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Traditional foods towards product innovation: strategies to improve food safety and shelf-life of ricotta cheese

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ABSTRACT

The main objective of the present thesis was to innovate the traditional ricotta cheeses produced in Sardinia (Italy). Innovation of traditional products can be obtained changing the manufacturing process through the introduction of production steps, ingredients or packaging. The purpose of this modernization process is to maintain the greater perceived quality and positive attitude of consumers toward traditional products, which include sensory properties and cultural heritage linked to the place of origin. At the same time there is the need to gain a competitive advantage on large scale market seeking to meet consumers' demand of food safety and convenience. Therefore, the studies illustrated in the present thesis demonstrate the feasibility of introducing innovation strategies in the production of traditional ovine ricotta cheese. In particular are discussed four scientific studies dealing with several aspects of food quality: determination of durability based on evolution of spoilage and pathogen microorganism during the shelf-life; modification of product composition in order to take into account consumers' special dietary needs (i.e. intolerance or allergies); use of protective cultures to control food spoilage during shelf-life.

CHAPTER 1 - Introduction

WHEY CHEESES

Types of ricotta cheeses in Sardinia

Whey cheeses are defined as the solid, semi-solid, or soft products, which are principally obtained through either the concentration of whey or the coagulation of whey by heat with or without the addition of acid (Codex Alimentarius Codex Standard 284-1971). In turn the whey is the fluid product obtained during the manufacture of cheese, casein or similar products by separation from the curd after coagulation of milk and/or of products obtained from milk (CODEX STAN 289-1995). Worldwide the main use of the whey is the production of whey cheeses, whey drinks and fermented whey drinks (FAO, 2018). Therefore whey cheeses are obtained by the aggregation and precipitation of whey proteins as consequence of thermal denaturation. The production of whey cheeses can start from the whey as such or added with small amount of whole milk; in this case there coprecipitation of casein contributes in the manufacturing of these specialties dairy products. Whey cheeses are manufactured all over the world either at industrial and artisanal level using different methods according to local recipes (Pintado et al., 2001).

The most popular and renowned whey cheese in the world is the ricotta, originally from Italy, which has now became popular in many countries, especially in the USA. In Latin American and the Hispanic communities in North America the term ricotta identifies an unripened soft cheese known also with the name "*Requeson*". Ricotta is a fresh, unripened grainy cheese which is widely appreciated for its white color, soft and creamy texture and mild taste. However, other than ricotta, a large variety of traditional whey cheeses are manufacture in Europe, both soft and semi-hard, ripened and unripened, mainly form sheep milk, particularly in countries of the Mediterranean basin. These typical products include: Mizithra, Anthotyros and Manouri (Greece), Anari (Cyprus), Requesón (Spain), Requeijao (Portugal), Brocciu (France), Urdă (Balkans region) (Casti et al., 2016). Each of these products is produced with a distinctive traditional production process which confer unique characteristics linked with the geographical origin. In Sardinia (Italy) ricotta is produced from the whey remaining after the production of sheep's milk cheeses, mainly hard semi-cooked cheeses such as Pecorino Romano PDO ad Pecorino Sardo PDO.

The manufacturing of ricotta is similar at both industrial and artisanal level, which follow the traditional batch production process based on the heat coagulation of ovine whey (Pala et al., 2016). Different types of ricotta cheeses are included as an item in the "List of Traditional Agri-Food Products" of the Italian Ministry of Agricultural, Food and Forestry Policies (Ministerial Decree 8 September 1999, n. 350; Italian Republic). Among the product obtained from the ovine whey are: a) "a*rrescottu spongiau*" (fermented ovine ricotta cheese); b) "*ricotta fresca ovina*" (fresh ovine ricotta cheese); c) several types of salted ricotta cheeses with names which differ according to the shape: "toscanella" (cylindrical shaped), "ricotta testa di morto" (ball shaped) and ricotta moliterna (truncated cone shaped); d) "*ricotta mustia*" (salted and smoked ricotta). The manufacturing process is similar for all types of ricotta up until the curds coagulation after which the processing steps differ according to the type of ricotta and regional recipes. The main steps adopted in Sardinian industrial cheese-making plants for the production of ricotta cheese with the traditional batch production method are described below (Laore, 2015). The whey remaining after the daily cheese production is filtered by centrifugal separators to remove curd residues and pre-heated at 60-70°C with the use of a plate heat exchanger.

Alternatively, if the whey is not transformed immediately, it is stored refrigerated into stainless silo tank until use. The whey, is transferred into large open kettles of ca. 1,200-1,500 L of capacity, where it can be blended with the addition of whole milk or cream milk, in order to enhance product texture and increase yield. The whey or whey blend is heated or by direct steam injection or by indirect heating until temperature rises to ca. 80 °C. During heating the whey is slowly mixed with the use of a manual stirrer to avoid breaking up the small bits of curd that have formed. As the flocculated proteins start to rise on the whey surface the heating is interrupted and the mixture held for ca. 10-20 minutes. The clotted proteins are collected using stainless steel straining skimmer and transferred to drain into perforated molds of different shape and size. The successive steps vary according to the type of ricotta cheese produced.

"*Ricotta fresca*" (or fresh ricotta) is an unripened fresh whey cheese, also known as "*ricotta gentile*" when obtained for the remaining whey of Pecorino Romano PDO production. The curd is placed into truncated cone polypropylene basket and allowed to drain and to cool until the inner temperature drops to ca. 65-70°C. Ricotta basket are then placed in cold room (3±1°C) where an inner temperature <7 °C is reached within 10 h

(LAORE, 2014). *Ricotta fresca* is stored refrigerated until packaging which takes place within 24 h after the production. The final product is a truncated cone shaped chees of ca. 1.5-1.7 kg weight, simply wrapped in food paper or packaged with the use of Modified Atmosphere Packaging. The shelf-life of *ricotta fresca* vary from business operator to business operator and according to the packaging method from 5 days uo to 21 days.

"*Ricotta salata*" (or salted ricotta) is a traditional salted variety of ricotta which is ripened for variable length of time. After flocculated whey proteins have risen, clots are transferred into plastic cylindrical molds lined with cheese cloth. The curd is then pressed at room temperature to enhance drainage for up to 24 h and placed in cold room (10-12°C) for 5-15 days. "*Ricottone*" is the term used to generically indicate all types of salted and ripened ricotta cheeses. Traditionally, ricotta salata is a cylindrical shaped cheese referred to with the term "*Ricotta toscanella*" with an average weight of approximately 3 kg. Ricotta is salted either by adding the salt in the whey before heating or on the finished product by dry salting or brine-salting. Another typology of ricotta salata is the "*Greek ricotta*" which is salted and hung to dry from cheese cloths so that it obtains the shape of a ball. Ricotta salata wheels are individually packed in vacuum bags as a whole or after

cutting into small wedges of ca. 200-300 g. When of small truncated cone shape the salted ricotta is called "*Ricotta moliterna*" average weight of ca. 1.2-1.5 kg. Salted ricotta cheese shelf-life vary from several weeks up to 3-6 months.

"*Ricotta mustia*" is a term used to identify a traditional typology of smoked ricotta. As other salted ricotta cheeses, *ricotta mustia* is pressed, dry salted and smoked. Traditionally *ricotta mustia* is hot smoked in a fireplace but at industrial level is performed a cold smoking (25-30°C) in a smoking chamber. The final product is a wheel of ca. 0.8-2.0 kg. The packaging is in wrap paper and the shelf-life varies from 30 days up to two months.

Chemical composition

The composition of whey cheeses vary upon several factors conditioning the composition of the whey such as the species of origin (bovine, caprine or ovine), the type of cheese the whey residue from (soft cheese retain more milk component during separation of the curd as compared to hard cheeses), the stage of lactation, the breed, the farming system and so on. Also technological expect may impact on the final composition (i.e. addition of milk or cream, heat temperature, pressing, salting, smoking and

seasoning). Intrinsic factors (pH and a_W) and chemical composition (moisture, fat, proteins and salt) vary also depending on the different types of ricotta cheeses.

Ricotta fresca. The pH ranges from 6.50-6.70; the a_W ranges from 0.986-0.994. Centesimal composition values range between 71.23%-79.22% for moisture; 8.96%-18.13% for fat; 8.08-10.11% for proteins (Pala et al. 2016; Pulina et al., 2017; Spanu et al. 2017, 2018).

Ricotta salata. The pH ranges from 6.30-6.54; the a_W ranges from 0.946-0.978. Centesimal composition values range between 53.65%-58.28% for moisture; 19.55%-23.74% for fat; 12.65-18.39% for proteins and 2.60-4.56% for salt (Spanu et al., 2012, 2013; Spanu et al., 2015a,b; Casti et al., 2016; Spanu et al. 2016).

Ricotta mustia. The pH ranges from 5.40-6.60; the a_W ranges from 0.970-0.989. Centesimal composition values range between 62.0%-77.0% for moisture; 9.0%-23.0% for fat; 9.0-14.0% for proteins and 1.1-3.6% for salt (Pulina et al., 2016; unpublished data).

Microflora

The physic-chemical characteristics of ricotta cheeses, which exhibit high moisture content, pH, water activity, nutrients and the absence of preservatives, make of these products an excellent substrate for the growth of spoilage and pathogen microorganisms (De Santis and Mazzette, 2002). As consequence of heating at 80 °C, the microflora is drastically reduced since most microorganism's vegetative forms are killed (Pintado et al., 2001). Therefore the residual microflora of whey cheeses at clotting is represented by thermoduric bacteria and spores survived at high temperatures (De Santis et al., 1999). Within the first 24 h after production, before packaging, whey cheeses are susceptible to recontamination. Due to the nature of the batch production system, which includes handling of the curd after clotting, in an open product area, which exposes ricotta to secondary (i.e. post-process) contamination originating from the equipment and the processing environment (Greenwood et al., 1991; Ibba et al., 2013). The risk of contamination is more elevated in the process steps downstream curd flocculation, such as molding, salting, chilling, ripening and packaging (Pala et al., 2016).

The main sources of contamination are represented by food contact (i.e. drainage tables and utensils) and non-food contact surfaces (i.e. floor drains, processing equipment). The contaminants can persist within niches in the processing environment protected by a structured biofilm ecosystem (Simões et al., 2009). From niches microorganism find their way into exposed food through direct contact, aerosol, dripping or water splashes and by means of operators (Kousta et al., 2010). Even with the application of good hygiene practice (GHP) and good manufacturing practice (GMP), environmental contamination of ricotta cannot be avoided but only limited. During the first 24 h of refrigerated storage different groups of microorganisms such as coliforms, coagulase-negative staphylococci and psychrotrophic *B. cereus* strains are able to grow (Pintado and Malcata, 2000). However, under refrigerated storage other groups of spoilage and pathogen psychrotrophic microorganisms which can overgrowth the other microorganisms (Champagne et al., 1994; Carrascosa et al., 2015). Common contaminants of ricotta cheese include *Pseudomonas* spp, *Enterobacteriaceae*, yeast and molds, *L. monocytogenes*, *B. cereus* and *Arcobacter* spp (Pintado et al., 2001; De Santis

and Mazzette, 2002; De Santis et al., 2008; Ibba et al., 2013; Scarano et al., 2014; Spanu et al., 2016; Tirloni et al., 2017).

Pseudomonas spp. In the absence, or poor presence, of natural microflora such as in ricotta submitted to high temperature during processing, *Pseudomonas* spp. benefits of the selective advantage of low temperature and long storage, overgrowing other microorganisms and reducing the risk of pathogens growth (Buchanan and Bagi, 1999; Carrascosa et al., 2015). Hence, *Pseudomonas* spp. represents the main obstacle to extend the durability of *ricotta fresca*. During refrigerated storage *Pseudomonas* can reach counts as high as 7 $\log_{10}$ cfu g^{-1} (Pala et al., 2016), levels compatible with negative effects on sensory properties (i.e. flavor and texture) of the product (Leriche et al., 2004). Some *Pseudomonas* spp. can cause important discoloration due to the secretion of yellow-green pyoverdin (Meyer et al., 2002) and of a blue pigment (Cantoni et al., 2003; Martin et al., 2011; Andreani et al., 2015). The level of implementation of hygiene during production impacts on the level of initial contamination of *ricotta fresca*. When hygiene management measures (e.g. cleaning and disinfection, personnel hygiene and training, etc.) are well implemented, *Pseudomonas* spp. were not detectable in the first 24 h after production

while failure in hygienic practices hesitated in counts above 3 $\log_{10}$ cfu g^{-1} in ca. 30% of the samples (Pala et al., 2016).

Enterobacteriaceae. No microbiological criteria has been defined by Regulation (EC) No 2073/205 (European Commission, 2005) for *Enterobacteriaceae* in whey cheeses that undergone heat treatment. However, the presence of these important indicator microorganisms suggests a recontamination from the equipment or the processing environment and that good manufacturing and good hygienic practices should be improved. Two different scenarios could be described for *ricotta fresca* and *ricotta salata*. In *ricotta fresca*, a low level of contamination has been observed within the first 24 h after production, with a prevalence of 11.1% and counts ranging between 2-3 $\log_{10}$ cfu g^{-1} which increased during refrigerated storage up to 82.2% and 5-6 $\log_{10}$ cfu g^{-1} (lPala et al., 2016; Spanu et al., 2017, 2018). In ricotta salata cheese Enterobacteriaceae contamination was observed both on the rind (indicating a possible recontamination from the processing environment) and in the inner paste (suggesting a lack of hygiene practice of the personnel during the phase of molding). On the rind the prevalence of contamination was 81.4% with a mean level ranging from 4 to 5 $\log_{10}$ cfu g^{-1} while on the

inner paste was 7.4% and ca. 3 $\log_{10}$ cfu g^{-1}. During refrigerated storage of vacuum packed ricotta salata for three up to six months was observed a progressive reduction in the prevalence and level of contamination, respectively to 50% and < 3 $\log_{10}$ cfu g^{-1} on the rind and to 6.6% and 1 $\log_{10}$ cfu g^{-1} in the inner paste (Spanu et al., 2012, 2013). Another investigation conducted on ricotta salata stored refrigerated (Casti et al., 2016) showed instead an increase over time in the prevalence and level of *Enterobacteriaceae* contamination on the rind respectively from 66.6% to 100% and from 4 up to 5 $\log_{10}$ cfu g^{-1}. These results can be explained with the fact that in the latter study the salt was added by brine-salting instead of dry salting, increasing the risk of contamination of the brine solution.

B. cereus. The presence of *B. cereus* in sheep ricotta cheese produced in in Sardinia has been reported (Cosentino et al., 1997; De Santis et al., 2008; Fadda et al., 2012). The presence of spore forming microorganisms in whey cheeses represents a serious concern for human health (Heyndrickx et al., 2002,). As consequence of heat treatment spores can germinate and vegetative cells are facilitated in their growth by the absence of competing microflora (Scheldeman et al., 2006). Despite the frequent recovery of *B. cereus* in dairy

products they have been seldom associated with human illness (EFSA, 2015). This low incidence is explained by the fact that the production of the emetic toxin (cerulide) requires the growth of the microorganism in the food. Under the refrigerated storage of ricotta cheeses the level of contamination do not reach the threshold that leads to significant toxin production. *B. cereus* mesophilic strains need temperature >10 °C to growth. A possible concern may be represented by *B. cereus* psychrotrophic strains that are able to grow at 4-5 °C but they growth is slow and therefore it is necessary a long storing for the microorganism to reach levels compatible with toxin production (Spanu, 2016). Furthermore, psychrotrophic strains are considered not able to produce emetic toxins (Carlin et al., 2006). Therefore, they represent a threat to consumer safety only as a consequence of improper and prolonged cold storage.

L. monocytogenes. The association between ovine ricotta produced in Sardinia and *L. monocytogenes* contamination has been documented and leaded, in several circumstances, to voluntary recall by international companies. In 2008 case of listeriosis associated to the consumption of *ricotta salata* cheese has been reported by the European Commission (RASFF, 2008). In 2012 a fatal case, with 4 deaths and 20 hospitalization,

occurred in the USA after the ingestion of ricotta salata imported from Italy (CDC, 2012). Milk pasteurization or thermization and whey heating inactivate *Listeria* cells to level of approximately 3-6 log (ICMSF, 1996). Therefore, the contamination of ricotta origins from the processing environment after the thermal treatments, mainly during operation following curd collection (Spanu et al., 2012; Ibba et al., 2013). The contamination of ricotta salata is almost exclusively limited to the rind, with a reported prevalence of ca. 20% (Ibba et al., 2013; Lioliou et al., 2001; Pintado & Malcata, 2000; Spanu, Scarano, Ibba, Spanu, & De Santis, 2015). The pH and a_W of ricotta cheese support the possible growth of *L. monocytogenes* to level potentially harmful to human health (Spanu et al., 2012). The strict application of good hygienic practices do not guarantee to prevent the contamination from occurring (Tompkin et al., 1999), therefore there is the need to implement alternative strategies to control the superficial contamination of the product such as post lethality treatment (FSIS, 2014). Water bath heat treatment have been validated in ricotta salata cheese, allowing to obtain 5-6 log reduction of L. monocytogenes count on the ricotta surface (Spanu et al., 2013; Spanu et al., 2015 a,b). However, such treatment are effective on the product rind and not on the inner paste,

hence implying that good hygienic practices should always be followed during manufacturing in order to prevent the risk of contamination of the paste.

INNOVATION OF TRADITIONAL FOOD PRODUCTS

At global level the demand for traditional products has increased over the years, since they are perceived by consumers as higher quality products (Shakeel-Ur-Rehman, et al., 2003). The protection of traditional products is of great importance for food business operator for both preserving the cultural heritage associated with such products and to create competitiveness on the market (Albayrak and Gunes, 2010). A strategy pursued by industries is to improve the safety, the healthiness ad convenience of traditional products introducing innovation of the traditional products. This strategy will allow food business operator to gain a competitive advantage and expand the market share of traditional product which is generally confined in a niche (Friar, 1995; Guerrero et al., 2009). The innovation of traditional products can be obtained in different ways: a) by introducing production steps in manufacturing process; b) adding new ingredients; c) changing the packaging of the product; d) conferring environmental sustainability features. The innovated traditional products are intended to be placed on the large scale

market where there is an increasing demand of safety and convenience (reduced size packaging, longer shelf-life, etc.) of the products. Product innovation has to be balanced with preservation of the culture, tradition, sensory properties and health benefits (Lipan et al., 2017). In Sardinia, the traditional ricotta cheese has been innovated at both artisanal and industrial level. Examples of efforts conducted by foods business operator of Sardinia include the conduction of shelf-life studies to support with scientific evidences the durability and the safety of the ricotta cheese they place on the market, application of processing steps such as post-lethality treatments to control recontamination, introduction of new packaging methods (Spanu et al., 2013; Pala et al., 2016).

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CHAPTER 2 - Thesis scope

The scope of the present thesis is aimed to introduce innovative strategies in the production of traditional ricotta cheeses aimed to: a) provide scientific evidences and safety assurance during the shelf-life; b) support the definition of the shelf-life based on microbiological profile; c) meet consumers' demand for special dietary needs (i.e. intolerance or allergies).

The first contribution to the present thesis (Chapter 3) is a study already published in the Italian Journal of Food Safety (2016) Volume 5, Issue 2, pp 57-60 entitled: "Evolution of the microbiological profile of vacuum packed sheep's ricotta salata wheels during shelf-life", of which the candidate is co-author. The study is a durability study conducted on vacuum packed ricotta salata cheese aimed to assess the evolution of spoilage and pathogen microorganisms. The results obtained provide valuable information to the food business operator to support the definition of the shelf-life based on scientific evidences.

The second contribution to the thesis (Chapter 4) is a study already published in Food Microbiology (2016) Volume 58, pp 135-138 entitled: "Occurrence and behavior of *Bacillus cereus* in naturally contaminated ricotta salata cheese during refrigerated

storage", of which the candidate is co-author. The study investigate the evolution of a serious microbiological concern in ricotta cheese such as *B. cereus*. The results provide evidence that the spores could overcome the heat treatment applied during processing and vegetative cell can be recovered from the product. The refrigerated storage represents a critical control measure not only to prevent the growth of the microorganism but to kill the vegetative cells.

The third contribution (Chapter 5) is a study already published in Italian Journal of Food Safety (2017) Volume 6, Issue 1, pp 33-39 entitled: "Production of farmstead lactose-free *Pecorino di Osilo* and *ricotta* cheese from sheep milk", of which the candidate is co-author. The study is the implementation at artisanal level of product innovation of a traditional ricotta cheese. In particular it validated the introduction of the lactase enzyme as ingredient in the formulation of ricotta in order to be suitable to consumers with special dietary needs. The results demonstrated that also at farm levels it is feasible to introduce innovation in the production process, preserving the sensory properties and cultural heritage associated with artisanal production.

The fourth contribution (Chapter 6) is a study already published in Food Microbiology (2017) Volume 66, pp 72-76, entitled: "Testing commercial biopreservative against spoilage microorganisms in map packed ricotta *fresca* cheese" of which the candidate is co-author. The study validate the use of innovative measures to control the growth of spoilage microorganisms in ricotta fresca cheese. In particular was validated the use of protective culture to contrast the growth of pseudomonas spp. The results demonstrated that the innovation introduced in the process can improve the microbiological quality of fresh ricotta cheese during the shelf-life.

CHAPTER 3 - Evolution of the microbiological profile of vacuum packed sheep's ricotta salata wheels during shelf-life.

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Abstract

Ricotta salata is a salted variety of ricotta traditionally made in Sardinia (Italy) from the whey remaining after the production of Pecorino Romano PDO or other sheep's milk cheeses. Ricotta salata is very critical for the possible growth of pathogenic and spoilage microorganisms. Sporadic cases of listeriosis associated with ricotta salata have been reported over recent years. The objective of the present study was to assess the evolution of spoilage and pathogen microorganism of vacuum packed ricotta salata during the entire product shelf-life. The durability study was conducted on 18 vacuum packed ricotta salata samples analyzed at the beginning of the shelf-life and after 60 and 90 days of refrigerated storage. Pathogen as *L. monocytogenes* and *B. cereus* were never detected. During shelf-life total bacterial counts ranged between 7.90±0.64 cfu g^{-1} and 9.19±0.58 cfu g^{-1} on the rind and between 2.95±0.68 cfu g^{-1} and 4.27±1.10 cfu g^{-1} in the inner paste, while *Enterobacteriaceae* ranged between 4.22±0.66 cfu g^{-1} and 5.30±0.73 cfu g^{-1} on the rind and 3.13±1.80 cfu g^{-1} and 2.80±0.88 cfu g^{-1} in the inner paste. Considered the technology, the intrinsic properties and the almost absence of competing microflora, ricotta salata can support the growth of spoilage and pathogen microorganism

originating from the processing environment. Control of contaminants in Ricotta salata relies on the implementation of hygiene during processing, in the reduction of superficial contaminants through application of post-lethality treatments and in preventing their growth by strict maintenance of refrigeration temperature during storage.

Introduction

Ricotta salata or Ricottone or Ricotta "*Toscanella*" is a salted variety of ricotta traditionally made in Sardinia (Italy) is from the whey remaining after the production of Pecorino Romano PDO (protected designation of origin) or other sheep's milk cheeses. A wide variety of whey cheeses are manufactured from sheep's milk in several European countries, especially in the Mediterranean basin: i.e. Mizithra, Anthotyros and Manouri (Greece), Anari (Cyprus), Requesón (Spain), Requeijao (Portugal), Broccio (France), Urdă (Balkans region) each with its own distinctive technology. In most of the cases these are fresh and soft or semi-soft traditional products made in small-scale operations and sell in local markets. The manufacturing of ricotta salata in industrial cheese-making plants follows the traditional batch process. The whey is filtered and preheated at 60-70°C with the use of a plate heat exchanger. The whey can be added with pasteurized cream or milk

to enrich the solid content. The blend is then transferred in large open kettles (1,200-1,500 l capacity) and heated to temperature above 85 °C and held for 30 min. After the flocculated protein rises to the surface, clots are collected using perforated ladder and transferred into plastic cylindrical molds lined with cheese cloth. The curd is then pressed to enhance drainage for 24 h and transferred in cold room (10-12°C) for about 10 days. Salting can be made adding directly sodium chloride to the whey or to the curd during molding or by dry salting during refrigerated storage or in brine after molding. Ricotta salata is dried until the moisture content is ca 50% if intended to be used for grating or of ca 55-60% if used as it is. The main steps of the process flow are summarized in the diagram in figure 1.

After the production process the mean fat and protein content are respectively 28-33% and 14-23%, the pH ranges from 6.1 to 6.9 while the a_W from 0.940 and 0.970 (Spanu et al., 2012; Spanu et al., 2013).The final product is a firm wheel of ca 3 kg weigh which is individually packed in vacuum bags as a whole or after cutting into wedges. Ricotta salata is stored at refrigeration temperature and the attributed shelf-life differs from three weeks up to several months, depending on the food business operators (C. Spanu et al.,

2015a). Ricotta salata is often used in salads or many other dishes sliced, crumbled or grated without further heating.

In consideration of its physico-chemical properties and the presence of and edible rind, ricotta salata is very critical for the possible growth of pathogenic and spoilage microorganisms. Among pathogens of particular concern are those able to growth in ricotta salata at refrigeration temperatures, i.e. *L. monocytogenes* and psychrotrophic *Bacillus cereus* strains. Outbreaks and sporadic cases of listeriosis associated with ricotta salata have been reported over recent years (RASFF, 2008; CDC, 2012). Evidence that *L. monocytogenes* contamination of ricotta salata origins from the processing environment and that this product supports the growth of the pathogen during refrigerated storage have been documented (Spanu et al., 2012; Ibba et al., 2013; Spanu et al., 2015b). Despite the frequent contamination of ricotta salata with *B. cereus* and the recovery of potentially pathogenic strain, no cases of *B. Cereus* human illness have been associated with the consumption of ricotta salata (De Santis et al., 2008). Considered the high moisture content, the high pH and the availability of nutrients such products are very susceptible to microbiological spoilage by *Enterobacteriaceae*, yeast, molds and *Pseudomonas*. The

presence of pathogens contaminants such as *L. monocytogenes* and *Bacillus cereus* has also been observed. However, little research has been conducted to describe the evolution of the microbiological profile of Ricotta salata during the shelf-life (Spanu et al., 2013).

The objective of the present study was to assess and characterize the evolution of spoilage and pathogen microorganism of vacuum packed ricotta salata under the foreseeable storage conditions during the entire product shelf-life.

Materials and methods

Ricotta salata samples

The study was conducted on vacuum packed ricotta salata wheels (table 1) which were provided by a local cheese-making plant using the whey remaining after the manufacturing of sheep's milk cheeses. A total of 18 samples were randomly selected from 3 different batches (6 ricotta salata for each batch) produced within one month. Moisture content of Ricotta salata batches was monitored after a drying period of approximately 10-15 days in cold room. Only samples with a moisture content ≤ 50% were selected for the experiment and sent to the laboratory. Immediately after they arrival

samples were labeled according to their use for the experiment and stored at refrigeration temperature (4±2°C) until analysis were performed.

Experimental design

The durability study was conducted analyzing ricotta salata samples at different time during the shelf-life. Sampling times were: within 24 h after the arrival of ricotta salata wheels defined as time zero (T_0), time 60 (T_{60}) and time 90 (T_{90}) respectively 60 days and 90 days after the arrival of the samples. In order to avoid temperature abuse during the storage period, the temperature of the cold room was monitored in continuous and recorded daily. Ricotta samples were analyzed for the determination of the microbiological profile, physico-chemical properties and composition. In table 1 are reported the number of samples and the analysis performed at each sampling time.

Microbiological profile

From each ricotta salata sample were aseptically collected 25 g aliquots of the rind (2 cm in depth) and of the inner paste. Aliquots were analyzed for the enumeration of total viable count (TVC) at 30°C (ISO 4833:2003), *Listeria monocytogenes* (ISO 11290-

1:1996), *Bacillus Cereus* (EN/ISO 7932:2004), *Enterobacteriaceae* (ISO 21528-2), yeasts and moulds (ISO 6611/IDF 94, 2004).

Physico-chemical properties and composition

PH and a_W were measured using pH meter GLP22 (Crison Instruments SA, Barcelona, Spain) and water activity meter Aqualab 4 TE (Decagon, Pullman, WA, USA), respectively. Near infrared transmittance (NIT) compositional analyzer (FOSS, Eden Prairie, MN, USA) was used for the determination of centesimal composition which included analysis of moisture, fat, protein, salt and total solids.

Statistical analysis

Differences in mean mesophilic bacteria counts ($\log_{10}$ cfu g^{-1}), intrinsic properties (mean ± SD) and composition (% ± SD) between sampling times were compared using Fisher's least significant difference (LSD) test. All statistical analyses were performed with R Studio software, version 0.98.1103 – © 2009-2014 RStudio, Inc.

Results

A total of 18 vacuum packed ricotta salata cheese wheels were analyzed to assess the presence of pathogen and spoilage microflora and for the determination of intrinsic properties and composition.

Microbiological profile

Pathogen microorganism such as *L. monocytogenes* and *B. cereus* were always below the detection limit (10 $\log_{10}$ cfu g^{-1} and 100 $\log_{10}$ cfu g^{-1}, respectively) either on the rind and in the inner paste. The mean TVC at T_0 was ca 8 $\log_{10}$ cfu g^{-1} on the rind and ca 3 $\log_{10}$ cfu g^{-1} in the inner paste, no significant increase during the storage was observed. The mean *Enterobacteriaceae* count was always greater on the rind as compared to the inner paste, with maximum concentration of ca 5 $\log_{10}$ cfu g^{-1} and 3 $\log_{10}$ cfu g^{-1} respectively. Yeasts and Moulds ranged between 3 and 5 log cfu g^{-1} on the rind and ca 2 log cfu g^{-1} on the inner paste. Table 2 show the results ($\log_{10}$ cfu g^{-1}; mean ± SD) of the targeted microbial groups at the different sampling times.

Intrinsic properties and composition

The pH of ricotta salata during refrigerated storage under vacuum packing decreased from an initial level (T_0) of 6.54±0.05 to a final level (T_{90}) of 5.68±0.16 (P<0.05). The a_w was stable for the entire duration of the study at level between 0.97±0.01 and 0.98±0.01 (P>0.05). Minor changes in composition (moisture, fat, proteins, salt and total solids) were observed during storage. Table 3 shows the intrinsic properties (mean ± SD) and composition (% ± SD) evolution during storage (T_0, T_{60}, T_{90}) of ricotta salata wheels.

Discussion

The durability of ricotta salata defined by food business operator is generally of several months under refrigerated storage. Therefore, ricotta salata is a ready to eat whey protein cheese that could be regarded as a refrigerated processed food of extended durability (REPFED). The shelf-life of ricotta salata depends upon a number of interacting factors other than storage temperature such as packaging conditions, product composition, presence of preservatives and competitive microflora. Production technology (whey heating which inactivates natural microflora and absence of starter

coltures), intrinsic properties (high pH and elevated water activity) altogether, determine the ability of the product to support the growth of pathogen and spoilage microorganism.

The origin of contamination can be from raw materials or from the environment and differs from one microorganism to another. The production of Ricotta salata, especially in the traditional batch manufacturing system, includes manual manipulation of the curd after floating, the exposition to environmental contamination from several food contact surfaces (drainage tables, plastic moulds, pressing equipment, drying shelves) and non-food contact surfaces (floors, drains, walls and ceiling). Contaminants can reach the product by means of aerosol, condenses, dripping or inappropriate practice of operators. The open nature of ricotta salata production make this a food particularly exposed to environmental contamination.

The enumeration of total viable count showed a total bacterial load ca 4 log greater on the rind as compared to the inner paste. This is the result of the exposition of the rind to the processing environment. This is confirmed by the high contamination with *Enterobacteriaceae* especially on the rind. Although no microbiological criteria is defined by Regulation EC 2073 (EC, 2005) for *Enterobacteriaceae* in whey cheeses that

has undergone heat treatment, the presence of these important indicator microorganisms reveals that in the cheese-making plants good hygienic and good manufacturing practices should be improved. In particular, the presence of *Enterobacteriaceae* in the inner paste indicates poor hygienic conditions in the phase of ricotta salata molding. This is a very critical step in ricotta production, in which manual manipulation can transfer contaminants from the processing environment into the inner product.

With reference to pathogenic microorganisms it is demonstrated that *L. monocytogenes* contamination origins from the processing environments. Food contact and non-food contact surfaces represent the main vehicles of contamination, mainly due to manipulation of the product after the production process. This explains the frequent recovery of *L. monocytogenes* on ricotta salata rind (Spanu et al., 2012; Ibba et al., 2013; Spanu et al., 2015b). Contamination of the inner paste is a rare finding and is generally associated with poor hygienic procedures during manual molding (Spanu et al., 2015a). Previous studies reported a prevalence of contamination ranging from 0.0% up to 30.0% of the samples (Pilo et al., 2007; Spanu et al., 2015b). In the present study *L. monocytogenes* was never detected (<0.04 cfu g^{-1}) both from the rind and the inner paste

for the entire shelf-life. This further confirms the importance of the strict implementation of hygiene during the production and storage steps.

The pathways of contamination of ricotta salata with sporeformers differs with respect to *L. monocytogenes* and other environmental contaminants. In fact, spore forming bacteria enter the dairy chain mainly with raw milk (Heyndrickx, 2011). The spores present in milk can resist to heat treatment applied to milk such as thermisation and pasteurization (Scheldeman et al., 2006). High temperature reached during whey heating (>80-85°C) activates the germination of residual spores, which are facilitated in their growth by the absence of other competitive microflora eliminated by heat treatments (Griffiths and Phillips, 1990). Despite it is almost impossible to avoid the contamination of spores from the dairy industry, cases of human illness associated to the consumption of dairy products contaminated with *B. cereus* is a rare finding (EFSA, 2015). *B. cereus* strains are generally not able to growth <10°C, only few psychrotrophic strains growth at temperature as low as 4-5°C. Therefore, temperature abuse are necessary during storage to lead to the multiplication of the vegetative cells. In a product such ricotta salata, characterized by an extended shelf-life, sporeformers have the time to reach level

potentially dangerous to human health (ICMSF, 1996). An essential strategy for the control of sporeformes is the strict cold-chain maintenance for the entire shelf-life of the product. During the 90 days of refrigerated storage *B. cereus* was never detected, indicating that a combination of the quality of raw material and temperature control during storage can contribute to the safety of ricotta salata.

Conclusion

The open nature of ricotta salata production and the presence of manual manipulation makes this product particularly exposed to environmental contamination. In addition, the absence of a competitive microflora, the richness in nutrient and the intrinsic properties are favorable conditions for microorganisms' growth of spoilage and pathogen microorganism. The strategies for the control of environmental contaminants are based on their prevention through the strict implementation of hygienic conditions during production process. Superficial contamination of the products can be reduced by the adoption of post-lethaly treatment (i.e. heath treatment) in vacuum packed product, allowing to extend ricotta salata shelf-life. As far as contaminants originating from raw ingredients, which are not eliminated during processing, such as sporeformers, their

control relies upon prevention of their growth through the maintenance of an unbroken chill chain for the storage.

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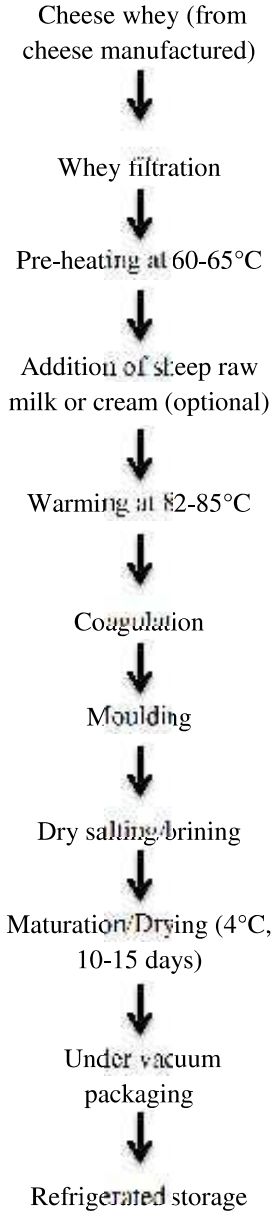
Figures

Figure 1: Process flow ricotta salata production



Tables

Table 1. Number of Ricotta salata wheels and analysis performed at each testing time[4].

Analysis	Sampling time		
	T_0	T_{60}	T_{90}
Microbiological profile	6	6	6
Intrinsic properties and composition	6	6	6

[4] Numbers of samples for each time refers to the three batches.

Table 2. Mean log microbial counts (log10 cfu g-1; mean ± SD) of Ricotta salata units during shelf-life1.

Parameters	**Samples**	**T_0**	**T_{60}**	**T_{90}**
TVC	Rind	7.90 ± 0.64^{a} (n =6/6)	7.79 ± 2.21^{a} (n =6/6)	9.19 ± 0.58^{a} (n =6/6)
	Paste	2.95 ± 0.68^{a} (n = 5/6)	4.51 ± 1.66^{a} (n =6/6)	4.27 ± 1.10^{a} (n =6/6)
Enterobatteriacee	Rind	4.22 ± 0.66^{b} (n =4/6)	5.16 ± 0.59^{ab} (n =5/6)	$5.30^{a} \pm 0.73$ (n =6/6)
	Paste	ND	3.13 ± 1.80^{a} (n =3/6)	2.80 ± 0.88^{a} (n =2/6)
Yeasts and Moulds	Rind	3.02 ± 0.36^{b} (n =3/6)	4.99 ± 0.81^{a} (n = 4/6)	4.01 ± 1.03^{ab} (n =4/6)
	Paste	2.00 ± 0.00^{a} (n =2/6)	1.67 ± 0.58^{a} (n =3/6)	2.30^{a} (n =1/6)

[1] Means in the same row with different superscript letters are significantly different ($P < 0.05$); values within brackets indicate the prevalence of positive samples. ND= not detected (below the detection limit of the method). T_0 was the packaging day, T_{60} and T_{90} were respectively 60 and 90 days production.

Table 3. Intrinsic properties (mean ± SD) and composition (% ± SD) evolution during storage of ricotta salata wheels[3].

Parameters	**T_0**	**T_{60}**	**T_{90}**
pH	6.54 ± 0.03[a]	6.04 ± 0.14[b]	5.68 ± 0.18[c]
a_w	0.973 ± 0.005[a]	0.977 ± 0.008[a]	0.978 ± 0.005[a]
% moisture	57.9 ± 1.85[a]	56.3 ± 0.88[a]	57.2 ± 1.81[a]
% total solids	42.13 ± 1.84[a]	44.65 ± 3.75[a]	43.33 ± 5.01[a]
% fat	19.55 ± 2.08[a]	21.24 ± 0.94[a]	20.08 ± 1.74[a]
% protein	14.84 ± 0.81[a]	15.18 ± 0.52[a]	15.17 ± 1.03[a]
% salt	3.42 ± 0.24[a]	3.10 ± 0.34[a]	3.38 ± 0.34[a]

[3]Means in the same row with different superscript letters are significantly different ($P < 0.05$). T_0 was the packaging day, T_{60} and T_{90} were respectively 60 and 90 days production.

CHAPTER 4 - Occurrence and behavior of *Bacillus cereus* in naturally contaminated ricotta salata cheese during refrigerated storage

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Abstract

The present study shows the fate of *B. cereus* in ricotta salata cheese during refrigerated storage for the entire shelf-life. A total of 144 ricotta salata wheels from nine naturally contaminated production batches were stored refrigerated and analyzed 10, 30, 60 and 90 days after production for the determination of total bacterial count, *B. cereus* spores and vegetative forms, intrinsic properties and composition. The presence of spores was sporadic while the prevalence and the level of *B. cereus* vegetative cells decreased respectively from 83.3% and 4.65±0.74 cfu g^{-1} at the beginning of the observation period to 33.3% and 1.99±0.55 cfu g^{-1} after 90 days. The production process of ricotta salata includes steps such as whey heating followed by slow cooling of clots, which expose to the risk of spore germination and successive growth to levels compatible with toxins production. The prolonged refrigerated storage was not favorable to sporulation, explaining the successive death of vegetative cells. Control of *B. cereus* in ricotta salata relies on the hygienic quality of raw milk and in preventing condition that support growth of the vegetative forms and the consequent cerulide production.

Keywords: *Bacillus cereus*; whey cheese; sheep's milk

Introduction

The *Bacillus cereus* group includes Gram-positive rod shaped spore-forming bacteria, which are widely distributed in the natural environment. Within the group, *B. cereus sensu stricto* is the most important organism due to its potential to cause food spoilage and food-borne illness (Kramer and Gilbert, 1989). *B. cereus* causes two clinical forms of foodborne illness: the emetic and the diarrheal syndrome (Granum and Lund, 1997). *B. cereus* is frequently isolated from raw milk and dairy products thus, representing a serious concern for the dairy industry (Svensson et al., 2006). Due to its ubiquitous nature and the extreme resistance of endospores to several harsh conditions (Nicholson, et al., 2000), it is almost impossible to avoid the contamination of dairy products. *B. cereus* can enter the dairy chain mainly through raw milk contaminated at farm level (Heyndrickx, 2011). However, contaminations may also arise from the food-processing environment (da Silva Fernandes et al., 2014).

Whey products processed at high temperatures and successively stored refrigerated are particularly exposed to the risk of *B. cereus* (Heyndrickx and Scheldeman, 2002). The endospores are activated by whey heating applied during protein

denaturation (>80°C) and vegetative cells are then facilitated in their growth by the absence of competing microbiota, inactivated by the heat treatment (Scheldeman et al., 2006). *B. cereus* psychotropic strains can grow to temperature as low as 4-5°C and during the refrigerated storage can reach levels potentially harmful for human health (Huck et al., 2007). A dose of 10^5-10^8 cells or spores per gram of food is generally considered necessary to cause illness (ICMSF, 1996; Granum and Lund, 1997). Dairy products have been seldom associated with human illness despite the frequent contamination with *B. cereus* (EFSA, 2005).

Ricotta salata is a traditional dry and salted sheep's milk whey cheese produced in Sardinia (Italy). Technology and microbiological profile of ricotta salata have been previously described (Spanu et al., 2015). The few published data existing on *B. cereus* contamination in ricotta salata produced in Sardinia reported a prevalence of ca. 15% and a contamination level ranging from 1 to 3 $\log_{10}$ cfu g^{-1} (Cosentino, et al., 1997; De Santis et al., 2008; Fadda et al., 2012). No published data are available on the fate of *B. cereus* in naturally contaminated ricotta salata stored under refrigerated conditions.

The present study reports a case of large *B. cereus* contamination of ricotta salata occurred in one sheep's milk cheese-making plant operating in Sardinia. During the period September-October 2014, a local food business operator observed the presence of *B. cereus* contamination in ricotta salata cheese tested as part of routine microbiological examination activities of the plant's food safety management system. The mean level of contamination was 5.57±0.15 $\log_{10}$ cfu g^{-1} in a batch. To date, no food safety criteria for *B. cereus* are applicable to foodstuffs placed on the market during their shelf-life (EC Regulation No. 2073/2005). However, contamination levels as high as 10^5-10^6 cfu/g pose a serious concern for consumer's health, due to possible food poisoning. The food business operator as corrective action withdrew the entire batch of ricotta salata samples from the market. The production batches, that after microbiological examination tested positive for the presence of *B. cereus*, were destined to a durability study.

The objective was to assess the evolution of *B. cereus* in naturally contaminated ricotta salata during the entire shelf-life, which is generally up to several months under refrigeration (Casti et al., 2016).

Materials and methods

2.1. Ricotta salata batches and samples

Ricotta salata batches to be used in the study were selected based on the natural occurrence of *B. cereus*. With this aim, during the period September-October 2014, ricotta salata production batches were tested on a daily basis for the presence of *B. cereus*. From each positive batch were randomly selected eight ricotta salata wheels. Samples were immediately vacuum packed in plastic bags and transported refrigerated to the laboratory where they were stored in cold room (4±2°C) until analyses were performed.

2.2. Experimental design

Two ricotta samples from each of nine different production batches were analyzed at four different times during the shelf-life. Sampling times were: within 24 h after the arrival of ricotta salata wheels defined as time zero (T_0), 30, 60 and 90 days after the production defined respectively as time 30 (T_{30}), time 60 (T_{60}) and time 90 (T_{90}). Duplicate samples were used for microbiological and physico-chemical analysis.

2.4. Microbiological analysis

Ricotta salata samples were analyzed for the determination of total aerobic mesophilic bacteria (ISO 4833, 2013) and enumeration of *B. cereus* (ISO 7932, 2004). The enumeration of both *B. cereus* vegetative cells and spores was determined before and after heating at 80°C for 10 min by plating two 0.1 mL aliquot on selective chromogenic culture media such as Mannitol Egg Yolk Polymyxin agar (MYP, Biolife, Milan, Italy) and Polymyxin Pyruvate Egg-Yolk Mannitol Bromothymol Blue (PEMBA, Oxoid) agar. Samples were incubated at 30°C in aerobic conditions for 24 h. From each positive sample were picked five presumptive *B. cereus* colonies, transferred onto Trypticase Soy Agar (TSA, Biolife) and incubated at 37°C for 24 h. Each isolate was submitted to phenotypic identification and successively confirmed by PCR (Oh et al., 2012).

2.5. Intrinsic properties and composition

PH and a_W were measured using pH meter GLP22 (Crison Instruments SA, Barcelona, Spain) and water activity meter Aqualab 4 TE (Decagon, Pullman, WA, USA), respectively. Determination of centesimal composition (% of moisture, fat, protein, salt

and total solids) was performed using the Near Infrared Transmittance (NIT) compositional analyzer (FOSS, Eden Prairie, MN, USA).

Results

3.1. Microbiological profile

A total of seventy-two ricotta samples were tested for microbiological examinations (18 for each of the 4 sampling times). The mean aerobic mesophilic count (log_{10} cfu g^{-1}; $\bar{x}$ ± SD) of ricotta salata analyzed at T_0, T_{30}, T_{60} and T_{90} was 5.17±1.39, 5.69±0.54, 5.99±0.67 and 5.62±0.87, respectively. The prevalence of *B. cereus* vegetative cells and the mean level of contamination decreased during the refrigerated storage (P<0.05). At T_0, the prevalence was 83.3% with counts ranging from 3.45 log_{10} cfu g^{-1} to 6.20 log_{10} cfu g^{-1}, while at T_{90} the observed prevalence was 33.3% with counts ranging from 1.30 log_{10} cfu g^{-1} to 2.56 log_{10} cfu g^{-1} (table 1). The mean reduction over time (ΔT) in *B. cereus* vegetative cells concentration (log_{10} cfu g^{-1}) was 0.38, 1.74 and 2.66 at T_{30}, T_{60} and T_{90}, respectively. The detection of *B. cereus* spores after heat activation was observed in one sample at T_{30} (2.30 log_{10} cfu g^{-1}) and one sample at T_{60} (2.0 log_{10} cfu g^{-1}) belonging to two different batches. Out of 49 total positive samples (68.0%) were

isolated 245 presumptive *B. cereus* strains of which 101 were confirmed by molecular identification.

3.2. Physico-chemical characteristics

Intrinsic properties values ranged for pH between 6.23 and 6.67 at T_0 and between 5.30 and 6.32 at T_{90}, while a_W values ranged between 0.964 and 0.986 at T_0 and 0.976 and 0.983 at T_{90}. The evolution of the mean centesimal composition values (%; $\bar{x} \pm$ SD) at different sampling times is reported in table 2.

Discussion

Despite raw milk is the main source of contamination of dairy product with sporeformers, their level is generally low, <1-10^2cfu mL^{-1} (Vissers et al., 2007). Seasonal variation has been reported with counts as high as 10^4 cfu mL^{-1} (Slaghuis et al., 1997; TeGiffel et al., 2002; Coorevits et al., 2008). The presence of *B. cereus* in ricotta salata is a rare finding, with maximum contamination level of ca. 3 $\log_{10}$ cfu g^{-1} (Cosentino et al., 1997; De Santis et al., 2008; Fadda et al., 2012; Spanu et al., 2012). The high level of contamination, up to 8.33 $\log_{10}$ cfu g^{-1}, observed in the present study and the large number of positive batches (nine) over a limited period of time (three months), should be

considered as an event strictly associated with the late summer and early fall production period. Microbiological testing of each production batch, conducted on a regular basis in the frame of the food business operator's HACCP procedures, showed no occurrence of *B. cereus* contamination during the rest of the year. This could be explained with the typical sheep's milk breeding systems adopted in Sardinia. Milk production is seasonal, starting from December until July. The peak of milk production is concentrated between January and May, with a decrease between June and August, when the sheep start entering in the dry period. Cheese-making during the dry period relies on the milk available provided by flocks adopting the out-of-season breeding system. Poor pasture quality during this season determines a decline in milk yield and microbiological quality (Sitzia et al., 2015). Due to economic reasons, raw milk is picked and transformed every three or five days instead that daily. In addition, during the winter and spring period sheeps mainly graze on grass pasture, while during the summer and fall period on stubble with concentrate and feedstuff supplement, which may increase the risk of transferring spores into raw milk.

Total bacterial count and yield records of the milk used to make the ricotta salata used in the present study, were obtained by the food business operator. Data confirmed differences in milk yield and microbiological quality over the milking season. In the period from January to June, the total bacterial count (geometric mean) of raw milk was ca. 140,000 cfu mL^{-1} with an average production of 2,150,000 l while in the out-of-season period the total bacterial count was ca. 1,100,000 cfu mL^{-1} with an average milk yield of 135,000 l. Therefore, the production of ricotta salata during the out-of-season period was characterized by risk factors that increase the likelihood of having high initial level of *B. cereus* contamination in the product. The greater relative decrease in *B. cereus* vegetative cells was observed after 60 days (-1.36 log_{10} cfu g^{-1}) and after 90 days (-0.92 log_{10} cfu g^{-1}) of refrigerated storage. Since psychrotrophic strains can grow at temperature as low as 4-5°C, contamination was likely due to mesophilic strains which minimum growth temperature is 15°C (ICMSF, 1996).

Growth and survival characteristics of *B. cereus* vary widely between strains and depend upon a complex series of interacting factors such as temperature, pH, water activity (NaCl concentration), nutrients and presence of competitive microbiota. *B. cereus*

is generally a poor competitor in unpasteurized products (Andersson, Ronner, & Granum, 1995). The high total bacterial count (ca. 6 log), combined with the decrease of pH values (from 6.49 to 5.63) observed over time, suggest the possible presence of contaminants from the whey or the environment that may have exerted a possible competitive action. A previous study, conducted on vacuum packed ricotta salata, showed a mean aerobic mesophilic bacteria count (log_{10} cfu g^{-1}) after 2 months and after 4 months of refrigerated storage ranging from 7.56±0.85 and 4.57±0.62 on the rind and from 3.64±0.71 and 2.95±0.65 on the inner paste, respectively (Spanu et al., 2013). At the beginning of the ricotta salata storage *B. cereus* is present mainly in its vegetative form, as consequence of heat activation of spores occurred during whey heating. The successive phases of pressing and salting of the warm clots, expose the product to temperature at risk for the growth of the vegetative forms to levels compatible with the emetic toxin (cerulide) production. The reduction in *B. cereus* vegetative cells count over the storage period suggests the death of the microorganism rather than sporulation, since no grow was observed in samples analyzed after pasteurization. The sporulation is a complex process which occurs as response to stress such as starvation, high cell density (10^6-10^7 cfu g^{-1})

or DNA damage and is regulated by hundreds of genes (Eichenberger et al., 2003; Piggot and Hilbert, 2004). Borge et al. (2001) concluded that vegetative cells are unlikely to develop endospores in refrigerated media.

The high levels of contamination observed in the present study indicates that ricotta salata may be a risk for human health due to the potential presence of *B. cereus* enterotoxins in the product. In fact, cerulide is highly resistant to heat, low pH, and proteolytic activity of pepsin and trypsin (Kramer and Gilbert, 1989; Rajkovic et al., 2008). The low contamination level observed in the product after long refrigerated storage could lead to the wrong conclusion that the product is safe, while cerulide still persists. More investigation is needed in order to assess whether the origin of the contamination is from ingredients, processing environment or from packaging materials and to determine the pathogenicity of the strains.

Conclusion

Ricotta salata production process includes critical phases such as heat coagulation and slow cooling of clots, which support the activation of *B. cereus* spores and the successive growth of vegetative cells, in the absence of competing microbiota. The

present investigation demonstrates that the level of *B. cereus* vegetative cells in naturally contaminated ricotta salata decreases during refrigerated storage, while the presence of spores is a rare finding. The control of *B. cereus* in ricotta salata relies on one hand on limiting the level of spores in raw milk, and as consequence in the whey, and on the other hand in preventing germination and successive growth of vegetative cells.

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Tables

Table 1. Evolution of *B. cereus* (log_{10} cfu g^{-1}; mean±SD) in nine batches of vacuum-packed Ricotta salata stored refrigerated until 90 days after production.

Batch	T0	T30	T60	T90
1	4.51±0.00[a] (n = 1/2)	2.79±0.79[a] (n = 2/2)	N.D. (n = 2/2)	N.D. (n = 2/2)
2	4.37±0.37a (n = 2/2)	5.04 ±0.00a (n = 1/2)	2.94±1.00a (n = 2/2)	N.D. (n = 2/2)
3	4.68±0.24a (n = 2/2)	4.49±0.20a (n = 2/2)	3.26±0.93ab (n = 2/2)	1.30±0.00b (n = 1/2)
4	4.50±0.33a (n = 2/2)	4.68±0.20a (n = 2/2)	2.15±0.00b (n = 1/2)	1.78±0.68b (n = 2/2)
5	4.66±0.35a (n = 2/2)	3.85±0.21a (n = 2/2)	N.D. (n = 2/2)	N.D. (n = 2/2)
6	4.88±0.00a (n = 1/2)	3.81±0.74a (n = 2/2)	3.45±0.16a (n = 2/2)	N.D. (n = 2/2)
7	4.0±0.00a (n = 1/2)	3.62±0.00b (n = 1/2)	2.38±0.00a (n = 2/2)	N.D. (n = 2/2)
8	6.19±0.14a (n = 2/2)	4.83±0.43b (n = 2/2)	3.78±0.00bc (n = 1/2)	2.56±0.00c (n = 1/2)
9	3.77±0.46ab (n = 2/2)	5.43±0.89b (n = 2/2)	2.46±1.67a (n = 2/2)	2.26±0.00a (n = 2/2)
total	4.65±0.74a (n =15/18)	4.27±0.90a (n =16/18)	2.91±0.84b (n =12/18)	1.99±0.55c (n =6/18)

The sampling time (T_0, T_{30}, T_{60} and T_{90}) were respectively the day of packaging and 30, 60 and 90 days after the production. Means in the same row with different superscript letters are significantly different ($P < 0.05$); values within brackets indicate the prevalence of positive samples. N.D = data not definable, below the detection limit of the method.

Table 2. Intrinsic properties (mean ± SD) and composition (% ± SD) evolution during storage of ricotta salata wheels.

	sampling times			
parameter	T_0	T_{30}	T_{60}	T_{90}
pH	6.49±0.10^{a}	6.18±0.10^{b}	5.73±0.14^{c}	5.63±0.28^{c}
aw	0.978±0.001^{ab}	0.976±0.002^{a}	0.976±0.002^{a}	0.980±0.001^{b}
% moisture	58.28±2.91^{a}	58.56±3.29^{a}	57.41±2.99^{ab}	56.23±2.55^{b}
% total solids	41.72±2.93^{a}	41.44±3.26^{a}	42.59±2.99^{ab}	43.77±2.49^{b}
% fat	23.74±3.92^{a}	23.13±4.28^{a}	23.32±3.73^{a}	23.39±3.86^{a}
% protein	12.65±1.12^{a}	12.83±1.16^{a}	13.01±0.84^{a}	13.36±1.14^{a}
% salt	2.60±0.30^{ab}	2.73±0.24^{b}	2.49±0.28^{c}	2.01±0.49^{d}

The sampling time (T0, T30, T60 and T90) were respectively the day of packaging and 30, 60 and 90 days after the production. Means in the same row with different superscript letters are significantly different ($P < 0.05$).

CHAPTER 5 – Production of farmstead lactose-free *Pecorino di Osilo* and *ricotta* cheese from sheep milk

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Abstract

The present work was aimed to define and validate farmstead production of lactose-free *Pecorino di Osilo* cheese, fresh Ricotta cheese, salted and smoked Ricotta cheese (*Ricotta mustia*). The enzymatic activity of the commercial preparation containing lactase (1.1 g/mL), preliminarily tested using a spectrophotometric titration, showed activity equal to 4,950±40 Neutral Lactase Units/g. The amount of lactase required to obtain the lactose-free milk was then established in triplicate laboratory trials, by adding the enzyme at concentrations of 0.7, 0.9 and 1.1 g/L in flasks containing 160 mL of raw sheep's milk. Samples were incubated under conditions expected during milk storage and cheese-making. The residual lactose content in milk was determined by enzymatic method. The addition of lactase at concentration of 1.1 g/L of milk reduced the lactose concentration below the limit of detection (0.06 g/L). The procedure was validated at a dairy farm, using three different batches of bulk raw sheep's lactose-free milk which were transformed into Pecorino di Osilo cheese. The resulting whey was used to produce fresh and Ricotta mustia cheese. Raw milk and whey samples were always below lactose detection limit. The residual lactose was measured in Pecorino di Osilo cheese, after 24

hours and 30 days from production; in fresh Ricotta cheese, after 48 hours; in Ricotta mustia cheese after 7 days. The determination of lactose content in cheese samples was conducted by a GC-FID method, which showed a LOD and limit of quantification (LOQ) respectively of 1.8 and 5.6 mg/kg for cheese, and 1.35 and 4.2 mg/kg for both ricotta cheeses. The lactose concentration was always below the relevant LOD values in all samples. The mean concentration of galactose and glucose were respectively 13,000±2,000 mg/Kg and 11,000±2,000 mg/Kg in fresh Pecorino di Osilo, 1,100±300 mg/Kg and 1,200±300 mg/Kg in fresh Ricotta and 950±400 mg/Kg and 750±250 mg/Kg in Ricotta mustia. The results of the present study showed that the production of farmstead lactose-free Pecorino di Osilo cheese and ricotta cheeses from raw sheep's milk is easily achievable. The main issue for farmstead production of artisanal lactose-free products is the implementation of permanent procedures based on HACCP principles aimed to guarantee the effectiveness of the process and to acquire analytical evidences to demonstrate the fulfilment of law requirements for labelling.

Introduction

Lactose, the main saccharide of milk, is composed of D-galactose and D-glucose joined by a β-1.4-glycosidic linkage. The intestinal absorption of lactose by the epithelium of the human colon needs the enzymatic hydrolysis into monosaccharides by β-galactosidase (EC 3.2.1.23). This enzyme is found most abundantly in the villus enterocytes of the jejunum (Lule et al, 2016). In mammals the lactase production progressively ceases after weaning and the adults lose their ability to digest lactose, while humans can keep lactase production also in adults (Jelen and Tossavainen, 2003). Hypolactasia, lactose maldigestion or, less properly, malabsorption, are synonyms indicating a deficiency in the lactase enzyme (Kies A.K., 2014). Lactose maldigesters are those human adults with a genetic deficiency in lactase production (primary adult hypolactasia). A large part of the human population is represented by lactase non-persistent people, as they undergone to a genetic programmed reduction in the lactase synthesis between 5 and 14 years of age, to level as low as 5-10% of the production observed in infants. Secondary lactase deficiency (or acquired hypolactasia) could also be caused by damage of the intestinal mucosa due to chemotherapy, acute viral or

bacterial infection and infestation by parasites. A rare congenital form (due to autosomal recessive disorder) and a developmental lactase deficiency, occurring in premature babies with less than 34 weeks of gestation, have been also described (Lule et al., 2016). Undigested lactose can be fermented by intestine microflora producing gaseous by-products like hydrogen, carbon dioxide and methane. In addition, the osmotic gradient created by the undigested lactose results in an influx of liquids which causes diarrhea. Clinical symptoms of lactose intolerance (LI) include abdominal pain, cramps, abdominal bloating, asthenia, loose stools or diarrhea (sometimes constipation), occurring after 1-2 hours from the ingestion of foods containing lactose (Beyerlein et al., 2008; Lule et al., 2016). Since LI depends by the amount of lactose ingested and the severity of symptoms varies from person to person, the dose of lactose necessary to cause illness cannot be defined. Symptoms of LI have been described after the ingestion of as little as 6 g of lactose, but it is generally necessary a single dose of 12 g to cause mild symptoms. If assumed distributed during the day, higher doses of lactose can be tolerated (EFSA, 2010). It was estimated that approximately 70% of the world's population produce low level of lactase and that the frequency of LI varies largely between geographical areas

and populations (Kies A.K., 2014, Lule et al., 2016). Casellas et al. (2010) demonstrated that symptoms experienced by patients after the ingestion of lactose-containing foods are associated with LI even with no support of a lactose malabsorption diagnosis.

Low-lactose and lactose-free products are obtained in the dairy industry by the hydrolysis of lactose in milk using the enzyme beta-galactosidase (lactase), by chromatographic separations or by use of membrane separation, that could be also combined with lactose hydrolysis techniques (Jelen and Tossavainen, 2003). The hydrolysis of lactose, obtained by means of free or immobilized lactase (Horner et al., 2011), can influence both the technological and the sensorial properties of products. Lactose hydrolysis could also results in transgalactosylation reactions, in which glucose and galactose released by hydrolysis served as galactosyl acceptors, producing oligosaccharides with prebiotic activity. Lactase can be of animal, plant and microbial origin. The latter is the most common and is generally obtained from fungi (A. niger, A. oryzae), yeast (Kluyveromyces lactis, Kluyveromyces marxianus, Saccharomyces fragilis) and bacteria (LAB, Bifidobacterium spp., Bacillus spp., E. coli) (Oliveira C. et al, 2011). The molecular weight, the amino-acids chain length, the position of the active

site, the pH and the temperature of optimal activity of beta-galactosidase could significantly vary when changing the origin of the enzyme (Mlichová et al., 2006). Lactase from bacteria and yeasts are the most commonly used in the dairy industry since they have the optimal activity at pH between 6 and 7. The use of beta-galactosidase from fungal origin, which shows the highest activity at pH between 3 and 4, is generally associated to hydrolysis in acid milk and cream (Chen et al., 2009). Mesophilic lactase show the highest activity at ca. 30 °C, while thermostable lactases are effective until 50-65 °C and temperature < 15°C are needed for cold-active lactases (Mlichová et al., 2006; Horner et al., 2011).

The EU regulatory framework governing lactose-free foods (or low lactose foods) has just been changed. On July 20th 2016 entered in force the Regulation (EU) No 609/2013 on food intended for infants and young children, food for special medical purposes and total diet replacement for weight control. It has repealed the Directive 2009/39/EC of the European Parliament on foodstuffs intended for particular nutritional uses. On the other hand, the lactose-free product labelling is ruled by art. 36 (voluntary food information) of Regulation (EU) No 1169/2011 on the provision of food information

to consumers. Waiting for a complete harmonization of the laws concerning this topic in the European Union, in Italy is possible labelling a food as “lactose-free” if its lactose amount is <0.1g/100 g or mL, whereas the label indicating “low in lactose” is admitted for fluid and fermented milk when the lactose concentration is less than 0.5 g/100 g or mL (Health Ministry, 2015). These values are referred also to a “naturally lactose-free” or a “naturally low in lactose” indications, that characterize dairy product in which the lactose is naturally hydrolyzed during the manufacturing process (e.g. cheeses with extended ripening) (Health Ministry, 2016a). An approval process for dairy industries that produce products without lactose is no longer required by the Competent Authority as before, but the adoption of a specific HACCP procedure by Food Business Operators is considered advisable (Health Ministry, 2016b). The increase of the consumer’s awareness about food allergy and intolerance drove the interest and continuous growth of lactose-free products on the market. The overall global demand of lactose-free milk and dairy products showed an increasing trend over the last years, with + 8% in USA market in 2015 (Baroke S., 2015). As a consequence, the dairy industry have expanded the array of low-lactose or lactose-free product available, previously limited only to fluid milk,

yogurt, cheeses, whey-products, butter and ice-creams. In this context, also the artisanal cheese making has introduced the lactose free milk into the traditional process of milk transformation.

The island of Sardinia (Italy) is one the most productive areas for sheep's milk, accounting for about 66% of total Italian production (ISTAT, 2015). Almost all the Sardinian production is transformed into cheeses and other valuable niche dairy products. Pecorino di Osilo is one of the most renowned sheep cheeses of Sardinia. It is a raw or semi-cooked sheep cheese, available in different versions depending from the ripening duration (from 2-3 weeks to 6 months). The fresh Ricotta cheese is a sheep cheese made from whey resulting from the Pecorino cheeses production. Sheep's whey, sometimes added of fresh, raw milk or cream, is heated at 80-82°C to obtain the denaturation of globulins and albumins, which coagulate in a fine curd. Curd is separated by the liquid by means of perforated scoop or filtering the mixture with a fine linen cloth, which retains it. The Ricotta mustia cheese is pressed, salted, dried and then light smoked. Pecorino, fresh Ricotta cheese and Ricotta mustia cheese are also included in the list of traditional Italian food products (MIPAAF, 2016). The present research was carried out in the frame

of a project aimed to obtain lactose-free products, in cooperation with a Sardinian farmstead cheesemaker. Commercial perspectives for assessing the "lactose-free version" of these products are related to the opportunity of selling Pecorino di Osilo cheese just after a short ripening for cooking or ricotta cheeses for direct consumption and, only for the fresh one, for bakery or filled pasta producers.

Hence, the principal objectives of the work were: a) to establish the lactase amount able to obtain lactose-free raw sheep's milk in farmstead production conditions; b) to validate the whole production process of Pecorino di Osilo cheese, fresh Ricotta and Ricotta mustia cheeses obtained from lactose-free sheep's milk and whey.

Materials and methods

Commercial β-galactosidase enzyme

The commercially available β-galactosidase enzyme HA-Lactase (Chr. Hansen, Hoersholm, Denmark) produced by Kluyveromyces lactis was used in the present study. According to supplier indication, the enzymatic concentration of HA-Lactase is 1.1g/mL, with an activity level of 5,200 Neutral Lactase Unit (NLU)/g. The recommended dosage for the enzyme in milk is between 500 and 4,000 NLU/L, and the optimal hydrolytic

activity is ensured working at temperature between 35 and 45 °C and a pH value between 6 and 7.

Enzymatic hydrolysis of lactose in sheep's milk

The conditions of enzymatic hydrolysis of lactose in sheep's milk were preliminarily assessed in a laboratory scale. Here, the compatibility of the principal variables of process (i.e. the concentration of lactase, the temperature of milk and its time of exposition to lactase) has been optimized on the basis of the needs of farmstead cheesemaker during the phases of milk storage and cheese making process. A laboratory trial was planned considering that the Pecorino di Osilo cheese is typically produced with the milk harvested from two consecutive milking (morning milking and evening milking). The production procedure at the farm prescribes that the amount of lactase needed to hydrolyze the lactose of the entire milk daily production is added immediately after the morning milking. The daily milk production is estimated on the average amount of the previous week and, if necessary, during the evening milking the lactase is eventually adjusted to the effective milk yield.

The laboratory trial was carried out adding decreasing amount of commercial lactase to a first aliquot of 80 mL of refrigerated raw sheep milk in 250 mL glass flasks. After 12 h (time T12), the second 80 mL-aliquot of the refrigerated raw sheep was added in each flask. The lactase was added to the milk (two aliquots, 160 mL) to reach the final concentrations, respectively, of 1.1 g/L (lactase amount 160 μL), 0.9 g/L (130.9 μL) and 0.7 g/L (101.8 μL). Finally, all flasks were first stored at 8 °C for further 12 hours (time T24), then they were heated and held at 35 °C for 65 min before the analysis, in order to simulate conditions adopted during the cheese-making. The experiment was carried out in triplicate for each lactase concentration. Three flasks containing each 160 mL of untreated raw milk were used as controls.

Technology of production of Lactose-free Pecorino di Osilo cheese.

The farmstead Pecorino di Osilo sheep cheese was produced in a local farm from April to May 2015. The raw milk collected during morning milking was stored in a refrigerated tank at 8 °C. According to the laboratory protocol, all the lactase (35 mL) necessary for the estimated full daily milk production (350 L) was added just after the morning milking to obtain an enzyme concentration of 1.1 g/L (as determined with the

preliminary assay). After 12 hours, the milk of the evening milking was added in the same tank, and the milk was stored for further 12 hours. Cheese production was made in a tinned copper cauldron. Here, a portion of the raw delactosed milk (315 L) was pre-heated at 35 °C and inoculated with a starter culture, then coagulated with 5 g/100L of calf rennet powder (Caglificio Clerici spa, Italy). The clotting takes place in 20 min followed by curd firming of approximately 12 min. The curd is cut into rice-grain sized particles and heated at 42-43 °C for ca. 15 min. Curds are molded and held in a warm chamber (42-43 °C for 3-4 h) for the acidification. Cheese is salted for 24 h in saturated sodium chloride brine at 10-12 °C; in this timelapse, NaCl is added to the rising surface of the wheel. Ripening was conducted in cave at constant temperature between 12 and 14 °C for a period between 20 days and 4-6 months. Cheese wheels were periodically turned over and spread on the surface with a mixture of olive oil and vinegar. The average weight of each cheese wheel was of 1.7 Kg.

Technology of production of lactose-free fresh Ricotta and Ricotta mustia cheeses

The whey remaining after the production of cheese (230 L) is added with sodium chloride (25 g), heated in large open kettles and stirred at a low speed. When the

temperature rises up to 55-60 °C the whey is blended with 35 L of lactose free raw milk per kettle. Heating of the whey/milk mixture is continued to 78-80 °C when the curd starts floating; after this point, heating was held for few min. Curds are scooped with perforated ladles and transferred into polyethylene perforated baskets of conical-truncated shape, allowed to cool for ca. 4 hours then refrigerated at 4 °C. The average weight of each basket of fresh Ricotta was ca. 0.4 Kg.

The production of Ricotta mustia cheese differs from fresh Ricotta cheese only in the use, unlike of the baskets, of perforated polyethylene cylindrical molds in order to better drain the curd. After ca. 60 min the curd is extracted, the molds are lined with cheese cloths and the curds put back into the molds and cooled at 4 °C for about 5 hours. The curd is then pressed for 8-10 hours with the use of a wooden cheese press. Ricotta is then removed from cloths and is dry salted for 12 hours and exposed to smoke of wood for 3 hours within a traditional smoking chamber. The Ricotta wheels are dried with natural air flow and stored at 4 °C and immediately marketed or ripened. The average weight of each Ricotta mustia wheel was 1.0 Kg.

Analysis

Determination of the β-galactosidase activity

The activity of the commercial β-galactosidase enzyme was assayed using the official spectrophotometric method proposed by AOAC (AOAC, 1998). In the presence of lactase, the colorless o-nitrophenyl-β-D-galactopyranoside (ONPG) is hydrolyzed into galactose and o-nitrophenol (ONP), a yellow compound (Miller, 1972). In this way the enzymatic activity is obtained by measuring the absorbance of the ONP chromophore. In particular, 1 NLU is the quantity of enzyme that liberates 1.30 μM ONP under assay conditions (AOAC, 1998).

The β-galactosidase was dissolved in a buffered solution (0.2 M in K3PO4, 0.1 mM in C10H14N2Na2O8.2H2O, 0.1 mM in MgCl2; pH 6.50±0.05; acronym: PEM) to obtain a working solution with an enzymatic activity of 0.075±0.010 NLU/mL. Test tubes were prepared in triplicate mixing 1 mL of this working solution and an aqueous solution of o-nitrophenyl-β-D-galactopyranoside, (5 mL, 5.2 μM/mL). This mixture was hence dispensed into bovine serum albumin (1% solution in PEM) coated polystyrene tubes. Tubes were first placed in a thermostatic shaking water bath kept at 30±1°C for 10 min

at 250 rpm, and then 2 mL of 1 M aqueous solution of Na2CO3 were added in order to stop the reactions. ONP absorbance was read at 420 nm on a spectrophotometer Shimadzu UV-1700. Calibration was conducted using standard solutions of ONP in the concentration range between 0.02 and 0.14 μM/mL. The excellent linearity of the method was proved by the high values of the correlation coefficient (R2), typically over 0.99. Finally, the enzymatic activity was computed using the following equation:

$$NLU/g = (A \times 8 \times f)/(4.65 \times 10 \times 1.30)$$

where A is the absorbance, corrected for test blank; 8 is the volume (mL) of incubation mixture after termination; f is the total dilution factor of test solution; a is the absorptivity (4.65); 10 is the incubation time (min) and 1.30 is the factor used in NLU definition.

2.2.2 Analytical characterization of sheep's milk

The determination of proteins, fats, lactose and pH in raw bulk milk samples was conducted using Fourier transform infrared spectrometry (MilkoScan 6000, Fosselectric, Hillerød, Denmark). Calibration was developed according to ISO 9622:2013/IDF 141:2013 (ISO, 2013). Somatic cell count was performed by flow cytometry counter (Fossomatic 5000 Fosselectric, Hillerød, Denmark) according to ISO

13366:2006/IDF148:2006 (ISO, 2006). Total bacterial count was conducted by automatic flow cytometry determination method using Bactoscan FC (Fosselectric, Hillerød, Denmark). In delactosed milk samples, the determination of residual lactose content was performed by enzymatic method using differential pH measurement according to ISO 26462:2010 (IDF 214:2010).

Analytical characterization of farmstead lactose-free sheep's cheeses.

Pecorino di Osilo cheese samples were analyzed after 30 days from production, fresh Ricotta after 48 h whereas Ricotta mustia 2 and 7 days after production. Three samples for each batch of production were analyzed. The determination of proteins, fats, moisture and NaCl was conducted by Near-infrared reflectance spectroscopy (NIRS) (FoodScan Lab, FossElectric, Hillerød, Denmark). The pH was determined with potentiometric measurement (GLP 22, Crison Instruments, Alella, Spain). Determination of aW was conducted using Aqua Lab (DecagonDevices, Inc., Pullman, USA).

GC-FID determination of lactose, glucose and galactose in farmstead lactose-free sheep's cheeses.

All samples were kept in the dark and stored at +4°C until analysis, which has been performed after 24 hours from the production for Pecorino di Osilo cheese, after 2 days from the production for fresh Ricotta cheese and after 7 days from production for Ricotta mustia cheese. The reagents used were all analytical grade. D-lactose, D-glucose, D-galactose, methyl-α-D-galactopyranoside, pyridine, heptane, trimethylsilylimidazole (TMSI), trimethylchlorosilane (TMCS), methanol and ethanol were purchased from Sigma Aldrich (Milan Italy). Ultrapure (Type 1) water (specific resistance 18 MΩ) was always used throughout the analyses. Due the complexity of the matrix, a proper pretreatment of sample was needed to remove proteins, which have to be separated from saccharides before their derivatization. In close analogy to what experienced (Idda et al., 2016; Troyano et al., 1991; Troyano et al., 1994; Troyano et al., 1996), the proteins were separated from analytes by precipitation with lower alcohols such as methanol or ethanol. An appropriate choice of the nature of the alcohol and of the amount of the sample (0.5000 and 10.000 g, respectively) allowed us to ensure flexibility to the method here proposed.

Below are reported the proposed procedures of sample pretreatment used respectively for Pecorino di Osilo cheese and Ricotta.

Sample pretreatment for Pecorino di Osilo sheep's cheese.

10g of Pecorino di Osilo cheese were mixed with 0,500 mL of a 1 % (w/v) internal standard aqueous solution and suspended into 25mL of a 40% (v/v) methanol/water solution. The mixture was first homogenized for 10 minutes and then sonicated at 42°C for 10 minutes into an ultrasonic bath. The mixture was then centrifuged at 2,000 rpm for 15 min and the supernatant was separated by the residue which was treated again with another 25mL aliquot of a 40% (v/v) methanol/water solution, according to the procedure described above. Both the obtained solutions were collected in a 50-mL volumetric flask and a pure 40% (v/v) methanol/water solution was added to a final volume of 50 mL. Later, a 2 mL aliquot of this solution was diluted with pure methanol up to 25mL, the solution was centrifuged at 2,000 rpm for 15 min, the supernatant was dried under vacuum at 50°C and the residue was dissolved in 1.2 mL of anhydrous pyridine.

Sample pretreatment for Fresh Ricotta and Ricotta mustia sheep's cheeses

0.5g of Ricotta were mixed with 0.1 mL of a 0.05% (w/v) internal standard ethanolic solution and diluted to 10 mL with ethanol; after 1 hour at room temperature the mixture was centrifuged at 2,000 rpm for 15 min; 8 mL of the supernatant were dried under vacuum at 50°C and the residue was dissolved in 0.8 mL of anhydrous pyridine.

Samples derivatization

0.1 mL of pyridine solution obtained after the sample pretreatment step described in 2.7.1.1 or in 2.7.1.2 was kept at 0°C for 30 minutes, then it was sequentially treated with 0.1 mL of a mixture 2:1 (v/v) of TMSI/TMCS, 0.1 mL of heptane and 0.2 mL of water. Finally, 1 µL of this solution was injected into the GC column. Each sample was analyzed at least in duplicate.

GC instrumental conditions

Gas chromatographic analysis was performed on a Hewlett–Packard 5890 Series II Gas Chromatograph (Agilent Technologies, Milan Italy) equipped with a Flame Ionization Detector. Injector and detector temperatures were 300°C. Chromatographic separation was carried out in a Supelco Low Bleed SLBTM-5ms, 30 m x 0.32 mm x 0.25

μm capillary column by using Nitrogen as carrier gas. Oven was held at 50°C for 2 min, then it was heated first at 10°C/min up to 130°C and then at 5°C/min up to 300°C. Temperature of the oven was held at 300°C for 5 min, and then it was raised at 30°C/min up to 360°C. This final temperature was held for 5 min. Data were elaborated using a HP ChemStation software (Agilent Technologies, Milan Italy).

LOD and LOQ values of lactose

LOD and LOQ for lactose have been evaluated according literature methods (Ribani et al., 2007). For Ricotta samples, LOD is 1.8 mg/Kg, whereas LOQ is 5.6 mg/Kg. The LOD and LOQ values for Pecorino di Osilo were 1.35 mg/Kg and 4.2 mg/Kg, respectively

Quantification of glucose and galactose

Quantification of glucose and galactose was carried out in two samples for each batch of Pecorino di Osilo cheese, fresh Ricotta cheese and Ricotta mustia cheese. Attribution of the chromatographic peaks of glucose and galactose in cheese samples were obtained both by comparing the retention times and spiking the peaks with a

standard solution containing known amounts of analyte of interest. For each analyte, analytical data are reported as sum of concentrations of all anomers.

Results

The hydrolysis of ONPG showed a β-galactosidase activity of 4,950±40 NLU/g which was slightly greater than the minimal activity (4,800 NLU/g) indicated by the producer. Table 1 reports the gross composition (proteins, fats, lactose), somatic cells and total bacterial count of the untreated raw sheep's milk used for the study (negative control) and of milk treated with different amounts of lactase. As expected, the lactose concentration decreased according to the increase of lactase concentration added to raw milk. The addition of lactase at concentration of 0.7 g/L reduced the lactose concentration in milk of ca. 97% while a concentration of lactase of 1.1 g/L was needed in order to reduce the concentration of lactose below its LOD. The highest lactase concentration (1.1 g/L) was then chosen to accomplish all farmstead productions. In table 2 is reported milk characterization, including also the pH value, carried out on raw sheep's milk used in each of the three experimental cheesemaking processes. No significant change in the milk

proteins composition and pH was observed between batches while an increase in fat content was observed from batch 1 to batch 3.

In Table 3 are reported the results of physico-chemical and composition analysis on Pecorino di Osilo cheese, fresh Ricotta and Ricotta mustia cheeses. Table 4 shows the amounts of lactose, galactose and glucose found in fresh Pecorino di Osilo cheese.

Discussion

The average lactose content in raw in sheep milk (4.5-5.0 g/L) is usually slightly higher than reported in literature for cow's milk (4.6-4.7 g/L) (Cosseddu et al., 2008). The amount of lactose in sheep's milk is generally constant in mid lactation and it is not expected to affect in this period the standardization of the production of free-lactose products, while a reduction of its content occurs in colostrum and in the end of the lactation (Haenlein and Wendorff, 2006). However, the production of farmstead cheese and ricotta cheese using lactose-free raw milk should take into account structural and management practices limitation, especially of lactase use in raw milk. A structural limitation is represented by the availability of milk storage equipment which, in most of the sheep dairy farms, are represented exclusively by the refrigerated tank. One of the

most representative issues of this research was the control of the temperature of sheep's milk before processing. The optimal temperature of milk storage is the compromise between the opposite needs of limit the growth of both psychrotrophic and pathogenic bacteria and preserving the activity of lactase, taking also into account regulatory provisions. The prolonged refrigeration of milk before transformation supports the possible growth of psychrotrophic bacterium which negatively affects cheese yield (de Garnica, 2011; Maciel et al., 2015). Hence, the temperature of 8 °C for milk storing was selected mainly in order to limit the growth of psychrotrophic microflora and to preserve the activity of lactase. The lactase of K. lactis source shows an increase in the activity of 85-95% at 38 °C as compared to that measured at 4-5°C (Mlichová et al., 2006; Horner et al., 2011). In the cheese making process the increase of temperature during milk warming (35 °C) and the cooking of curd (42 °C), allow the complete hydrolysis of the residual lactose before that the curd acidification could limit the activity of the lactase, when pH drops below 5.5. Laboratory experimentation demonstrated that a lactase concentration of 1.1 g/L is needed to almost completely hydrolyze the lactose in sheep's milk, reducing its concentration below the LOD of the enzymatic method (0.06 g/100

ml). The high concentration of lactase needed was probably due to the specific operative conditions rather than the use of raw milk or the particular lactose concentration in sheep milk. In cow's milk, no differences have been observed in lactase activity in relation to the type of milk (raw or pasteurized) or in relation to milk fat content (Horner et al., 2011). With the aim to preserve as much as possible all organoleptic and quality features of the considered artisanal cheeses, the only modification here proposed in the manufacturing of lactose-free variants of Pecorino di Osilo, fresh Ricotta and Ricotta mustia cheeses, is the replacement of raw sheep's milk with the lactose-free sheep's milk. For evaluating traces of lactose in these cheeses, a sensitive GC-FID method specifically devoted to accurately measure lactose and its hydrolysis products (i.e. galactose and glucose) has been developed and used. The lactose LOD value in Pecorino di Osilo cheese was 1.35 mg/Kg, whereas t in Ricotta cheeses was 1.8 mg/Kg (i.e. more than two orders of magnitude less than LOD with the enzymatic method). Lactose was measured after 24 hours and 30 days from its production in Pecorino di Osilo cheese, after 2 days from the date of production in fresh Ricotta and after 7 days from production in Ricotta mustia. All samples analyzed showed always a lactose concentration below the relevant LOD. In

addition, the GC-FID method has allowed us to evaluate the amounts of galactose and glucose in all samples within the first seven days after production. The concentrations of monosaccharides found in the three dairy products considered in this study are rather different among them. Galactose and glucose are ten time more abundant in fresh Pecorino di Osilo cheese than in Ricotta cheeses. The amounts of monosaccharides found in fresh cheese are coherent with the amount contained in the whey retained in this cheese. In addition, the very short ripening time elapsed from production has probably prevented the further degradation of these compounds. The average concentration of galactose and glucose in fresh Pecorino di Osilo is 13,000±2,000 mg/Kg and 11,000±2,000 mg/Kg, respectively.

However, the high variability of these data does not allow us to find a statistically significant difference. In fresh Ricotta, the concentration of galactose and glucose was respectively 1,100±300 mg/Kg and 1,200±300 mg/Kg, whereas in Ricotta mustia were 950±400 mg/Kg and 750±250 mg/Kg. The overall quantity of monosaccharides observed in fresh Ricotta is ca. 35% higher than in Ricotta mustia. Glucose is the monosaccharide that prevails in the fresh Ricotta, while galactose is more abundant than glucose in Ricotta

mustia (P> 0.05). The amount of galactose and glucose found in both Ricotta cheeses are much lower than those expected on the basis of the high amount in the whey remaining after ricotta cheese production. It is evident that in milk whey and then in both of Ricotta cheeses some degradation phenomena occurred and they are active in reducing the concentrations of these monosaccharides.

Finally, our results demonstrated that the residual lactose in the Pecorino di Osilo cheese, in the fresh Ricotta and in the Ricotta mustia are always below the relevant detection limits calculated for the GC-FID method. It should be noted that the very low LOD values which characterized the chromatographic method used to attempt the quantification of lactose in dairy products, are the best guarantee for claiming lactose-free the Pecorino di Osilo cheese, the fresh Ricotta cheese and the Ricotta mustia cheese, because they contains a lactose content less than 1/500 the limit of 100 mg/100 g fixed for this purpose by the Italian law (Health Ministry, 2015).

Conclusions

In the present work were defined the conditions of use of lactase for the artisanal production of raw milk sheep cheese and ricotta. The technology of production of traditional Pecorino di Osilo, fresh and Ricotta mustia cheeses were applied in an experimental trial conducted in a farmstead cheese making facility in order to obtain lactose-free products. The results obtained showed the feasibility of obtaining lactose free product at artisanal level. The major issues for farmstead cheese-makers are to develop adequate procedures, in the frame of their HACCP plan, to guarantee the effectiveness of the process and to acquire analytical data to demonstrate in the final product the compliance of the residual lactose concentrations with the legislation requirements for the lactose-free labelling.

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Tables

Table 1. Mean (±SD) macro constituents of raw sheep's milk lactose, somatic cell count (SCC), total bacterial count (TBC) of raw sheep milk with no addition of lactase (Negative Control) and with the addition of 0.7, 0.9 and 1.1 g/L of lactase.

Milk samples	Proteins g/100mL	Fat g/100mL	Lactose g/100mL	SCC Log_{10}/mL	TBC Log10 cfu/mL
Negative control[1]	5.49±0.00	6.39 ± 0.01	5.01 ± 0.02	5.90 ± 0.00	4.74 ± 0.02
0.7 g/L	5.66±0.01	6.43 ± 0.01	0.16 ± 0.05	5.90 ± 0.01	4.68 ± 0.02
0.9 g/L	5.67 ± 0.01	6.44 ± 0.01	0.07 ± 0.03	5.90 ± 0.02	4.68 ± 0.02
1.1 g/L	5.68 ± 0.01	6.43 ± 0.01	<0.06	5.89 ± 0.01	4.70 ± 0.04

Table 2. Gross composition, lactose, somatic cell count (SCC), total bacterial count (TBC) and pH of refrigerated bulk tank raw sheep milk samples added with 1.1 g/L of lactase

Batch	Proteins g/100mL	Fat g/100mL	lactose g/100mL	SCC Log_{10}/mL	TBC Log_{10}/mL	pH
1	5.52	5.70	<0.06	5.80	4.81	6.69
2	5.48	5.90	< 0.06	5.77	4.67	6.69
3	5.55	6.21	< 0.06	5.89	5.59	6.67

Table 3. Physico-chemical composition of "Pecorino di Osilo cheese" (30 days of ripening), fresh Ricotta cheese (48 hours after production) and Ricotta mustia cheese (7 days after production).

	Pecorino di Osilo			Fresh ricotta			Ricotta mustia		
parameter/batch	1	2	3	1	2	3	1	2	3
pH	5.30 ± 0.03	5.14 ± 0.01	5.13 ± 0.01	6.54 ± 0.02	6.50 ± 0.02	6.65 ± 0.03	5.4 ± 0.3	5.8 ± 0.2	6.0 ± 0.3
a_w	0.97 ± 0.00	0.97 ± 0.00	0.96 ± 0.00	0.990 ± 0.005	0.988 ± 0.006	0.988 ± 0.006	0.970 ± 0.004	0.972 ± 0.003	0.976 ± 0.002
moisture (g/100g)	37.05 ± 0.20	37.6 ± 0.3	37.1 ± 0.5	75 ± 1.0	76 ± 2.0	73 ± 2.0	65 ± 1.0	62 ± 1.0	64 ± 1.0
fat (g/100g)	30.35 ± 0.15	30.5 ± 0.2	31.4 ± 0.5	12.7 ± 0.4	12 ± 2.0	12 ± 1.0	15 ± 1.0	19 ± 0.1	18.3 ± 0.7
proteins (g/100g)	30.0 ± 0.2	29.05 ± 0.15	29.0 ± 0.3	9.2 ± 0.4	8.8 ± 0.1	9.5 ± 0.4	13.4 ± 0.4	12.9 ± 0.3	12.7 ± 0.4
lactose (mg/Kg)	< 1.35[1]	< 1.35[1]	< 1.35[1]	<1.8[1]	<1.8[1]	<1.8[1]	<1.8[1]	<1.8[1]	<1.8[1]
NaCl (g/100g)	1.31 ± 0.01	1.35 ± 0.04	1.41 ± 0.03	-	-	-	1.31 ± 0.01	1.35 ± 0.04	1.41 ± 0.03

1: < LOD

Table 4. Average amounts of lactose, galactose and glucose in sheep's cheese produced by delactosed milk (Analyses performed by means of the GC-FID method).

	Samples n.	Ripening time (days)	Lactose (mg/Kg)	Galactose[2] (mg/Kg ± SD)	Glucose[2] (mg/Kg ±SD)	Galactose/ glucose ratio
Fresh ricotta	6	2	<1.8[1]	1,100 ± 300	1,200 ± 300	0.92
Ricotta *mustia*	6	7	<1.8[1]	950 ± 400	750 ± 250	1.27
Pecorino di Osilo	6	0	<1.35[1]	13,000 ± 2000	11,000 ± 2000	1.18

1 LOD value for the considered matrix; 2 expressed as sum of anomers

CHAPTER 6 - Testing commercial biopreservative against spoilage microorganisms in map packed ricotta *fresca* cheese.

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Abstract

Ricotta *fresca* cheese is susceptible to secondary contamination and is able to support the growth of pathogens or spoilage psychotrophic bacteria during storage. The aim of the present study was to evaluate which among three commercial biopreservatives was suitable to be used to control the growth of spoilage microrganisms in sheep's milk MAP ricotta *fresca* cheese. 144 Ricotta *fresca* cheese samples were inoculated either with the bioprotective culture Lyofast FPR 2 (including *Enterococcus faecium*, *Lactobacillus plantarum* e *Lactobacillus rhamnosus*) or Lyofast CNBAL (*Carnobacterium* spp) or the fermentate MicroGARD 430. Not inoculated control and experimental ricotta were MAP packed (30% CO_2 and 70% N_2) and stored at 4°C. Triplicate samples were analyzed after 5 h and 7, 14 and 21 days after inoculation for total bacterial count, mesophilic lactic acid bacteria, *Enterobacteriaceae*, *Pseudomonas* spp, *Listeria monocytogenes*, moulds and yeasts. Among the tested biopreservatives only *Carnobacterium* spp was able to control *Pseudomonas* spp and *Enterobacteriaceae*. The maximum reduction in the concentration of *Pseudomonas* spp and *Enterobacteriaceae* was respectively 1.93 and 2.66 $\log_{10}$ cfu/g,

observed 14 days after production. Therefore, *Carnobacterium* spp was selected as the culture of choice to conduct a challenge study against *Pseudomonas* spp.

Introduction

Ricotta fresca is a traditional whey cheese produced by heat coagulation of sheep's milk whey. In Sardinia (Italy) it is generally manufactured from the whey remaining after the production of hard semi-cooked cheeses (Pecorino Romano PDO and Pecorino Sardo PDO). The industrial production follows the traditional batch production process (Pala *et al.*, 2016). *Ricotta fresca* intended for large-scale retail are commercialized in modified atmosphere packaging (MAP), under refrigeration temperature, with a shelf life, determined under the responsibility of the Food Business Operator, varying from 14 up to 21 days. The batch production process exposes *Ricotta fresca* to post-process contamination originating from the dairy plant environment (Greenwood *et al.,* 1991). Due to its naturally poor competitive microflora (Pintado *et al.,* 2002), to its composition, inherent physical and chemical properties and the absence of preservatives, *Ricotta fresca* is an excellent substrate for the growth of pathogens or spoilage psychotropic bacteria during refrigerated storage. Psychotropic microorganisms in refrigerated whey cheeses are mainly represented by *Pseudomonas* spp, yeasts, molds

and *Enterobacteriaceae* (Pintado *et al.,* 2001; De Santis and Mazzette, 2002; Pala *et al.,* 2016).

The improvement of the hygiene management procedures is a measure that could only reduce the level of initial contamination of ricotta surface. Therefore, the use of bio preservatives (i.e. nisin, other bacteriocins, fermentates or bioprotective cultures), alone or combined with other treatments, has been proposed to compete with contaminants and to preserve the quality and safety of dairy products and other foods (Sobrino-López & Martín-Belloso, 2008; Elsser-Gravesen, & Elsser-Gravesen, 2013). Shelf life extension of whey cheeses using bio preservatives have been previously tested against *Listeria monocytogenes* (Davies et al., 1997; Samelis et al., 2003; Martins et al., 2010). However, to date no available studies investigated the use of biopreservatives against psychotropic spoilage microorganism in sheep ricotta cheese.

The present study was conducted as a preliminary investigation to assess the potential use of biopreservatives to control the growth of spoilage microorganism on the surface of MAP *ricotta fresca* during refrigerated storage. The main objective of the present study was to select which commercial biopreservative, among those available on

the market, presents the best adaptation to *ricotta fresca* substrate and is able to control the growth of psychotropic microorganisms. The biopreservative of choice will be used for a successive challenge study against *Pseudomonas* spp.

Materials and methods

Biopreservatives

The protective cultures and the fermentate were selected, among available products on the market, based on the proven activity against spoilage and pathogen microorganisms, their ability to grow at refrigeration temperature and the low development of acidity and aroma in the product. Of the two commercial protective cultures tested, one was Lyofast FPR 2 (Clerici-Sacco Group, Como, Italy) consisting of bacteriocins producing *Enterococcus faecium*, *Lactobacillus plantarum* and *Lactobacillus rhamnosus* in the ratio 1:1:1 with an optimum growth temperature of 37 °C (range between 4°C and 48 °C). The second was Lyofast CNBAL (Clerici-Sacco Group, Como, Italy) consisting of a selected strain of *Carnobacterium spp* producing bacteriocins with an optimum growth temperature between 25-45 °C. Both are lyophilized protective cultures indicated for surface treatment of cheeses to inhibit unwanted bacteria, including

also *Listeria* spp. The fermentate, the MicroGARD 430 (Danisco, New Century, KS, USA), a microbial fermentation complex obtained through the fermentation of milk by *Propionibacterium shermanii*, is a cultured grade A non-fat dry milk powder. The antimicrobial activity derives from the production of diacetyl, lactic, acetic and propionic acids produced during fermentation (Stasweski and Jagus, 2008). This type of fermentate has been successfully used to control spoilage and pathogenic microorganisms in cottage cheese and other dairy products (Al-Zoreky et al., 1991).

2.2. Samples

144 Ricotta *fresca* cheese samples were obtained from a local industrial sheep cheese making plant. Ricotta *fresca* were truncated cone shaped (ca.7.5 cm wide at the top and ca. 5.5 cm wide at the bottom) weighing approximately 1.1 kg. 48 ricotta *fresca* samples were randomly selected from each of three different batches (each batch was manufactured in a different day of production). The day after production ricotta fresca samples were packed in rigid polypropylene trays sealed with high-barrier peelable laminated films. Films were made of bioriented polyamide and cast polypropylene with O_2 T.R. (20°- 65 % R.H.) of ~ 30 cc/m2, 24h. Samples were transported refrigerated to

the laboratory. Culture one samples (FRP) were ricotta *fresca* treated with Lyofast FPR 2, culture two samples (CNBAL) were ricotta *fresca* treated with Lyofast CNBAL and Fermentate samples (FERM) were ricotta *fresca* treated with MicroGARD 430. Blank samples (BS) were untreated ricotta *fresca*. According to manufacture's instruction protective cultures were individually rehydrated by dilution in distilled water immediately before their use to a final concentration of 10^6 cfu mL^{-1} (confirmed by count on agar plates) while the fermentate was resuspended in distilled water in order of 0.5-1% of the samples weight. The surface area of Ricotta *fresca* samples to treat was estimated in ca. 292 cm^2, corresponding to ca. 30 g. After the removal of the film 2.5 mL of Lyofast FPR 2 and Lyofast CNBAL were sprayed respectively on the surface of FPR and CNBAL samples and 4 mL of MicroGARD 430 final suspension distributed on the surface of FERM samples. Each inoculum was evenly sprayed on the upper exposed surface of *Ricotta fresca cheese* samples, and repacked in MAP (30% CO_2 and 70% N_2) using the FP Basic Sec tray sealer (Ilpra, Vigevano, Italy). The experimental design describing sample units, testing times and related analysis is summarized in table 1.

2.3. Microbiological profile intrinsic properties and composition analysis

For each batch, triplicate samples of ricotta *fresca* were analyzed for the determination of microbiological profile, intrinsic properties and composition 5 h (T_0), 7, 14 and 21 days (T_7, T_{14}, T_{21}) after the addition of the biopreservatives. The preparation of the initial suspension and decimal dilution for microbiological examination was conducted according to ISO 6887-1:1999. Briefly, 25 g of samples were aseptically collected from ricotta surface and weighted into a sterile plastic stomacher bag. After the addition of 225 mL of Buffered Peptone Water were homogenized using a stomacher. Transfer of 1 mL of the initial suspension into a tube containing 9 mL of sterile diluent was performed to obtain the 10^{-2} dilution. If required, these operations were repeated using the 10^{-2} to obtain further serial decimal dilution. For the enumeration of aerobic mesophilic bacteria, mesophilic lactic acid bacteria and *Enterobacteriaceae*, yeasts and moulds the pour-plating procedure was used. Briefly, 1 mL of each decimal dilution was aseptically transferred into sterile Petri dishes. Then, 12 to 15 ml of the appropriate medium were poured at 44-47°C into each Petri dish. After complete solidification of Plate Count Agar and MRS medium at pH 5.7 (Biolife, Milan, Italy), respectively for the

enumeration of aerobic mesophilic bacteria (ISO 4833:2003) and mesophilic lactic acid bacteria (ISO 15214: 1998), plates were incubated at 30°C ± 1°C for 72 h ± 3 h. For the enumeration of *Enterobacteriaceae* the plate count technique without resuscitation was used, incubating Violet Red Bile Agar plates (Biolife) at 35-37°C for 24 h (ISO 21528-1:2004). For the enumeration of yeast and molds (ISO 6611/IDF094:2004) Chloramphenicol Yeast Glucose Agar plates (Biolife) were incubated at 25 ± 1°C and the colonies counted on each plate after 3, 4 and 5 days of incubation. For the enumeration of *Pseudomonas* spp (ISO/TS 11059:2009), 0.1 mL of each decimal dilution were spread over the surface of Pseudomonas Agar Plates added with PP supplement (Biolife) and incubated at 25 °C ± 1°C for 48 h ± 2 h. The detection of *Listeria monocytogenes* was conducted with the two steps enrichment (ISO 11290-1: 1996). The initial suspension was prepared in Fraser Broth Half Concentration (Biolife), after incubation at 30°C for 24 h (pre-enrichment) 0.1 mL were subcultured into 10 mL of Fraser Broth and incubated at 37°C for 24-48 h (enrichment). From both, pre-enrichment and enrichment broth, 0.1 mL were streaked onto Agar Listeria Ottaviani Agosti (ALOA, Biolife) and Oxford (Oxoid, Basingstoke, UK) agar plates and incubated at 37 °C for up to 48 ±3 h.

Enumeration was a conducted according to ISO 11290-2:1998, streaking 1 mL volume of the initial dilution both onto 3 ALOA and 3 Oxford agar plates and incubated at 37 °C for up to 48 ±3 h. Samples inoculated with Lyofast CNBAL at T_0 were also analyzed for the enumeration of *Carnobacterium spp* using MRS modified by increasing the pH to 8.5, omitting acetate, and substituting glucose for sucrose (Hammes et al., 1992).

2.4. Intrinsic properties, composition and headspace gas analysis

PH and a_w were measured using pH meter GLP22 (Crison Instruments SA, Barcelona, Spain) and water activity meter Aqualab 4TE (Decagon, Pullman, WA, USA) respectively. Fat, moisture, protein and total solids were analyzed by using the compositional FoodScan™ device (FOSS, Analytic, Hillerød, Denmark), which uses the near-infrared spectrophotometer system. After accurate mixing of Ricotta *fresca* samples, the sample cups were filled up, placed in the instrument and read in the light spectrum ranging from 850 to 1050 nm. The composition of the headspace gas mixture was conducted on ricotta *fresca* samples on the sealed packages prior to other analysis. Measure of combined residual O_2 % and CO_2 % were obtained piercing the lid using a sterile needle connected to the Dansensor gas analyser (PBI Dansensor, Ringsted,

Denmark). In order to avoid leaks occurring from penetration of the needle through the packaging film, 15 Ø mm septum (PBI Dansensor) were applied on the film lid prior to measurement of headspace gas composition.

Statistical analysis

Differences among average microbiological group counts (log_{10} cfu g^{-1}), headspace gas concentration (%), intrinsic properties and centesimal composition (%) over time (T_0, T_7, T_{14} and T_{21}) and among treatments (BS, CNBAL FRP2 and FERM) within one time point were compared using Fisher's least significant difference test. Statistical analyses were performed with Statgraphics Centurion XVI software (Stat Point Technologies, Warrenton, VA, USA).

Results

Microbiological profile

Ricotta fresca cheese total bacterial count in control samples at T_0 was < 3 log_{10} cfu g^{-1} and increased after 21 days of refrigerated storage above 7 log_{10} cfu g^{-1} while the mesophilic lactic acid bacteria were below the detection limit (≥ 10 cfu g^{-1}) at T_0 and ca 5 log_{10} cfu g^{-1} at T_{21}. During refrigerated storage a significant increase (P<0.01) of

spoilage microorganisms to level as high as 6 log_{10} and 8 log_{10} was observed for *Enterobacteriaceae* and *Pseudomonas* spp, respectively. Yeast and molds were occasionally reported, with maximum values around 4 log_{10} at T_{21}. The complete microbiological profile with mean counts (log_{10} cfu g^{-1}; $\bar{x} \pm$ SD) over time is reported in table 2. *L. monocytogenes* was never detected on either blank samples and ricotta inoculated with biopreservatives. *Carnobacterium* spp. log_{10} counts were 6.28 ± 0.35 at T_0, 6.64 ± 1.56 at T_7, 8.03 ± 0.39 at T_{14} and 8.59 ± 0.47 at T_{21} showing a significant increase after T_{14} ($P< 0.05$).

Physico-chemical characteristics and MAP gas composition

In blank samples the pH showed a slight decrease over time, from 6.67 at T_0 to 6.52 at T_{21} ($P<0.05$) while no significant difference was observed in the a_w. In blank samples the O_2 content in the headspace increased from the initial level of 0.87% up to 1.80% at T_7, to decrease again as low as 0.42 at T_{21}. Instead, the CO_2 content decreased from T_0 to T_{21} respectively from 13.05% to 6.78%. No significant difference was observed among blank samples and ricotta inoculated with biopreservatives in moisture,

fat and protein composition. Intrinsic properties, composition and gas composition in the headspace ($\bar{x} \pm$ SD) during the refrigerated storage are reported in table 3.

Discussion

The microflora in the whey used to manufacture *Ricotta fresca cheese* is drastically reduced as consequence of high temperature applied during thermal denaturation of whey proteins. Considered the high moisture and pH, low salt content and the availability of nutrients, *Ricotta fresca* is an excellent substrate for the growth of spoilage and pathogenic microorganisms such as *Pseudomonas* spp., *Enterobacteriaceae*, *Listeria monocytogenes*, *B. cereus* and *Arcobacter* spp. (De Santis and Mazzette, 2002; De Santis *et al.* 2008; Ibba *et al.*, 2013; Scarano *et al.*; 2014; Spanu *et al.*, 2016). However, a large part of Ricotta cheese microflora at the end of the shelf life is generally represented by *Pseudomonas* spp, that overgrows other microbial contaminants, including pathogens (Pala *et al.*, 2016). The fermentate showed no activity against the growth of microbiota in ricotta during refrigerated storage. In fact, total bacterial counts, LAB, *Enterobacteriaceae*, *Pseudomonas* spp., yeast and molds showed no significant differences between blank samples and samples inoculated with FERM. The higher

counts at T_0 of mesophilic LAB (ca 5 $\log_{10}$) in *Ricotta fresca cheese* samples inoculated with FRP as compared to control samples and ricotta inoculated with the other bio preservatives was expected. FRP cultures despite refrigeration demonstrated during storage an increase, though it was statistically significant, of less than 1 $\log_{10}$. However, FRP showed no control against *Enterobacteriaceae* and *Pseudomonas* spp which, at the end of the storage, were ca 1 $\log_{10}$ higher respect to blank samples. In ricotta samples inoculated with CNBAL mesophilic LAB counts were always lower as compared to the other samples. This result could be explained with the fact that for the isolation and cultivation of LAB the De Man, Rogosa and Sharpe (MRS) agar is generally used, in which it has been observed that most of the *Carnobacterium* spp are not able to growth (Hammes et al., 1992). This could lead to a significant underestimation of its concentration in foods. In the present study *Carnobacterium* spp showed a good adaptive response to the experimental condition of inoculum and storage, showing an increase in its mean counts of approximately of 2 $\log_{10}$ from T_0 to T_{21}. The competitive activity of CNBAL was effective in reducing *Pseudomonas* spp and *Enterobacteriaceae* at the end of the shelf life of at least 1 $\log_{10}$. However, it should be noticed that the effect of CNBAL

was greater after 14 days were the difference with blank samples was respectively of 1.93 log_{10} for *Pseudomonas* and 2.66 log_{10} for *Enterobacteriaceae*. The genus *Carnobacterium* comprises several species of which *Carnobacterium divergens* and *C. maltaromaticum* are the only two frequently recovered from foods. These are able to produce a wide array of bacteriocins such as Carnobacteriocin A, Carnobacteriocin BM1, Carnobacteriocin B2, Piscicolin 126, Piscicocin CS526, Divercin V41,Divergicin M35, Divergicin A, Divergicin 750, Carnocin H ans Carnocin UI149 (Leisner *et al.*, 2007).

The objective of the present study was to investigate the adaptability of protective cultures to refrigerated *ricotta fresca* as a possible strategy to control the growth of unwanted microbiota. However, the growth of LAB cultures could potentially affect the sensory properties of the product. In particular, acidification would be detrimental for a fresh and mild taste product such as *ricotta fresca*. The conduction of sensory analysis was not among the aims of the study. However, evidence that *Carnobacterium* spp did not acidified Ricotta fresca during its growth is provided by the evolution of the pH during the refrigerated storage. The pH values of blank samples were comparable with samples inoculated with biopreservatives. Other than on organoleptic features, an excessive

reduction of the pH could have exerted an inhibition of the production of some carnobacteriocins (Ahn & Stiles, 1990). Despite the growth of *Carnobacterium* spp, the level of *Pseudomonas* spp contamination at T_{14} was already as high as 6 log_{10}, level compatible with possible alteration of the product and in some circumstances (pigment production and discoloration of the product) beyond the acceptability of consumers. In a previous study (Pala *et al.*, 2016) we have already pointed out that, the evolution of Pseudomonas during the shelf life in refrigerated *ricotta fresca* is greatly dependent upon the initial contamination level. This in turn is greatly influenced by the correct implementation of good hygienic practices and procedure based on the HACCP principles during manufacturing. It has been stressed that the use of bioprotective cultures is intended as an additional measure to control spoilage microorganisms, once prerequisite programs have been correctly implemented at the processing plant. The gas mixture chosen for MAP packaging of ricotta *fresca* (30% CO_2 and 70% N_2) is the composition generally used in Sardinian industrial cheesemaking plants. As previously demonstrated, the concentration of CO_2 in the headspace at T_0 differs from the level used during packaging, as a result of gas solving in the product while the reduction of O_2% during

storage is associated with the growth of aerobic mesophilic microorganisms (Pala *et al.*, 2016).

Conclusion

The present study was specifically designed to provide preliminary information on the possible use of biopresarvatives to control the growth of psychotropic spoilage microorganism's in MAP packaged *ricotta fresca*. Since no information was previously available on the adaptation of biopreservatives on sheep's milk *ricotta fresca*, the primary objective of the study was to select among three commercial products which one was suitable as biopreservative. *Carnobacterium* spp. inoculated on the surface finished product showed a good adaptation to grow in *ricotta fresca* and promising results in controlling superficial contamination with spoilage microorganisms. However, the present investigation was conducted on naturally contaminated ricotta samples. Therefore, CNBAL was the protective culture of choice to conduct a challenge test specifically designed to assess the effect of *Carnobacterium* spp against *Pseudomonas* spp.

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Tables

Table 1. Number of ricotta fresca samples and analysis performed at each sampling time.

Analysis	Biopreservative treatment	Sampling time T_0	T_7	T_{14}	T_{21}	Total
- Microbiological profile: aerobic mesophilic bacteria; mesophilic lactic acid bacteria; *Enterobacteriacae;* *Pseudomonas* spp; Yeast and molds.	BS	9	9	9	9	36
	CNBAL	9	9	9	9	36
	FRP2	9	9	9	9	36
- Intrinsic properties: pH and a_W; composition (%): moisture; fat; protein.	FERM	9	9	9	9	36
- Headspace composition (%): CO_2; O_2						
Total		36	36	36	36	144

T_0 = day of inoculum; T_7, T_{14} and T_{21}= respectively 7, 14 and 21 days of storage after the inoculum. BS (Blank Samples): not inoculated units; CNBAL: samples inoculated with *Carnobacterium* spp protective culture; FRP2: samples inoculated with *Enterococcus faecium*, *Lactobacillus plantarum* and *Lactobacillus rhamnosus* protective culture; FERM: samples inoculated with the microbial fermentation complex.

Table 2. Microbiological profile (log_{10} cfu g^{-1}; $\bar{x}$ ± SD) of ricotta fresca by time (days after) and sample type.

Microbial group	Biopreservative treatment	Day of storage			
		T_0	T_7	T_{14}	T_{21}
Aerobic mesophilic bacteria	BS	2.72 ± 0.44^{a1} (n = 9/9)	4.90 ± 1.36^{b1} (n = 9/9)	6.01 ± 0.56^{c1} (n = 9/9)	6.90 ± 0.86^{d1} (n = 9/9)
	CNBAL	3.18 ± 1.95^{a1} (n = 9/9)	6.53 ± 0.85^{b2} (n = 9/9)	6.87 ± 1.13^{b23} (n = 9/9)	8.87 ± 0.38^{c2} (n = 9/9)
	FRP2	5.11 ± 0.62^{a2} (n = 9/9)	6.63 ± 0.91^{b2} (n = 9/9)	7.35 ± 0.51^{c3} (n = 9/9)	7.85 ± 0.22^{c1} (n = 9/9)
	FERM	2.91 ± 0.51^{a1} (n = 9/9)	5.21 ± 1.05^{b1} (n = 9/9)	6.11 ± 0.82^{c12} (n = 9/9)	6.92 ± 0.51^{d1} (n = 9/9)
mesophilic lactic acid bacteria	BS	ND	3.55 ± 0.49^{a1} (n = 9/9)	4.33 ± 0.71^{b1} (n = 9/9)	4.92 ± 0.67^{b12} (n = 9/9)
	CNBAL	2.13 ± 0.76^{a1} (n = 4/9)	3.30 ± 0.93^{b1} (n = 9/9)	3.76 ± 0.62^{b1} (n = 9/9)	3.32 ± 0.60^{b3} (n = 9/9)
	FRP2	5.01 ± 0.72^{a2} (n = 9/9)	5.35 ± 0.78^{ab2} (n = 9/9)	5.16 ± 0.44^{a2} (n = 9/9)	5.77 ± 0.40^{b1} (n = 9/9)
	FERM	1.52 ± 0.24^{a1} (n = 3/9)	2.86 ± 0.05^{ab1} (n = 2/9)	4.05 ± 0.68^{b1} (n = 7/9)	4.58 ± 1.68^{b2} (n = 7/9)
Enterobacteriacae	BS	2.20 ± 1.02^{a1} (n = 4/9)	4.05 ± 0.86^{b1} (n = 5/9)	4.43 ± 0.99^{b1} (n = 7/9)	5.34 ± 0.97^{b1} (n = 8/9)
	CNBAL	ND	1.95 ± 0.00^{ab3} (n = 1/9)	1.77 ± 1.15^{a2} (n = 6/9)	3.90 ± 0.42^{b3} (n = 5/9)
	FRP2	2.03 ± 0.00^{a1} (n = 2/9)	3.79 ± 0.67^{b12} (n = 9/9)	5.41 ± 0.75^{c3} (n = 9/9)	6.29 ± 0.47^{d2} (n = 9/9)
	FERM	3.78 ± 2.02^{a1} (n = 4/9)	3.21 ± 0.82^{a23} (n = 6/9)	4.24 ± 0.91^{a1} (n = 6/9)	5.84 ± 0.59^{b12} (n = 7/9)
Pseudomonas spp	BS	2.64 ± 0.59^{a1} (n = 5/9)	4.89 ± 1.21^{b1} (n = 9/9)	6.52 ± 0.99^{c1} (n = 9/9)	6.83 ± 0.91^{c1} (n = 9/9)
	CNBAL	2.43 ± 0.18^{a1} (n = 4/9)	2.59 ± 0.67^{a2} (n = 9/9)	4.59 ± 0.65^{b2} (n = 9/9)	5.27 ± 0.64^{b2} (n = 9/9)
	FRP2	2.53 ± 0.51^{a1} (n = 5/9)	5.89 ± 0.64^{b3} (n = 9/9)	6.81 ± 0.82^{c1} (n = 9/9)	7.01 ± 0.53^{c1} (n = 9/9)
	FERM	2.69 ± 0.27^{a1} (n = 6/9)	5.02 ± 0.81^{b1} (n = 9/9)	6.33 ± 0.94^{c1} (n = 9/9)	7.26 ± 0.31^{d1} (n = 9/9)
Yeast and molds	BS	ND	2.78 ± 0.40^{a12} (n = 4/9)	3.62 ± 0.33^{b1} (n = 3/9)	3.43 ± 0.76^{ab1} (n = 5/9)
	CNBAL	2.36 ± 0.10^{a1} (n = 3/9)	2.15 ± 0.21^{a2} (n = 2/9)	3.00 ± 0.00^{b1} (n = 1/9)	ND
	FRP2	2.00 ± 0.00^{a1} (n = 1/9)	3.01 ± 0.49^{ab12} (n = 3/9)	3.52 ± 0.38^{b1} (n = 5/9)	3.64 ± 0.73^{b1} (n = 3/9)
	FERM	ND	3.97 ± 0.42^{a3} (n = 3/9)	3.88 ± 0.68^{a1} (n = 9/9)	3.19 ± 1.14^{a1} (n = 8/9)

Table 3a. Intrinsic properties and composition (mean±SD) of Ricotta fresca cheese at different testing times

Parameters	Biopreservative treatment	T_0	T_7	T_{14}	T_{21}
pH	BS	6.67 ± 0.1^{a1}	6.58 ± 0.05^{bc1}	6.61 ± 0.07^{ab1}	6.52 ± 0.11^{c1}
	CNBAL	6.66 ± 0.12^{a1}	6.66 ± 0.11^{a2}	6.66 ± 0.09^{ab1}	6.54 ± 0.04^{c1}
	FRP2	6.67 ± 0.06^{a1}	6.59 ± 0.05^{b12}	6.55 ± 0.07^{b1}	6.31 ± 0.07^{c2}
	FERM	6.68 ± 0.10^{a1}	6.56 ± 0.04^{bc1}	6.60 ± 0.08^{ab1}	6.49 ± 0.14^{c1}
a_w	BS	0.990 ± 0.003^{a1}	0.996 ± 0.006^{a1}	0.993 ± 0.006^{a12}	0.993 ± 0.006^{a12}
	CNBAL	0.991 ± 0.005^{a1}	0.989 ± 0.008^{a1}	0.986 ± 0.001^{a2}	0.985 ± 0.001^{a2}
	FRP2	0.995 ± 0.004^{a1}	0.995 ± 0.001^{a1}	0.997 ± 0.001^{a1}	0.997 ± 0.002^{a1}
	FERM	0.994 ± 0.003^{a1}	0.994 ± 0.001^{a1}	0.992 ± 0.005^{a12}	0.993 ± 0.008^{a12}

T_0 = day of inoculum; T_7, T_{14} and T_{21}= respectively 7, 14 and 21 days of storage after the inoculum. BS (Blank Samples): not inoculated units; CNBAL: samples inoculated with *Carnobacterium* spp protective culture; FRP2: samples inoculated with *Enterococcus faecium*, *Lactobacillus plantarum* and *Lactobacillus rhamnosus* protective culture; FERM: samples inoculated with the microbial fermentation complex. Means in the same row with different superscript letter were significantly different ($P<0.05$); means in the same column among biopreservative treatments with different superscript number were significantly different ($P<0.05$).

Table 3b. Intrinsic properties and composition (mean±SD) of Ricotta fresca cheese at different testing times

Parameters	Biopreservative treatment	T_0	T_7	T_{14}	T_{21}
Moisture (%)	BS	71.23 ± 3.52^{a1}	73.37 ± 2.10^{a1}	73.56 ± 2.08^{a1}	74.70 ± 0.91^{a1}
	CNBAL	72.02 ± 3.38^{a12}	71.97 ± 3.82^{a1}	71.20 ± 3.92^{a1}	71.68 ± 2.91^{a1}
	FRP2	77.43 ± 3.11^{a2}	73.27 ± 2.83^{a1}	74.35 ± 3.54^{a1}	72.22 ± 2.09^{a1}
	FERM	74.26 ± 2.74^{a12}	74.61 ± 0.83^{a1}	74.13 ± 2.01^{a1}	73.46 ± 1.29^{a1}
Fat (%)	BS	18.13 ± 5.80^{a1}	14.31 ± 2.31^{a1}	13.30 ± 2.60^{a1}	14.66 ± 2.96^{a1}
	CNBAL	18.03 ± 4.48^{a12}	17.48 ± 4.46^{a1}	17.15 ± 3.95^{a1}	15.42 ± 3.29^{a1}
	FRP2	11.03 ± 2.59^{a2}	17.15 ± 1.16^{c1}	13.09 ± 2.98^{ab1}	15.11 ± 1.08^{bc1}
	FERM	13.78 ± 1.62^{a12}	12.43 ± 2.10^{a1}	13.59 ± 1.88^{a1}	14.67 ± 2.53^{a1}
Protein (%)	BS	9.81 ± 0.78^{a1}	9.97 ± 1.04^{a1}	10.23 ± 1.36^{a1}	8.94 ± 2.61^{a1}
	CNBAL	9.38 ± 0.45^{a1}	9.33 ± 0.75^{a1}	9.46 ± 0.39^{ab1}	10.46 ± 0.48^{b1}
	FRP2	10.05 ± 0.02^{a1}	11.15 ± 1.07^{a1}	9.99 ± 0.73^{a1}	10.86 ± 0.24^{a1}
	FERM	9.77 ± 1.50^{a1}	10.32 ± 1.37^{a1}	9.87 ± 0.76^{a1}	10.19 ± 0.81^{a1}
O_2%	BS	0.87 ± 0.49^{a1}	1.80 ± 1.18^{b1}	1.05 ± 0.82^{a1}	0.42 ± 0.78^{a1}
	CNBAL	0.99 ± 0.58^{ab1}	1.12 ± 1.65^{b1}	0.14 ± 0.17^{ab2}	0.02 ± 0.01^{b1}
	FRP2	0.99 ± 0.54^{a1}	1.89 ± 1.47^{b1}	0.31 ± 0.25^{ac2}	0.004 ± 0.01^{c1}
	FERM	1.01 ± 0.63^{a1}	1.66 ± 0.32^{b1}	0.75 ± 0.91^{a12}	0.51 ± 0.84^{a1}
$C0_2$%	BS	13.05 ± 2.88^{a1}	6.20 ± 2.09^{b1}	5.50 ± 2.96^{b1}	6.78 ± 2.89^{b1}
	CNBAL	13.55 ± 2.42^{a1}	5.50 ± 2.02^{b1}	5.00 ± 2.08^{b1}	5.18 ± 1.91^{b1}
	FRP2	13.68 ± 2.23^{a1}	6.96 ± 1.55^{c1}	7.50 ± 1.73^{c1}	10.22 ± 1.72^{b2}
	FERM	13.24 ± 2.21^{a1}	6.47 ± 2.10^{b1}	5.79 ± 2.67^{b1}	6.98 ± 4.15^{b1}