Use of Carnobacterium spp protective culture in MAP packed Ricotta fresca cheese to control Pseudomonas spp

Highlights

- *Pseudomonas* spp is the main spoilage microorganism in refrigerated ricotta fresca
- A microbiological challenge test was conducted on MAP ricotta fresca samples
- Carnobacterium spp commercial protective culture was tested against *Pseudomonas* spp
- Inoculation of Carnobacterium spp protective culture reduced *Pseudomonas* spp growth

Use of *Carnobacterium* spp protective culture in MAP packed Ricotta *fresca* cheese to control *Pseudomonas* spp

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Abstract

Ricotta *fresca* is a whey cheese susceptible of secondary contamination, mainly from *Pseudomonas* spp. The extension of the shelf life of refrigerated *ricotta fresca* could be obtained using protective cultures inhibiting the growth of this spoilage microorganism. A commercial biopreservative, Lyofast CNBAL, comprising *Carnobacterium* spp was tested against *Pseudomonas* spp. The surface of *ricotta fresca* samples were inoculated either with *Pseudomonas* spp or *Pseudomonas* and *Carnobacterium* spp. Samples were MAP packed, stored at 4°C and analyzed the day of the inoculum and 7, 14 and 21 days after the contamination. Microbiological analyses included total bacterial count, mesophilic lactic acid bacteria, *Enterobacteriaceae*, *Pseudomonas* spp, *Listeria monocytogenes*, moulds and yeasts. *Pseudomonas* mean initial contamination level was comparable in blank and artificially inoculated samples, respectively with values of 2.15±0.21 and 2.34±0.26 log cfu g^{-1}. *Carnobacterium* spp. significantly reduced the growth of *Pseudomonas* spp respectively of 1.28 log and 0.83 log after 14 and 21 days of refrigerated storage. Intrinsic properties and physico-chemical composition were also investigated. Limited variation of pH was observed in samples inoculated with the protective cultures, indicating low acidification properties of

Carnobacterium spp. Instead, no significant differences were observed for a_W, moisture, fat and proteins during storage and between inoculated and control samples.

Keywords: *Carnobacterium* spp.; protective cultures; *Pseudomonas* spp; ricotta; MAP.

1. Introduction

According to the Codex Standard for whey cheeses, these are 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). Whey cheeses are manufactured all over the world at artisanal and industrial level using different protocols depending on the country of origin. Ricotta, originally from Italy is the most renowned whey cheese in the world (Pintado & Malcata, 2000). However, in European countries, especially in the Mediterranean basin, wide varieties of whey cheeses are manufactured, mainly from ovine whey. These whey cheeses fall under several designation, e.g. Anthotyros (Greece), Anari (Cyprus), Requesón (Spain), Requeijao (Portugal), Broccio (France), Urdă (Balkans region). Ricotta *fresca* is a traditional whey cheese, which in Sardinia is produced by heat coagulation of sheep's milk whey remaining after the production of *Pecorino* type cheeses. The discontinuous manufacturing method include whey heating into open kettles to 80-82 °C. At this temperature the flocculated proteins rise and float on the surface. The curd is then scooped and transferred into perforated plastic baskets. Ricotta baskets are allowed to drain and to cool into a cold room. The detailed manufacturing process of Ricotta *fresca* has been previously described (Pala et al., 2016). *Ricotta fresca* is packed either in food wrapping paper or in polypropylene trays that are heat sealed with plastic lidding film. Depending on the food business operator, atmospheric air or modified atmosphere packaging (MAP) is used. MAP conditions largely vary from one operator to another; with 30% CO_2 and 70% N_2 being the most used gas mixture used. The shelf life is designated under the responsibility of the

food business operator and varies from 5-7 days for wrapped Ricotta up to 21 days for MAP Ricotta. *Ricotta fresca* is poor in natural microflora since it is drastically reduced by the high temperature applied during the thermal denaturation of whey proteins (Pintado, Macedo & Malcata, 2001). *Ricotta fresca* is exposed to post-process contamination originating from the cheese-making environment, particularly in the steps between molding and packaging (Greenwood, Roberts & Burden, 1991; Ibba et al., 2013). Given the high moisture (70-80%), elevated pH (6.10-6.80) and the water activity (0.974-0.991) and the refrigerated storage (De Santis, Mazzette, Scintu, Deriu, & Carta, 1999), *Ricotta fresca* is an excellent substrate for the possible growth of several psychotropic pathogenic and spoilage microorganisms (De Santis & Mazzette, 2002). Common contaminants of Ricotta fresca include *Pseudomonas* spp., *Enterobacteriaceae*, *Listeria monocytogenes*, *B. cereus* and *Arcobacter* spp., yeast and moulds (Pintado, Macedo & Malcata, 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). However, *Pseudomonas* spp. benefits of the selective advantage of the combination of low temperature and long storage to overgrow other microorganisms, reducing the risk of pathogens growth (Buchanan & Bagi, 1999; Carrascosa et al., 2015). Therefore, *Pseudomonas* spp. represents the main obstacle to extend *Ricotta fresca* shelf life (Pala et al., 2016). Growth of *Pseudomonas* spp. to level as high as 7 $\log_{10}$ cfu g^{-1} negatively affect the sensory properties of the product (Leriche *et al.,* 2004). Important discoloration of the product derives by the secretion of yellow-green pyoverdin (Meyer et al., 2002) and of a blue pigment (Cantoni, Stella, Cozzi, Iacumin, & Comi, 2003; Martin, Murphy, Ralyea, Wiedmann & Boor, 2011; Andreani et al., 2015) from some *Pseudomonas* spp. In order to reduce the initial microbial load at production, it is essential the strict application of good hygiene and good manufacturing procedures. A previous investigation, conducted on Sardinian cheese-making plant manufacturing *Ricotta fresca*, showed that there is an association between secondary contamination with *Pseudomonas* spp. and the level of implementation of the food safety management system at plant level (Pala et al., 2016). There is the

need of additional strategies to control the growth of *Pseudomonas* spp., especially in industrial MAP *Ricotta fresca*. The use of bio preservatives to extend the shelf life of whey cheeses has been previously tested (Davies, Bevis, & Delves-Broughton, 1997; Samelis, Kakouri, Rogga, Savvaidis, & Kontominas, 2003; Martins, Cerqueira, Souza, do Carmo Avides, & Vicente et al., 2010). Carnobacteria are Gram positive rod shaped lactic acid bacteria (LAB) isolated from different echological niches. The genus *Carnobacterium*, is able to produce bacteriocins therefore, it has been used as protective cultures to provide durability and safety to various types of foods (Elsser-Gravesen & Elsser-Gravesen, 2013). Of the species included in the genus *Carnobacterium*, only two, *C. divergens* and *C. maltaromaticum*, are frequently isolated from natural environment and dairy products (Leisner, Laursen, Prevost, Drider, & Dalgaard, 2007; Afzal et al., 2010). *Carnobacterium* spp, due to similar growth conditions (pH and temperature variation) of psychrotrophic spoilage and pathogen microorganisms, can improve the durability of chilled foods by reducing or inhibiting the growth of *Pseudomonas* spp. To date, little investigation has been conducted to validate the efficacy of bio preservatives against *Pseudomonas* spp. in sheep *Ricotta fresca* cheese. A commercial protective culture, consisting of a selected strain of *Carnobacterium spp* producing bacteriocins, the Lyofast CNBAL (Clerici-Sacco Group, Como, Italy) was sprayed on *Ricotta fresca* surface (Spanu et al., 2017). The study demonstrated a good adaptation to the substrate with growth during refrigerated storage with no acidification of the product. *Carnobacterium* spp. showed a competitive activity against *Pseudomonas* spp. in *Ricotta fresca*. However, the experiment was conducted on naturally contaminated samples. The main objective of the present study was to evaluate, as a possible innovation in the manufacturing process of *Ricotta fresca*, the use of a protective culture to control the superficial contamination against spoilage microorganisms. With this aim, the efficacy of *Carnobacterium* spp against the growth of *Pseudomonas* spp was tested in artificially contaminated *Ricotta fresca*.

2. Materials and methods

2.1. Samples

The study was conducted on 162 *Ricotta fresca* samples provided by a local industrial cheese-making plant using the whey remaining after the production of hard sheep's milk cheese (e.g. *Pecorino* type cheeses). The samples were truncated cone shaped cheese with an upper base ca.7.5 cm wide and a lower base ca. 5 cm wide, weighing approximately 1.1 kg. Fifty-five *Ricotta fresca* samples were randomly selected from each of three different batches (each batch was manufactured in a different day of production). Samples were packed in MAP (gas mixture was 30% CO_2 and 70% N_2) using 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/m^2, 24 h. After packaging samples were transported refrigerated to the laboratory within 24 from the production. Within each batch, samples were randomly allocated to the following treatment: artificial inoculation with *Pseudomonas fluorescens* (RfPf), artificial inoculation with *Pseudomonas fluorescens* and *Carnobacterium* spp (RfPf+C), no treatment (RfB). Part of the samples (Ph-Ch) were used for the determination of intrinsic properties (pH and a_W), percentage composition (moisture, fat, protein) and headspace gas composition (CO_2 and O_2).

2.2 Artificial inoculation

The RfPf units (n. 36) were the units artificially inoculated with *Pseudomonas fluorescens*, which served as positive control samples. The preparation of the inoculum was conducted according to the guidelines prepared by NACMCF (2010). A mixture of five *Pseudomonas fluorescens* wild type strains was used to challenge *Ricotta fresca* units. The strains were recovered from naturally contaminated *Ricotta fresca* during a previous investigation (Pala et al., 2016). In order to avoid overrepresentation of clones, isolates were submitted to genetic characterization using pulsed-field gel electrophoresis (PFGE). Only strains with different restriction profile were included in the preparation of the inoculum. All the strains were stored at −80 °C in Brain Heart Infusion (BHI)

broth (Oxoid, Basingstoke, UK) with glycerol (20% v/v). In order to account for natural contamination levels and allow the enumeration of the inoculum, the target level was 10^2 cfu g^{-1} of product surface (Spanu et al., 2014). Preliminary trials were conducted in order to determine the growth condition necessary to standardize the level of inoculum and to adapt strains to refrigeration temperature. Each strains was subcultured by streaking onto the non selective trypticase soy agar medium (TSA, Biolife, Milan, Italy) and incubated for 24 h at 25 °C. A single colony was picked and transferred into tubes containing the non-selective brain heart infusion broth, BHI (Oxoid, Basingstoke, UK). Tubes were incubated overnight at 25°C in a shaking water bath to obtain cells in the same physiological state (late exponential or early stationary phase). Adaptation of the strains to refrigeration was conducted by subculturing in BHI broth at 4±2 °C until cells were in the exponential growth phase (Uyttendaele et al., 2004). Equal volumes of each individual culture were mixed in a sterile flask. From the mixture were prepared adequate serial decimal dilution in phosphate buffered saline (Biolife) to adjust the concentration to 10^4 cfu mL^{-1}. After the removal of the sealing film, an aliquot of 1.5-2.0 mL of inoculum was evenly sprayed on the surface of Ricotta fresca (ca. 290 cm^2, corresponding to ca. 40-50 g of product). All concentrations were confirmed by plate count on TSA (Biolife).

After the inoculum of *Pseudomonas fluorescens*, the RfP+C units (n. 36) were also inoculated with the protective cultures 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. The commercial formulation is a freeze-dried powder, which, according to manufacturer's instructions, was diluted in Phosphate-buffered saline (PBS) solution (Oxoid) to obtain 10^6 cfu g^{-1} of product. With this aim, the suspension was adjusted to a concentration of 10^8 cfu mL^{-1} and 1.5-2.0 mL were evenly sprayed on the *Ricotta fresca* surface. *Carnobacterium* spp concentrations were confirmed by count on agar plates. After the inoculation, all samples (included untreated blank samples) were repacked in MAP (30% CO2 and 70% N2) using the FP Basic Sec

tray sealer (Ilpra, Vigevano, Italy). Blank samples were packed under the same conditions of the other samples with no further manipulation. All samples were stored under refrigeration (+ 4°C) until analysis were performed.

2.3 Experimental design

Triplicate samples of each of the three batches of *Ricotta fresca* were analyzed the day of inoculation (T_0), and after different days of refrigerated storage, respectively 7 (T_7), 14 (T_{14}) and 21 (T_{21}). At each time-point RfPf, RfPf+C and RfB samples were analyzed for the determination of the microbiological profile. In order to mimic the evolution of the microbiological profile under the foreseeable condition during retail selling, a subset of RfPf+C samples were opened seven days after the inoculation and analyzed after three further days of storage (T_{10}) at 8±2°C (abuse temperature). *Ricotta fresca* samples were also analyzed for the determination of the intrinsic properties, composition and headspace gas analysis. The detailed design including sample units, testing times and related analysis is described in table 1.

2.3. Microbiological profile

Ricotta fresca samples submitted to microbiological examination were prepared according to ISO 6887-1:1999. Briefly, the initial suspension and decimal dilution was conducted after the sterile collection of to 25 g of ricotta surface into a sterile plastic stomacher bag. 225 mL of Buffered Peptone Water were added and the sample homogenized using a stomacher. Decimal serial dilutions were obtained by transferring 1 mL of the initial suspension into a tube containing 9 mL of sterile diluent. If required, these operations were repeated using the 10^{-2} to obtain further serial decimal dilution. The pour-plating procedure was used for the enumeration of aerobic mesophilic bacteria, mesophilic lactic acid bacteria and *Enterobacteriaceae*, yeasts and moulds. Plate Count Agar and MRS medium at pH 5.7 (Biolife, Milan, Italy) were incubated at 30°C ± 1°C for 72 h ± 3 h respectively for the enumeration of aerobic mesophilic bacteria (ISO 4833:2003) and mesophilic lactic acid bacteria (ISO 15214: 1998). The enumeration of *Enterobacteriaceae* was conducted

using the plate count technique without resuscitation, incubating Violet Red Bile Agar plates (Biolife) at 35-37°C for 24 h (ISO 21528-1:2004). Enumeration of yeast and molds was conducted using Chloramphenicol Yeast Glucose Agar plates (Biolife) incubated at 25 ± 1°C up to 5 days (ISO 6611/IDF094:2004). 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. For the detection of *Listeria monocytogenes* (ISO 2004a) the initial suspension was supplemented with Fraser Half Selective Supplement (Biolife) and incubated at 30 °C for 24 h (primary enrichment). After incubation 0.1 mL of the primary enrichment 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 of *L. monocytogenes* (ISO 2004b) was a conducted streaking 1 mL volume of the initial dilution onto both three ALOA and three Oxford agar plates and incubated at 37 °C for up to 48 ±3 h. The enumeration of *Carnobacterium spp* was conducted using MRS modified by increasing the pH to 8.5, omitting acetate, and substituting glucose for sucrose (Hammes et al., 1992).

2.5. Pseudomonas fluorescens characterization

A total of five colonies from each sample containing typical colonies on Pseudomonas Agar Plates were confirmed with two different PCR protocols, one used to identify *Pseudomonas fluorescens* (Scarpellini et al., 2004) and the other to identify *Pseudomonas* spp and *Pseudomonas aeruginosa* (De Vos et al., 1997). Among the confirmed *Pseudomonas fluorescens* strains, up to three isolates per samples were randomly selected and analyzed using pulsed-field gel electrophoresis (PFGE). After DNA extraction, agarose plugs were digested with enzyme restriction *Spe*I (Roche Diagnostics,

Laval, Quebec, Canada) (Maslow, 1993). PFGE was performed using the protocol described by (Gershman et al., 2008). The obtained restriction profiles were analyzed by visual examination to distinguish inoculated strains among each other and from strains originating by natural contamination.

2.5. Intrinsic properties, composition and headspace gas analysis

Determination of pH and a_w was conducted using pH meter GLP22 (Crison Instruments SA, Barcelona, Spain) and water activity meter Aqualab 4TE (Decagon, Pullman, WA, USA), respectively. The near-infrared spectrophotometer system for the determination of centesimal composition (%) of fat, moisture, protein and total solids was used. Ricotta *fresca* samples were accurately mixed, placed into Petri dishes and read in the light spectrum of 850-1050 nm using the compositional FoodScan™ device (FOSS, Analytic, Hillerød, Denmark). The headspace gas composition was conducted on MAP ricotta *fresca* samples on the sealed packages before performing 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). To avoid gas leaks during the penetration of needle, 15 Ø mm septum (PBI Dansensor), were applied on the film lid before measurements of headspace gas composition.

2.6. 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 types of samples (RfB, RfPf and RfPf+C) 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). The effectiveness of *Carnobacterium* spp in controlling the growth of *Pseudomonas fluorescens* was evaluated computing the minimum difference (Δ) between of the $\log_{10}$ cfu g^{-1} between RfPf and RfPf+C samples at each time point (T_7, T_{14} and T_{21}).

3. Results

3.1. Microbiological profile

In *Ricotta fresca* blank samples the total bacterial count was 3.12±0.71 log_{10} cfu g^{-1} at T_0 and gradually increased during refrigerated storage up to 6.34±0.87 log_{10} cfu g^{-1} at T_{21}. The mesophilic lactic acid bacteria ranged between 2.25±0.06 log_{10} cfu g^{-1} at T_0 and 3.83±1.06 log_{10} cfu g^{-1} at T_{21}. *Enterobacteriaceae* were observed in one samples both at T_0 and at T_7 with counts of ca. 2 log_{10} cfu g^{-1}; their concentration increased to 3.48±0.58 log_{10} cfu g^{-1} at T_{21}. *Pseudomonas aeruginosa* was never detected both in inoculated and blank samples. Natural contamination with *Pseudomonas* spp was observed in two samples out of nine with a mean count of 2.15±0.21 log_{10} cfu g^{-1} at T_0 and increased to 6.48±0.78 log_{10} cfu g^{-1} at T_{21}. In *ricotta fresca* samples inoculated with the protective culture, *Carnobacterium* spp. grew from the initial concentration of 6.36 ± 0.32 log_{10} cfu g^{-1} at T_0 to 8.78 ± 0.25 log_{10} cfu g^{-1} at T_{21}. The comparison between *ricotta fresca* samples inoculated with *Pseudomonas* spp plus the protective culture and samples inoculated only with *Pseudomonas* spp showed similar level of initial contamination, ca. 2.30 log_{10} cfu g^{-1}. After 14 and 21 days of refrigerated storage Pseudomonas concentration was respectively 1.28 and 0.83 log_{10} cfu g^{-1} lower in ricotta fresca inoculated with *Carnobacterium* spp protective culture ($P<0.05$).

Yeast and molds were occasionally reported, with maximum values below 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. Microbiological analysis conducted on RfPf+Copen samples analyzed at T_{10} showed the following counts (within brackets positive samples over tested samples): total bacterial count 8.40 ± 0.63 log_{10} cfu g^{-1} (n = 9/9), mesophilic lactic acid bacteria 3.12 ± 0.41 log_{10} cfu g^{-1} (n = 6/9), *Carnobacterium spp* 6.90± 1.89 log_{10} cfu g^{-1} (n = 9/9), *Enterobacteriaceae* 2.15 log_{10} cfu g^{-1} (n = 1/9), *Pseudomonas* spp 7.65 ± 0.51 log_{10} cfu g^{-1} (n = 9/9), yeast and molds 2.86 ± 0.43 log_{10} cfu g^{-1} (n = 5/9), while *L. monocytogenes* was never detected.

3.2. *Physico-chemical characteristics and MAP gas composition*

A moderate decrease of pH was observed over time in all *ricotta fresca* samples. However, significant difference between blank samples and in samples inoculated with *Carnobacterium spp* were observed only at T_{21}, showing respectively values of 6.60±0.04 and 6.48±0.07 ($P<0.05$). The a_w was stable over time and between sample types ranging from 0.984±0.001 and 0.987±0.001. No significant difference was observed among blank samples and ricotta inoculated with biopreservatives in moisture, fat and protein composition, showing respectively a mean content of 72.38±3.15, 15.67±3.99 and 10.70±0.93. The O_2 content in the headspace increased from the initial level of 0.52±0.24% up to 1.30±1.28% at T_7, to decrease again as low as 0.03±0.05 at T_{21}. Instead, the CO_2 content decreased from T_0 to T_{21} respectively from 16.71±0.61% to 7.16%. No significant difference in gas composition were observed between blank and inoculated *ricotta fresca* samples. Intrinsic properties, composition and gas composition in the headspace ($\bar{x} \pm$ SD) during the refrigerated storage are reported in table 3.

3.3. *Pseudomonas spp natural and artificial contamination*

From RfPf+C a total of 108 were confirmed as *Pseudomonas fluorescens* strains and typable by PFGE. The comparison of the restriction profiles obtained showed that 103 strains (95.4%) belonged to one of the five *Pseudomonas fluorescens* strains of the mix used for the artificial contamination. Five strains (4.6%), showing a different restriction profile, were from natural contamination.

4. Discussion

Ricotta fresca cheese is particularly susceptible of secondary contamination from the processing environment and represents an excellent substrate for the growth of psychotropic pathogenic and spoilage microorganisms (De Santis and Mazzette, 2002; De Santis *et al*. 2008; Ibba *et al*., 2013; Scarano *et al*.; 2014; Spanu *et al*., 2016). Therefore, it is necessary to provide *ricotta fresca* with a

protection against the growth of unwanted bacteria. A possible strategy is the use of microbiological cultures aimed to control the multiplication of contaminants. Previous investigation has been conducted to assess the feasibility of using different biopreservatives in *ricotta fresca* (Spanu et al., 2017). The study, conducted on naturally contaminated ricotta, indicated *Carnobacterium* spp as a possible protective culture to be used to control the growth of *Pseudomonas* spp. The present study was aimed to validate the efficacy of *Carnobacterium* spp inoculated on the surface of ricotta fresca before packaging to control the growth of *Pseudomonas* spp in artificially contaminated samples. The microbiological profile of controls (not inoculated) samples showed a natural intitial contamination level of *Pseudomonas* spp of ca. 2 log cfu g^{-1}. *Pseudomonas* spp grew to level as high as 7 log cfu g^{-1} after 21 days of refrigerated storage. *Carnobacterium* spp showed a good adaptation to the substrate showing an increase in mean counts of ca. 2 log cfu g^{-1} during refrigerated storage. The use of *Carnobacterium* spp was effective in reducing *Pseudomonas* spp concentration respectively of 1.28 log cfu g^{-1} and 0.83 log cfu g^{-1} after 14 and 21 days of storage. These results are in agreement with previous studies conducted on naturally contaminated sheep *ricotta fresca* (Spanu et al., 2017). This is explained by the level of artificial contamination used in the present study, which was comparable with the natural level of *Pseudomonas* contamination. In turn, this finding highlight the need of considering *ricotta fresca* as a "high risk" production and of improving the procedures based on the HACCP principles put in place in Sardinian cheesemaking plants. Despite the growth of *Carnobacterium* spp, little difference was observed in pH value between ricotta fresca inoculated with the protective culture and control samples. Variation of pH over time was comparable with previous observations (Pala et al., 2016; Spanu et al., 2017). No significant differences were observed in a_W and composition between blank samples and samples inoculated with *Carnobacterium* spp. However, the possible impact on sensory properties of the protective culture should be further investigated. In the present study sensory analysis were not performed because the level of *Pseudomonas* spp contamination at T_{14} was already as high as 6

$\log_{10}$, compatible with possible alteration of the product. Before performing sensory analysis it is necessary first to guarantee the safety of the product and the absence of any alteration that could compromise acceptability of consumers.

The evolution of the gas composition in the headspace followed a trend which was expected. After MAP packaging a residual concentration of ca. 0,52% O_2 persist in the headspace, which increases during the first week of storage to above 1.0% and successively decrease until ca. 0.03% at T_{21}. This could be explained with the release into the headspace of O_2 incorporated in the food matrix at first, than the successive reduction is attributable to the consumption of O_2 during the successive growth of the microorganisms. The differences in the CO_2 content in the headspace with respect to the concentration used in the gas mixture was the result of prompt gas solving in the product, while the successive CO_2 reduction could be related to permeability of the plastic lidding film.

To mimic foreseeable condition during retail selling after 7 days of refrigerated storage *Ricotta fresca* samples were opened by removal of the film lid and exposing the product to atmospheric air. Samples were then submitted to three days of thermal abuse (T_{10}) at 8±2°C. Abused samples showed a significant increase in aerobic mesophilic bacteria count and of *Pseudomonas* spp that reached respectively, level as high as 8 log cfu g^{-1} and 7.65 log cfu g^{-1}. This further confirms the importance of the cold chain in preserving the microbiological durability of *ricotta fresca*.

5. Conclusion

The use of *Carnobacterium* spp as a protective culture, sprayed on the surface of ricotta fresca during refrigerated storage, demonstrated to be effective to obtain a reduction of ca 1 log of *Pseudomonas* spp growth. Despite *Carnobacterium* spp does not serve a single step against *Pseudomonas*, it may be part of an integrated approach where several combined control measures limit the growth of the microorganism to an acceptable level.

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Table 1. Experimental design: number of *Ricotta fresca* samples and types of analysis performed at each sampling time.

Analysis	type of samples	Sampling time T_0	T_7	T_{10}	T_{14}	T_{21}	Total
- Microbiological profile: aerobic mesophilic bacteria; mesophilic lactic acid bacteria; *Enterobacteriacae; Pseudomonas* spp; L. monocytogenes; Carnobacterium spp;yeast and molds.	RfB	9	9	-	9	9	36
	RfPf	9	9	-	9	9	36
	RfPf+C	9	9	-	9	9	36
	RfPf+C_{open}	-	-	9	-	-	9
- Intrinsic properties: pH and a_W; composition (%): moisture; fat; protein; - Headspace composition (%): CO_2; O_2	Ph-Ch	9	9	9	9	9	45
Total		36	36	18	36	36	162

T_0 = day of inoculum; T_7, T_{10}, T_{14}, and T_{21}= respectively 7, 10, 14 and 21 days of storage after the inoculum. RfB: not inoculated blank samples; RfPf: samples inoculated with *Pseudomonas fluorescens*; RfPf+C: samples inoculated with both *Pseudomonas fluorescens* and *Carnobacterium* spp; RfPf+C_{open}: samples inoculated with both *Pseudomonas fluorescens* and *Carnobacterium* spp and stored refrigerated after the removal of the film lid; Ph-Ch: samples used for the determination of intrinsic properties, composition and headspace gas.

Table 2. Evolution of the microbiological profile (log_{10} cfu g^{-1}; $\bar{x}$ ± SD) of ricotta during refrigerated storage by sample type.

Microbial group	Type of samples	Day of storage			
		T_0	T_7	T_{14}	T_{21}
Aerobic mesophilic bacteria	RfB	3.1 ± 0.7^{a1} (n = 9/9)	4.7 ± 0.8^{b1} (n = 9/9)	5.7 ± 0.8^{c1} (n = 9/9)	6.3 ± 0.9^{c1} (n = 9/9)
	RfPf	3.0 ± 0.7^{a1} (n = 9/9)	5.0 ± 0.5^{b2} (n = 9/9)	7.6 ± 0.5^{c1} (n = 9/9)	8.3 ± 0.3^{d2} (n = 9/9)
	RfPf+C	2.8 ± 1.3^{a1} (n = 9/9)	6.9 ± 1.1^{b2} (n = 9/9)	8.6 ± 0.4^{c2} (n = 9/9)	9.1 ± 1.3^{c3} (n = 9/9)
mesophilic lactic acid bacteria	RfB	2.3 ± 0.1^{ab1} (n = 4/9)	1.7 ± 0.6^{a1} (n = 6/9)	2.8 ± 0.9^{b12} (n = 6/9)	3.8 ± 1.1^{c1} (n = 9/9)
	RfPf	2.1 ± 0.5^{a1} (n = 6/9)	3.3 ± 0.8^{b2} (n = 8/9)	3.7 ± 0.8^{b2} (n = 8/9)	5.0 ± 0.1^{c2} (n = 9/9)
	RfPf+C	3.5 ± 1.1^{a2} (n = 6/9)	3.0 ± 1.0^{ab2} (n = 8/9)	2.5 ± 0.4^{a1} (n = 5/9)	2.9 ± 0.9^{a3} (n = 9/9)
Enterobacteriacae	RfB	ND	ND	2.6 ± 1.0^{a1} (n = 3/9)	3.2 ± 0.9^{a1} (n = 9/9)
	RfPf	2.0 ± 0.0^{a} (n = 1/9)	1.9 ± 0.0^{a} (n = 1/9)	2.4 ± 0.9^{a1} (n = 5/9)	3.9 ± 0.6^{a1} (n = 5/9)
	RfPf+C	ND	1.9 ± 0.0^{ab} (n = 1/9)	1.5 ± 0.8^{a1} (n = 4/9)	3.1 ± 0.7^{b1} (n = 6/9)
Pseudomonas spp	RfB	2.1 ± 0.2^{a1} (n = 2/9)	4.5 ± 1.0^{b1} (n = 8/9)	5.6 ± 1.3^{c1} (n = 8/9)	6.5 ± 0.8^{c1} (n = 6/9)
	RfPf	2.3 ± 0.4^{a1} (n = 9/9)	5.3 ± 0.6^{b2} (n = 9/9)	8.2 ± 0.3^{c2} (n = 9/9)	8.6 ± 0.4^{c2} (n = 9/9)
	RfPf+C	2.3 ± 0.3^{a1} (n = 9/9)	4.8 ± 0.5^{b12} (n = 9/9)	6.9 ± 0.7^{c3} (n = 9/9)	7.8 ± 0.6^{d3} (n = 9/9)
Yeast and molds	RfB	ND	ND	ND	ND
	RfPf	2.7 ± 0.3^{a1} (n = 3/9)	3.2 ± 0.6^{a1} (n = 4/9)	3.5 ± 0.7^{a1} (n = 8/9)	3.6 ± 0.9^{a1} (n = 9/9)
	RfPf+C	2.4 ± 0.1^{ab1} (n = 3/9)	2.8 ± 0.1^{ab1} (n = 2/9)	2.5 ± 1.0^{a2} (n = 8/9)	3.9 ± 0.9^{b1} (n = 3/9)
Carnobacterium spp	RfB	2.6 ± 0.5^{a1} (n = 4/9)	3.1 ± 0.9^{ab1} (n = 7/9)	3.2 ± 1.1^{ab1} (n = 9/9)	4.2 ± 1.2^{b1} (n = 9/9)
	RfPf	2.1 ± 0.5^{a1} (n = 7/9)	3.4 ± 0.4^{b1} (n = 8/9)	4.1 ± 0.4^{c2} (n = 9/9)	4.8 ± 0.9^{d1} (n = 9/9)
	RfPf+C	6.4 ± 0.3^{a2} (n = 9/9)	6.9 ± 0.6^{b2} (n = 9/9)	7.9 ± 0.6^{c3} (n = 9/9)	8.8 ± 0.3^{d2} (n = 9/9)

T_0 = day of inoculum; T_7, T_{10}, T_{14}, and T_{21}= respectively 7, 10, 14 and 21 days of storage after the inoculum. RfB: not inoculated blank samples; RfPf: samples inoculated with *Pseudomonas fluorescens*; RfPf+C: samples inoculated with both *Pseudomonas fluorescens* and *Carnobacterium* spp;
Means in the same row with different superscript letter were significantly different (P<0.05); means in the same column among type of samples with different superscript number were significantly different (P<0.05). Values within brackets indicate the prevalence of positive samples.

Table 3. Intrinsic properties, physico-chemical characteristics and MAP gas composition ($\bar{x}$ ±SD) of ricotta during refrigerated storage by sample type.

		Day of storage			
Parameters	Type of samples	T_0	T_7	T_{14}	T_{21}
pH	RfB	6.7±0.1^{a1}	6.6±0.2^{a1}	6.6±0.1^{a1}	6.6±0.0^{a1}
	RfPf	6.8±0.1^{a1}	6.6±0.1^{ab1}	6.6±0.0^{b1}	6.6±0.0^{ab1}
	RfPf+C	6.8±0.1^{a1}	6.7±0.1^{ab1}	6.6±0.1^{bc1}	6.5±0.1^{c2}
a_w	RfB	0.986 ± 0.001^{a1}	0.987 ± 0.001^{a1}	0.987 ± 0.001^{a1}	0.987 ± 0.001^{a1}
	RfPf	0.987 ± 0.001^{a1}	0.986 ± 0.001^{b1}	0.986 ± 0.001^{a1}	0.987 ± 0.001^{ab1}
	RfPf+C	0.987 ± 0.001^{a1}	0.984 ± 0.001^{b1}	0.985 ± 0.001^{b1}	0.986 ± 0.001^{ab1}
Moisture (%)	RfB	71.8±4.9^{a1}	71.4±2.8^{a1}	72.6±2.8^{a1}	71.9±2.4^{a1}
	RfPf	70.9±3.0^{a1}	72.0±5.0^{a1}	72.6±5.7^{a1}	74.3±1.3^{a1}
	RfPf+C	73.0±4.0^{a1}	72.0±3.8^{a1}	71.2±3.9^{a1}	74.2±1.4^{a1}
Fat (%)	RfB	17.3±5.7^{a1}	16.9±4.3^{a1}	13.9±2.0^{a1}	15.6±2.6^{a1}
	RfPf	17.3±4.4^{a1}	15.8±5.6^{a1}	15.2±6.7^{a1}	15.0±2.6^{a1}
	RfPf+C	15.8±6.8^{a1}	16.9±4.7^{a1}	17.3±3.9^{a1}	12.6±1.8^{a1}
Protein (%)	RfB	10.1±1.2^{a1}	10.6±0.4^{a1}	11.0±0.8^{a1}	11.2±1.0^{a1}
	RfPf	11.1±1.2^{a1}	10.8±1.5^{a1}	10.3±0.1^{a1}	11.4±1.6^{a1}
	RfPf+C	10.2±1.2^{a1}	10.0±0.8^{a1}	10.2±1.2^{a1}	10.7± 0.3^{a1}
O_2%	RfB	0.7± 0.3^{ab1}	1.1 ±0.3^{ab1}	1.8±0.6^{b1}	0.0 ± 0.0^{a1}
	RfPf	0.5±0.2^{a1}	1.2±0.1^{b1}	0.1±0.2^{c2}	0.1±0.1^{c1}
	RfPf+C	0.4±0.1^{a1}	1.6±2.5^{a1}	0.2±0.2^{a2}	0.0±0.0^{a1}
$C0_2$%	RfB	16.4±0.5^{a1}	8.1±1.5^{b1}	5.9±0.5^{c1}	6.7±0.6^{bc1}
	RfPf	16.9±0.1^{a1}	7.9±1.0^{b1}	6.6±0.2^{c1}	7.2±0.2^{bc12}
	RfPf+C	16.8±1.0^{a1}	8.5±0.8^{b1}	7.0±1.1^{b1}	7.6± 0.4^{b2}

T_0 = day of inoculum; T_7, T_{10}, T_{14}, and T_{21}= respectively 7, 10, 14 and 21 days of storage after the inoculum. RfB: not inoculated blank samples; RfPf: samples inoculated with *Pseudomonas fluorescens*; RfPf+C: samples inoculated with both *Pseudomonas fluorescens* and *Carnobacterium* spp; Means in the same row with different superscript letter were significantly different (P<0.05); means in the same column among type of samples with different superscript number were significantly different (P<0.05).