacteriocin adsorption by producing cells and observed that at pH 4 the adsorbed nisin on *La. lactis* is ca. 60%, while at pH 5 is about 80%. As a culture medium, the high nutritive content of cow's milk provides for excellent growth of *La. lactis* and for the cell's release of antimicrobial compounds into the medium. However, no correlation was demonstrated between the cell concentration of *La. lactis* and the production of antimicrobial compounds: the microbial counts (expressed as log cfu/mL) were about 8 log cfu/mL after

**TABLE 1.** ANTI-LISTERIA ACTIVITY, pH AND MICROBIAL CONCENTRATION FOR EVERY TRANSFER AFTER 30 H OF GROWTH OF *LACTOCOCCUS LACTIS* ATCC 11454 AND CRA 26 TRANSFERRED TO A NEW EXPERIMENTAL MEDIA

| Experimental media | | | | | | |
|---|---|---|---|---|---|---|
| Pre-inoculum | Inoculum | Strain (*L. lactis*) | Transfer | Anti-listeria activity (AU/mL) | pH | Microbial count (log cfu/mL) |
| M17 | M17 | ATCC 11454 | 1 | 400 | 5.83 ± 0.01 | 8.60 ± 0.06 |
| | | | 2 | 400 | 5.77 ± 0.01 | 8.34 ± 0.04 |
| | | | 3 | 400 | 5.75 ± 0.03 | 8.56 ± 0.05 |
| | | | 4 | 400 | 5.74 ± 0.02 | 8.71 ± 0.09 |
| | | | 5 | 400 | 5.70 ± 0.02 | 8.70 ± 0.05 |
| | | CRA 26 | 1 | 200 | 5.78 ± 0.02 | 8.21 ± 0.047 |
| | | | 2 | 200 | 5.75 ± 0.01 | N.D. |
| | | | 3 | 400 | 5.74 ± 0.02 | 8.75 ± 0.05 |
| | | | 4 | 400 | 5.72 ± 0.01 | 8.67 ± 0.06 |
| | | | 5 | 400 | 5.68 ± 0.03 | N.D. |
| M17 | 25% M17 +25% skimmed milk | ATCC 11454 | 1 | 800 | 4.35 ± 0.01 | 8.70 ± 0.07 |
| | | | 2 | 800 | 4.17 ± 0.00 | 8.48 ± 0.07 |
| | | | 3 | 800 | 4.14 ± 0.02 | 8.71 ± 0.04 |
| | | | 4 | 800 | 4.17 ± 0.02 | 8.73 ± 0.09 |
| | | | 5 | 800 | 4.15 ± 0.02 | 8.69 ± 0.02 |
| | | CRA 26 | 1 | 400 | 4.21 ± 0.03 | 8.44 ± 0.04 |
| | | | 2 | 400 | 4.21 ± 0.01 | N.D. |
| | | | 3 | 800 | 4.20 ± 0.04 | 8.71 ± 0.05 |
| | | | 4 | 800 | 4.20 ± 0.03 | 8.67 ± 0.04 |
| | | | 5 | 800 | 4.22 ± 0.01 | N.D. |
| MRS | MRS | ATCC 11454 | 1 | 200 | 5.12 ± 0.02 | 8.05 ± 0.05 |
| | | | 2 | 200 | 5.10 ± 0.01 | 8.10 ± 0.07 |
| | | | 3 | 200 | 5.10 ± 0.00 | N.D. |
| | | | 4 | 200 | 5.08 ± 0.01 | N.D. |
| | | | 5 | 200 | 5.09 ± 0.01 | 8.02 ± 0.06 |
| | | CRA 26 | 1 | 200 | 5.01 ± 0.00 | 8.01 ± 0.07 |
| | | | 2 | 200 | 5.01 ± 0.01 | N.D. |
| | | | 3 | 200 | 5.05 ± 0.03 | 8.02 ± 0.06 |
| | | | 4 | 200 | 5.03 ± 0.02 | 8.03 ± 0.09 |
| | | | 5 | 200 | 5.01 ± 0.02 | N.D. |
| MRS | 25% MRS +25% skimmed milk | ATCC 11454 | 1 | 800 | 4.21 ± 0.03 | 8.72 ± 0.01 |
| | | | 2 | 800 | 4.14 ± 0.03 | 8.63 ± 0.02 |
| | | | 3 | 800 | 4.30 ± 0.04 | 8.55 ± 0.00 |
| | | | 4 | 800 | 4.10 ± 0.03 | 8.56 ± 1.16 |
| | | | 5 | 800 | 4.15 ± 0.03 | 8.71 ± 0.04 |
| | | CRA 26 | 1 | 800 | 4.07 ± 0.03 | 8.88 ± 0.12 |
| | | | 2 | 800 | 4.06 ± 0.05 | N.D. |
| | | | 3 | 800 | 4.18 ± 0.05 | 8.86 ± 0.26 |
| | | | 4 | 800 | 4.10 ± 0.03 | 8.43 ± 0.08 |
| | | | 5 | 800 | 4.15 ± 0.02 | N.D. |

Data represent the average values ± standard deviation of three replicates samples for three experiments
AU, arbitrary unit; cfu, colony-forming units; MRS, de Man Rogosa Sharpe; N.D., not determined.

each transfer. These results underline that antimicrobial activity is not directly related to cell number because higher cell populations can provide lower anti-listeria activity. The CFSs achieve after the first transfer of each lactic strain in MRS broth with added milk were concentrated twice in order to improve the anti-listeria activity of the supernatant and to obtain a concentrated CFS (CCFS).

To produce anti-listeria films for food packaging, aliquots of CCFS (1,600 AU/mL) were sprayed on the surface of the plastic films (25 μL/cm$^2$). Figure 1a,b shows the anti-listeria activity of activated films: in the agar plate, the superficial growth of the indicator strain *Li. monocytogenes* ATCC 19115 was limited to the area surrounding the activated film that could clearly inhibit the development of the pathogen. In contrast, the pathogen could grow homogeneously on the surface of the plate and underneath the untreated film used as control (Fig. 1c). The spray-coating method yielded positive results, in agreement with Mauriello *et al.* (2004),

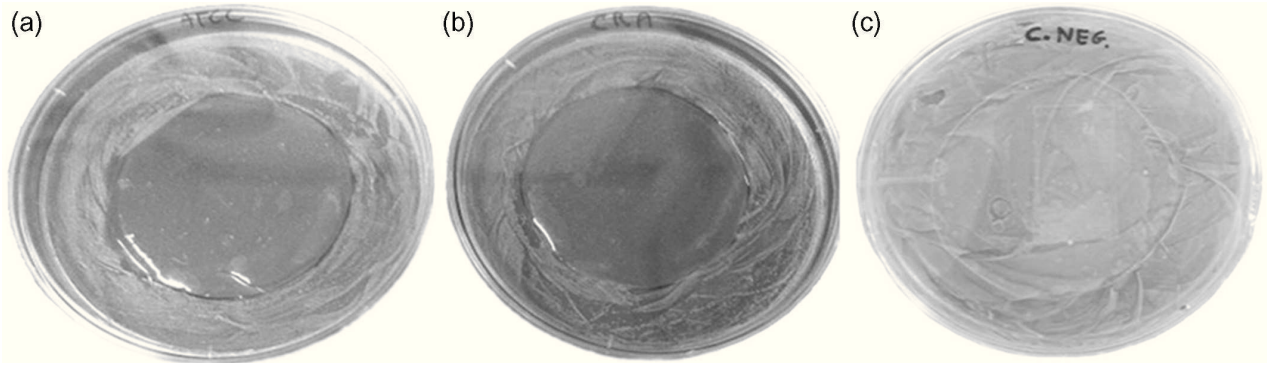


**FIG. 1.** ANTIMICROBIAL ACTIVITY OF FILM SPRAY ACTIVATED WITH CONCENTRATED CELL-FREE SUPERNATANT (CCFS) SOLUTION AT 1,600 ARBITRARY UNITS PER MILLILITER AGAINST THE INDICATOR STRAIN *LISTERIA MONOCYTOGENES* ATCC 19115 SPREAD INOCULATED ON TRYPTIC SOY AGAR PLATES
(a) Film treated with CCFS of *Lactococcus lactis* ATCC 11454; (b) film treated with CCFS of *La. lactis* CRA 26; (c) untreated film.

and proved to be effective in containing the surface development of pathogen on agar plates (Fig. 1).

To evaluate the quantitative anti-listeria activity of activated films in food, the films produced (treated and untreated) were used as packaging for sliced cheese (Fig. 2), artificially inoculated with *Li. monocytogenes* ATCC 19454, and stored at 12C for 20 days. Pathogen counts observed are reported in Fig. 3a. After 15 days of storage, mold growth was observed on the surface of the cheese (data not shown), and therefore, it was not possible to perform the analysis on the cheese incubated at 12C for 18 and 20 days. This result indicates that the activated film tested in our study did not show anti-mold activity. After 1 day of storage, *Li. monocytogenes* counts decreased in each sample, but no significant differences ($P > 0.05$) were observed between the control samples (packed with untreated film) and the samples packed with CCFS of *La. lactis* ATCC 11454. Instead, the film activated with CCFS of *La. lactis* CRA 26 showed a significant ($P < 0.05$) anti-listeria activity already after the first day of storage, with an average decrease of pathogen count of 1.02 log cfu/g (range of 0.78–1.27 log cfu/g) and the difference in respect to the control samples remains significant until the 15th day. At the end of the storage time at 12C, a significant decrease ($P < 0.05$) in *Li. monocytogenes* ATCC 19115 counts was observed in cheese packed in films treated with CCFS of *La. lactis* ATCC 11454 and CRA 26, with an average decrease of 2.11 and 2.13 log cfu/g, respectively. In recent years, many studies were made regarding the use of activated film during the shelf life of the cheese (Conte *et al.* 2009; Di Pierro *et al.* 2011). For example, a cellulose-based packaging, including nisin, was assayed as an insert interleaved between slices of cheddar cheese packaged under a modified atmosphere (Scannell *et al.* 2000). In that case, the population of *Li. innocua* and *Staphylococcus aureus* dropped dramatically in the first week of refrigeration conditions and, as a result, the shelf life of cheddar cheese was significantly extended. Lucera *et al.* (2014) demonstrated that a coating with potassium sorbate (3%) showed a certain inhibition on microbial proliferation in mozzarella cheese stored at 8C and samples remained acceptable for 8 days with respect to the control that was refused after about 4 days.

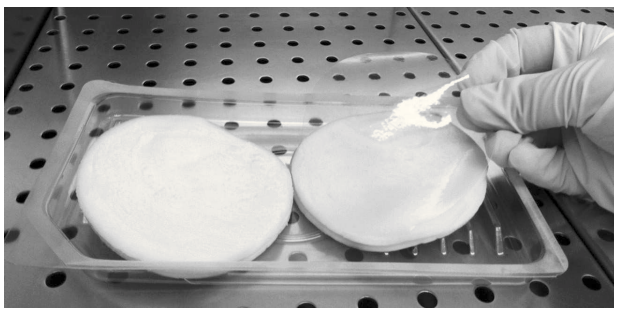

**FIG. 2.** ARTIFICIALLY CONTAMINATED SLICED CHEESE PACKED WITH ACTIVATED FILM

The data obtained in our study are in agreement with those of other researchers who have observed results in reducing bacteria populations within food samples wrapped in packaging materials containing bacteriocins (Ming *et al.* 1997; Lee *et al.* 2003; Iseppi *et al.* 2008).

During the assay, we also investigated if the treated films cause changes in the indigenous microbiota (LAB counts) (Fig. 3b). The average LAB count on the control cheese at day 0 was $7.26 \pm 0.17$ log cfu/g (range 7.04–7.44) and decreased moderately ($P < 0.05$) in 15 days of storage, until it reached $7.05 \pm 0.05$ log cfu/g (range 7.01–7.12). No significant differences were observed in LAB counts during the storage of cheese packaged with films treated or untreated. In fact, at day 0, the average LAB counts on cheese packed

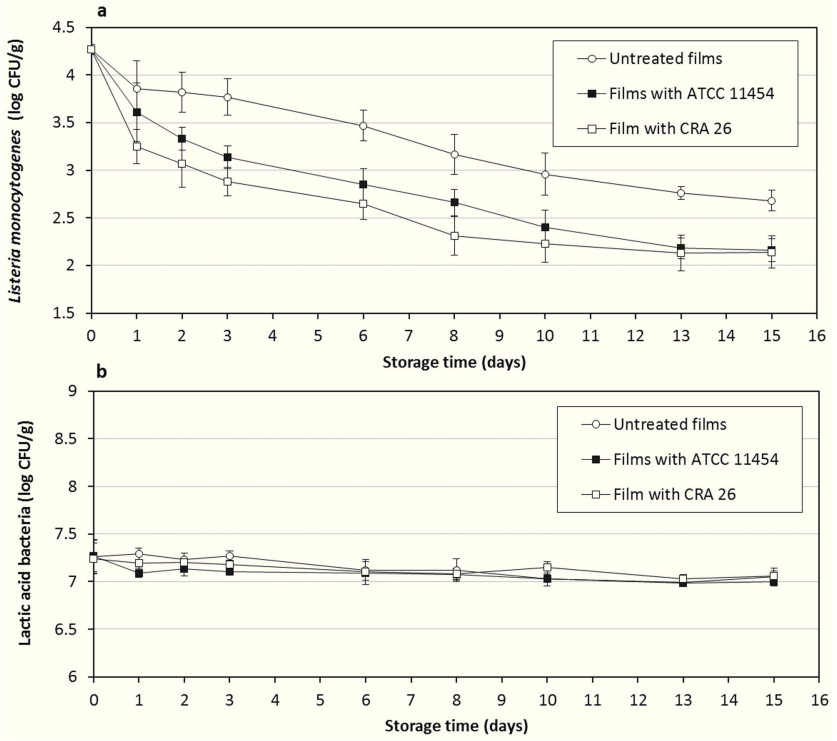


**FIG. 3.** BEHAVIOR OF *LISTERIA MONOCYTOGENES* ATCC 19115 (A) AND LACTIC ACID BACTERIA (B) OBSERVED ON ARTIFICIALLY CONTAMINATED SLICED CHEESE PACKED IN TREATED OR UNTREATED FILMS AND STORED AT 12C FOR 15 DAYS Data observed are reports as average values (log colony-forming units per gram) and error bars (standard deviation) of three replicates samples for three experiments.

with film coated by CCFS of *La. lactis* ATCC 11454 and CRA 26 were 7.27 ± 0.17 and 7.24 ± 0.16 log cfu/g, respectively, and after 15 days of storage the average counts were 7.00 ± 0.05 and 7.06 ± 0.08 log cfu/g, respectively.

The bacteriocins are characterized by a narrow inhibitory spectrum against closely related bacteria: many LAB produce bacteriocins inhibitory to other bacteria usually related (Jack *et al.* 1995). The fact that bacteriocins also inhibit the useful microbiota may limit their practical application (Giraffa *et al.* 1995), but in our study, no significant differences were observed in LAB counts of cheese packaged with films either treated or untreated. These results highlighted that the bioactive plastic films developed in this study not only inhibits listeria growth, but does not have an adverse effect on the lactic microbiota, and this contributes to preserve the quality of the cheese. Bacteriocins are bacterially produced antimicrobial peptides with narrow or broad host ranges. Many bacteriocins are produced by food-grade LAB, a phenomenon that offers food scientists the possibility of directing or preventing the development of specific bacterial species in food. This can be particularly useful in the preservation or food safety applications. In fact, bacteriocins can be used in helping processors extend their control over the food flora after manufacture. Therefore, the production of bacteriocins by LAB is not only advantageous to the bacteria themselves but could also be exploited by the food industry as a tool to control undesirable bacteria in a natural manner, which is likely to be more acceptable to consumers (Deegan *et al.* 2006).

In conclusion, our results confirm the use of LAB as natural preservatives that can be added directly in the food (fermented food) or on the packaging material (activated film) in order to counteract the development of pathogens possibly present in the food as a contaminant and, thus, to improve the quality and the shelf life of products. The results presented in the this study suggest that the concentrated supernatant of *La. lactis* can be used for the packaging of semi-hard sliced cheese in order to improve the food safety without altering the product itself.

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## CONFLICT OF INTERESTS

The authors declare no potential conflict of interests.

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# 7. THE PRESENCE, GENETIC DIVERSITY AND BEHAVIOUR OF *Listeria monocytogenes* IN BLUE-VEINED CHEESE RINDS DURING THE SHELF LIFE

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# The presence, genetic diversity and behaviour of *Listeria monocytogenes* in blue-veined cheese rinds during the shelf life

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ABSTRACT

Blue-veined cheeses may allow *Listeria monocytogenes* survival and multiplication due to the biochemical characteristics of the cheese and the growth characteristics of the pathogen. Because of the availability of a considerable number of samples, we wanted this study to take a large view of the microbiota and of the incidence and genetic diversity of *L. monocytogenes* in blue-veined cheese rinds. Moreover, we wanted to determine if the pathogen present on the rinds at the end of ripening represented a risk to the consumer if the cheese should exceed the domestic storage limit imposed by Commission Regulation (EC) No 2073/2005 for food safety criteria.

The rind microflora showed a high level of heterogeneity, and the incidence of *L. monocytogenes* (never found in the paste samples) was 55%, with an increased presence and concentration associated with a longer ripening time. Amplified Fragment Length Polymorphism (AFLP) was used to characterise *L. monocytogenes* isolated from blue-veined cheese rinds and highlighted the heterogeneity among the strains, demonstrating its suitability for studying the biodiversity of the pathogen in this environment. *L. monocytogenes* was able to grow during the shelf life of the cheese and was influenced by the refrigeration temperatures and the physicochemical characteristics of the cheese.



## 1. Introduction

Among food-borne pathogens, *Listeria monocytogenes* represents one of the most serious food safety concerns. Numerous outbreaks and sporadic cases of listeriosis associated with foods have been reported since 1980 in various parts of the world (Norton & Braden, 2007). In particular, dairy products are often a source of this infection (Bille et al., 2006; De Buyser, Dufour, Maire, & Lafarge, 2001). The survival and growth of *L. monocytogenes* in a dairy environment depends on the manufacturing, ripening and storage conditions used for the cheeses, even when the cheese is stored at refrigeration temperatures (Pintado, Oliveira, Pampulha, & Ferriera, 2005). In this regard, research into soft-ripened and blue-veined cheeses is an important field (Carminati, Perrone, Giraffa, Neviani, & Mucchetti, 2004; Cocolin et al., 2009; Lomonaco et al., 2009; Manfreda, De Cesare, Stella, Cozzi, & Cantoni, 2005).

Blue-veined cheeses are characterised by a pH range from 4.5 to 6.5 and a sodium chloride content of 2.3–7%. Moreover, biochemical changes due to the proteolytic activity of moulds during ripening can increase the pH, thus influencing the evolution of the microflora and favouring potential contamination (Mucchetti & Neviani, 2006). Although the application of a heat treatment is generally sufficient to inactivate the pathogen present in raw milk, the pathogen can reoccur as a consequence of cross-contamination during handling and contact with the ripening environment in post-production steps. Contamination during processing and the characteristics of the *L. monocytogenes* and of the product make it possible for the pathogen to survive and multiply (Ryser, 2007). The contamination, if present, seems to be limited to the rind (GOLIS Project, 2004), however, *L. monocytogenes* can still be a threat to consumer health because the contamination can be transferred to the internal paste during slicing.

Epidemiologic data indicate that the foods involved in listeriosis outbreaks have a contamination significantly higher than 100 cfu $g^{-1}$ (FAO/WHO, 2004). Due to these data, the Commission Regulation (EC) No 2073/2005 established a maximum level of 100 cfu $g^{-1}$ as the food safety criteria for tolerable levels of *L. monocytogenes* in ready-to-eat foods. This limit is allowed at the

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end of production on the condition that the pathogen does not grow during the shelf-life and reach higher levels by the time of consumption. *L. monocytogenes* must not be present in 25 g samples. When the behaviour of the pathogen in a product under particular conditions is unknown, studies to gather the experimental data concerning the implicated product are recommended (Codex Alimentarius, 2002). The refrigerated storage of blue-veined cheeses may not be sufficient to inhibit the growth of *L. monocytogenes* because this pathogen is able to grow at a temperature of 1 °C (Chan & Wiedmann, 2009). However, other factors, such as the $a_w$, free fatty acid concentration and competitive microflora, may influence the growth of *L. monocytogenes* in this type of cheese. For this purpose, microbiological challenge testing (MCT) to evaluate if an inoculated organism can grow in a specific product and to determine the point at which the growth reaches unacceptable levels in a specific product (Notermans & in't Veld, 1994) is an important tool for documenting the behaviour of *L. monocytogenes* in ready-to-eat (RTE) foods.

The primary aim of this research was to determine the incidence and genetic diversity of *L. monocytogenes* in blue-veined cheese rinds using a large number of available samples. Moreover, we wanted to know if *L. monocytogenes* present on the rinds at the end of the ripening could exceed the limit imposed by Commission Regulation (EC) No 2073/2005 for food safety criteria during domestic storage and thus represent a risk to consumers.

For these purposes, we investigated (i) the microbiota of the rinds of Italian blue-veined, mould-ripened cheeses associated with the detection of *L. monocytogenes* in the paste and rind and (ii) the genetic diversity among the *L. monocytogenes* isolates determined by AFLP as well as (iii) used MCT to study the behaviour of *L. monocytogenes* on cheese rinds during the shelf life.

## 2. Materials and methods

### 2.1. Sampling and physicochemical analysis of cheeses

Two series of blue-veined cheeses manufactured with pasteurised milk were considered: CI, entire cheese ripened for 50 days and CII, entire cheese ripened for 80 days. A total of 58 CI and 62 CII samples were recovered from October 2009 to April 2010 from the same cheese factory. 48 CI and 52 CII upper rinds were fully scratched with a sterile scraper and maintained at 4 °C for several hours prior to performing microbiological analyses. The remaining cheeses (10 CI and 10 CII) were sliced in 150 g portions, packed under a modified atmosphere and maintained at 4 °C until the microbiological challenge testing.

The moisture, protein, fat, ash and NaCl content (International Dairy Federation 1964a, 1964b, 1970, 1972, 1981), the pH (Beckman Coulter) and the $a_w$ (AquaLab CX-3) were determined for the 48 CI and 52 CII rind samples used for microbiological analysis. Analyses were performed in duplicate, and the average values for each series ± standard deviation were calculated.

### 2.2. Microbiological analysis

48 CI and 52 CII samples were microbiologically analysed. Five g of each rind sample were blended 1:10 in Ringer's solution (Oxoid, Basingstoke, UK) and decimally diluted for bacterial enumerations using the following media: total mesophilic bacteria on Plate Count Agar (Oxoid) at 30 °C for 48 h; yeasts and moulds on Malt Extract Agar (Oxoid) at 28 °C for 7 days; mesophilic lactic acid bacteria on De Man, Rogosa, Sharp Agar (Oxoid) at 30 °C for 48 h; Enterobacteriaceae on Violet Red Bile Glucose Agar (Oxoid) at 37 °C for 24 h; total coliforms and *Escherichia coli* on Coli ID (BioMérieux, Firenze, Italy) at 37 °C for 24 h; and *L. monocytogenes* on Ottaviani & Agosti (ALOA) (Biolife Italiana, Milan, Italy) at 37 °C for 48 h. All plates were incubated under aerobic conditions. The colony counts were performed in duplicates, and the average values were calculated.

In addition to the quantitative analysis, a qualitative determination of the presence of *L. monocytogenes* on the cheese pastes and rinds was performed to detect bacteria that were present at levels below the limit of detection of the quantitative analysis. For primary enrichment, 25 g of each sample were blended in 225 ml half-concentrated Fraser Broth (HFB; Oxoid) and incubated at 30 °C for 24 h. After the primary enrichment, 0.1 ml of the culture was transferred to 10 ml Fraser Broth (FB) (Oxoid) and incubated at 37 °C for 48 h for a secondary enrichment. Subcultures on ALOA (Biolife Italiana) (Vlaemynck, Lafarge, & Scotter, 2000) were made from both enrichment stages and incubated at 37 °C for 48 h to check for the presence of colonies.

### 2.3. Isolation

A total of 121 colonies (39 recovered from CI rind samples and 82 from CII rinds samples) were selected from ALOA, isolated through two purification steps on ALOA incubated at 37 °C for 48 h in aerobic conditions and maintained in Tryptone Soya Broth (TSB) plus 20% glycerol at −80 °C after purification.

### 2.4. Phenotypic and genotypic identification of L. monocytogenes

Three reference strains (*L. monocytogenes* LMG$^T$13305, *L. monocytogenes* LMG$^T$21264 and *Listeria innocua* Seeliger LMG$^T$11387) and 121 isolates were analysed by catalase and haemolysis tests (Carminati et al., 2004).

Of these isolates, 41 that tested positive in the phenotypic tests were identified by PCR. Strains were cultured twice at 37 °C for 18 h in TSB (Oxoid) in aerobic conditions, and the total genomic DNA was extracted and purified from 2 ml cultures according to the method of Marmur (1961). Two PCR protocols were performed for the identification of *Listeria* spp. (Border, Howard, Plastow, & Siggens, 1990) and *L. monocytogenes* (Bansal, 1996).

### 2.5. Genotypic characterisation by amplified fragment length polymorphism (AFLP) analysis

The AFLP protocol was based on the method published by Lazzi et al. (2009) using an AFLP Microbial Fingerprinting Kit (Applied Biosystem-Pe Corporation, Foster City, California, USA) with modification of the primers. In particular, four combinations of preselective and selective primers (Table 1) were tested on seven isolates and the reference strains.

Peaks representing AFLP fragments from 50 to 500 bp were reported in binary format with "1" for the presence of a band and "0" for its absence. Hierarchical cluster analysis was performed with Statistica 6 software (Statsoft Italia, Padova, Italia) using the

**Table 1**
Combinations of primers tested for preselective and selective amplifications.

| Combinations | Preselective amplification | Selective amplification |
|---|---|---|
| 1 | EcoRI-0 | EcoRI-A |
| | MseI-0 | MseI-A |
| 2 | EcoRI-0 | EcoRI-A |
| | MseI-0 | MseI-C |
| 3 | EcoRI-0 | EcoRI-A |
| | MseI-0 | MseI-T |
| 4 | EcoRI-0 | EcoRI-A |
| | MseI-A | MseI-AC |

Euclidean distance and unweighted pair group method with arithmetic mean (UPGMA) setting. The repeatability of the AFLP technique was determined using by three separate AFLP experiments on *L. monocytogenes* LMG21264$^T$ and *L. monocytogenes* LMG13305$^T$ strains.

### 2.6. Microbiological challenge testing

To represent the behaviour of naturally contaminating strains during the shelf life of the products, five *L. monocytogenes* strains (Lm4, Lm50, Lm67, Lm71, Lm122) that are genetically different by AFLP were used in a mixture to artificially contaminate cheese samples.

Each frozen stock culture was transferred twice (2% inoculum) into TSB (Oxoid) and incubated at 37 °C for 18 h in aerobic conditions. The cultures were then mixed in equal volumes. The mixed inoculum was decimally diluted in quarter-strength Ringer's solution (Oxoid).

Contamination ranging between 1 log cfu g$^{-1}$ and 2 log cfu g$^{-1}$ was performed by spreading the appropriate dilution of the mixture on the rinds of 24 slices and allowing them to dry under the flow of a biohazard cabinet (Faster, Milan, Italy) for a few hours to allow the attachment of the cells to the rind. Contaminated samples were incubated in refrigerators at either 4 °C or 8 °C for 55 days.

24 CI and 24 CII rinds were previously qualitatively checked to test the absence of naturally occurring *L. monocytogenes* in the rinds of samples used for MCT. Moreover, ten non-inoculated slices of both cheeses (control) were incubated at both temperatures to monitor the absence of naturally occurring *L. monocytogenes* during the shelf life.

After 0, 5, 10, 15, 30 and 55 days of incubation at both temperatures, control samples and contaminated samples were analysed to check for the presence *L. monocytogenes* as previously described (2.2).

## 3. Results and discussion

### 3.1. Physicochemical characteristics of cheeses

The physicochemical analyses of the samples are reported in Table 2.

Overall, CII was characterised by a high protein, fat, ash and NaCl content compared to CI, and CI showed a significantly higher $a_w$ value than CII. The higher pH of CII may be linked to the longer ripening period of the cheese. Gobbetti, Burzigotti, Smacchi, Corsetti, and De Angelis (1997) previously reported an increase from a pH of 6.09 at 49 days to 6.84 at 86 days of ripening in Gorgonzola cheese due to the production of $NH_3$ from the catabolism of amino acids and the metabolism of lactic acid by yeast and moulds.

**Table 2**
Physicochemical characterization of cheese I (CI) and cheese II (CII). Data represent the average values ± standard deviation calculated for 48 CI and 52 CII.

| | CI | CII |
|---|---|---|
| Moisture[a] | 50.61 ± 2.10 | 45.88 ± 3.00 |
| Proteins[a] | 18.76 ± 1.18 | 20.88 ± 1.46 |
| Fat[a] | 26.83 ± 1.18 | 28.83 ± 2.08 |
| Ash[a] | 2.96 ± 0.43 | 3.55 ± 0.63 |
| NaCl[a] | 1.75 ± 0.41 | 2.20 ± 0.52 |
| pH | 5.98 ± 0.64 | 6.8 ± 0.76 |
| $a_w$ | 0.97 ± 0.01 | 0.93 ± 0.01 |

[a] Average values expressed in g 100 g$^{-1}$ ± standard deviation.

### 3.2. Microbiological analysis

To identify the microbiota present in blue-veined cheese rinds, 48 CI and 52 CII rind samples were subjected to an enumeration of their total mesophilic bacteria, yeasts and moulds, mesophilic lactic acid bacteria, enterobacteriaceae, total coliforms and *E. coli* and *L. monocytogenes* counts.

The total mesophilic bacteria counts were similar in CI and CII, ranging from 6.5 to 8.9 log cfu g$^{-1}$ (Fig. 1) and are in agreement with data obtained by Gobbetti et al. (1997) during the ripening period of Gorgonzola. Cocolin et al. (2009) also noticed a great heterogeneity among studied samples, with values ranging from 3 log cfu g$^{-1}$ to 9 log cfu g$^{-1}$. The CII samples had lower numbers of mesophilic lactic acid bacteria than the CI samples, yielding between 5.0 and 8.4 log cfu g$^{-1}$ (Fig. 1). These results are 1–2 log cfu g$^{-1}$ higher than those previously reported in Italian blue-veined DOP cheeses (Gobbetti et al., 1997) and 2 log cfu g$^{-1}$ lower than those obtained by Cocolin et al. (2009). The average counts for yeasts and moulds were similar for CI and CII and ranged from 6.1 to 8.3 log cfu g$^{-1}$ (Fig. 1), in agreement with the results obtained by Gobbetti et al. (1997) and Cocolin et al. (2009) for Gorgonzola.

The high standard deviation values highlight the heterogeneity of the microflora among the samples and imply that the microbiota of these products are influenced by the manufacturing, ripening and environmental conditions (GOLIS project, 2004; Mucchetti & Neviani, 2006).

Enterobacteriaceae were recovered from only 9 samples (4 CI and 5 CII), and a maximum value of 2.51 log cfu g$^{-1}$ was recovered from CI (data not shown). Similarly, coliforms were identified in only 8 samples (6 CI and 2 CII), and they reached a peak level of 2.43 log cfu g$^{-1}$ in CII (data not shown). *E. coli* was not recovered from any samples (data not shown). The contamination levels of the hygienic index microflora comply with the process hygiene criteria of Commission Regulation (EC) 1441/2007, and in particular regarding *E. coli*, these levels can be considered satisfactory because the values are less than 2 log cfu g$^{-1}$ in all samples tested.

*L. monocytogenes* was not found in any of the 100 paste (CI and CII) samples. However, of the 100 rind (CI and CII) samples, 43 samples showed varying contamination levels after growth on ALOA: 12 of 43 rind samples showed contamination of 1–2 log cfu g$^{-1}$, 13 with 2–3 log cfu g$^{-1}$, 10 with 3–4 log cfu g$^{-1}$, and 8 showed a contamination level higher than 4 log cfu g$^{-1}$. For the remaining 57 rind samples, the pathogen was below the detection limit of the quantitative method (<1 log cfu g$^{-1}$), but qualitative analysis of 25 g of product confirmed the presence of *L. monocytogenes* in 12 samples. Overall, 45 rind samples of 25 g each tested negative for the pathogen.

When we analysed the differences among the two types of cheese, we found that 47.9% of the CI rinds were contaminated compared to the 61.5% of CII rinds (Fig. 2). In particular, the highest contamination levels, up to 4 log cfu g$^{-1}$, were recovered only from the CII rinds (Fig. 2) and affected 15% of the samples. These observations indicate that a longer shelf life and a higher pH value can allow for the growth of the pathogen and increased levels of contamination. The highest frequency of contamination for the CI rinds (18.8%) had a pathogen concentration of 1–2 log cfu g$^{-1}$. Similar to the results from the CI samples, the GOLIS project (2004) demonstrated the presence of *L. monocytogenes* in 47% of Gorgonzola cheese rind samples, and as we noticed for CII, a longer ripening time was associated with an increased frequency of contamination. Other authors (Manfreda et al., 2005; Pinto & Reali, 1996) observed a lower incidence of *L. monocytogenes* in Italian blue-veined cheeses (ranging from 2.1% to 14%), but they analysed only the core and not the rind where contamination usually occurs as a result of cross-contamination from ripening environments or during handling (Carminati et al., 2004; Cocolin et al., 2009).

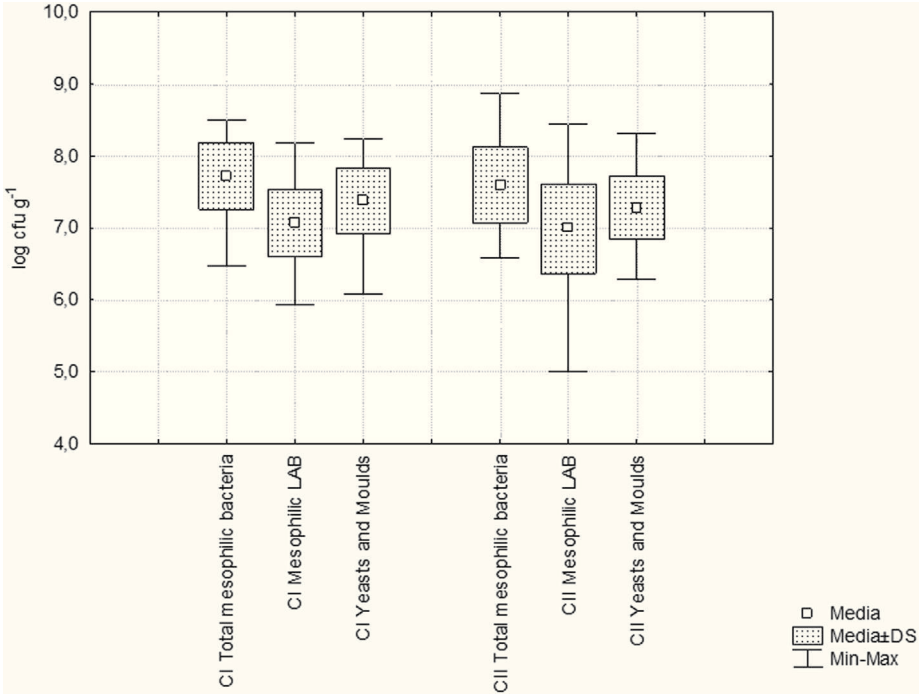


**Fig. 1.** Box and Whisker plot of total mesophilic bacteria, mesophilic lactic acid bacteria and yeasts & mould counted for 48 CI and 52 CII rind samples.

### 3.3. *Identification and genotypic characterisation of L. monocytogenes*

To study the genetic diversity of *L. monocytogenes* strains recovered from the blue-veined cheeses studied, 121 colonies (39 isolated from CI samples and 82 from CII samples) grown on ALOA medium were selected, purified and phenotypically analysed. Of these, 41 (12 isolated from CI rinds and 29 from CII rinds) were positive for $H_2O_2$ production and the presence of haemolysis zones. Moreover, to confirm the species, these isolates were genotypically analysed by PCR, and all of the isolates contained listeriolysin O (*hlyA*), a gene that is specific to *L. monocytogenes* (Bansal, 1996).

The genomic variability of these *L. monocytogenes* isolates was studied by AFLP analysis. Several techniques such as ribotyping (Swaminathan et al., 1996), Pulsed-Field Gel Electrophoresis (PFGE) (Kerouanton et al., 1998), Randomly Amplified Polymorphic DNA (RAPD) (Vogel, Jorgensen, Ojeniyi, Huss, & Gram, 2001) and Amplified Fragment Length Polymorphism (AFLP) (Autio, Keto-Timonen, Lundén, Björkroth, & Korkeala, 2003) have been used for the molecular characterisation of pathogens associated with

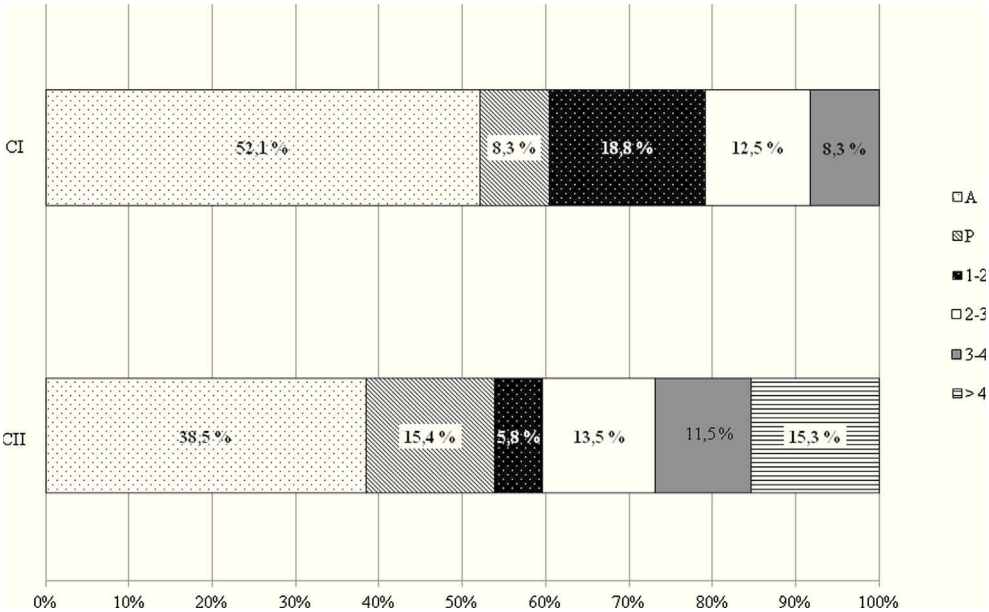


**Fig. 2.** *L. monocytogenes* in blue veined 50 days (at 2–8 °C) ripened cheese rinds (CI) and in blue veined 80 days (at 2–8 °C) ripened cheese rinds (CII): prevalence at different contamination levels. (A: absence in 25 g; P: presence in 25 g; numeric values expressed as intervals of log cfu $g^{-1}$.)

foods and food processing to analyse the route of contamination. Among these techniques, PFGE is often applied, but AFLP has been demonstrated to be a valuable tool that is highly discriminatory, sensitive and reproducible (Autio et al., 2003; Liu, 2006; Paun & Schönswetter, 2012).

In our study, a preliminary screening was performed on seven isolates and three reference strains to evaluate the performance of different primer combinations and select the most suitable protocol for our purposes.

Using four combinations of primers (Table 1), varying numbers of fragments (ranging in size from 50 to 500 bp) were visualised as peaks in the electropherograms after selective amplification. The patterns obtained using the preselective primers pair containing one-nucleotide extensions (MseIA) were simpler than those obtained using a preselective primer with no extra nucleotide (EcoRI0, MseI0). The combination EcoRI0/MseI0–EcoRIA/MseIC generated optimal AFLP profiles that had an adequate number of fragments (approximately 90), were well distributed in the range of interest and yielded intense signals (data not shown); therefore, this combination was chosen for AFLP analysis of the 41 strains.

Moreover, the enzyme combination that we used (EcoRI/MseI) was previously employed by others for *L. monocytogenes* typing (Aarts, Hakemulder, & Van Hoef, 1999; Mikasova, Oravcova, Kaclikova, Kuchta, & Drahovska, 2005), and it produced positive results in terms of band numbers and distribution.

The comparison between AFLP electropherograms revealed a marked variability among the strains that were genotyped on the basis of polymorphic peaks. Overall, 134 peaks representing AFLP fragments were obtained from the strain profiles. Of these, 84 were non-common polymorphic peaks and ranged from a minimum of 11 in strain Lm84 to a maximum of 38 in strain Lm67 (data not shown).

The repeatability level of the AFLP technique, corresponding to a genetic distance of 3, was determined by three independent AFLP experiments on the *L. monocytogenes* reference strains LMG 21264T and LMG 13305T (data not shown). This value was chosen as a cut-off point to group the strains. Fig. 3 shows the UPGMA dendrogram of 41 *L. monocytogenes* AFLP profiles. Similar to what has been observed in other AFLP studies (Aarts et al., 1999; Keto-Timonen, Autio, & Korkeala, 2003; Mikasova et al., 2005; Parisi et al., 2010), we identified two major clusters, A and B, containing 36 and 4 isolates, respectively, that diverged by approximately 5 linkage distances. Cluster A included 10 isolates from CI and 26 from CII. Cluster B included 4 isolates (2 from both the cheeses). Considering the repeatability level, the dendrogram was characterised by 13 clusters that could not be completely correlated to the type of cheese that the isolates were obtained from; only clusters 2 and 6 were formed exclusively by strains isolated from CII. The *L. monocytogenes* biotype did not seem to be correlated with the environmental conditions or the ripening time, but it showed a high degree of biodiversity. Moreover, 9 of 13 clusters were formed by single strains (4 isolated from CI and 5 from CII) that showed unique patterns. Interestingly, strain Lm4, which was isolated from CII, appeared genetically distinct and revealed a higher genetic distance in comparison with all of the other strains.

In contrast, other authors have observed low strain heterogeneity in *L. monocytogenes* strains isolated from Italian blue-veined cheese rinds using the following techniques: RAPD (Carminati et al., 2004), PFGE (Lomonaco et al., 2009) and ribotyping (Manfreda et al., 2005). To the best of our knowledge, this is the first time that AFLP has been used to characterise *L. monocytogenes* strains isolated from blue-veined cheese rinds, and we have demonstrated that it is suitable for studying the biodiversity of the pathogen in this food environment. The advantages of this technique in terms of reproducibility and throughput compared to other fingerprinting techniques have already been recorded in studies that typed *L. monocytogenes* isolated from other foods and environments (Guerra, Bernardo, & McLauchlin, 2002; Keto-Timonen et al., 2003; Parisi et al., 2010).

### 3.4. Microbiological challenge testing

It was previously reported that the cheese surface can become contaminated with *L. monocytogenes* after a set of procedures involved in cheese ripening, such as dry salting, wheel piercing, wet brushing and handling in general. This contamination generally does not involve the paste, but it may be transferred during cutting and portioning (Carminati et al., 2004; Manfreda et al., 2005). In our

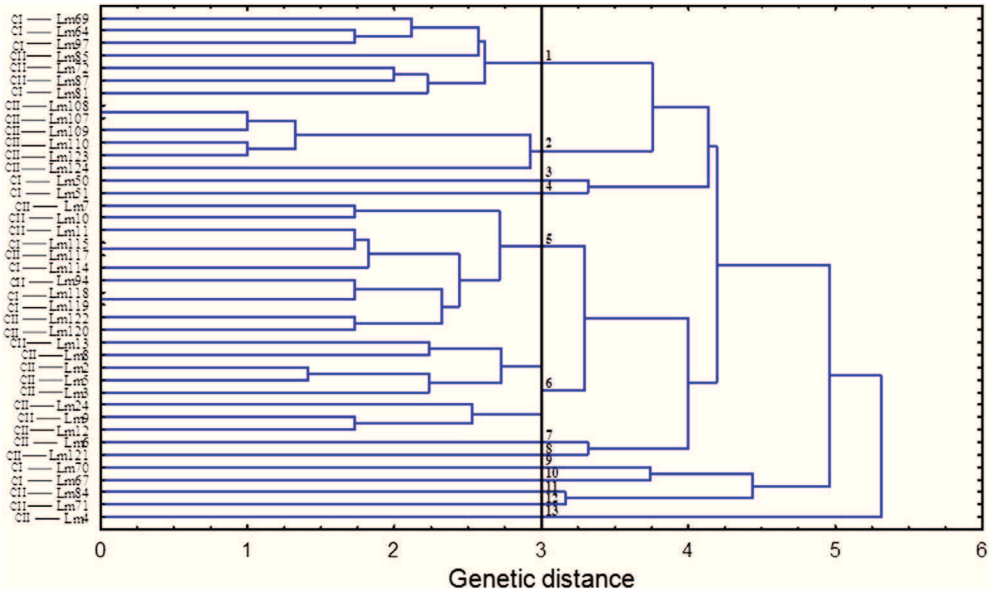


**Fig. 3.** UPGMA dendrogram deriving from AFLP patterns of the 41 *L. monocytogenes* strains using the primer combination EcoRI0/MseI0–EcoRIA/MseIC. On the left side of the figure are indicated the names of the strains and the kind of cheese. The repeatability value is indicated by the vertical line. The numbers reported represent the clusters formed at the cut-off point.

study, no *L. monocytogenes* was found in the paste of any of the samples, but the rinds were contaminated at a frequency of more than 50% with variable contaminations levels. The Commission Regulation (EC) 1441/2007 states that a maximum level of 2 log cfu $g^{-1}$ of *L. monocytogenes* is acceptable in RTE foods that do not support the growth of the pathogen during the shelf life of the food. The majority of the contaminated rind samples analysed in our study showed this level of contamination. For these reasons, an MCT was performed for rinds with contamination levels between 1 and 2 log cfu $g^{-1}$.

Both CI and CII were considered to evaluate the behaviour of the pathogen during the shelf life. In particular, contaminated CI and CII samples were stored at 4 °C or 8 °C for 55 days to determine if differences in domestic storage conditions could influence the pathogen's behaviour.

No naturally occurring *L. monocytogenes* were recovered from any of the samples analysed, and the absence of the pathogen in non-inoculated slices was confirmed throughout the storage period (data not shown).

Regarding CI, *L. monocytogenes* was not too affected by the temperature in the first 30 days of storage; in fact, it rapidly increased by 1.80 and 1.70 log cfu $g^{-1}$ after 10 days and by 2.48 and 2.93 log cfu $g^{-1}$ after 30 days at 4 °C and 8 °C, respectively (Fig. 4). Only after 55 days were differences due to the temperature registered; in samples stored at 8 °C, the pathogen further increased to a maximum value of 4.94 ± 0.17 log cfu $g^{-1}$, and samples stored at 4 °C showed a slight decrease to 3.00 ± 0.49 log cfu $g^{-1}$ (Fig. 4). Thus, data obtained for CI at both temperatures showed that the maximum level of *L. monocytogenes* present at the end of production exceed safety levels if the rind is considered edible because the contamination of the samples with 1.44 log cfu $g^{-1}$ of *L. monocytogenes* exceeds the limit imposed by Commission Regulation (EC) 2073/2005. However, we emphasise that these data relate only to the rind and, currently, we are unable to show similar data for the paste.

Data obtained for CII showed a different trend (Fig. 4). For the first 30 days of storage, *L. monocytogenes* maintained low contamination levels at both temperatures. Only after 55 days in samples stored at 8 °C did the pathogen reach levels of 4.63 ± 0.26 log cfu $g^{-1}$, near the values obtained for CI. On the other hand, storage at 4 °C resulted in only a slight increase to 0.62 log cfu $g^{-1}$ after 55 days. Interestingly, reducing the shelf life of CII to 30 days could avoid the growth of the pathogen over the limits of food safety criteria. Moreover, the maintenance of a correct refrigeration temperature represents a very important control tool. In fact, the slight increase in the pathogen numbers at 4 °C on day 55 is unlikely to be dangerous for the consumer because the storage period in a natural atmosphere, representative of domestic conservation, is not usually so long. Different studies have demonstrated that the modification of the atmosphere, in particular an increase in $CO_2$ levels, can inhibit *L. monocytogenes* growth (Whitley, Muir, & Waites, 2000).

In Queso Blanco cheese ($a_w$ 0.97), *L. monocytogenes* grew logarithmically in 15 days at 5 °C (Uhlich et al., 2006), highlighting a behaviour similar to that observed for CI in our research. A trend similar to our CII results was observed during the refrigerated storage of soft cheeses in which the pathogen was capable of surviving and decreased by 1 log cfu $g^{-1}$ after two weeks (Cataldo et al., 2007). In mould-ripened soft lactic cheeses, the pathogen survived in the paste during 42 days of ripening even with a level of milk contamination as low as 10 cfu $ml^{-1}$ (Morgan, Bonnin, Mallereau, & Perrin, 2001). In this case, the absence of pathogen growth may be due to the low inoculum size that has previously been shown to influence the probability of growth, particularly under stress conditions (Augustin, Brouillaud-Delattre, Rosso, & Carlier, 2000; Robinson et al., 2001).

The behaviour of *L. monocytogenes* in a complex food ecosystem is regulated by different physicochemical conditions, stress factors, microbial interactions and nutrient availability. Thus, modifications in the storage temperature or cheese composition can alter

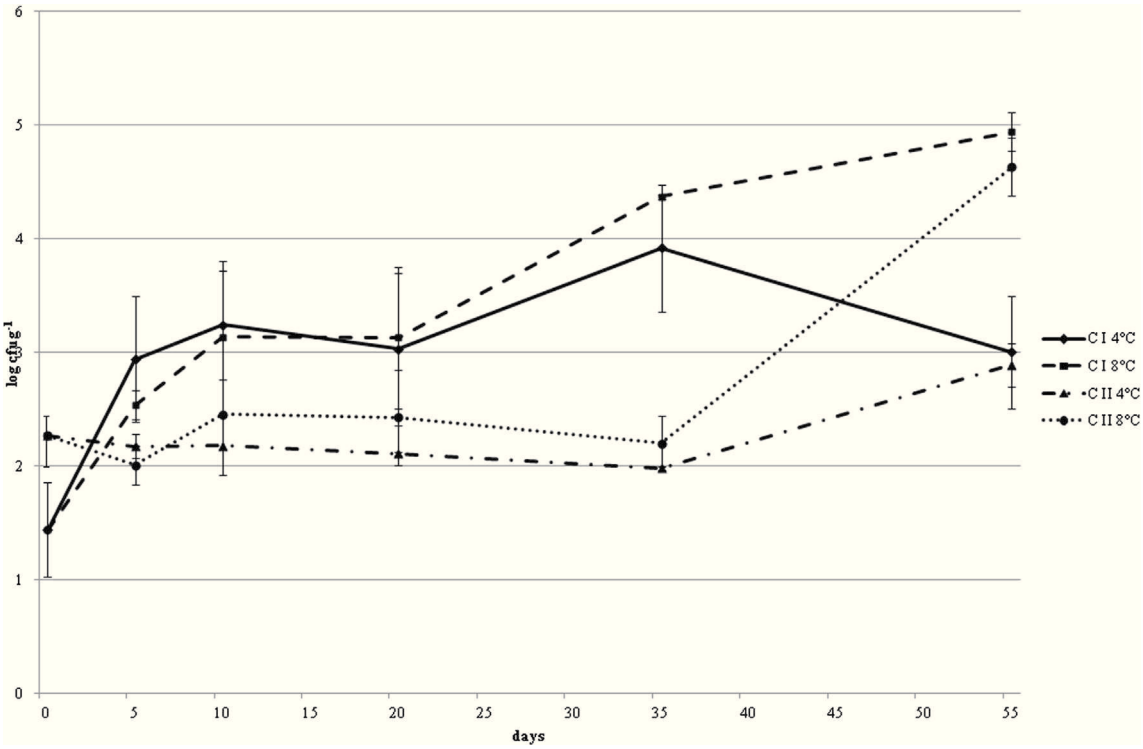


**Fig. 4.** The in vivo behaviour of *L. monocytogenes* in cheese I (CI) and cheese II (CII) the cheese samples were artificially contaminated with a mixture of five strains of *L. monocytogenes* (Lm4, Lm 50, Lm67, Lm71, Lm122) and incubated for 0, 5, 10, 15, 30 and 55 days at two different temperature (4 °C and 8 °C).

pathogen growth that, in cheeses, is controlled by several preservation factors such as pH, water activity, salt concentration and $O_2$ concentration. In blue-veined cheeses, the proteolysis of casein leads to the release of ammonia and the utilisation of lactic acid with a resulting increase in the pH that contributes to the creation of conditions favourable for pathogen growth. On the other hand, factors such as the high salt concentration and the presence of free fatty acids have been suggested to inhibit *L. monocytogenes* multiplication during ripening (Papageorgiou & Marth, 1989).

Thus, the higher NaCl percentage, the lower $a_w$, the lower moisture content and the higher pH (index of a higher proteolysis) of CII compared to CI may be responsible for the stronger inhibition of *L. monocytogenes* growth observed in our study for CII.

## 4. Conclusions

The microflora of a considerable number of blue-veined cheese rinds analysed in our study was characterised by a great heterogeneity of the different bacterial groups and highlighted the influence of the raw milk composition and the manufacturing, ripening and environmental conditions on the dynamics between microorganisms. Although *L. monocytogenes* was never recovered in the paste, its incidence on the rinds was important and correlated with increased ripening time of the products. Despite the increased possibility of rind contamination in long-ripened products, such as that demonstrated by the presence of *L. monocytogenes* near the limit imposed by Commission Regulation (EC) No 2073/2005 for food safety, CII demonstrated the ability to inhibit the growth of the pathogen during the shelf life of cheese slices maintained at refrigeration temperatures in domestic conditions.

In summary, the variability in the growth of *L. monocytogenes* in these products can result in contamination levels that are considered hazardous and exceed the limit allowed by food safety criteria. It would be interesting to apply product MCT protocols to other blue-veined cheeses with different chemical and physical compositions to gather more information regarding the "in vivo" behaviour of pathogenic microorganisms and to identify proper food safety criteria according to product characteristics.

## Disclosure

Authors declare that no conflict of interests exist. All authors have approved the final article.

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# 8. CUTTING PROCEDURES MIGHT BE RESPONSIBLE FOR *Listeria monocytogenes* CONTAMINATION OF FOODS: THE CASE OF GORGONZOLA CHEESE

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# Cutting procedures might be responsible for *Listeria monocytogenes* contamination of foods: The case of Gorgonzola cheese

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ABSTRACT

Commission Regulation (EC) No. 2073/2005 established, as a food safety criterion for tolerable levels of *Listeria monocytogenes* in ready-to-eat foods which do not support the growth of the pathogen or with shelf life below 5 days, a maximum of 100 cfu $g^{-1}$. Blue-veined cheeses are among these foods because their rinds can be contaminated, and the pathogen can be transferred to the paste during slicing. The aim of this research was to investigate whether cutting procedures could be responsible for cheese paste contamination. Considering that the Commission Regulation limit is allowed when the pathogen does not grow during the shelf life, we also wanted to verify whether, in the case of positive dragging, *L. monocytogenes* was able to grow on cut slices beyond the limit imposed, thereby becoming a risk for consumers during storage at 4 °C. Gorgonzola cheese was chosen for this investigation. The cutting simulation on artificially inoculated wheel rinds indicated that greater rind contamination corresponded to a higher percentage of contaminated paste samples. The growth of *L. monocytogenes* transferred to cut slices was variable relative to the physicochemical characteristics of the cheese, to the contamination level and to the time of storage. In particular, the sweet typology was able to support the growth of *L. monocytogenes* in the shelf life conditions considered and the quick overcoming of the limit imposed by food safety criteria would not ensure the safety for consumption.



## 1. Introduction

Gorgonzola, one of the most important Italian cheeses, awarded with the “Protected Denomination of Origin” (PDO) from the European Commission (EU Regulation, 1996), represents an optimal food substrate for the growth of *Listeria monocytogenes* (Lomonaco, Patti, Knabel, & Civera, 2012). Gorgonzola is a blue-veined, mould-ripened cheese, made with pasteurised cow's milk and characterised by a final pH ranging from 4.5 to 6.5 and a sodium chloride content of 2.3–7%. During ripening, biochemical changes due to the proteolytic activity of *Penicillium roqueforti*, that develops as an internal blue-green mould, cause an increase in pH, influencing the microflora's evolution and favouring possible contamination by ubiquitous dairy microorganisms and food-borne pathogens, among which *L. monocytogenes* has raised particular concern (Mucchetti & Neviani, 2006). In 2006 Gianfranceschi et al. (2006) described the first case of listeriosis associated with the consumption of contaminated Gorgonzola cheese in Italy. Although the pasteurization of milk inactivates the pathogen, it can enter the product after contact with the environment during ripening (Cocolin et al., 2009). Contamination during processing principally depends on the ripening and storing conditions of the cheeses, and together with the characteristics of the *L. monocytogenes* and of the product, these conditions can make pathogen survival and multiplication possible (Ryser, 2007). This contamination, if present, consists of a small number of cells and is limited to the rind (Carminati, Gatti, Bonvini, Neviani, & Mucchetti, 2004; GOLIS Project, 2004), which has been declared not edible by the Consortium for the Protection of Gorgonzola Cheese. However, *L. monocytogenes* can still be a threat to consumer health because contamination can be transferred to the internal paste during slicing. In fact, it is know how the bacterial transfer from surfaces to

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food or food to food, called “cross-contamination”, can occurs via hands or hands contact surfaces as well as equipment like cutting boards or knifes. Different studies on pathogen cross-contamination of foods have been documented (Cogan, Bloomfield, & Humphrey, 1999; Wachtel, McEvoy, Luo, Williams-Campbell, & Solomon, 2003) and, in general, it is estimated that cross-contamination is involved in 39% of food-borne outbreaks (Evans et al., 1998).

Epidemiologic data have indicated that the foods involved in listeriosis outbreaks showed a contamination significantly greater than 100 cfu $g^{-1}$ (FAO/WHO, 2004). For this reason, Commission Regulation (EC) No. 2073/2005 established, as a food safety criteria for tolerable levels of *L. monocytogenes* in ready-to-eat (RTE) foods, a maximum level of 100 cfu $g^{-1}$. This limit is allowed at the end of production on the condition that the pathogen does not grow during the shelf life and exceed this level at consumption. If this criterion is not met, *L. monocytogenes* must not be present in 25 g samples. When the behaviour of the pathogen in a product under particular conditions is not known, the acquisition is recommended of experimental data concerning the implicated product (Codex Alimentarius, 2002). Microbiological challenge testing (MCT), which evaluates whether an inoculated organism can grow and at which point the growth reaches unacceptable levels in a specific product (Notermans & in't Veld, 1994), has become an important tool to document the behaviour of *L. monocytogenes* in RTE foods. Since the publication, in 2008, of the EURL Lm Technical Guidance document for conducting shelf-life studies on *L. monocytogenes* in ready-to-eat foods to the guidance document of 2004 (European Commission, 2014), many studies have applied challenge test to determine the ability of different foodstuffs to support *L. monocytogenes* growth (Álvarez-Ordóñez, Leong, Hickey, Beaufort, & Jordan, 2015).

The aim of this research was to investigate whether cutting could be responsible for the contamination of Gorgonzola cheese slices with *L. monocytogenes*. Moreover, we wanted to verify whether, in case of positive dragging, *L. monocytogenes* was also able to grow in cut slices, exceeding the limit imposed by Commission Regulation (EC) No. 2073/2005 and thus posing a risk to the consumer during storing.

For these purposes, two different MCTs were applied. The first MCT simulated Gorgonzola cheese cutting procedures to study the possible transfer of *L. monocytogenes* from the rind, at different levels of contamination, to the cheese paste. The second MCT examined the behaviour of *L. monocytogenes* transferred to cut slices over the shelf life of the cheese for 55 days of storage at 4 °C.

## 2. Materials and methods

### 2.1. Samples and physicochemical analysis of cheeses

Five Gorgonzola cheeses types (which differs in term of sweet or piquant typology and days of ripening), provided by the Consortium for the Protection of Gorgonzola Cheese, were considered for the experiments within the first MCT (MCT I): a sweet Gorgonzola ripened for 60 days (S60), a sweet Gorgonzola ripened for 80 days (S80), two piquant Gorgonzolas (of two different producers) ripened for 80 days (P80a and P80b) and a piquant Gorgonzola ripened for 120 days (P120).

In order to represent as closely as possible the behaviour of *L. monocytogenes* in Gorgonzola cheese paste, a representative product for each typology (the sweet and the piquant one) was considered for the experiments within the second MCT (MCT II): a sweet Gorgonzola ripened for 60 days (SG) and a piquant Gorgonzola ripened for 80 days (PG).

Trials were repeated three times for both MCT I and MCT II.

All of the cheeses used in this study complied with the dimensions characteristic for this product in accordance with the product specifications: 13–20 cm height, 20–32 cm diameter and 6–13 kg weight.

The moisture (International Dairy Federation, 1970), proteins (International Dairy Federation, 1964a), fat (International Dairy Federation, 1981), ash (International Dairy Federation, 1964b), NaCl content (International Dairy Federation, 1972), pH (Beckman Coulter), and $a_w$ (AquaLab CX-3) were determined at the beginning of the shelf life for the SG and PG used for studying *L. monocytogenes* behaviour in cheese paste. Analysis was performed of 7 SGs and 7 PGs, belonging to the same batch of production of the cheeses used for MCT II, and average values ± standard deviations were calculated separately for each series.

### 2.2. Strains

Five *L. monocytogenes* strains (Lm4, Lm50, Lm67, Lm71, Lm122), belonging to strains collected by the Department of Food Science of the University of Parma, previously isolated from blue-veined cheese rinds, were used in a mixture to contaminate artificially the cheese samples in both MCT I and MCT II. The strains were chosen according to a previous genotypic characterisation obtained by the mean of amplified fragment length polymorphism PCR (AFLP) (Bernini et al., 2013). Moreover, a *Listeria innocua* strain (LIN9), provided by the Agriculture Research Council, Fodder and Dairy Productions Research Centre, CRA-FLC, of Lodi (Italy), where it was previously isolated from blue-veined cheese rinds and identified, was used in preliminary test aimed to standardise the contamination procedures of cheese rind and cheese paste.

Each frozen stock culture was transferred twice (2% inoculums v/v) in Tryptone Soya Broth (TSB) (Oxoid, Basingstoke, UK), incubated at 37 °C for 18 h in aerobic conditions. The *L. monocytogenes* single strain cultures were then mixed in equal volumes in order to obtain a mixed inoculum (Lmix). The Lmix was decimally diluted in quarter Ringer's solution (Oxoid) and was used for contamination of the cheeses. Different initial inoculums levels were calculated in order to reach a *L. monocytogenes* contamination of 3, 2 and 1 log cfu $g^{-1}$ on the rind for MCT I and 3, 2, 1, 0, −1 and −2 log cfu $g^{-1}$ on the paste for MCT II.

### 2.3. L. monocytogenes detection and enumeration

*L. monocytogenes* was detected both by qualitative and quantitative analyses according to ISO 11290-1 (1996) and ISO 11290-2 (1998) respectively. For quantitative determination, different aliquots of samples were taken in relation to the overall volume of the samples to be considered: in particular 5 g aliquots for MCT I and 25 g aliquots for MCT II were blended at a 1:10 ratio in Ringer's solution (Oxoid). The suspensions were then stored at room temperature for 1 h and then decimally diluted for *L. monocytogenes* enumeration on Agar Listeria according to Ottaviani & Agosti (ALOA), supplemented with ALOA enrichment-selective supplements (Biolife Italiana, Teramo, Italy) and incubated at 37 °C for 24–48 h. All of the plates were incubated in aerobic conditions. Typical *L. monocytogenes* colonies were confirmed by rapid test Monocytogenes ID Disk (Biolife) following manufacturer instructions. The colony counts were performed in triplicates, and the average values ± standard deviations were calculated (International Standards Organization 11290-2, 1998) as representative of each sample. In addition to the quantitative analysis, qualitative determination of the presence of *L. monocytogenes* in the samples was performed to detect bacteria that were present at levels less than the limit of detection of the quantitative analysis. For primary enrichment, 25 g of each sample

were blended in 225 ml of Fraser Broth added to Half Fraser supplement (HFB) (Oxoid) and were incubated at 30 °C for 24 h. Then, 0.1 ml of the first enrichment culture were transferred to 10 ml of Fraser Broth added to Fraser supplement (FB) (Oxoid) and were aerobically incubated at 37 °C for 48 h for secondary enrichment. Subcultures of both enrichment stages were plated on ALOA (Biolife Italiana) (Leclercq, 2004; Vlaemynck, Lafarge, & Scotter, 2000) and incubated at 37 °C for 24–48 h to determine the presence of colonies (International Standards Organization 11290-1, 1996).

### 2.4. Set up of cheese contamination procedures

Preliminary test were performed with *L. innocua* LIN9 on five cheeses in order to standardise the rind contamination procedures for MCT I. In particular, the volume and dilution of the strain, the number of sprayed and the distance to be maintained from the sample were assessed according to Bernini et al. (2015).

Preliminary test with *L. innocua* LIN9 were also performed before to proceed with MCT II in order to standardise the procedures of cheese paste contamination. In particular, six contamination levels had to be assessed: A: 3 log cfu $g^{-1}$; B: 2 log cfu $g^{-1}$; C: 1 log cfu $g^{-1}$; D: 0 log cfu $g^{-1}$; E: −1 log cfu $g^{-1}$; F: −2 log cfu $g^{-1}$. Once established the volume and dilution of the strain, the number of sprayed and the distance to be maintained from the sample in order to reach contamination levels A, B and C, the culture solution used for contamination level A was 1000, 10000 and 100000 fold diluted in order to obtain contamination levels D, E and F respectively. While contamination levels A, B and C were checked by plate count, contamination levels D, E, and F were under the detection limit of the technique (10 cfu $g^{-1}$) and the pathogen contamination of paste samples was only assessed by qualitative detection.

### 2.5. Microbiological challenge testing I (MCT I): effects of cutting procedures on L. monocytogenes cheese paste contamination

Three experimental trials for each cheese type (S60, S80, P80a, P80b and P120) were carried out to study the effects of different cutting methodologies and contamination levels on the transfer of *L. monocytogenes* from rind to cheese paste.

Each cheese was contaminated by spreading the appropriate dilution of the Lmix on the upper and lateral rind sides to obtain an estimated *L. monocytogenes* concentration ranging between 1 log cfu $g^{-1}$ (at the limit of the detection of the plate count method) and 3 log cfu $g^{-1}$. In particular, a 10000 fold diluted Lmix was used to contaminate cheeses S60 and P80a rinds in order to reach a theoretical contamination of 3 log cfu $g^{-1}$, a 100000 fold dilution was used to have an estimated contamination of 2 log cfu $g^{-1}$ cells on cheese S80 rind and a contamination of 1 log cfu $g^{-1}$ was calculated for cheeses P80b and P120 rinds by spreading the 1000000 fold dilution of the Lmix. The cheeses were left to dry in a refrigerated chamber (4 °C) for a few hours to allow for the attachment of the cells to the rind. After drying, three cutting steps, characterised by different cutting procedures, were applied with the aim of simulating industrial, point of sale and domestic cutting. The first cutting step (I), reproducing an industrial process, was performed on the entire cheese wheel. Following the vertical positioning of the cheese, the cutter tool that was used made a transversal cut in the wheel to obtain two half cheeses (Fig. 1A). The second cutting step (II), made with the same cutter tool used for step I, was performed on one of the two half cheeses previously obtained and allowed for the obtaining of two cheese eighths and eight cheese slices, simulating an industrial cut (Fig. 1B). The third cutting step (III), simulating a point of sale and/or domestic cut, was performed with a knife on the eight cheese slices, to obtain five portions for each slice (Fig. 1C).

A total number of 220 paste samples (44 for each cheese) were collected for each trial from the cut cheeses, maintaining a distance of 5 mm from the rind layer, and they were analysed to determine the presence of *L. monocytogenes*. In particular, all the cheese paste surface that came in contact with the cutting tools was analysed by dividing the area in many little samples in order to allow an accurate mapping of the pathogen cells localization. The sampling scheme, analogous for each cheese, is represented in Fig. 2. Briefly, from one of the two halves of each cheese obtained after step I (Fig. 1A), 12 paste samples were collected (samples 1 to 12) (Fig. 2A). The second half of the cheese was cut into four parts (representing cheese eighths). Two of the parts were cut into four slices each (step II) to obtain a total of eight slices (Fig. 1B). Two eighths were sampled in duplicate (samples 15, 16, 17, and 18) together with four slices (samples from 21 to 28) (Fig. 2B). The remaining four slices were equally positioned vertically or horizontally, cut with a knife (step III) into five parts each (Fig. 1C) and collected (samples from 29 to 48) (Fig. 2C).

Before cutting, in four area of the rind (samples 13, 14, 19 and 20), the amount of Lmix artificially inoculated on the rinds was evaluated as average values ± standard deviations of the three trials and separately for each cheese types (Fig. 2a,b).

Moreover, for each cheese typology, 5 paste samples originating from the same cheese vat, were qualitatively checked for the absence of *L. monocytogenes* in order to consider these cheese typologies suitable for the challenge test.

*L. monocytogenes* cell transfer from rind to paste after cutting was expressed as percentage of contaminated samples (%) and calculated as follows: $Nf/N_0{*}100$, where $N_f$ is the number of samples found positive (presence in 25 g) after cutting and $N_0$ is the number of all the samples representative of the whole cheese paste area exposed to cutting tools.

### 2.6. Microbiological challenge testing II (MCT II): L. monocytogenes behaviour in cheese paste during storage at 4 °C

Three experimental trials involving a SG and a PG cheese types were carried out to evaluate the behaviour of *L. monocytogenes* in Gorgonzola cheese paste during a 55-day shelf life at 4 °C, with particular reference to the initial contamination level. Ten cheese wheels for each type, derived from the same cheese vat, were provided by a local manufacturer such as for sale: sliced in portions of 150 g and packed without vacuum. Samples were maintained at 4 °C until artificial contamination with *L. monocytogenes*. The pathogen contamination at paste level was performed within a few hours by spreading the appropriate dilution of the Lmix on the paste of each cheese slice and then leaving it to dry under the flow of a biohazard cabinet (Faster, Milan, Italy) to allow for the attachment of the microbial cells to the paste. With the aim of restoring the microaerophilic conditions characteristic of the inside of the cheese, each inoculated slice was overlapped with a non-contaminated one. The samples thus obtained were finally packaged as originally, stored at 4 °C and analysed over the shelf life considered.

Overall, 96 samples (48 for each cheese type) for each trial, representing duplicates of six estimated contamination levels (A: 3 log cfu $g^{-1}$; B: 2 log cfu $g^{-1}$; C: 1 log cfu $g^{-1}$; D: 0 log cfu $g^{-1}$; E: −1 log cfu $g^{-1}$; F: −2 log cfu $g^{-1}$) and four sampling times (0, 15, 30 and 55 days) were assessed both quantitatively and qualitatively for *L. monocytogenes*.

Eight SG cheese slices and 8 PG cheese slices were previously checked qualitatively to test for the absence of naturally occurring *L. monocytogenes* in the paste of the samples used for MCT II. Moreover, 8 non-inoculated slices of both cheeses (control

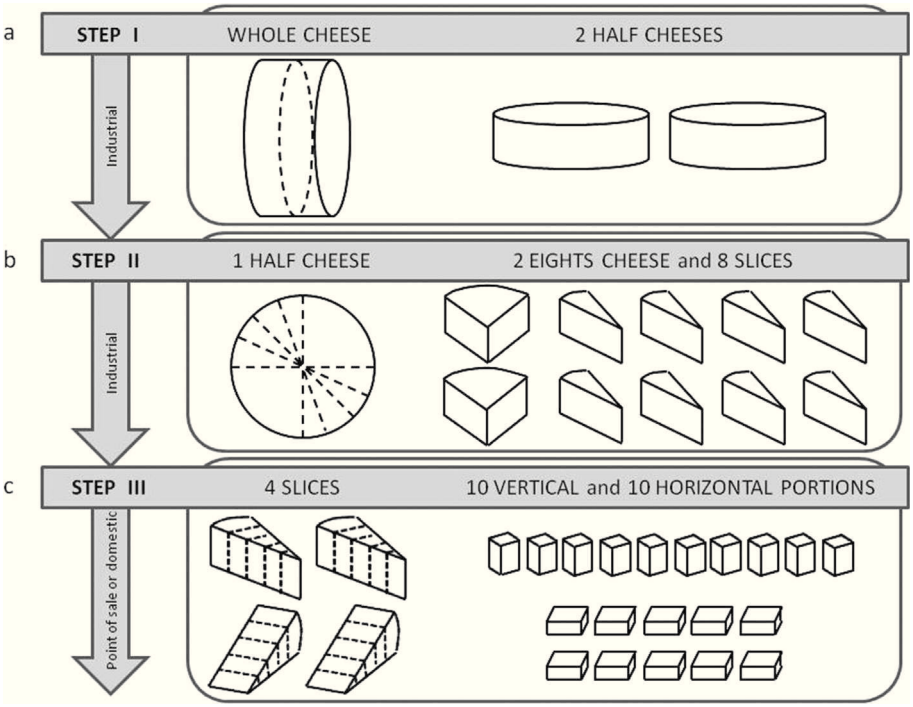


**Fig. 1.** Gorgonzola cheese cutting scheme. The three cutting steps (I, II and III) are reported. The dashed lines indicate the cutting zones.

samples) were incubated at 4 °C to monitor for the absence of naturally occurring *L. monocytogenes* during the shelf life. After 0, 15, 30 and 55 days of incubation at 4 °C, control samples and contaminated samples were analysed in triplicate to check for the presence of *L. monocytogenes* as previously described.

### 2.7. Statistical analysis

The means and standard deviations of *L. monocytogenes* counts and the physicochemical parameters of the cheeses were processed with SPSS (Version 21.0, SPSS Inc., Chicago, IL, USA) statistical software.

Pearson's correlation coefficient was calculated to evaluate the relationship between the levels of rind contamination and the percentages of contaminated cheese paste samples after cutting.

Student's t test ($P < 0.01$) was used to identify the significant differences between the bacterial counts at the same sampling times in SG and PG Gorgonzola cheeses. One-way analysis of variance (ANOVA) and Tukey's honest significant difference test (HSD) were chosen at a 95% confidence level ($P < 0.05$) to identify differences among the same samples at different sampling times and among different samples at the same sampling time.

## 3. Results and discussion

### 3.1. Physicochemical characteristics of cheeses

The physicochemical analysis of cheeses SG and PG, representing the sweet and piquant typologies chosen for the study of *L. monocytogenes* behaviour in the paste, are reported in Table 1.

Overall, PG was characterised by higher proteins, fat, ash and NaCl content compared to SG, which showed a higher $a_w$ value. The higher pH of PG compared to SG might have been linked to the longer ripening time and also to the use of different mold secondary starters showing more intensive proteolytic activities. Gobbetti, Burzigotti, Smacchi, Corsetti, and De Angelis (1997)

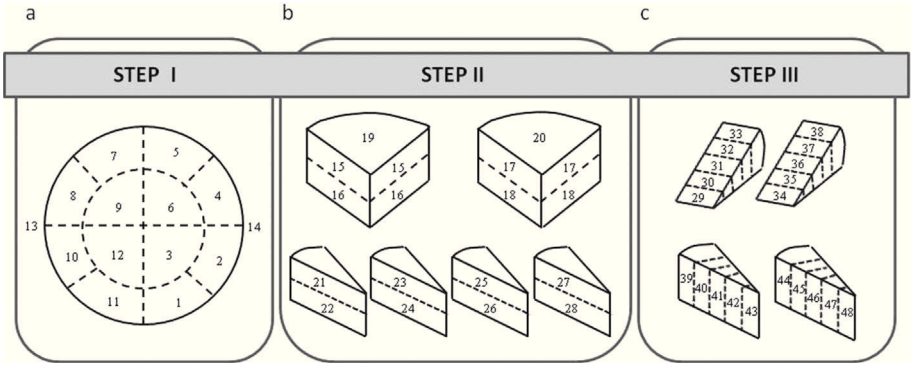


**Fig. 2.** Gorgonzola cheese sampling scheme after the three cutting steps (I, II and III). 48 sections are reported, representing 44 paste samples for the analysis of *L. monocytogenes* cells transfer and 4 rind samples (numbers 13, 14, 19 and 20) for the checking of the pathogen initial contamination level.

**Table 1**
Physicochemical characterisation of sweet Gorgonzola ripened for 60 days (SG) and piquant Gorgonzola ripened for 80 days (PG). Data represent the average values ± standard deviations calculated for 7 SG and 7 PG cheeses.

| | SG | PG |
|---|---|---|
| Moisture[a] | 49.87 ± 1.90 | 44.53 ± 2.92 |
| Proteins[a] | 18.32 ± 1.30 | 21.75 ± 1.55 |
| Fat[a] | 27.02 ± 1.45 | 29.68 ± 2.15 |
| Ash[a] | 3.10 ± 0.24 | 3.45 ± 0.59 |
| NaCl[a] | 1.98 ± 0.38 | 2.31 ± 0.48 |
| pH | 5.86 ± 0.58 | 6.92 ± 0.46 |
| $a_w$ | 0.97 ± 0.03 | 0.92 ± 0.01 |

[a] Average values expressed in g 100 $g^{-1}$ ± standard deviation.

already noted an increase in pH from 6.09 to 6.84 from 49 to 86 days of ripening of Gorgonzola cheese due to the production of $NH_3$ through the catabolism of amino acids, combined with the metabolism of lactic acid by yeasts and moulds.

### 3.2. Contamination test

Preliminary test carried out with *L. innocua* LIN9 allowed to evaluate the correct procedures to follow in order to obtain an homogeneous distribution of microorganisms on the cheese rind and paste and to reach the contamination level desired for each MCT. Regarding contamination levels under the detection limit of plate count technique, the number of cfu $g^{-1}$ was only referred to an estimated value based on the dilution protocol. Within the same level of contamination, no significant differences ($P > 0.05$) in microbial concentrations were detected among the different area of cheese sampled (data not shown).

### 3.3. Effects of cheese cutting on L. monocytogenes paste contamination

Blue-veined cheese rinds, such as that of Gorgonzola cheese, can become contaminated by *L. monocytogenes* after a set of procedures involved in cheese ripening, such as dry salting, wheel piercing, wet brushing and cheese handling. About this, the GOLIS Project (2004) emphasised the presence of a small number of *L. monocytogenes* cells in 47% of the samples of Gorgonzola cheese rinds.

The results obtained by MCT I confirmed that the cutting procedures could drag *L. monocytogenes* from the rind to the paste. Moreover, these findings showed that paste contamination depended on the level of rind contamination.

The protocol for contamination applied in this experiment was corrected to allow for the reaching of expected levels of artificial rind contamination for all five of the cheeses before cutting (Fig. 3). The most contaminated cheese was P80a, with 2.98 ± 0.10 log cfu $g^{-1}$, while cheeses P120 and P80b showed lower contamination levels (Fig. 3).

*L. monocytogenes* was not detected by selective enrichment in the control paste samples tested in order to consider the five cheese types suitable for the challenge test. All 220 paste samples (44 for each cheese) analysed for each of the three trials were checked for both *L. monocytogenes* enumeration on ALOA and the qualitative presence of the pathogen.

In all cheese paste samples *L. monocytogenes* was below the detection limit of the plate count method (<1 log cfu $g^{-1}$). The absence of a measurable cells transfer in term of cfu $g^{-1}$ did not allow to calculate the ratio between cells in the paste after cutting and cells in the rind before cutting, in order to estimate quantitatively how cross-contamination occurred after each cutting procedure. Anyway, positive results that confirmed the presence of

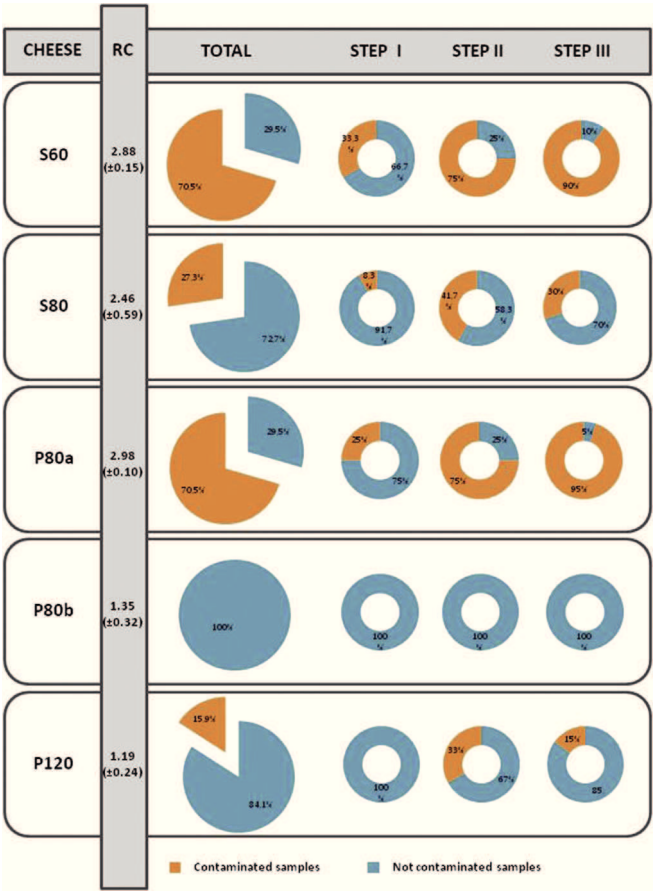


**Fig. 3.** Percentages of *L. monocytogenes* contaminated paste samples at the end of the cutting procedures (Total) and after each of the three cutting steps (Step I, II and III) relative to different cheese rind artificial contamination levels (RC) for the five cheese typologies considered (S60, S80, P80a, P80b and P120). RC are calculated as average values of three trial for each cheese typology and expressed as log cfu $g^{-1}$ ± standard deviation.

*L. monocytogenes* in 25 g paste samples were obtained by qualitative analysis. The analysis of the whole cheese paste area, divided into sections, exposed to the cutting tools provided a map of positive and negative samples. This allowed to calculate the percentages of contaminated paste (%), representative of pathogen transfer, thus highlighting different percentages of paste contamination among differently contaminated cheeses (Fig. 3). According to Pearson's correlation coefficient, a strong association emerged between the levels of artificial rind contamination and the percentage of contaminated cheese paste samples after the three cutting steps ($r = 0.85$).

In fact, the highest rind contamination levels (2 and 3 log cfu $g^{-1}$) corresponded a high percentage of paste contamination after cutting. In particular, the highest number of contaminated paste samples, corresponding to 70.5% of the whole, was recovered for cheeses S60 and P80a, corresponding to the highest rind contamination levels. Absence of the pathogen was detected in the paste when the rind contamination level decreased to 1 log cfu $g^{-1}$ (Table 2). Lower *L. monocytogenes* rind contamination reflected a lower percentage of pathogen presence in the paste at the end of all of the cutting steps: 27.3% and 15.9% of the samples were contaminated after the cutting of cheeses S80 and P120, respectively, while *L. monocytogenes* was not detected in 25 g of cheese P80b.

Considering the different cutting steps, the first horizontal cut

**Table 2**
Behaviour of *L. monocytogenes* in sweet Gorgonzola ripened for 60 days (SG) and piquant Gorgonzola ripened for 80 days (PG) cheese paste over 55 days of storage at 4 °C relative to different expected artificial paste contamination levels.

| Cheese | Days | Rind contamination level | | | | | |
|---|---|---|---|---|---|---|---|
| | | A (3 log cfu $g^{-1}$) | B (2 log cfu $g^{-1}$) | C (1 log cfu $g^{-1}$) | D (0 log cfu $g^{-1}$) | E (−1 log cfu $g^{-1}$) | F (−2 log cfu $g^{-1}$) |
| SG | 0 | 3.34 ± 0.08 Ba | 2.38 ± 0.02 Db | 1.46 ± 0.104 Cc* | 0.36 ± 0.33 Cd | + | + |
| | 15 | 6.21 ± 0.27 Aa* | 4.30 ± 0.05 Cc* | 5.61 ± 0.10 Ab* | 4.05 ± 0.17 Bc | + | + |
| | 30 | 6.18 ± 0.34 Aa* | 5.72 ± 0.22 Aa* | 5.05 ± 0.13 Ab* | 4.85 ± 0.14 Abc | 4.29 ± 0.16 c | + |
| | 55 | 6.21 ± 0.08 Aa* | 4.73 ± 0.02 Bb* | 3.74 ± 0.65 Bb | 3.96 ± 0.43 Bb* | 3.61 ± 0.59 b | + |
| PG | 0 | 3.38 ± 0.08 Aa | 2.63 ± 0.38 Ab | 1.06 ± 0.06 Bc | + | + | + |
| | 15 | 3.23 ± 0.00 Aa | 2.41 ± 0.06 Ab | 1.59 ± 0.07 Ac | + | + | + |
| | 30 | 2.86 ± 0.01 Ba | 2.53 ± 0.34 Aa | 0.84 ± 0.06 Cb | + | + | + |
| | 55 | 2.83 ± 0.26 Ba | 2.33 ± 0.31 Aa | + | 0.96 ± 0.24 b | − | − |

Values with different capital-case letters (A–D) within the same column in the group (SG or PG) differ significantly ($P < 0.05$).
Values with different lower-case letters (a–d) within the same row in the group (SG or PG) differ significantly ($P < 0.05$).
Values with asterisks (*) within the same time point between the two different groups (SG and PG) differ significantly ($P < 0.01$).
+: presence in 25 g.
−: absence in 25 g.

(step I), made by an industrial cutter to obtain two half cheeses, resulted in contamination of 33.3% and 25% of the S60 and P80a paste samples, respectively (Table 2). It caused the contamination of only 8.3% of the paste samples for S80, and this contamination was located on the outer part of the sampled surface (sampling points 7, 8, 10 and 11 in Fig. 2), very close to where the cutting began, and it did not affect the inner and more distant parts, thus confirming the presence to be compatible with *L. monocytogenes* being transferred from the rind. *L. monocytogenes* was not transferred to the paste of cheeses P80b and P120 after horizontal cutting.

Regarding cutting step II, the vertical cut performed with an industrial cutter tool necessary to obtain cheese eighths and slices, led to the contamination of 75% of the paste samples of cheeses S60 and P80a, which presented the highest rind initial contamination levels. Lower *L. monocytogenes* rind contamination resulted lower transfer to samples of cheeses S80 and P120, with 41.7% and 33%, respectively, of samples having a pathogen presence after cutting.

In cheeses S60 and P80a, the highest percentage of transfer was caused by knife cutting (step III), with 90% and 95% of the portions of the two cheeses contaminated, respectively.

The contamination rates were 30% and 15%, respectively, in cheeses S80 and P120.

Only for cheese P80b no *L. monocytogenes* paste contamination was detected after the three cutting steps, demonstrating how a low pathogen concentration on the rind could limit the cells transferring to the paste.

Considering cheeses S60 and P80a, representing a sweet and a piquant typologies, artificially contaminated with a similar *L. monocytogenes* concentration, no differences in paste contamination emerged that could be attributable to the different physicochemical characteristics of the sweet and piquant typologies. Anyway, few informations are available so far to allow to discuss an eventual relation between cheese parameters (in particular the higher pH and NaCl content and the lower $a_w$ of the piquant typology) and cross contamination.

Overall, even if to our knowledge this is the first investigation presenting data that regard pathogen cell transfer from cheese rind to paste, some researches concerning meats and salami (Lin et al., 2006; Vorst, Todd, & Ryser, 2006) confirm that cutting procedures are a critical point related to food safety. In particular, as also emerged in the present work, Lin et al. (2006) found that higher *L. monocytogenes* cell numbers inoculated on the slicer blade resulted in more *L. monocytogenes*-positive sliced meat samples. Moreover this topic has also raised interests for surface transfer modelling (Sheen, 2008).

### 3.4. Behaviour of *L. monocytogenes* in cheese paste during storage at 4 °C

To evaluate whether paste contamination, even if low, could represent a risk to the consumer, a second microbiological challenge testing (MCT II) was applied. In this case, sweet (SG) and a piquant (PG) varieties of Gorgonzola cheese were considered to assess the behaviour of *L. monocytogenes* at different contamination levels in cheese paste over 55 days of storage at 4 °C, as the condition recommended by manufacturers.

*L. monocytogenes*, not artificially added, was not detected in any of the 8 SG and 8 PG control paste samples analysed at the beginning of the MCT II. The absence of *L. monocytogenes* in non-inoculated slices was also confirmed throughout the 55 days of storage (data not shown).

MCT II demonstrated that SG strongly supported *L. monocytogenes* growth when the pathogen was initially present at contamination levels A, B, C, D and E (Table 2). In fact, a significant increase ($P < 0.05$) in *L. monocytogenes* was achieved during the shelf life at 4 °C. In particular, in the case of the highest rind contamination (A), after 15 days of storage at 4 °C the pathogen increased by approximately 3 log cfu $g^{-1}$ and then remained stable until 55 days (Table 2). A similar increase was also obtained with initial rind contamination level B, but it required 30 days, and the pathogen then started to decrease significantly ($P < 0.05$). A maximum increase of 3–4 log cfu $g^{-1}$ was reached, starting from rind contamination levels C and D, after 15 and 30 days, respectively. However, as already noticed for contamination level B, after 55 days, *L. monocytogenes* started to decrease, probably as a consequence of the stress in the matrix to which a lower number of cells has been difficult to respond. Anyway, at the end of the considered shelf life, the pathogen was still beyond the limit imposed by Commission Regulation No. 2073/2005. Additionally, when initially present at −1 log cfu $g^{-1}$ (E), *L. monocytogenes* was able to multiply, but it required 30 days to exceed the food safety criteria. Only with the lowest estimated rind contamination F (−2 log cfu $g^{-1}$) the pathogen was unable to reach a level detectable by the counting method, but its presence 25 g samples was confirmed by the enrichment technique over the entire storage period storage (Table 2).

Different behaviour of contamination with *L. monocytogenes* was observed in PG (Table 2). Considering the highest rind contamination levels A and B, *L. monocytogenes* counts did not change significantly during the first 15 days of storage at 4 °C ($P \geq 0.05$), while the pathogen significantly decreased ($P < 0.05$) after 30 days. In the case of initial contamination level B,

the pathogen counts did not change ($P > 0.05$) throughout the shelf life considered, while when *L. monocytogenes* was present at an initial estimated contamination level of 1 log cfu $g^{-1}$ (C), the pathogen grew for 15 days ($P < 0.05$) and then started to decrease significantly ($P < 0.05$) after 30 days until it could be detected only by the enrichment method after 55 days. Considering initial contamination level D, the pathogen was recovered by plate counts only after 55 days; before this time, it was less than the plate count detection limit although it was present in 25 g of the samples. In cheese samples with a −1 and −2 log cfu $g^{-1}$ level of initial contamination (E and F), *L. monocytogenes* never grew beyond the detection limit for the count, and after 55 days, it could no longer be detected by the enrichment method either (Table 2).

A different behaviour of *L. monocytogenes* was evidenced during the shelf life at 4 °C, according to the type of cheese. In fact, significant differences ($P < 0.01$) between bacterial counts within the same sampling time between SG and PQ were confirmed by Student's t test (Table 2).

As already observed in MCT I, the behaviour of *L. monocytogenes* during storage at 4 °C also depended both on the level of contamination and on the type of cheese. Growth of pathogens in cheese is controlled by several preservation factors, such as pH, $a_w$, salt content, and $O_2$ concentration (Papageorgiou & Marth, 1989). In mould-ripened cheeses most of these factors change during ripening. In particular, proteolysis of casein results in the release of ammonia and utilisation of lactic acid, with a consequent increase in pH that contributes to creating favourable conditions for growth (Gobbetti et al., 1997). Moreover, it has been suggested that high salt concentrations and the presence of free fatty acids could inhibit the multiplication of *L. monocytogenes* during the ripening of blue-veined cheeses (Papageorgiou & Marth, 1989). The high NaCl percentage, the low $a_w$ and the low moisture content of PG, compared to SG, could be correlated with the greater inhibition of the growth of *L. monocytogenes*.

In SG *L. monocytogenes* was able to grow in cheese paste during shelf life in refrigerated condition. This behaviour, in the presence of paste contaminations at the end of production of 2 log cfu $g^{-1}$ (B), 1 log cfu $g^{-1}$ (C) and 0 log cfu $g^{-1}$ (D), led to the overcoming of the limit imposed at consumption by Commission Regulation No. 2073/2005 after only two weeks.

Previously, Bernini et al. (2013) demonstrated by microbiological challenge testing that *L. monocytogenes* artificially inoculated on the rinds was able to survive and grow over 55 days of storage at both 4 and 8 °C. A similar behaviour, with a logarithmical duplication of the pathogen within 15 days at 5 °C, was already observed in Queso Blanco cheese (Uhlich et al., 2006), with the $a_w$ (0.97) and pH (6.80) values of which were similar to those of SG. *L. monocytogenes* was also capable of surviving during refrigerated storage of soft cheeses, decreasing to only 1 log cfu $g^{-1}$ after two weeks (Cataldo et al., 2007). In mould-ripened soft cheeses, it has been demonstrated that the pathogen survived in the paste during 42 days of ripening, even with a level of milk contamination as low as 1 log cfu $g^{-1}$ (Morgan, Bonnin, Mallereau, & Perrin, 2001).

Interestingly, in PG *L. monocytogenes* was not able to grow and furthermore the pathogen slightly decreased when present in cheese paste at contamination levels A, C, E and F. So, even if we can not extend this statement to all piquant Gorgonzola cheeses, we may assume that the typology considered in this study is not able of supporting *L. monocytogenes* growth in the storage conditions presented. In contrast, longer storage usually applies modified atmospheres, and different studies have demonstrated that the modification of the atmosphere, in particular an increase in $CO_2$ levels, could inhibit *L. monocytogenes* growth (Whitley, Muir, & Waites, 2000).

Regarding the different contamination levels, we observed that low levels reduced *L. monocytogenes* growth and, particularly with cheese PG, the pathogen, if present with an estimated initial contamination of few cells per gram, could not be detected by the enrichment method until the end of the storage period. Similarly, no *L. monocytogenes* was detected after only 18 days of 5 °C storage of a traditional Greek soft cheese (Kagkli, Iliopoulos, Stergiou, Lazaridou, & Nychas, 2009). In agreement with these findings, it has previously been shown that the probability of growth of *L. monocytogenes* could be influenced by inoculum size (Pascual, Robinson, Ocia, Aboaba, & Mackey, 2001; Robinson et al., 2001) and that the lag time inversely depended on this size, particularly under stress conditions (Augustin, Brouillaud-Delattre, Rosso, & Carlier, 2000).

Overall, the data obtained emphasised that *L. monocytogenes* could survive in Gorgonzola cheese paste over 55 days of storage at 4 °C. Moreover, the pathogen could even grow in presence of low salt content and high $a_w$, as in the case of the soft variety of Gorgonzola. *L. monocytogenes* survival and growth during the shelf life also depend on the number of cells initially present in the paste and that are transferred from the rind during cutting procedures. Pathogen transfer can occur and can involve different percentages and areas of cheese paste relative to the number of pathogen cells located on the rind, with a higher rind contamination corresponding to a higher percentage of contaminated paste samples.

## 4. Conclusions

The microbiological challenge tests applied in this study underlined how the Gorgonzola cheese cutting procedures could constitute a critical point for *L. monocytogenes* being transferred to the paste. Once reached the paste, the pathogenic cells, even if to a different extent in relation to the physicochemical characteristics of the cheeses and to the time of storage at 4 °C, could also overcome the limit imposed by food safety criteria and the safety for consumption may not be longer ensured. Overall, if on one side the pathogenic cells transfer was demonstrated, on the other one we can state that an high paste contamination in terms of cfu $g^{-1}$ was not conveyed. On the contrary, an high paste contamination could result after cut from a really high rind contamination that can be considered unusual.

Implementation of Good Manufacturing Practices focused on the control of Gorgonzola cheese rind contamination and the possibility of reducing this eventual contamination by the application of post-processing treatments, such as high pressures and infra red technologies, as already discussed by several researchers (Bernini et al., 2015; Carminati et al., 2004; Mucchetti, Bonvini, Francolino, Neviani, & Carminati, 2008), could be therefore be important tools for increasing cheese safety. To avoid risk to the consumer, the Consortium for the Protection of Gorgonzola Cheese has already declared the rind to be not edible.

## Disclosures

Authors declare that no conflict of interests exist. All of the authors approved the final article.

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# 9. A MULTI-SAMPLING APPROACH TO EVALUATE AN INFRARED SURFACE TREATMENT FOR REDUCING *Listeria monocytogenes* CONTAMINATION ON WHOLE GORGONZOLA CHEESE RINDS

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# A multi-sampling approach to evaluate an infrared surface treatment for reducing *Listeria monocytogenes* contamination on whole Gorgonzola cheese rinds

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ABSTRACT

The microbial ecology of Gorgonzola cheese rind is the focus of many studies because the surface can be contaminated by pathogenic microorganisms. Among food-borne pathogens, particular attention is focused on the behaviour of *Listeria monocytogenes* that is able to grow at refrigeration temperatures and it could also grow during ripening. The Consortium for the Protection of Gorgonzola Cheese declares the rind not edible but the pathogen may also be transferred during cutting and portioning. Therefore, the decontamination of rinds is important to increasing cheese safety. To achieve this goal, many different strategies have been proposed. In this study, the application of an infrared surface treatment to decontaminate cheese rinds is proposed. The presence of *L. monocytogenes*, which was artificially inoculated in cheese rinds together with cheese rind microflora, and the cheese rind microflora were monitored before and after the treatment of 32 samples of Gorgonzola cheese rinds.

The infrared surface treatment provided good reduction of the rind microflora, and *L. monocytogenes* was particularly affected by this. The treatment, applied to cheeses at the end of ripening, does not interfere with the ripening process and offers the advantages of short time exposures and easy installation of the equipment in cheese plants. Moreover, this study demonstrated that the sampling method affects the detection of cheese rind microflora. In fact, a non-destructive sampling method, based on a sponge and often used for surface sampling but never before applied to ready to eat food sampling, was compared with a traditional but destructive method, based on rind scraping. Regarding *L. monocytogenes*, the sponge method allowed to estimate even only 5.71 ± 0.79 log cfu $g^{-1}$ of cells reduction after the treatment while the higher reduction when considering the rind scraping method was 4.06 ± 3.38 log cfu $g^{-1}$. The sponge method, combined with the classic scraping one, besides offering the great advantage of not being destructive, allowed to differentiate the effect that the treatment has on the microflora located on the surface from those in deeper layers.



## 1. Introduction

Gorgonzola, one of the most appreciated "Protected Denomination of Origin" (PDO) Italian cheeses, is a blue-veined, mould-ripened cheese made with pasteurised cow's milk inoculated with *Lactobacillus* and *Streptococcus* starter cultures and *Penicillium roqueforti* that develops as an internal blue-green mould (Gripon & Hubert, 2002; Mucchetti & Neviani, 2006). Gorgonzola ripening is characterised by a complex microbial community that is formed during production and is mainly composed of mesophilic and thermophilic lactic acid bacteria, lactococci, enterococci, yeast and moulds, coliforms, staphylococci and micrococci (Gobbetti, Burzigotti, Smacchi, Corsetti, & De Angelis, 1997). In particular, the microbial ecology of Gorgonzola cheese rinds has been the topic

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of several studies (Cocolin et al., 2009; Fontana, Cappa, Rebecchi, & Cocconcelli, 2010) because the surface could be contaminated by food-borne pathogenic microorganisms. If environmental contamination occurs, pathogens could survive and grow on cheese surface during ripening. These microorganisms, which are introduced from the environment, can develop during ripening when the pH rises due to typical ripening that proceeds from outside to inside and *P. roqueforti* proteolytic activity (Mucchetti & Neviani, 2006).

Among food-borne pathogens, particular attention is focused on *Listeria monocytogenes* (Carminati, Gatti, Bonvini, Neviani, & Mucchetti, 2004; Cocolin et al., 2009; Lomonaco et al., 2009; Manfreda, De Cesare, Stella, Cozzi, & Cantoni, 2005). Although the application of a heat treatment is sufficient to inactivate the pathogen present in raw milk, *L. monocytogenes* can also occur as a consequence of a cross-contamination in ripening environments during ripening and storage steps (Cocolin et al., 2009). If *L. monocytogenes* contaminates blue-veined cheese rinds at the end of production, the pathogen is able to grow over the limit set by Commission Regulation (EC) No 2073/2005 at refrigeration temperatures (Bernini et al., 2013). To avoid the risk of *L. monocytogenes* ingestion, the Consortium for the Protection of Gorgonzola Cheese declared the rind inedible (UE Notice 2008/C 111/17) but the internalisation of pathogenic cells may happens during cutting and portioning.

Therefore, the decontamination of rinds is important to increasing cheese safety. Non-thermal approaches have been extensively studied as food processing alternatives capable of decontaminating food surfaces (Lianou & Koutsoumanis, 2013). Many strategies have been proposed to control *L. monocytogenes* in Gorgonzola and the rinds of soft cheeses. Some of them are based on the activity of bacteriophages (Schellekens, Wouters, Hagens, & Hugenholtz, 2007) and on the use of antimicrobial producing strains as surface starter cultures (Carminati, Neviani, Ottogalli, & Giraffa, 1999; Izquierdo, Marchioni, Aoude-Werner, Hasselmann, & Ennahar, 2009; Siafaras, Hatzikamari, Litopoulou-Tzanetaki, & Tzanetakis, 2008). Other authors investigated the effects of physical treatments, such as high-pressure processing (HPP) (Carminati et al., 2004; Cheftel, 2005), on *L. monocytogenes*. Additionally, atmospheric pressure plasma (Song et al., 2009) was investigated; it led to variable reductions but showed limitations because of the restriction in volumes and sizes of the food. Infrared technology has also been considered for food treatment (Krishnamurthy, Khurana, Jun, Irudayaraj, & Demirci, 2008; Ramaswamy, Krishnamurthy, & Jun, 2012, chap. 15) and it has been investigated by different authors as a possible application for decontamination of different products such as turkey frankfurters, hotdogs, shell eggs and strawberries (Huang, 2004; Huang & Sites, 2008; James, Lechevalier, & Ketteringham, 2002; Tanaka et al., 2007).

One challenge that is independent of the decontamination technology is the detection of the surface microflora. Generally, food sampling destroys the sample due to product fractionation. Therefore, the traditional techniques cannot be easily applied on a large scale because they cannot satisfy the industrial requirement of maintaining product integrity. Although no ideal food sampling method exists, the Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA) recommends sponges as the environmental sampling technique for *L. monocytogenes* (Food Safety and Inspection Service, 2013). In agreement with these tips, several studies had already applied non-destructive swabbing methods for carcasses sampling (Lindblad, 2007; Martínez, Celda, Anastasio, García, & López-Mendoza, 2010).

In the present study the application of an infrared surface treatment is proposed for the first time for cheese rinds decontamination. The effect of an infrared surface treatment patented by the Biraghi S.p.A. Milk Industry (Cavallermaggiore, Cuneo, Italy) on *L. monocytogenes* and microflora reduction in Gorgonzola cheese wheel rinds was evaluated.

Moreover, a non-destructive sampling method, based on sponges and commonly used for surface sampling but never applied to food sampling, was compared with a traditional, destructive method, based on rind scraping.

## 2. Material and methods

### 2.1. Infrared surface treatment

The infrared surface treatment (Patent Number TO2007A000822) was applied homogeneously to the whole cheese rinds at the end of ripening so as not to interfere with the product specifications. The method is based on a temperature effect that results from the exposure of the cheeses to low and high temperatures. The treatment, realised in an apparatus projected and setup for commercial installation and application, was performed in three steps: *i) cold pre-treatment*. The cheeses are cooled in a forced air cooling tunnel to reach a temperature of 0–2 °C at a rind depth of 4 mm, without freezing the product. The exposure is maintained for 30–240 min depending on the size and creaminess of the cheeses. *ii) heat treatment*. The cheeses are rapidly heated to 80–85 °C in the surface layer (4 mm) via the application of an infrared source. The radiation is performed for 5–120 s per $cm^2$. To obtain good penetration of the infrared radiation into the surface layer, the lamp consists of a radiating quartz tubular element (1500 W, 230 V) with an emission spectrum from 1.5 to 3.5 μm and a peak at 2.3 μm *iii) cooling treatment*. The cheeses are cooled to 2–4 °C in a forced air cooling tunnel, and the final temperature is maintained for 30–240 min.

The characteristic temperatures of each step are reached in 15–30 s to prevent adaptation by the microorganisms. All of the tested cheeses complied with the following dimensions and are characteristic for this product in accordance with the product specifications: 13–20 cm height, 20–32 cm diameter, 6–13 kg weight.

### 2.2. L. monocytogenes strains

To represent the behaviour of naturally contaminating strains and to avoid variations in growth and survival, a mixed inoculum (Lmix) of five *L. monocytogenes* strains (Lm2, Lm3c, Lm8, Lm60 and Lm90) was used to evaluate the effect of the infrared treatment on microbial reduction. Moreover, a *Listeria innocua* strain (LIN9) was used in preliminary test aimed to standardise the contamination procedures of the upper cheese rind. All the strains were kindly provided by the Agriculture Research Council, Fodder and Dairy Productions Research Centre, CRA-FLC, of Lodi (Italy), where they were previously isolated from blue-veined cheese rinds and identified.

Each frozen stock culture was transferred twice as a 2% (v/v) inoculum in Tryptone Soya Broth (TSB) (Oxoid, Basingstoke, England) and incubated at 37 °C for 18 h in aerobic conditions before use. Regarding *L. monocytogenes* strains, they were mixed in equal volumes to obtain an Lmix containing 8 log cfu $g^{-1}$. The Lmix was then diluted 10-fold in quarter strength Ringer's solution (Oxoid) for cheese contamination.

### 2.3. Cheese rind contamination and sampling

Preliminary test were performed on five cheeses in order to standardise the contamination procedures and assure a homogeneous distribution of microorganisms on the cheese

surface. In particular, a constant volume of *L. innocua* LIN9 was sprayed toward a constant number of sprayed and maintaining the same distance from the sample. The contamination was performed in a refrigerated room, and the cheeses were left to dry for 4 h to allow the attachment of the cells to the rind. After contamination, four area for each upper cheese rind were sampled (for a total amount of 20 samples collected) and analysed by plate counts in order to determine the distribution of the microorganisms.

To evaluate the efficacy of the infrared surface treatment, the contamination procedure was performed on 32 Gorgonzola cheese wheels, derived from different manufacturing processes: 14 sweet Gorgonzola (SG) ripened for 60 days and 18 piquant Gorgonzola (PG) ripened for 80 days. Cheeses, as stated by the manufacturer, were characterised by fat 27 g 100 $g^{-1}$, proteins 19 g 100 $g^{-1}$, NaCl 1.9 g 100 $g^{-1}$, moisture 51%, dry matter 49%, $a_w$ 0.95%, pH 6.2. The upper rind of each cheese wheel was contaminated by spreading the appropriate dilution of the Lmix to obtain trials at different contamination levels: 13 cheeses were contaminated with 5 log cfu $g^{-1}$ (Lmix5), 10 with 6 log cfu $g^{-1}$ (Lmix6), 5 with 7 log cfu $g^{-1}$ (Lmix7) and 4 with 8 log cfu $g^{-1}$ (Lmix8).

Additionally, 5 sweet and 5 piquant Gorgonzola cheeses were not artificially inoculated and were qualitatively checked to test for the absence of naturally occurring *L. monocytogenes* in the rinds.

For each artificially contaminated cheeses, before (t0) and after (tf) the application of the infrared surface treatment, a quarter of the contaminated upper cheese rind was gently scraped with a knife (rind scraping method, RS) and a quarter was scrubbed with a sponge bag wet with buffered peptone water (Biogenetics, Padova, Italy) (sponge bag method, SB) (Fig. 1). This sampling procedure does not involve the removal of pieces of cheese and allowed to maintain the integrity of the whole cheese during the treatment as in a real condition. Moreover, the use of the same cheese to assess the microflora before and after the treatment avoided mistakes in the evaluation of reduction due to initial microbial variability among cheeses.

### 2.4. Microbiological analysis

All of the collected rind samples were blended 1:10 in Ringer's solution and diluted 10-fold for the following bacterial counts: total mesophilic bacteria (TMB) on Plate Count Agar (PCA) (Oxoid, Basingstoke, England) incubated at 30 °C for 48 h, mesophilic lactic acid bacteria (MLAB) on MRS Agar (Oxoid) incubated at 30 °C for 48 h, yeasts and moulds (Y&M) on Malt Extract Agar (MEA) (Oxoid) incubated at 28 °C for 7 days and *L. monocytogenes* (Lm) on Agar *Listeria* acc. to Ottaviani & Agosti (ALOA) supplemented with ALOA enrichment-selective supplements (Biolife Italiana, Teramo, Italy) incubated at 37 °C for 48 h. Typical *L. monocytogenes* colonies were confirmed by rapid test Monocytogenes ID Disk (Biolife) following manufacturer instructions. The colony counts were performed in triplicate, the average values ± standard deviation were calculated and were considered as representative of each sample.

Qualitative determination was performed to detect *L. monocytogenes* below the limit of quantitative analysis for samples collected after the treatment, according to ISO 11290 (1996). Subcultures of both enrichment stages were spread plated on ALOA (Biolife Italiana) (Vlaemynck, Lafarge, & Scotter, 2000) and incubated at 37 °C for 48 h to check for the presence of colonies. Typical *L. monocytogenes* colonies were confirmed as previously described.

The efficacy (E %) of the infrared surface treatment on the reduction of TMB, MLAB, Y&M and Lm (considered within each inoculation level: Lmix5, Lmix6; Lmix7, Lmix8) was determined, according to Mucchetti, Bonvini, Francolino, Neviani, and Carminati (2008), using the following formula: $E\ \% = 100 - (n_t/n_o)^*100$. Moreover, percentages of cell recovery (R %) after the application of the infrared radiation treatment were calculated for TMB, MLAB, Y&M and Lm (considered within each inoculation level: Lmix5, Lmix6; Lmix7, Lmix8) as follows: $R\ \% = (n_t/n_0)^*100$. In both cases, $n_0$ = cfu $g^{-1}$ of the samples before the treatment and $n_t$ = cfu $g^{-1}$ of the samples after the treatment. Regarding R % of *L. monocytogenes*, $n_0$ = theoretical cfu $g^{-1}$ of *L. monocytogenes* inoculated in the samples.

### 2.5. Statistical analysis

The means and standard deviations were calculated using SPSS (Version 21.0, SPSS Inc., Chicago, IL, USA) statistical software. First, for each sample counted in triplicate, means and standard deviations were calculated as representative of the sample. Then, these representative values determined for each sample were used to calculate means and standard deviations among each group of bacterial counts (TMB, MLAB, Y&M and Lm). Regarding *L. monocytogenes*, data were considered within each inoculation level (13 trials for Lmix5, 10 for Lmix6, 5 for Lmix7 and 4 for Lmix8). A Student's t test ($P < 0.05$) was used to determine the differences between the bacterial counts before (t0) and after (tf) the treatment, between the sweet (SG) and piquant (PG) Gorgonzola cheeses for the two sampling methods (RS and SB) for each bacterial count (TMB, MLAB, Y&M and Lm) and between the different *L. monocytogenes* initial contamination levels (Lmix5, Lmix6, Lmix7 and Lmix8) for the two sampling methods (RS and SB).

## 3. Results and discussion

Preliminary test carried out on five cheeses which have been contaminated on the upper rind with *L. innocua* LIN9 allowed to evaluate the homogeneous distribution of microorganisms on the cheese surface. In fact, no significative differences ($P > 0.05$) in microbial concentrations were detected among the four area of cheese surface sampled (data not shown). So, the contamination procedure followed was used to contaminate with *L. monocytogenes* the 32 Gorgonzola cheese rinds in order to evaluate, in different trials for each contamination level (Lmix5, Lmix6, Lmix7 and Lmix8), the reduction of the pathogen after the treatment.

### 3.1. Effect of the infrared surface treatment on L. monocytogenes reduction in cheese rinds

Artificial contamination of the 32 Gorgonzola cheese rinds with *L. monocytogenes* was performed to verify the efficacy of the infrared surface treatment on the reduction of the pathogen located in the surface of this product. The absence of naturally occurring *L. monocytogenes* in the rinds was verified on 10 Gorgonzola cheeses (5 sweet and 5 piquant) by qualitative analysis as described in 2.3 paragraph (data not shown). The *L. monocytogenes* counts at t0, detected using both sampling methods, confirmed that the contamination introduced the expected levels of contamination. The microbial counts of *L. monocytogenes* after the application of the infrared surface treatment (tf) were significantly different ($P < 0.05$) than those obtained before the treatment (t0), but the Lm reduction was affected by the sampling method (Fig. 2). Moreover, even considering the same sampling method, a great variability in the values of treated samples was observed and an in-depth into the causes would be interesting in future studies. A Student's t-test showed that the reduction obtained on samples with different initial contamination levels (Lmix5, Lmix6, Lmix7, Lmix8) was not significantly different ($P > 0.05$) for the two sampling methods.

The pathogen was reduced, on average, by more than 4 log cfu $g^{-1}$ for the SB method and 2 log cfu $g^{-1}$ for the RS method

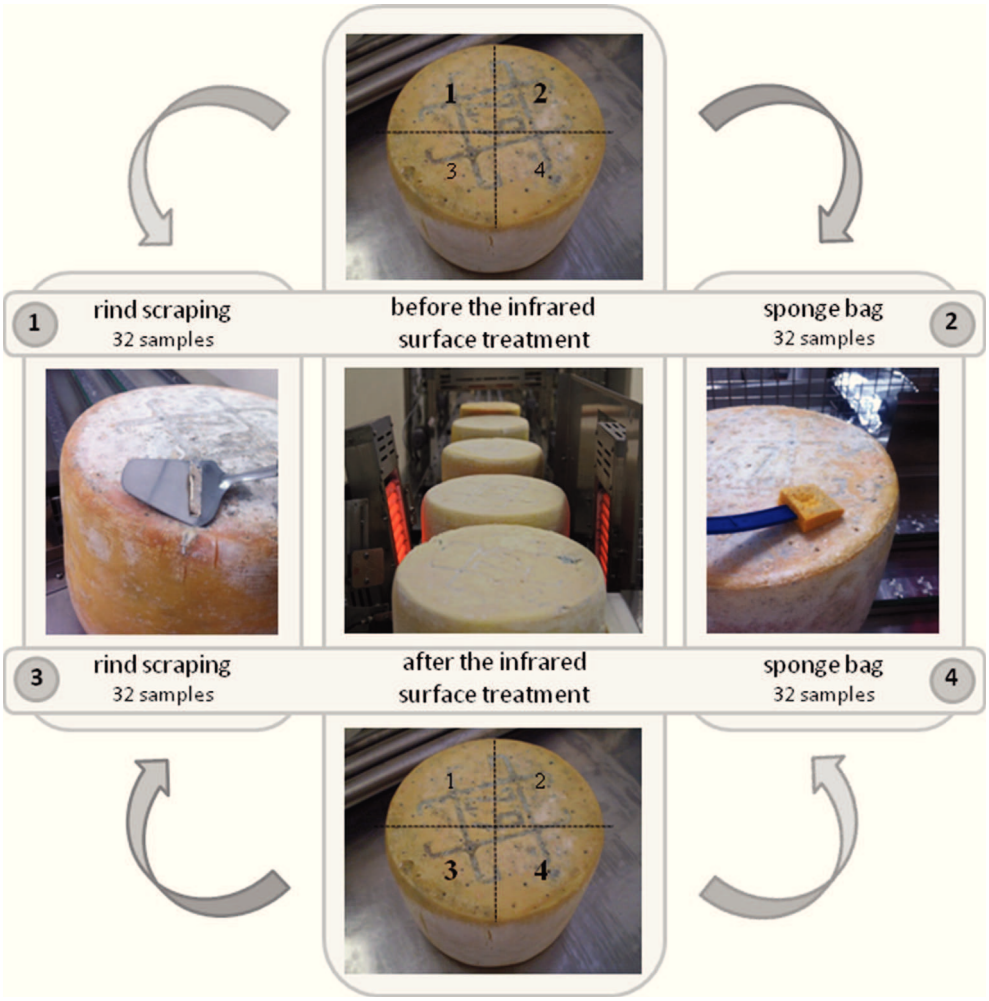


**Fig. 1.** Gorgonzola cheese rind sampling scheme before (t0) and after (tf) the infrared surface treatment according to the rind scrape (RS) and sponge bag (SB) sampling methods.

(Table 1). The best decontaminating effect of the treatment was obtained for the more superficially located *L. monocytogenes* cells, and we could hypothesise that *L. monocytogenes* artificially added on the rind localised also a few millimetres into the rind. This result confirmed, as expected, the ability of *L. monocytogenes* to colonise the entire surface layer of the cheese. This ability may be dependent on the strain and environment in which it is located and needs to be investigated further.

The efficacy of the treatment varied from 99.892 ± 0.232% for Lmix6 to 99.999 ± 0.001% for Lmix7 when it was estimated using the SB method. The efficacy was lower varying from 89.678 ± 15.105% to 98.947 ± 1.489% when estimated using the RS method. The high standard deviation for the RS method indicates the great variability due to the heterogeneity among the samples (Table 1). These data are confirmed by percentages of cell recovery (R %) after treatment: in fact very low recoveries, varying from 0.002 ± 0.003% to 0.257 ± 0.496% were detected by SB methods, while higher percentages of cells (corresponding to lower efficacy of the treatment) were recovered when estimated by RS method (from 2.000 ± 2.828% to 31.433 ± 50.766%) (Table 1).

The main methods currently used to remove *Listeria* from cheese surfaces are based on high pressures. The performances of these methods are variable, and the efficacy of each treatment can be improved by optimising the method. Mucchetti et al. (2008) obtained a variable decontaminating effect, ranging from 72% to 99%, on *L. innocua* using a method based on high pressure washing, but only the highest pressures or a prolonged treatment time were able to remove *Listeria* cells. Additionally, Carminati et al. (2004) obtained a reduction greater than 99.999% by pressurising cheese rinds, but the method required a long time and expensive equipment. A higher heterogeneity among the reductions was realised for *L. monocytogenes* inoculated on cheese slices using atmospheric plasma, although this method has many limitations due to volume, size and shape irregularities of the product (Song et al., 2009). The pathogen reduction obtained in the present study applying an infrared surface treatment was comparable to, or even better than, the best results obtained in those studies. One of the main difficulties in the commercial application of infrared technology is to find the balance among a good efficacy on microbial reduction and the maintaining of the qualitative characteristics of the products. Other authors investigated the effect of infrared treatments on *L. monocytogenes* and inactivation of other organisms in foods other than cheese, even if with different results in relation to the different product characteristics. When the technique was applied on hot-dogs a reduction of more than 5 log cfu $g^{-1}$ was obtained (Huang & Sites, 2008), while the feasibility of the method appeared low when used for strawberry decontamination. The maximum surface temperature of 50 °C, achievable without product damaging, did not allow a proper inactivation of fungal contamination (Tanaka et al., 2007).

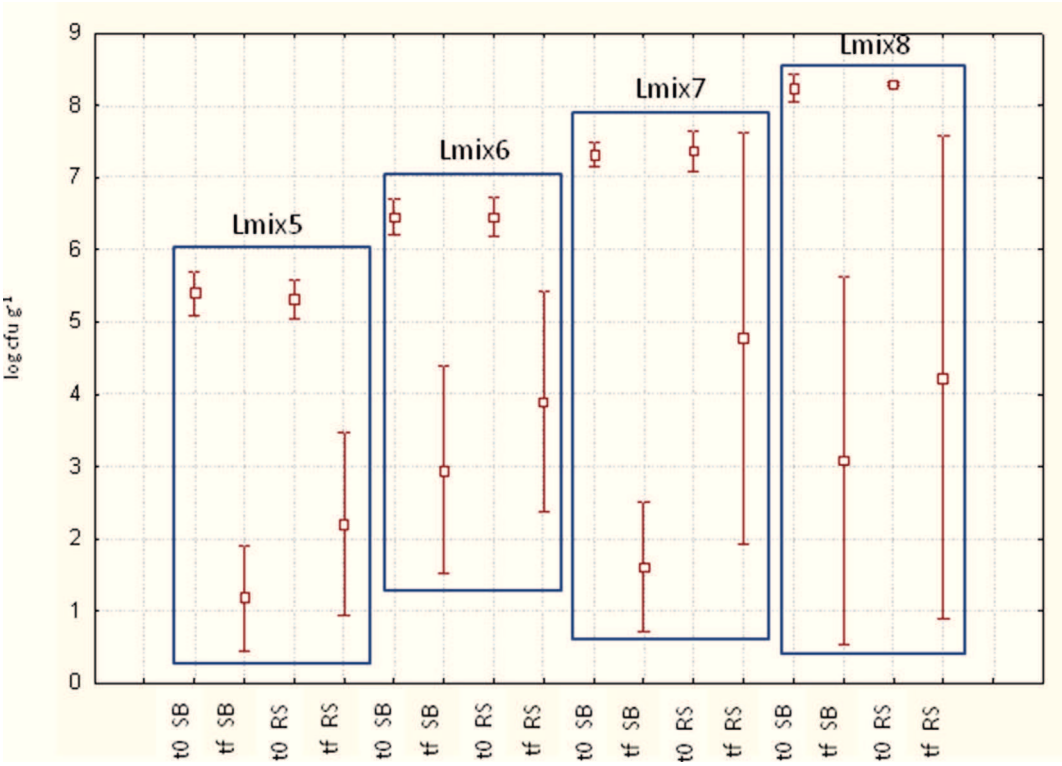


**Fig. 2.** *L. monocytogenes* artificially inoculated on Gorgonzola cheese rinds at different contaminating levels (Lmix5, Lmix6, Lmix7 and Lmix8), calculated on 13, 10, 5 and 4 cheese rinds respectively, before (t0) and after (tf) the infrared surface treatment, according to the sponge bag (SB) and the rind scraping (RS) sampling methods. The squares represent the averages and the bars represent the minimum and maximum values.

**Table 1**
Effect of the infrared surface treatment on Gorgonzola cheese rinds. Data, collected according to the sponge bag (SB) and the rind scraping (RS) sampling methods, regard the total mesophilic bacteria (TMB), mesophilic lactic acid bacteria (MLAB), yeasts and moulds (Y&M), calculated on 32 cheese rinds, and *L. monocytogenes* artificially inoculated at different contamination levels (Lmix5, Lmix6, Lmix7 and Lmix8), calculated on 13, 10, 5 and 4 cheese rinds respectively. The data are represented as: differences in microbial counts before and after the treatment (Δ log cfu $g^{-1}$); efficacy of the treatment for microbial inactivation (E %); percentage of cell recovery after the application of the infrared radiation treatment (R %). The data are shown as the average values ± standard deviations calculated considering the average values ± standard deviations of each sample counted in triplicate.

| | SB | | | RS | | |
|---|---|---|---|---|---|---|
| | Δ log cfu $g^{-1}$ | E % | R % | Δ log cfu $g^{-1}$ | E % | R % |
| TMB | 2.24 ± 0.76 | 98.484 ± 1.668 | 1.516 ± 1.668 | 1.60 ± 0.51 | 95.915 ± 3.576 | 4.085 ± 3.576 |
| MLAB | 2.56 ± 1.06 | 98.134 ± 3.628 | 1.866 ± 3.628 | 1.45 ± 0.59 | 93.116 ± 7.912 | 6.884 ± 7.912 |
| Y&M | 2.78 ± 1.04 | 98.777 ± 2.619 | 1.223 ± 2.619 | 1.51 ± 0.49 | 94.899 ± 5.084 | 5.101 ± 5.084 |
| Lmix5 | 4.22 ± 0.73 | 99.968 ± 0.077 | 0.080 ± 0.194 | 3.10 ± 1.22 | 93.870 ± 21.202 | 7.540 ± 24.839 |
| Lmix6 | 3.50 ± 1.49 | 99.892 ± 0.232 | 0.207 ± 0.415 | 2.55 ± 1.57 | 92.237 ± 16.350 | 12.906 ± 23.115 |
| Lmix7 | 5.71 ± 0.79 | 99.999 ± 0.001 | 0.002 ± 0.003 | 2.59 ± 2.76 | 89.678 ± 15.105 | 31.433 ± 50.766 |
| Lmix8 | 5.15 ± 2.36 | 99.908 ± 0.177 | 0.257 ± 0.496 | 4.06 ± 3.38 | 98.947 ± 1.489 | 2.000 ± 2.828 |

### *3.2. Effect of the infrared surface treatment on the microbial reduction in cheese rinds*

Great variability was observed in the Gorgonzola cheese rind microbial counts (TMB, MLAB and Y&M) before the treatment, which is in agreement with the literature (Bernini et al., 2013; Cocolin et al., 2009; Gobbetti et al., 1997). This variability regarded samples collected with the same method, confirming that the microbiota of these products is influenced by the manufacturing, ripening and environmental conditions as well as by microbial techniques and analytical procedures when considering variability between laboratories. However, in this study the bacterial detection limit changed depending on the sampling method. The TMB before the treatment were 7.78 ± 0.48 log cfu $g^{-1}$ and 9.23 ± 0.25 log cfu $g^{-1}$, for the SB and RS methods, respectively (Fig. 2). The MLAB detected by the SB method before the treatment (6.94 ± 0.55 log cfu $g^{-1}$) were 2 log cfu $g^{-1}$ lower than the RS method (8.69 ± 0.41 log cfu $g^{-1}$) (Fig. 3), which is consistent with the results of Bernini et al. (2013), whereas Gobbetti et al. (1997) and Cocolin et al. (2009) detected, sampling by rind scraping, higher and lower counts, respectively. Similarly, Y&M, detected before the treatment, were lower by SB (7.36 ± 0.54 log cfu $g^{-1}$) than by RS (9.08 ± 0.36 log cfu $g^{-1}$) (Fig. 3).

These differences could be due to the microbial colonisation of the layer located just a few millimetres below the surface, which can be sampled only by the rind scraping method. When considering the effect of the infrared surface treatment on the microflora, the RS method allowed for the detection of a smaller reduction after the treatment than the SB method. In fact, for most of the SB samples, the microorganisms decreased from 2 to 4 log cfu $g^{-1}$ and

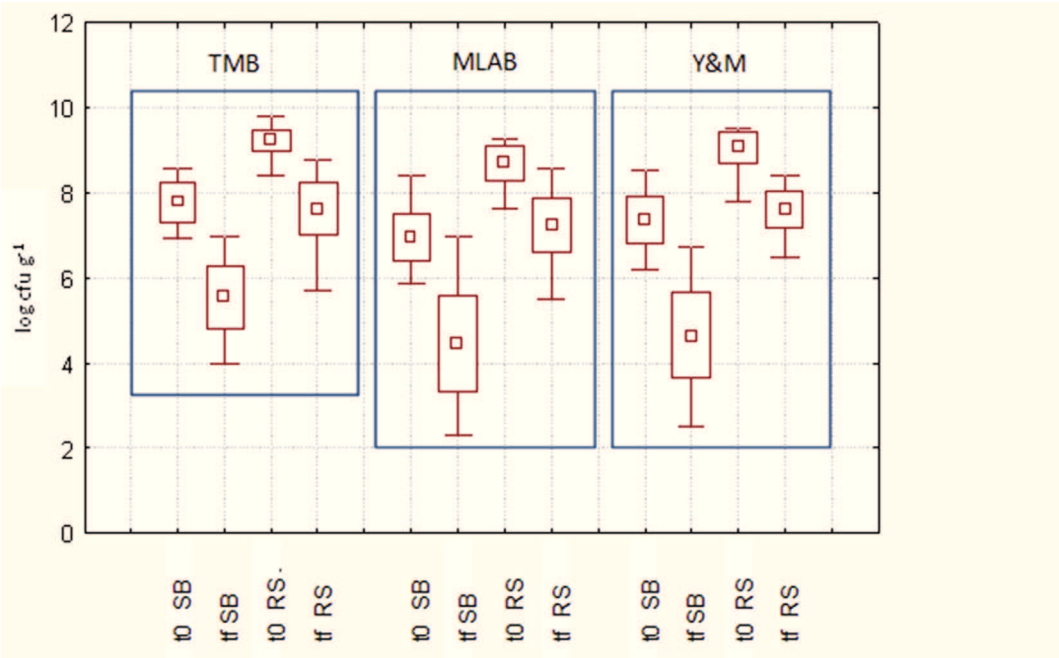


**Fig. 3.** Total mesophilic bacteria (TMB), mesophilic lactic acid bacteria (MLAB) and yeasts and moulds (Y&M) counted for 32 Gorgonzola cheese rinds before (t0) and after (tf) the infrared surface treatment, according to the sponge bag (SB) and the rind scraping (RS) sampling methods. The boxes represent the averages ± standard deviations, the squares inside the boxes represent the average and the bars represent the minimum and maximum values.

in some samples the reduction was greater than 4 log cfu $g^{-1}$. Moreover, regarding MLAB and Y&M, counts after treatment revealed an increased variability that could be due to the heterogeneity of microorganisms located on the rind that differently respond to the treatment. In contrast none of the RS samples reached this high of a decontamination level, and almost all samples showed a reduction of less than 2 log cfu $g^{-1}$ (Table 1). However, regardless of the sampling approach, all microbial counts before and after the treatment were significantly different ($P < 0.05$). The efficacy of the infrared surface treatment (E %) on the TMB, MLAB and Y&M, evaluated as percent reductions of the average values ± standard deviation, was different depending on the sampling method (Table 1). In particular, as already revealed by *L. monocytogenes* data, higher E% were detected by SB method in comparison to the RS one and, therefore, also in this case percentages of recovery (R %) were higher in RS samples (Table 1).

This result can be explained by considering that the microbial cells that are located below the surface are recovered only by the scraping method. These cells are less influenced by the treatment, which is more effective on the surface of the cheese. Thus, the efficacy of the treatment when evaluated using the RS method is lower than when estimated using the SB method. A Student's t-test showed that the efficacy of the treatment on the TMB count was significantly different ($P < 0.05$) between the sweet and piquant cheeses (data not shown). In particular, the reduction of TMB in piquant cheese was higher, most likely due to the different surface characteristics of the two cheese varieties. On the other hand the efficacy of the treatment on the MLAB and Y&M counts was not significantly different ($P > 0.05$) between the sweet and piquant cheeses for the two sampling methods.

In the light of what has been observed for both *L. monocytogenes* and cheese rind microflora when the efficacies of different treatments are compared, it is important to pay attention to the variances between the strains, growth step, characteristics of the food and sampling methods (Cheftel, 2005; Datta & Deeth, 1999; O'Reilly, O'Connor, Kelly, Beresford, & Murphy, 2000). In this research, it was demonstrated that the sampling method affected the detection of the cheese rind microflora, influencing the efficacy of the treatment. The SB sampling method estimated a high efficacy and offers the advantage of being non-destructive, which allows for applications on a large scale.

The SB sampling method recovered the microflora located on the surface and, as proposed in this study, gave interesting results. In fact, it has been used with the classic method to differentiate the microflora on the surface from the cells in deeper layers in a product, such as Gorgonzola cheese rind, that has irregular and rough surface that could present niches for microorganisms. So, it can be hypothesised that highly irregular and porous sample surfaces correspond to a more heterogeneous distribution of microorganisms, which can be found a few millimetres deep into the cheese.

The role of the microflora involved in cheese ripening is not compromised by the treatment since it is applied at the end of the ripening process and is focused on 4 mm of the rind that is declared not edible. So this application does not contradicts production regulations imposed by the Consortium for the Protection of Gorgonzola cheese and, moreover, offers the advantages of a short exposure time and easy installation of the equipment in cheese plants. In conclusion, this study demonstrated that, in the processing conditions tested, the infrared surface treatment, applied for the first time to cheese surfaces, allows for the control of the cheese rind microflora and, in particular, of *L. monocytogenes* up to a few millimetres into the sample. Considering that in the eventuality of a contamination of Gorgonzola and blue-veined cheese rinds a low number of *L. monocytogenes* cells is involved, the efficacy of the treatment confirmed in this study could really improve cheese safety. In fact, the treatment has the potential to reduce pathogenic cells below the limit of food safety criteria for ready to eat foods imposed by Commission Regulation 2013/2005, that is a maximum of 2 log cfu $g^{-1}$ at consumption. Therefore, it is reasonable to consider this infrared surface treatment a candidate for the superficial decontamination of this product as well as other.

## Disclosure

All authors have approved the final article.

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# 10. *Listeria monocytogenes* IN GORGONZOLA CHEESES: STUDY THE BEHAVIOUR THROUGHOUT THE PROCESS AND GROWTH PREDICT DURING THE SHELF LIFE

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# *Listeria monocytogenes* in Gorgonzola cheese: study of the behaviour throughout the process and growth prediction during shelf life

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**Abstract**

As reported on *RASFF*'s portal, in the first 9 months of 2016, a total of 13 "alerts / information for attention" were issued concerning the presence of *Listeria monocytogenes* in mould cheeses throughout Europe. This study analyzes the behavior of *L. monocytogenes* in soft blue-veined cheese when contaminated at different time points. In the first challenge test, the pasteurized milk was contaminated and the complete cheese manufacture (cheesemaking, ripening) and shelf life was simulated. After a decrease during the first days of the cheesemaking, the pH remained constant for 5 weeks and then it increased rapidly reaching the final values of 6.64 ± 0.14 in the core and 5.76 ± 0.02 on the rind. At the same time, the pathogen concentration decreased (about 2 log cfu $g^{-1}$), although during the last week a rapid pathogen growth was observed corresponding to the rise in pH values. When the cheese was stored at thermal abuse condition (8-12 °C), the pathogen concentration on the rind was 4.83 ± 0.31 log cfu $g^{-1}$ and after 66 days (about 9 weeks) no significant difference ($p>0.05$) was observed; whereas, a growth from 5.39 ± 0.45 to 7.14 ± 0.54 log cfu $g^{-1}$ was observed in the core. As regards the second challenge test, the maximum specific growth rates ($\mu_{max}$) of *L. monocytogenes* estimated in 3 batches of commercial sliced cheese stored at 8 °C ranged from 0.007 $h^{-1}$ to 0.061 $h^{-1}$. Therefore, the square root model was used to predict the $\mu_{max}$ at a chosen temperature and to establish the time necessary to reach the European critical legal limit of 100 cfu $g^{-1}$, in different storage scenarios. The predictions obtained in this study can be applied to any time-temperature profile, and in particular to the conditions to which the product is most likely to be subject in normal use, up to its final consumption. This study can be considered a valuable tool

also aimed at supporting the monitoring surveys carried out by officers of the Regional Veterinary Authority.

**Key words**: Mould cheeses, *Listeria monocytogenes,* Challenge test, Predictive microbiology.

## 1. Introduction

The contamination of some varieties of cheeses with *Listeria monocytogenes* is an important problem for consumers' health, as well as for the consequent substantial industrial financial losses (Cocolin et al., 2009; de Cesare et al., 2007; Lomonaco et al., 2009; Rudolf and Scherer, 2001). The presence of *L. monocytogenes* in cheeses can be due to various factors, such as contaminated raw milk, unsatisfactory pasteurization treatment or contamination after the heat treatment (i.e. in ripening rooms or during transportation from the processing plant to ripening facilities) (Lomonaco et al., 2009). Among dairy products, ripened soft cheeses, such as smear or mould surface-ripened, and blue-veined cheeses are known to be the most frequently contaminated (Beckers et al., 1987; Bernini et al., 2013; Griffiths, 1989). As reported by the *Rapid Alert System for Food and Feed* portal (http://ec.europa.eu/food/safety/rasff/index_en.htm), in the first 9 months of 2016, a total of 45 “alerts / information for attention” were issued concerning the presence of *L. monocytogenes* in milk and milk products, 13 of which involved mould cheeses (EC, 2010).

When pasteurized milk is used, contamination mainly concerns the rinds, as a consequence of cross-contaminations during handling and contact with the ripening environments (Canillac and Mourey, 1993; Carminati et al., 2000; Frye and Donnelly

2005; Terplan, 1990). In fact, during cheese ripening, complex microbial communities develop on the surface of some types of cheeses. The development of moulds occurs during the first days of ripening: yeasts metabolize the lactate completely into $CO_2$ and $H_2O$ forming alkaline metabolites, such as ammonia, inducing a pH increase on the surface (Bonaïti et al., 2004; van den Tempel and Nielsen, 2000). This deacidification can increase the possibilities of survival and growth of *L. monocytogenes* on the cheese rind.

One of the most well-known blue-veined cheeses is Gorgonzola, a Protected Designation of Origin (PDO) Italian product. Gorgonzola is a mould-ripened cheese with a washed rind, characterised by a pH ranging from 4.5 to 6.5 and a chloride content of 2.3-7%. It is made with pasteurized cow's milk inoculated with *Lactobacillus* and *Streptococcus* starter cultures, together with *Penicillium roqueforti* that develops as an internal blue-green mould (Mucchetti and Neviani 2006; Gripon and Hubert, 2002). Given the economic importance of Gorgonzola cheese (CPGC, 2014) and its worldwide commercialization, it is of great interest to understand the behaviour of *L. monocytogenes* in these cheeses so as to ensure consumers' safety. In the case of Gorgonzola cheese, the contamination, if present, is limited to the rind, it contains a small number of cells and does not affect the internal portion (Bernini et al., 2013). However, it is not possible to exclude the transferring of the pathogen to the internal portion during cutting and portioning; the dragging is directly proportional to the contamination level of the rinds (Bernini et al., 2016).

The EC Regulation 2073 (EC, 2005) states that Food Business Operators (FBO) are required to carry out studies aimed at assessing the growth of *L. monocytogenes* in specific products during shelf life, under reasonably foreseeable conditions of storage and distribution. The mentioned regulation establishes that 100 cfu $g^{-1}$ for

Ready To Eat (RTE) foods "able to support the growth of *L. monocytogenes*" is the critical limit that must be satisfied (EC, 2005). The EC and the Codex Alimentarius (1999) endorse the use of predictive microbiology in order to ensure food safety by predicting pathogen dynamics with reference to growth, survival or death. Predicting the behaviour of microorganisms in foods is a challenge carried out by many public and privately employed microbiologists for the benefit of consumers' health and well-being (Havelaar et al., 2010; McMeekin et al., 2006).

In this study, two different challenge tests were carried out with *L. monocytogenes*. The first challenge test was carried out assuming a contamination at the beginning of the Gorgonzola cheese process. The pathogen behaviour was assessed in the core and on the rind both during the cheesemaking and ripening process and during the shelf life of sliced cheese stored at a dynamic temperature (8-12 °C). In the second challenge test the cheeses were contaminated during cutting, and precisely at the end of the process to evaluate pathogen behaviour during shelf life at a constant temperature (8 °C) in commercial slices of Gorgonzola cheese. The objectives were i) to investigate the effect of physicochemical and microbiological changes on the behaviour of *L. monocytogenes* during the Gorgonzola process in order to increase knowledge concerning the impact of the process on the survival of pathogenic microorganisms, and ii) to calculate the specific growth rate of *L. monocytogenes* in sliced Gorgonzola cheese during shelf life at a constant temperature so as to predict the pathogen behaviour at a dynamic storage temperature in accordance with the *Technical Guidance Document on shelf-life studies for L. monocytogenes in ready-to-eat foods* (EURL, 2014).

## 2. Materials and methods

### *2.1. Bacterial cultures*

In the first challenge test, three *L. monocytogenes* strains (registered: ATCC® 19115™; wild: Lm273250 and Lm242382/9) were used to contaminate the pasteurized milk. The bacterial cultures were prepared following what indicated by Dalzini et al. (2015). Before use, the individual strains were combined in equal volumes in order to obtain a multi-strain cocktail. In the second challenge test, two *L. monocytogenes* bacterial cultures (ATCC® 19115™ and Lm273250) were prepared according to EURL technical document (2014) and used separately to contaminate the sliced cheese. All wild strains, belonging to IZSLER's collection (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy), were previously isolated from the soft cheeses.

### *2.2. Physicochemical and microbiological evolution during the manufacturing of Gorgonzola cheese: cheesemaking, ripening and shelf life.*

#### *2.2.1 Milk inoculation*

In order to assess the behaviour of *L. monocytogenes* and the changes in physicochemical and microbiological properties during the Gorgonzola cheese process (cheesemaking and ripening), a total of 400 l of milk were used in this experiment. The milk was provided by two different local farms and transported to IZSLER's laboratory where it was pasteurized (63 °C for 30 minutes). The milk was inoculated once cooled to 32 °C. One batch of milk (200 l) was inoculated with 1% v/v of multi-strain cocktail of *L. monocytogenes*, in order to give a final concentration ranging from 4 to 5 log cfu $ml^{-1}$, and was used to manufacture contaminated cheeses.

Another batch of milk (200 l) was inoculated (1% v:v) with sterile physiological solution and was used to manufacture control cheeses.

*2.2.2. Gorgonzola process*

The Gorgonzola cheese was manufactured according to traditional production specifications. The milk was inoculated with starter culture (*Streptococcus cremoris*, *Streptococcus lactis*, *Penicillum roqueforti*) and cheese rennet (92% chymosin-8% pepsin, 1 ml in 100 l), provided by local manufacturers, and incubated at 21 °C for the acidification and coagulation steps. After 30 minutes, the coagulum was cut into 2 cm cubes and let stand for 60 min to induce syneresis. The curd was transferred into round moulds (25 cm diameter and 18 cm height), and let stand at 18-20 °C for 72 h, turning the moulds every 2h. The ripening of the cheese begins with the manual salting of the rind. On day 15 and 25 of ripening, the cheese rind was pierced with needles to facilitate paste aeration and the development of the moulds inoculated. The ripening of the Gorgonzola cheese was carried out for 9 weeks (63 days) at 4 ± 2 °C with 90-95% Relative Humidity (RH). To evaluate the behaviour of *L. monocytogenes* during shelf life at dynamic temperatures (8-12 °C), the cheese was cut into slices (200 g each), packaged in plastic trays and stored for 9 weeks. Dynamic temperatures were studied, so as to simulate different stages of the cold chain. Therefore, the packaged cheese was stored at 8 °C for one week to simulate the stage from the manufacture to the arrival at the display cabinet, and then at 12 °C for 8 weeks to simulate a retail stage and consumers' storage, in accordance with EURL's technical document (EURL, 2014).

*2.3. Growth rate of L. monocytogenes in commercial sliced Gorgonzola cheese*

To simulate a contamination with *L. monocytogenes* when cutting Gorgonzola cheese at the end of ripening, three batches (30 packages of 200 g each per batch) of packaged sliced Gorgonzola cheese on plastic trays were provided by a local manufacturer and used for this study. The trays were opened aseptically and the slices were inoculated on the top surface with 1 % v/w of *L. monocytogenes* suspension to a final concentration of 1.5-2 log cfu $g^{-1}$ (contaminated samples) or with 1% v/wt of sterile physiological solution (control samples). The inoculum was distributed over the entire surface with a sterile L-shaped plastic cell spreader (Incofar, Modena, Italy) and the slices were then re-packaged and stored at a static temperature of 8 °C for 14 weeks (100 days) in accordance with the shelf life assigned by the local manufacturer who provided the products.

*2.4. Sampling*

During the cheesemaking, two replicates of milk, curd and cheese were analysed. During the ripening and shelf life of the cheese made in the first challenge test, interior (core) and surface (rind) samples were collected and analysed separately. Rind sampling was carried out by scraping in depth up to a maximum of 2 mm. The core samples corresponded to cubes taken from the very heart of the cheese. During the shelf life of the commercial sliced Gorgonzola, samples for analysis were obtained by scraping the surface of the inoculated slices in depth up to a maximum of 2 mm.

*2.5. Microbiological and physicochemical analyses*

The microbiological analyses on milk were performed through direct plate count. Subsequently, in the case of curd and cheese, 25 g of contaminated and control samples were first transferred separately into plastic one-chamber filter stomacher bags (NEOMED, Milano, Italy) and then homogenized 1:3 (w:v) in sterile Peptone Water (PW, CONDA, Madrid, Spain) for 3 min using a Stomacher 400 blender (Seward Medical, London, UK). Decimal dilutions in sterile PW were then prepared. For the enumeration of *L. monocytogenes* in contaminated samples, appropriate dilutions were surface-plated onto duplicate plates of differential-chromogenic ALOA agar (Microbiol Diagnostici, Cagliari, Italy) (ISO, 1998). Typical pathogen colonies were counted after aerobic incubation at 37 °C for 24-48 h. Mesophilic Lactic Acid Bacteria (LAB) and moulds were enumerated in control samples by pour plating 1 ml of appropriate dilution in de Man, Rogosa and Sharpe Agar (MRSA) (Microbiol Diagnostici, Cagliari, Italy) and by surface-plated 0.1 ml of dilution on Dichloran Rose Bengal Chloramphenicol Agar (DRBC, Oxoid, Milano, Italy) respectively. Typical colonies of each microorganism were counted after the incubation of plates at 37 °C for 48-72 h (MRSA) or at 25 °C for 5 days (DRBC).

The physicochemical analyses were performed on 10 g of control samples examined for each sampling time. The pH was measured on 10 g of control samples using an HI 223 Calibration checkTM Microprocessor pH meter (Hanna Instrument, USA) equipped with a Gel-Glass electrode (Hamilton, Switzerland). Water activity ($a_w$) was measured at 25 °C with the $a_w$ recorder AquaLab, series 3, Model TE (Decagon Devices, Inc., Pullman, USA) in accordance with ISO/FDIS 21807 (ISO/FDIS, 2004). The time/temperature profile during cheesemaking/ripening/shelf life was

monitored using electronic data loggers (cox tracer, Cox Technologies, Belmont N.C., USA).

2.6. *Data analysis*

Bacterial counts were converted to log cfu per gram. The individual means and standard deviations of microbiological and physicochemical results were determined on the basis of the average of one sample for each batch. The data were statistically analysed using the R statistical software version 2.7.0 (R Development Core Team, 2008). Differences among mean values were detected through the HSD Tukey's test and evaluations were based on a confidence interval of 95%.

During shelf life at 8 °C (static temperature), the fitting of *L. monocytogenes* growth curves was performed using the free software programme DMFit (www.ifr.ac.uk/safety/DMfit) to measure growth parameters [maximum specific growth rate ($\mu_{max}$), Lag time and maximum population ($y_{end}$)] using the model of Baranyi and Roberts (Baranyi and Roberts, 1994).

In a following step, the $\mu_{max}$ of *L. monocytogenes* at other temperatures were predicted as recommended by the EU technical guidance (EURL, 2014). The highest value estimated ($\mu_{max\ ref}$) using DMFit at $T_{ref}$, was utilized to calculate another $\mu_{max}$ at another temperature (T) making use of the square-root secondary model (Ratkowsky et al., 1982; Zwietering et al., 1996) and then calculated using the following equation:

$$\mu_{max} = \mu_{max\,ref} \frac{(T - T_{min})^2}{(T_{ref} - T_{min})^2}$$

where $T_{min}$ is a minimal growth temperature for *L. monocytogenes* (-1.5 °C) (EURL, 2014).

## 3. Results and discussion

### *3.1 Physicochemical and microbiological changes during the manufacture of Gorgonzola cheese: cheesemaking, ripening and shelf life.*

Although well-documented cases and outbreaks of listeriosis have been directly linked to the consumption of contaminated cheeses in other countries (Bula et al., 1994; Jacquet et al., 1995), Gianfranceschi et al. (2006) describe the first case of listeriosis associated with the consumption of a blue-veined cheese in Italy. Furthermore, Carminati et al. (2004) show that the fungal maturation and the pH fluctuations during the ripening of Gorgonzola cheese can permit the survival and reproduction of *Listeria* spp., mostly on rinds, contaminated as a result of cross contaminations from ripening environments or during handling. Gorgonzola cheese is made of pasteurized milk. However, irrespective of the origin of the contamination, it is a safety concern to follow the growth of this pathogen throughout the manufacture and shelf life of the cheese.

Considering a post-pasteurization contamination, in this study we aimed at assessing the behaviour of *L. monocytogenes* as a function of several physicochemical and microbiological changes throughout the Gorgonzola process. Phases (duration) and temperature profile, measured during each manufacturing step, are shown in Table 1. In the first hours of the process, the milk temperature decreased from 32 °C to 21 °C and remained constant for 72 hours, until the beginning of the ripening stage (Figure 1). The consumption of carbohydrates by the starter culture, due to the fermentation of the food matrix, leads to important chemical transformations.

Organic acids, especially Lactic Acid Bacteria, are produced by carbohydrates, leading to acidification, production of flavours and changes of $a_w$ values.

At the beginning of the cheesemaking, the LAB grow rapidly and produce acid in milk causing a decrease in pH values. In fact, in 72 hours LAB reached their maximum concentration of 7.47 ± 0.15 log cfu $g^{-1}$, while no significant changes were observed for the mould population after their inoculum in the milk. At the same time, pH and $a_w$ decreased from 6.65 ± 0.02 to 4.82 ± 0.03 and from 0.994 ± 0.001 to 0.967 ± 0.001 respectively, as shown in Table 2. Similar results were reported in Cabrales cheese, a typical Spanish blue veined cheese (Flórez et al., 2006).

Table 3 shows the changes of the most relevant physicochemical and microbiological properties in core and on rind during cheese ripening. The LAB concentration in the core and on the rind varied not significantly ($p>0.05$), from 6.65 ± 0.14 to 7.60 ± 0.13 log cfu $g^{-1}$ and from 6.07 ± 0.19 to 6.80 ± 0.89 log cfu $g^{-1}$ respectively, throughout the ripening time, in accordance with the published data obtained in similar products (Bernini et at., 2013; Bernini et al., 2015; Fontana et al., 2010; Gobbetti et al., 1997). During ripening, cheese was pierced on day 15 and 25 and the concentration of moulds increased in the core cheese reaching about 6.45 ± 0.39 log cfu $g^{-1}$ in 5 weeks. As reported byTrieu-Cout and Grippon (1983), the piercing of cheese permits the growth of moulds, which is generally visible inside the curd 2-3 weeks after manufacture, as confirmed in our study. During the last weeks of ripening, the concentration of moulds increased in the core, together with an increase in pH values. After a decrease during the first days of cheesemaking, the pH remained constant for 5 weeks and then it increased rapidly reaching the final values of 6.64 ± 0.14 in the core and 5.76 ± 0.02 on the rind. This can be due to the consumption of lactic acid by moulds and the proteolysis process which takes place during ripening,

freeing large quantities of alkaline compounds (amino acids and ammonia) which also contributes to the pH increase in the cheese core (Prieto et al., 2000). A similar pH evolution was also observed in other blue veined cheese types (Flórez et al., 2006; Prieto et al., 2000) and in the Gorgonzola cheese previously studied (Gobbetti et al., 1997; Seratlić et al., 2011).

The final $a_w$ values in the core cheese (0.962 ± 0.003) were slightly higher than those observed in other blue-veined cheeses (Flórez et al., 2006; Gobbetti et al., 1997; Prieto et al., 2000). However, Fernahndez-Salguero et al. (1986) observed higher values in other blue-veined cheese varieties, such as Bleu de Bresse, Danablu and Gorgonzola.

The pH changes in the core and on the rind seem to play a key role in the behaviour of *L. monocytogenes*. As shown in Figure 2, a reduction of about 2 log units was observed both in the core and on the rind until the 8th week during cheese ripening at 4 °C. These results are in accordance with those obtained by Le Marc et al. (2002), who observed that the combination of low temperatures and a pH with high organic acid concentrations could be effective in controlling the growth of *L. monocytogenes*. Also Papageorgiou and Marth (1989) in a study performed in a similar cheese product, reported that the growth of *L. monocytogenes* ceased when the pH of the cheese dropped below 5.0; on the other hand, when the environment became more favourable (the pH of the cheese increased), from days 50 to 120, a survival of the pathogen was observed. Similar results were reported by Morgan et al. (2001) in mould-ripened soft lactic cheeses where the pathogen survived in the paste during 42 days of ripening, even with a level of milk contamination as low as 10 cfu $ml^{-1}$. However, in our study, an increase of *Listeria* concentration occurred during the last week, corresponding to the rise in pH values. The maximum concentration was

reached in the core, where a higher increase in the pH was also observed up to 6.64 ± 0.14 compared to that of the rind, which remained at about 5.76 ± 0.02 (Table 3).

Similar results were obtained by Rosshaung et al. (2012) during their study on the behaviour of *L. monocytogenes* in soft blue-white cheese when contaminated at different time points. In their study, only when the cheese was inoculated in the first weeks (on day 13) and ripened at 13 °C, the data showed a long lag phase corresponding to a low pH, before the significant pathogen growth.

The rapid growth shown in our study cannot be explained only by the rise of the pH, but it is possible to assume that it is also due to the presence in cheese of compounds that promote pathogen growth. Many changes in the free amino acid profile occur during cheese ripening due to the proteolytic capacity of *Penicillum* species. Prieto et al. (2000) reported an increase in the total free amino acid concentration at the end of the ripening of Picón Bejes-Tresviso cheese, a Spanish blue veined cheese with an abundance of valine, leucine, lysine, phenylalanine and glutamic acid, which together accounted for 50% of the total free amino acids present. In addition, the development of yeasts during the ripening induces the production of growth factors by yeasts, which appears to promote the development of Gram positive, catalase positive, salt-tolerant microbial communities as reported by Corsetti et al. (2001).

To evaluate the effect of Gorgonzola manufacture added to a thermal abuse storage on the behavior of *L. monocytogenes*, the inoculated sliced cheeses were stored at dynamic temperature conditions: 8 °C for one week and 12 °C for 8 weeks, so as to simulate different cold chain stages in accordance with EURL (2014). Table 4 shows the changes of the most relevant physicochemical and microbiological properties in the core and on the rind during the shelf life of the control cheese. Although at the beginning of shelf life the pH of the rind was lower compared to that of the core, after

the third week the pH values became similar ($p>0.05$), but *Listeria* assumed different behaviors (Figure 2). During shelf life the pathogen concentration on the rind was 4.83 ± 0.31 log cfu $g^{-1}$ and no significant differences ($p>0.05$) were observed after 66 days (about 9 weeks); whereas, a growth from 5.39 ± 0.45 to 7.14 ± 0.54 log cfu $g^{-1}$ was observed in the core. These results were a further confirmation that it is not only the pH to govern the behavior of the pathogen. On the rind, the *L. monocytogenes* inactivation may be caused by $a_w$ dropping below 0.93, regardless of pH ($5.98 < pH < 6.61$). Genigeorgis et al. (1991) and Chatelard-Chauvin et al (2015) also observed *L. monocytogenes* showing less increase and inactivation on the rind than in the core respectively, in spite of a higher pH of the rind.

The presence of foodborne pathogens like *L. monocytogenes* in raw milk and bulk tank milk has been widely reported (Dalzini et al., 2016; D'Amico and Donnelly, 2010), but it does not survive high-temperature short-time pasteurization (Rosshaung et al., 2012). Therefore, *L. monocytogenes* occurrences in cheeses made from pasteurized milk are usually due to post-pasteurization contaminations. As previously mentioned, the surface of the cheese can become contaminated with *L. monocytogenes* after a set of procedures involved in cheese ripening, such as dry salting, wheel piercing, wet brushing and handling in general. This contamination generally does not involve the paste, but it may be transferred during cutting and portioning (Bernini et al., 2016; Carminati et al., 2004; Manfreda et al., 2005). Cheese contamination during portioning was also simulated in our study, through the inoculation of three commercial sliced cheese batches with two different *L. monocytogenes* strains (registered and wild). During shelf life at 8 °C for 14 weeks, no significant changes were observed for the principal microbiological and physicochemical parameters studied (Table 5). At 8 °C the sliced cheese supported a

rapid and prolific *L. monocytogenes* growth. The growth curves of *L. monocytogenes* in commercial Gorgonzola slices contaminated during the portioning and stored at 8 °C were obtained by fitting the observed growth data using the DMFit programme (Figure 3). As recommended by Buchanan and Phillips (1990), in order to obtain a better fit of the model with the experimental data, no-growth data were eliminated.

According to the Combase Predictor tool (part of the Modelling Toolbox of www.combase.cc) (Baranyi & Tamplin, 2004), in culture media with the same growth condition of the cheese (inoculums level: 3 log cfu $g^{-1}$; pH: 6.61; $a_w$: 0.978; temperature: 8 °C) a maximum *L. monocytogenes* population of 8.52 log cfu $g^{-1}$ was reached in about 340 hours. However, in our growth curve the pathogen concentration did not exceed the level of 8 cfu $g^{-1}$, but reached a maximum concentration of 6.8 log cfu $g^{-1}$ (registered strains inoculated in batch 3) and then the growth was suppressed (Figure 3). This was observed for each growth curve and may be due to a non-specific competition for nutrients by the pathogen and the indigenous microflora present at high concentration in the matrix. A similar competition was also observed by other authors between *L. monocytogenes* and the natural biofilm microflora of wooden shelves used for cheese ripening, when pathogen growth was completely suppressed and the natural microflora reached their maximum concentration (Guillier et al., 2008).

Once the growth curves were obtained, the DMFit programme was used also to calculate growth parameters for each condition tested (Table 6). The maximum specific growth rates ($\mu_{max}$) ranged from 0.007 $h^{-1}$ to 0.061 $h^{-1}$, in accordance with the values reported by Daminelli et al. (2013) in blue cheese where the $\mu_{max}$ of *L. monocytogenes* varied from 0.015 $h^{-1}$ at 6 °C to 0.08 $h^{-1}$ at 12 °C. As reported by Daminelli et al. (2014), a significant factor that can influence the pathogen behavior

in food is the origin of the strains used in the challenge tests, which can affect the ability of adaptation of the microorganism and the growth rate in the food matrix. In fact, our results show that the higher value of $\mu_{max}$ (0.061 $h^{-1}$) was observed just for the wild strain inoculated in Batch 3 (Table 6). The significant variation among values of $\mu_{max}$ for the different strains in the cheese matrix stresses the importance of testing various strains that have been isolated from the cheese and are thus adapted for cheese when challenge testing cheese.

Assuming a very simple linear primary model (without lag phase or stationary phase, which may lead to fail-safe result), our study estimated the time to reach 2 log cfu $g^{-1}$ (critical legal limit provided by EC Regulation 2073) starting from an initial contamination level of -1.39 log cfu $g^{-1}$ (presence in 25 g) for *L. monocytogenes*. Based on different $\mu_{max}$ calculated in our study, the shelf life of Gorgonzola cheese stored at 8 °C ranged from about 5 to 42 days considering the $\mu_{max}$ of 0.061 $h^{-1}$ (wild strain inoculated in Batch 3) and 0.007 $h^{-1}$ (registered strain inoculated in Batch 2) respectively (data not shown).

Secondary models usually describe variations in growth kinetic parameters with environmental factors (Bang et al. 2008). Therefore, the square root model (Ratkowsky et al., 1982; Zwietering et al., 1996) was used to predict the $\mu_{max}$ at a chosen temperature, and to establish the time for reaching the critical legal limit of 2 log cfu $g^{-1}$ in Gorgonzola cheese, in a different storage scenario. Considering an initial contamination level of -1.39 log cfu $g^{-1}$ (presence in 25 g), the legal limit for *L. monocytogenes* was exceeded after 14 days at the usual refrigerate temperature of 4 °C as reported by Bernini et al. (2016).

Since food safety is a responsibility of the Food Business Operator (FBO), it is up to the FBO, as regards these particular types of RTE foods, to demonstrate compliance

with the limit of 100 cfu $g^{-1}$ throughout shelf life. This triggers the following questions: How does the FBO i) decide if the product is able or unable to support the growth of *L. monocytogenes*, ii) demonstrate compliance with the 100 cfu $g^{-1}$ limit throughout the shelf-life, and iii) set its own specifications for *L. monocytogenes* for its particular food product at the end of the production line that should be low enough to guarantee that the limit of 100 cfu $g^{-1}$ does not exceed at the end of shelf-life (EC, 2005). The predictions obtained in our study can be applied to any time-temperature profile, and in particular to the conditions to which the product is most likely to be subjected in normal use, until its final consumption. Our study can be considered a valuable tool also to support the monitoring surveys carried out by officers of the Regional Veterinary Authority, especially when a Gorgonzola sample is found positive to the presence of *L. monocytogenes* during shelf life. In this case, knowing the product storage conditions (storage temperature profile and duration of shelf life) allows to predict the growth of *L. monocytogenes*, so as to assess the safety of the product before it reaches the final consumer.

In conclusion, Gorgonzola cheese is made from pasteurized milk, while *L. monocytogenes* occurrences in cheeses are usually due to post-pasteurization contaminations. Therefore, during the process there are many factors that influence the behaviour of *Listeria*, including a significant change of pH, due to the development of different microflora such as Lactic Acid Bacteria and moulds during the process. The presence of the pathogen in Gorgonzola paste due to cross-contamination during the process or due to a transfer contamination from the rind when cutting the cheese may represent a risk for consumers' safety. During shelf life, even if storage temperature is low (less than 8 °C), *L. monocytogenes* can grow reaching the legal limit (and safety limit) of 100 cfu $g^{-1}$ in a few weeks. Food

operators producing RTE food products that can be subject to the growth of *L. monocytogenes* are strongly recommended in the EC Regulation 2073 (EC, 2005) to "sample the processing areas and equipment for *L. monocytogenes* as part of their sampling scheme." As reported by Dufour (2011), testing for Listeria spp. is recommended because Listeria spp are more frequently found than *L. monocytogenes* alone, and it is considered a good process indicator to monitor the risk of *L. monocytogenes*. However, there is a real potential for *L. monocytogenes* to be present when *Listeria* spp. are detected, and appropriate control measures should be applied immediately when this occurs in the environment or product. Finally, as already discussed by several researchers (Bernini et al., 2015; Carminati et al., 2004; Mucchetti et al., 2008), the implementation of Good Manufacturing Practices focused on the control of Gorgonzola cheese rind contamination and the possibility to reduce this possible contamination by applying post-processing treatments - such as high pressure and infra red technologies - are important tools for increasing cheese safety.

## Disclosure

Authors declare that no conflict of interests exists.


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**Table 1.** Conditions of the manufacturing process of Gorgonzola cheese during cheesemaking.

| Manufacturing step | Duration (hours) (days) | Temperature (°C) |
|---|---|---|
| Addition starter culture and cheese rennet[a] | 0.5 (0.02) | 32 ± 2 |
| Acidification and coagulation | 0.5 (0.02) | 30 ± 2 |
| Cutting coagulum and sineresis | 1 (0.04) | 21 ± 2 |
| Moulding | 0.5 (0.02) | 21± 2 |
| Draining and inversion | 72 (3) | 18 ± 2 |
| Manual salting | 0.5 (0.02) | 4 ± 2 |
| Piercing | 0.5 (0.02) | 4 ± 2 |
| Ripening | 1488 (62) | 4 ± 2 |

**Table 2.** Changes of mesophilic Lactic Acid Bacteria (LAB) and mould log counts, pH and $a_w$ values during the cheesemaking of Gorgonzola cheese. The data represent the average values ± standard deviation of two samples.

| Parameter | Milk | Curd | Cheese (at 24 hours) | Cheese (at 72 hours) |
|---|---|---|---|---|
| LAB [a] | <1 A[b] | 4.91±0.19 B | 5.62±0.11 C | 7.47±0.15 D |
| Mould | <1 A[b] | 2.54 ± 0.07 B | 2.9 ± 0.12 B | 2.98 ± 0.12 B |
| pH | 6.65±0.02 A | 6.56±0.06 A | 4.93±0.01 B | 4.82±0.03 B |
| $a_w$ | 0.994±0.001 A | 0.973±0.002 B | 0.967±0.001 B | 0.965±0.003 B |

[a] Values are mean log cfu $g^{-1}$ ± standard deviation

[b] Means with different uppercase letters within a row are significantly different ($p$<0.05)

**Table 3.** Changes of mesophilic Lactic Acid Bacteria (LAB) and mould log counts, pH and $a_w$ values during the ripening of Gorgonzola cheese. The data represent the average values ± standard deviation of two samples.

| Parameters | | Cheese at different ripening times (weeks) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 7 | 8 | 9 |
| LAB [a] | | | | | | | | | |
| | core | 6.69±0.12 A | 6.65±0.14 A | 7.38±0.44 A | 7.38±0.44 A | 6.9±0.04 A | 7.14±0.04 A | 7.60±0.13 A | 7.56±0.36 A |
| | rind | 6.07±0.19 A | 5.79±0.06 A | 5.86±0.17 A | 5.99±0.01 A | 6.32±0.47 A | 6.30±0.64 A | 6.8±0.89 A | 6.53±0.24 A |
| Mould [a] | | | | | | | | | |
| | core | 2.77 ± 0.01A | 3.65 ± 0.01B | 3.87 ± 0.07C | ND | 6.45 ± 0.39D | 5.4 ± 0.4D | ND | 6.22 ± 0.02D |
| | rind | ND | ND | ND | ND | ND | ND | ND | ND |
| pH | | | | | | | | | |
| | core | 4.73±0.12 A | 4.69±0.09 A | 4.76±0.07 A | 4.73±0.04 A | 5.25±0.47 A | 6.57±0.04 B | 6.64±0.14 B | 6.8±0.02 B |
| | rind | 4.73±0.06 A | 4.70±0.12 A | 4.75±0.04 A | 4.74±0.11 A | 5.00±0.15 AB | 5.63±0.28 BC | 5.76±0.02 C | 5.82±0.41 C |
| $a_w$ | | | | | | | | | |
| | core | 0.965±0.001 A | 0.964±0.001 A | ND | 0.960±0.003 AB | 0.957±0.002 B | 0.963±0.002 AB | 0.962±0.003 AB | 0.964±0.003 A |
| | rind | ND | ND | ND | ND | 0.957±0.007 A | 0.939±0.016 A | 0.951±0.002 A | 0.943±0.004 A |

[a] Values are mean log cfu $g^{-1}$ ± standard deviation

[b] Means with different uppercase letters within a row are significantly different ($p$<0.05)

[c] ND: not determined

**Table 4.** Changes of mesophilic Lactic Acid Bacteria (LAB) and mould log counts, pH and $a_w$ values during the shelf life at 8-12 °C of Gorgonzola cheese. The data represent the average values ± standard deviation of two samples.

| Parameters | | Cheese at different shelf life times (weeks) | | | |
|---|---|---|---|---|---|
| | | 1 | 3 | 7 | 9 |
| LAB [a] | | | | | |
| | core | 7.58±0.37 A | 7.96±0.26 A | 7.91±0.37 A | 8.18±0.25 A |
| | rind | 5.70±0.53 A | 6.48±0.52 A | 7.17±0.91 A | 7.10±0.14 A |
| Mould [a] | | | | | |
| | core | 6.98 ± 0.50A | 6.95 ± 0.47A | 6.89 ± 0.44A | 5.39 ± 0.20B |
| | rind | ND | ND | ND | ND |
| pH | | | | | |
| | core | 6.88±0.31 A | 6.68±0.78 A | 6.45±0.98 A | 6.63±0.95 A |
| | rind | 5.98±0.40 A | 6.11±0.32 A | 6.40±1.24 A | 6.61±0.92 A |
| $a_w$ | | | | | |
| | core | 0.964±0.004 A | 0.967±0.012 A | 0.927±0.027 A | 0.953±0.025 A |
| | rind | ND | 0.962[d] | 0.933[d] | 0.914[d] |

[a] Values are mean log cfu $g^{-1}$ ± standard deviation

[b] Means with different uppercase letters within a row are significantly different ($p$<0.05)

[c] ND: not determined

[d] Value measured on a single replicate

**Table 5.** Mesophilic Lactic Acid Bacteria (LAB) and mould log count, pH and $a_w$ values measured on three batches of commercial Gorgonzola sliced cheese (not contaminated) during shelf life at 8 °C. The data represent the average values ± standard deviation of three samples for each batch.

| Parameters | Cheese at different shelf life times (weeks) | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 7 | 14 |
| LAB[a] | 7.61 ± 0.21A | 7.42 ± 0.33A | 7.77 ± 0.34A | 7.43 ± 0.69A | 7.62 ± 0.15A |
| Mould[a] | 7.67 ± 0.18A | 7.66 ± 0.28A | 7.52 ± 0.28A | 7.36 ± 0.45A | 6.3 ± 0.46B |
| pH | 6.61 ± 0.1A | 6.24 ± 0.21A | 6.21 ± 0.11A | 6.15 ± 0.11A | 6.26 ± 0.21A |
| $a_w$ | 0.978 ± 0.005A | ND | ND | 0.956 ± 0.004B | 0.947 ± 0.005B |

[a] Values are mean log cfu $g^{-1}$ ± standard deviation

[b] Means with different uppercase letters within a row are significantly different ($p < 0.05$)

[c] ND: not determined

**Table 6**. Output parameters estimated by the DMFit programme for each growth curve of two different *L. monocytogenes* strains (registered and wild) in three batches of commercial Gorgonzola. The specific growth rate ($\mu_{max}$) is the potential maximum rate of the model; Lag denotes the lag parameter as described by Baranyi and Roberts (1994); $y_0$ is the initial point of the sigmoid curve; $y_{end}$ is the upper asymptote of the sigmoid curve; SE is the standard error of fitting; $R^2$ is the adjusted R-square statistics of the fitting.

| Batch | Type strain | $\mu_{max}$ ($h^{-1}$) | Lag (h) | $y_0$ (log cfu $g^{-1}$) | $y_{end}$ (log cfu $g^{-1}$) | SE | $R^2$ |
|---|---|---|---|---|---|---|---|
| Batch 1 | Registered | 0.008 | | 2.7 | 4.49 | 0.57 | 0.63 |
| | Wild | 0.016 | | 2.72 | 6.07 | 0.69 | 0.8 |
| Batch 2 | Registered | 0.007 | | 1.58 | 5.49 | 0.39 | 0.95 |
| | Wild | 0.04 | 324.63 | 2.33 | 5.39 | 0.48 | 0.9 |
| Batch 3 | Registered | 0.032 | | 2.77 | 6.87 | 0.61 | 0.89 |
| | Wild | 0.061 | | 2.8 | 6.72 | 0.72 | 0.83 |

**Figure Legends**

**Figure 1**. Temperature profile monitored in the first challenge test during cheesemaking (A) and ripening (B) of Gorgonzola cheese.

**Figure 2.** The behavior of *L. monocytognese* (solid line) and pH changes (dashed line) evaluated in the core (closed symbols) and on the rind (open symbols) during ripening (A) and during shelf life (B) of Gorgonzola cheese during the first challenge test. Averages are represented with Standard Deviation as error bars (interpolations have a visual interest only).

**Figure 3.** Growth curves of *L. monocytogenes* ATCC 19115 (■) (registered strain) and *L. monocytogenes* Lm273250 (□) (wild strain) fitted by the DMFit programme according to the Baranyi and Roberts model (Baranyi and Robert, 1994), in three different batches of commercial Gorgonzola slices stored at 8°C: A (Batch 1), B (Batch 2) and C (Batch 3).

**Figure 1**

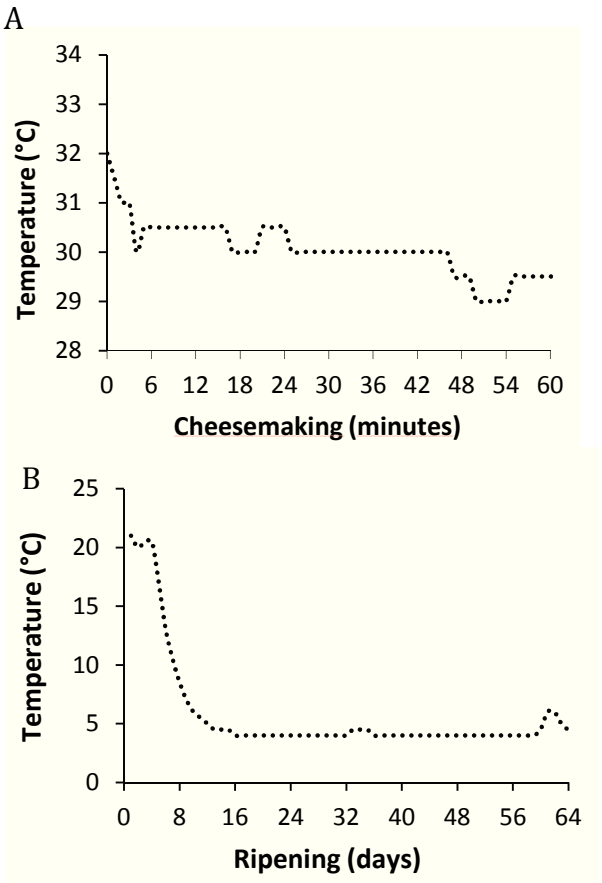
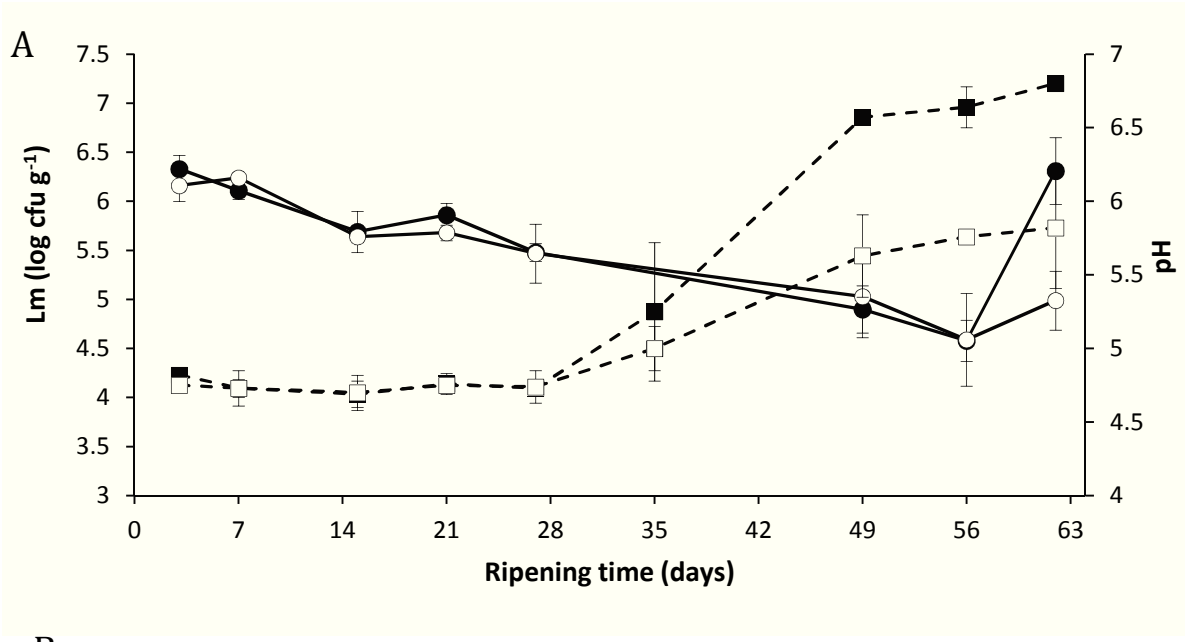

A
34
33
32
31
30
29
28
Temperature (°C)
0 6 12 18 24 30 36 42 48 54 60
Cheesemaking (minutes)
B
25
20
15
10
5
0
Temperature (°C)
0 8 16 24 32 40 48 56 64
Ripening (days)

**Figure 2**


A
Lm (log cfu g-1)
pH
Ripening time (days)
0
7
14
21
28
35
42
49
56
63
3
3.5
4
4.5
5
5.5
6
6.5
7
7.5
4
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5
5.5
6
6.5
7

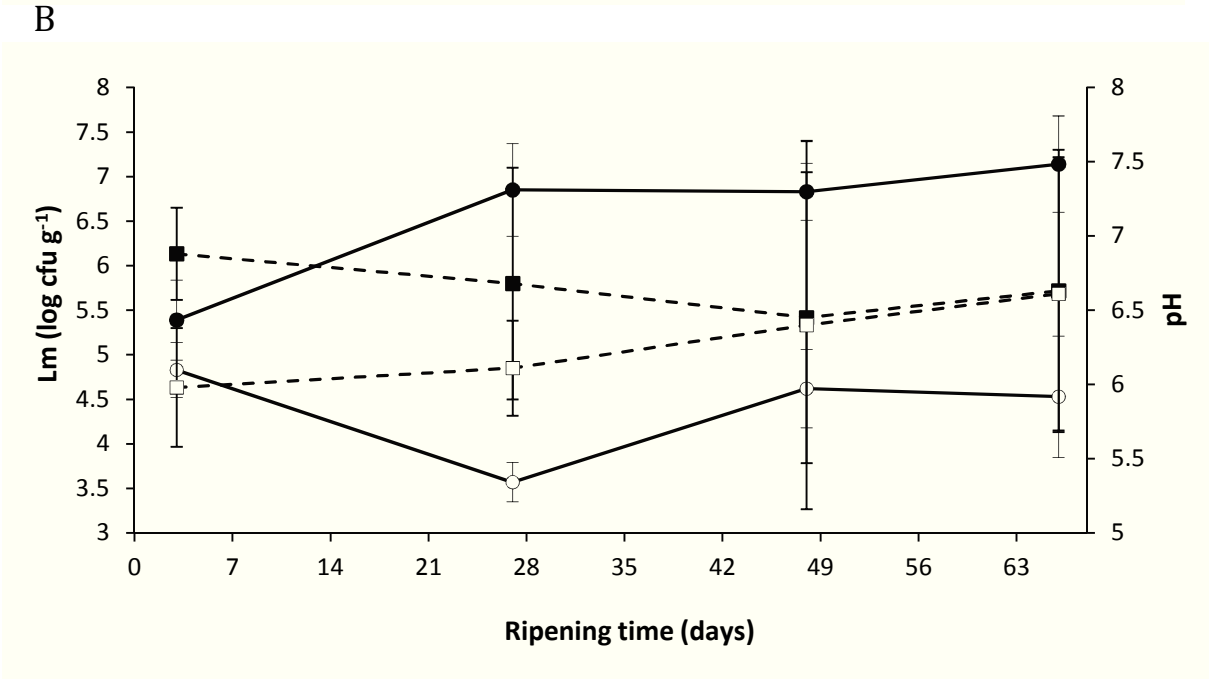
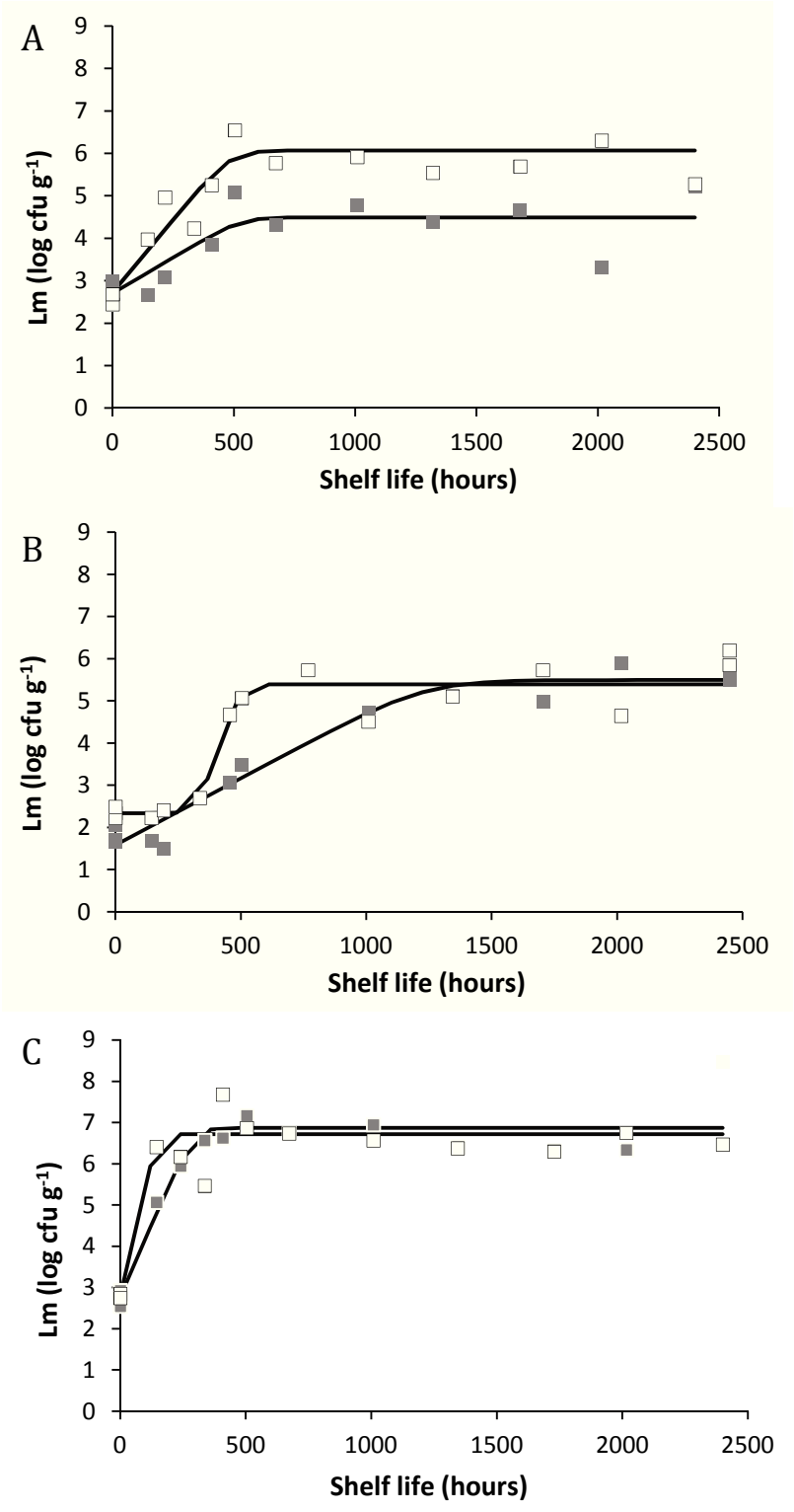

B
Lm (log cfu g-1)
pH
Ripening time (days)
0
7
14
21
28
35
42
49
56
63
3
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
5
5.5
6
6.5
7
7.5
8

**Figure 3**


A
Lm (log cfu g-1)
Shelf life (hours)
B
Lm (log cfu g-1)
Shelf life (hours)
C
Lm (log cfu g-1)
Shelf life (hours)

# 11. PREDICTIVE MODELLING OF MICROBIAL INTERACTIONS IN CHEESE: A TRADE-OFF BETWEEN MATHEMATICAL EXACTNESS AND EMPIRICAL PRAGMATISM

Elena Cosciani Cunico, József Baranyi, **Elena Dalzini**, Paolo Daminelli, Marina Nadia Losio, Giorgio Varisco

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IAFP's European Symposium on Food Safety, Budapest, Hungary, 7-9 May 2014.

# PREDICTIVE MODELLING OF MICROBIAL INTERACTIONS IN CHEESE: A TRADE-OFF BETWEEN MATHEMATICAL EXACTNESS AND EMPIRICAL PRAGMATISM

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The subject of this study is to provide a modelling framework for the biochemical reactions in the cheese ecosystem of soft ripened cheese, such as Gorgonzola cheese, characterized by a strong enzyme (protease) activity during maturation. An important agent in these reactions is *P. roqueforti*, the growth of which results in the increase of the pH, which could represent a hazard to microbial safety. Any reasonable model of such a dynamic system must be a set of differential equations.

The objective is to develop a framework which can serve as a template for predicting the behaviour of *Listeria monocytogenes* as a function of the dynamically changing ecological and physical environment in soft ripened cheese.

## Material and methods

The log cell concentrations of indigenous lactic acid bacteria (LAB) and that of inoculated *L. monocytogenes*, as well as the pH of the food, were measured during the process and shelf life of Gorgonzola cheese. Statistical analysis and numerical simulation was carried out within Microsoft Excel.

The used kinetic equations and the needed biochemical rates were partly from the literature (Figs.1,2,3), partly derived from own measurements. Data were recorded in Microsoft Excel spreadsheets. Runge-Kutta 2nd order solver was programmed in the sheet, to simulate the developed model.

## Results

The temporal variation of the mould biomass (log cell concentration) was fitted by the model of Baranyi and Roberts (1994). Source: Gobbetti et al. 1997 (Fig.1).

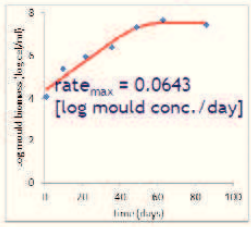


Fig. 1) ♦ log mould concentration (log cell/g) ; (—) Model of Baranyi and Roberts (1994) fitted to the data

Based on measurements, an approximately linear relationship was established between log(mould) and the soluble Nitrogen. It was shown that the one unit increase of the logarithm of the mould concentration results in 0.23 unit increase in the logarithm of the soluble Nitrogen, regardless whether it is water-soluble or pH 4.6 -soluble Nitrogen ($p<0.05$).

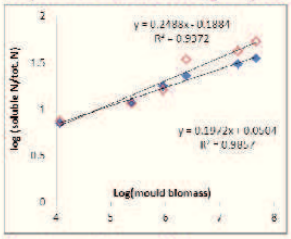


Fig. 2) linear relationship between ♦ log(mould biomass) and ◇ soluble Nitrogen

The logarithm of the concentration of $NH_3$ linearly depend on the soluble Nitrogen concentration, and finally the pH increased linearly with it.

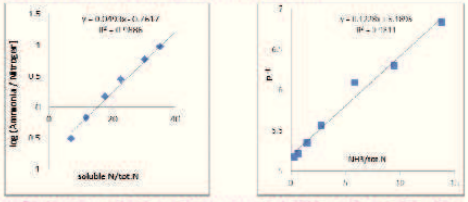


Fig. 3) linear relation between: (♦) $NH_3$ and soluble N; and ( ■ ) between $NH_3$ and pH .

Based on these, a compartments-in-chain model (Fig. 4) can be developed, from which it is evident why the pH increases so dramatically, at double exponential rate, as the mould grows:


Fig. 4) Chain of relevant effector - response variables.

The respective coefficients can be read from the figures. The resultant model in the first 60 days is:

$$\frac{d \log m}{dt} = 0.0643 \qquad \frac{d \log N_s}{d \log m} = 0.23$$

$$\frac{d \log[NH_3]}{dN_s} = 0.0493 \qquad \frac{d\,pH}{dNH_3} = 0.123$$

where $m$ is the mould biomass (g/g), $Ns$ is the soluble Nitrogen concentration (ppm), $NH_3$ is the free ammonia (ppm) that increases the $pH$.

## Validation

No sufficient data are available to predict mould growth independently of the experimental results used to estimate the above rate parameters, but the generated pH profile can be used to predict the behaviour of *Listeria monocytogenes* under varying temperature, based on a formerly developed empirical model describing its growth as a function of temperature and pH. Namely, we assumed that at low pH ($pH<5$), the organism dies at -0.001[logcfu/h] rate, at $pH>5.5$ it grows at 30% of the rate ComBase Predictor predicts (bias factor for this cheese), otherwise no growth or death occurs. Note that the above parameters were estimated from independent data, generated in constant environments. The results are summarized in Fig.5.

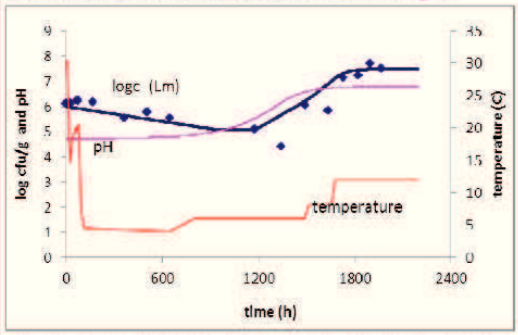


Fig.5) Model prediction and validation using the log cell data of L.monocytogenes during processing and shelf life of Gorgonzola cheese under varying temperature.

## Conclusion

Combination of mechanistic and empirical elements provides efficient models to describe the kinetics of complex food ecology under dynamic conditions.

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# 12. GENERAL CONCLUSIONS

Since food safety is a responsibility of the Food Business Operator (FBO), it is up to the FBO, as regards these particular types of RTE foods, to demonstrate compliance with the limit of 100 cfu $g^{-1}$ throughout shelf life. This triggers the following questions: how does the FBO i) decide if the product is able or unable to support the growth of *L. monocytogenes*, ii) demonstrate compliance with the 100 cfu $g^{-1}$ limit throughout the shelf-life, and iii) set its own specifications for *L. monocytogenes* for its particular food product at the end of the production line that should be low enough to guarantee that the limit of 100 cfu $g^{-1}$ does not exceed at the end of shelf-life (EC, 2005).

Traditional approaches to milk safety that rely heavily on regulatory inspection and sampling regimes cannot sufficiently guarantee consumer protection because: i) they are time-consuming, ii) 100% sampling and inspection is financially and logistically impossible and iii) temperature abuse in the chill-chain, a major cause of safety problems, is not being dealt with. An alternative approach to traditional methods is to rely on quantitative microbiology.

The presence of low levels of *L. monocytogenes* in raw milk destined for the production of raw milk cheeses characterized by a short ripening time, can constitute a threat to the consumer. Using high levels of starter culture bacteria is possible to inhibit the *Listeria* growth thanks to the competitions defined as Jameson effect.

Gorgonzola cheese is made from pasteurized milk, while *L. monocytogenes* occurrences in cheeses are usually due to post-pasteurization contaminations. Therefore, during the process there are many factors that influence the behaviour of *Listeria*, including a significant change of pH, due to the development of different microflora such as Lactic Acid Bacteria and moulds during the process. The presence of the pathogen in Gorgonzola paste due to cross-contamination during the process or due to a transfer contamination from the rind when cutting the cheese may represent a risk for consumers' safety. During shelf life, even if storage temperature is low (less than 8 °C), *L. monocytogenes* can grow reaching the legal limit (and safety limit) of 100 cfu $g^{-1}$ in a few weeks. Food operators producing RTE food products that can be subject to the growth of *L. monocytogenes* are strongly recommended in

the EC Regulation 2073 (EC, 2005) to “sample the processing areas and equipment for *L. monocytogenes* as part of their sampling scheme.” The implementation of Good Manufacturing Practices focused on the control of Gorgonzola cheese rind contamination and the possibility to reduce this possible contamination by applying post-processing treatments - such as high pressure and infra red technologies - are important tools for increasing cheese safety.

The develop of a simple mathematical model to describe the behavior of *Listeria* as a function of the environmental conditions, may be a very useful tool to support the monitoring surveys carried out by officers of the Regional Veterinary Authority. In fact, knowing the manufacturing or storage conditions allows to predict the growth of *L. monocytogenes*, as to assess the safety of the product before it reaches the final consumer.

# 13. ACKNOWLEDGEMENTS

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