

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

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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

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3 1 **Use of *Carnobacterium* spp protective culture in MAP packed Ricotta *fresca* cheese to control**
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5 2 ***Pseudomonas* spp**

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21
22 10 **Abstract**

23
24 11 Ricotta *fresca* is a whey cheese susceptible of secondary contamination, mainly from *Pseudomonas*
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26 12 spp. The extension of the shelf life of refrigerated *ricotta fresca* could be obtained using protective
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28 13 cultures inhibiting the growth of this spoilage microorganism. A commercial biopreservative,
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30 14 Lyofast CNBAL, comprising *Carnobacterium* spp was tested against *Pseudomonas* spp. The
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32 15 surface of *ricotta fresca* samples were inoculated either with *Pseudomonas* spp or *Pseudomonas*
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34 16 and *Carnobacterium* spp. Samples were MAP packed, stored at 4°C and analyzed the day of the
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36 17 inoculum and 7, 14 and 21 days after the contamination. Microbiological analyses included total
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38 18 bacterial count, mesophilic lactic acid bacteria, *Enterobacteriaceae*, *Pseudomonas* spp, *Listeria*
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40 19 *monocytogenes*, moulds and yeasts. *Pseudomonas* mean initial contamination level was comparable
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42 20 in blank and artificially inoculated samples, respectively with values of 2.15±0.21 and 2.34±0.26
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44 21 log cfu g⁻¹. *Carnobacterium* spp. significantly reduced the growth of *Pseudomonas* spp respectively
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46 22 of 1.28 log and 0.83 log after 14 and 21 days of refrigerated storage. Intrinsic properties and
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48 23 physico-chemical composition were also investigated. Limited variation of pH was observed in
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50 24 samples inoculated with the protective cultures, indicating low acidification properties of
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62 25 *Carnobacterium* spp. Instead, no significant differences were observed for a_w , moisture, fat and
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64 26 proteins during storage and between inoculated and control samples.
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69 28 Keywords: *Carnobacterium* spp.; protective cultures; *Pseudomonas* spp; ricotta; MAP.
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72 73 30 **1. Introduction**

74
75 31 According to the Codex Standard for whey cheeses, these are solid, semi-solid, or soft products,
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77 32 which are principally obtained through either the concentration of whey or the coagulation of whey
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79 33 by heat with or without the addition of acid (Codex Alimentarius Codex Standard 284-1971). Whey
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81 34 cheeses are manufactured all over the world at artisanal and industrial level using different
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83
84 35 protocols depending on the country of origin. Ricotta, originally from Italy is the most renowned
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86 36 whey cheese in the world (Pintado & Malcata, 2000). However, in European countries, especially in
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88 37 the Mediterranean basin, wide varieties of whey cheeses are manufactured, mainly from ovine
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90 38 whey. These whey cheeses fall under several designation, e.g. Anthotyros (Greece), Anari (Cyprus),
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92 39 Requesón (Spain), Requeijao (Portugal), Broccio (France), Urdă (Balkans region). Ricotta *fresca* is
93
94 40 a traditional whey cheese, which in Sardinia is produced by heat coagulation of sheep's milk whey
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96 41 remaining after the production of *Pecorino* type cheeses. The discontinuous manufacturing method
97
98 42 include whey heating into open kettles to 80-82 °C. At this temperature the flocculated proteins rise
99
100 43 and float on the surface. The curd is then scooped and transferred into perforated plastic baskets.
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102
103 44 Ricotta baskets are allowed to drain and to cool into a cold room. The detailed manufacturing
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105 45 process of Ricotta *fresca* has been previously described (Pala et al., 2016). Ricotta *fresca* is packed
106
107 46 either in food wrapping paper or in polypropylene trays that are heat sealed with plastic lidding
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109 47 film. Depending on the food business operator, atmospheric air or modified atmosphere packaging
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111 48 (MAP) is used. MAP conditions largely vary from one operator to another; with 30% CO₂ and 70%
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113 49 N₂ being the most used gas mixture used. The shelf life is designated under the responsibility of the
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121 50 food business operator and varies from 5-7 days for wrapped Ricotta up to 21 days for MAP
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123 51 Ricotta. *Ricotta fresca* is poor in natural microflora since it is drastically reduced by the high
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125 52 temperature applied during the thermal denaturation of whey proteins (Pintado, Macedo & Malcata,
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128 53 2001). *Ricotta fresca* is exposed to post-process contamination originating from the cheese-making
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130 54 environment, particularly in the steps between molding and packaging (Greenwood, Roberts &
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132 55 Burden, 1991; Ibba et al., 2013). Given the high moisture (70-80%), elevated pH (6.10-6.80) and
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134 56 the water activity (0.974-0.991) and the refrigerated storage (De Santis, Mazzette, Scintu, Deriu, &
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136 57 Carta, 1999), *Ricotta fresca* is an excellent substrate for the possible growth of several psychotropic
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138 58 pathogenic and spoilage microorganisms (De Santis & Mazzette, 2002). Common contaminants of
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140 59 Ricotta fresca include *Pseudomonas* spp., *Enterobacteriaceae*, *Listeria monocytogenes*, *B. cereus*
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142 60 and *Arcobacter* spp., yeast and moulds (Pintado, Macedo & Malcata, 2001; De Santis and Mazzette,
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144 61 2002; De Santis et al. 2008; Ibba et al., 2013; Scarano et al.; 2014; Spanu et al., 2016; Tirloni et al.,
145
146 62 2017). However, *Pseudomonas* spp. benefits of the selective advantage of the combination of low
147
148 63 temperature and long storage to overgrow other microorganisms, reducing the risk of pathogens
149
150 64 growth (Buchanan & Bagi, 1999; Carrascosa et al., 2015). Therefore, *Pseudomonas* spp. represents
151
152 65 the main obstacle to extend *Ricotta fresca* shelf life (Pala et al., 2016). Growth of *Pseudomonas*
153
154 66 spp. to level as high as $7 \log_{10} \text{ cfu g}^{-1}$ negatively affect the sensory properties of the product
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156 67 (Leriche et al., 2004). Important discoloration of the product derives by the secretion of yellow-
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158 68 green pyoverdin (Meyer et al., 2002) and of a blue pigment (Cantoni, Stella, Cozzi, Iacumin, &
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160 69 Comi, 2003; Martin, Murphy, Ralyea, Wiedmann & Boor, 2011; Andreani et al., 2015) from some
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162 70 *Pseudomonas* spp. In order to reduce the initial microbial load at production, it is essential the strict
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164 71 application of good hygiene and good manufacturing procedures. A previous investigation,
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166 72 conducted on Sardinian cheese-making plant manufacturing *Ricotta fresca*, showed that there is an
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168 73 association between secondary contamination with *Pseudomonas* spp. and the level of
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170 74 implementation of the food safety management system at plant level (Pala et al., 2016). There is the
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180 75 need of additional strategies to control the growth of *Pseudomonas* spp., especially in industrial
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182 76 MAP *Ricotta fresca*. The use of bio preservatives to extend the shelf life of whey cheeses has been
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184 77 previously tested (Davies, Bevis, & Delves-Broughton, 1997; Samelis, Kakouri, Rogga, Savvaidis,
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186 78 & Kontominas, 2003; Martins, Cerqueira, Souza, do Carmo Avides, & Vicente et al., 2010).
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189 79 Carnobacteria are Gram positive rod shaped lactic acid bacteria (LAB) isolated from different
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191 80 ecological niches. The genus *Carnobacterium*, is able to produce bacteriocins therefore, it has
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193 81 been used as protective cultures to provide durability and safety to various types of foods (Elsser-
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195 82 Gravesen & Elsser-Gravesen, 2013). Of the species included in the genus *Carnobacterium*, only
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197 83 two, *C. divergens* and *C. maltaromaticum*, are frequently isolated from natural environment and
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199 84 dairy products (Leisner, Laursen, Prevost, Drider, & Dalgaard, 2007; Afzal et al., 2010).
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201 85 *Carnobacterium* spp, due to similar growth conditions (pH and temperature variation) of
202
203 86 psychrotrophic spoilage and pathogen microorganisms, can improve the durability of chilled foods
204
205 87 by reducing or inhibiting the growth of *Pseudomonas* spp. To date, little investigation has been
206
207 88 conducted to validate the efficacy of bio preservatives against *Pseudomonas* spp. in sheep *Ricotta*
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209 89 *fresca* cheese. A commercial protective culture, consisting of a selected strain of *Carnobacterium*
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211 90 spp producing bacteriocins, the Lyofast CNBAL (Clerici-Sacco Group, Como, Italy) was sprayed
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213 91 on *Ricotta fresca* surface (Spanu et al., 2017). The study demonstrated a good adaptation to the
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215 92 substrate with growth during refrigerated storage with no acidification of the product.
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217 93 *Carnobacterium* spp. showed a competitive activity against *Pseudomonas* spp. in *Ricotta fresca*.
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219 94 However, the experiment was conducted on naturally contaminated samples. The main objective of
220
221 95 the present study was to evaluate, as a possible innovation in the manufacturing process of *Ricotta*
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223 96 *fresca*, the use of a protective culture to control the superficial contamination against spoilage
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225 97 microorganisms. With this aim, the efficacy of *Carnobacterium* spp against the growth of
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227 98 *Pseudomonas* spp was tested in artificially contaminated *Ricotta fresca*.
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100 2. Materials and methods

101 2.1. Samples

102 The study was conducted on 162 *Ricotta fresca* samples provided by a local industrial cheese-
103 making plant using the whey remaining after the production of hard sheep's milk cheese (e.g.
104 *Pecorino* type cheeses). The samples were truncated cone shaped cheese with an upper base ca. 7.5
105 cm wide and a lower base ca. 5 cm wide, weighing approximately 1.1 kg. Fifty-five *Ricotta fresca*
106 samples were randomly selected from each of three different batches (each batch was manufactured
107 in a different day of production). Samples were packed in MAP (gas mixture was 30% CO₂ and
108 70% N₂) using rigid polypropylene trays sealed with high-barrier peelable laminated films. Films
109 were made of bioriented polyamide and cast polypropylene with O₂ T.R. (20°- 65% R.H.) of ~30
110 cc/m², 24 h. After packaging samples were transported refrigerated to the laboratory within 24 from
111 the production. Within each batch, samples were randomly allocated to the following treatment:
112 artificial inoculation with *Pseudomonas fluorescens* (RfPf), artificial inoculation with *Pseudomonas*
113 *fluorescens* and *Carnobacterium* spp (RfPf+C), no treatment (RfB). Part of the samples (Ph-Ch)
114 were used for the determination of intrinsic properties (pH and a_w), percentage composition
115 (moisture, fat, protein) and headspace gas composition (CO₂ and O₂).

116 2.2 Artificial inoculation

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

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298 125 broth (Oxoid, Basingstoke, UK) with glycerol (20% v/v). In order to account for natural
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300 126 contamination levels and allow the enumeration of the inoculum, the target level was 10^2 cfu g⁻¹ of
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302 product surface (Spanu et al., 2014). Preliminary trials were conducted in order to determine the
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304 growth condition necessary to standardize the level of inoculum and to adapt strains to refrigeration
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306 temperature. Each strains was subcultured by streaking onto the non selective trypticase soy agar
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308 medium (TSA, Biolife, Milan, Italy) and incubated for 24 h at 25 °C. A single colony was picked
309 130
310 and transferred into tubes containing the non-selective brain heart infusion broth, BHI (Oxoid,
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312 Basingstoke, UK). Tubes were incubated overnight at 25°C in a shaking water bath to obtain cells
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314 in the same physiological state (late exponential or early stationary phase). Adaptation of the strains
315 133
316 to refrigeration was conducted by subculturing in BHI broth at 4±2 °C until cells were in the
317 134
318 exponential growth phase (Uyttendaele et al., 2004). Equal volumes of each individual culture were
319 135
320 mixed in a sterile flask. From the mixture were prepared adequate serial decimal dilution in
321 136
322 phosphate buffered saline (Biolife) to adjust the concentration to 10⁴ cfu mL⁻¹. After the removal of
323 137
324 the sealing film, an aliquot of 1.5-2.0 mL of inoculum was evenly sprayed on the surface of Ricotta
325 138
326 fresca (ca. 290 cm², corresponding to ca. 40-50 g of product). All concentrations were confirmed
327 139
328 by plate count on TSA (Biolife).
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332 141 After the inoculum of *Pseudomonas fluorescens*, the RfP+C units (n. 36) were also inoculated with
333 142
334 the protective cultures Lyofast CNBAL (Clerici-Sacco Group, Como, Italy) consisting of a selected
335 143
336 strain of *Carnobacterium* spp producing bacteriocins with an optimum growth temperature between
337 144
338 25-45 °C. The commercial formulation is a freeze-dried powder, which, according to
339 145
340 manufacturer's instructions, was diluted in Phosphate-buffered saline (PBS) solution (Oxoid) to
341 146
342 obtain 10⁶ cfu g⁻¹ of product. With this aim, the suspension was adjusted to a concentration of 10⁸
343 147
344 cfu mL⁻¹ and 1.5-2.0 mL were evenly sprayed on the *Ricotta fresca* surface. *Carnobacterium* spp
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346 concentrations were confirmed by count on agar plates. After the inoculation, all samples (included
347 149
348 untreated blank samples) were repacked in MAP (30% CO₂ and 70% N₂) using the FP Basic Sec
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150 tray sealer (Ilpra, Vigevano, Italy). Blank samples were packed under the same conditions of the
151 other samples with no further manipulation. All samples were stored under refrigeration (+ 4°C)
152 until analysis were performed.

2.3 Experimental design

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

2.3. Microbiological profile

164 *Ricotta fresca* samples submitted to microbiological examination were prepared according to ISO
165 6887-1:1999. Briefly, the initial suspension and decimal dilution was conducted after the sterile
166 collection of to 25 g of ricotta surface into a sterile plastic stomacher bag. 225 mL of Buffered
167 Peptone Water were added and the sample homogenized using a stomacher. Decimal serial dilutions
168 were obtained by transferring 1 mL of the initial suspension into a tube containing 9 mL of sterile
169 diluent. If required, these operations were repeated using the 10^{-2} to obtain further serial decimal
170 dilution. The pour-plating procedure was used for the enumeration of aerobic mesophilic bacteria,
171 mesophilic lactic acid bacteria and *Enterobacteriaceae*, yeasts and moulds. Plate Count Agar and
172 MRS medium at pH 5.7 (Biolife, Milan, Italy) were incubated at $30^\circ\text{C} \pm 1^\circ\text{C}$ for $72\text{ h} \pm 3\text{ h}$
173 respectively for the enumeration of aerobic mesophilic bacteria (ISO 4833:2003) and mesophilic
174 lactic acid bacteria (ISO 15214: 1998). The enumeration of *Enterobacteriaceae* was conducted

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416 175 using the plate count technique without resuscitation, incubating Violet Red Bile Agar plates
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418 176 (Biolife) at 35-37°C for 24 h (ISO 21528-1:2004). Enumeration of yeast and molds was conducted
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420
421 177 using Chloramphenicol Yeast Glucose Agar plates (Biolife) incubated at 25 ± 1°C up to 5 days
422
423 178 (ISO 6611/IDF094:2004). For the enumeration of *Pseudomonas* spp (ISO/TS 11059:2009), 0.1 mL
424
425 179 of each decimal dilution were spread over the surface of Pseudomonas Agar Plates added with PP
426
427 180 supplement (Biolife) and incubated at 25 °C ± 1°C for 48 h ± 2 h. For the detection of *Listeria*
428
429 181 *monocytogenes* (ISO 2004a) the initial suspension was supplemented with Fraser Half Selective
430
431 182 Supplement (Biolife) and incubated at 30 °C for 24 h (primary enrichment). After incubation 0.1
432
433 183 mL of the primary enrichment were subcultured into 10 mL of Fraser Broth and incubated at 37°C
434
435 184 for 24-48 h (enrichment). From both, pre-enrichment and enrichment broth, 0.1 mL were streaked
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437
438 185 onto Agar Listeria Ottaviani Agosti (ALOA, Biolife) and Oxford (Oxoid, Basingstoke, UK) agar
439
440 186 plates and incubated at 37 °C for up to 48 ±3 h. Enumeration of *L. monocytogenes* (ISO 2004b)
441
442 187 was a conducted streaking 1 mL volume of the initial dilution onto both three ALOA and three
443
444 188 Oxford agar plates and incubated at 37 °C for up to 48 ±3 h. The enumeration of *Carnobacterium*
445
446 189 *spp* was conducted using MRS modified by increasing the pH to 8.5, omitting acetate, and
447
448 190 substituting glucose for sucrose (Hammes et al., 1992).
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450 191 2.5. *Pseudomonas fluorescens* characterization

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452 192 A total of five colonies from each sample containing typical colonies on Pseudomonas Agar Plates
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454 193 were confirmed with two different PCR protocols, one used to identify *Pseudomonas fluorescens*
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456
457 194 (Scarpellini et al., 2004) and the other to identify *Pseudomonas* spp and *Pseudomonas aeruginosa*
458
459 195 (De Vos et al., 1997). Among the confirmed *Pseudomonas fluorescens* strains, up to three isolates
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461 196 per samples were randomly selected and analyzed using pulsed-field gel electrophoresis (PFGE).
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463 197 After DNA extraction, agarose plugs were digested with enzyme restriction *SpeI* (Roche
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465 198 Diagnostics,
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475 199 Laval, Quebec, Canada) (Maslow, 1993). PFGE was performed using the protocol described by
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477 200 (Gershman et al., 2008). The obtained restriction profiles were analyzed by visual examination to
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480 201 distinguish inoculated strains among each other and from strains originating by natural
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482 202 contamination.

483 484 203 *2.5. Intrinsic properties, composition and headspace gas analysis*

485
486 204 Determination of pH and a_w was conducted using pH meter GLP22 (Crison Instruments SA,
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488 205 Barcelona, Spain) and water activity meter Aqualab 4TE (Decagon, Pullman, WA, USA),
489
490 206 respectively. The near-infrared spectrophotometer system for the determination of centesimal
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492 207 composition (%) of fat, moisture, protein and total solids was used. Ricotta *fresca* samples were
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494 208 accurately mixed, placed into Petri dishes and read in the light spectrum of 850-1050 nm using the
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496 209 compositional FoodScan™ device (FOSS, Analytic, Hillerød, Denmark). The headspace gas
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499 210 composition was conducted on MAP ricotta *fresca* samples on the sealed packages before
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501 211 performing other analysis. Measure of combined residual O₂ % and CO₂ % were obtained piercing
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503 212 the lid using a sterile needle connected to the Dansensor gas analyser (PBI Dansensor, Ringsted,
504
505 213 Denmark). To avoid gas leaks during the penetration of needle, 15 Ø mm septum (PBI Dansensor),
506
507 214 were applied on the film lid before measurements of headspace gas composition.

509 215 *2.6. Statistical analysis*

511 216 Differences among average microbiological group counts (\log_{10} cfu g⁻¹), headspace gas
512
513 217 concentration (%), intrinsic properties and centesimal composition (%) over time (T₀, T₇, T₁₄ and
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515
516 218 T₂₁) and among types of samples (RfB, RfPf and RfPf+C) within one time point were compared
517
518 219 using Fisher's least significant difference test. Statistical analyses were performed with Statgraphics
519
520 220 Centurion XVI software (Stat Point Technologies, Warrenton, VA, USA). The effectiveness of
521
522 221 *Carnobacterium* spp in controlling the growth of *Pseudomonas fluorescens* was evaluated
523
524 222 computing the minimum difference (Δ) between of the \log_{10} cfu g⁻¹ between RfPf and RfPf+C
525
526 223 samples at each time point (T₇, T₁₄ and T₂₁).

3. Results

3.1. Microbiological profile

In *Ricotta fresca* blank samples the total bacterial count was $3.12 \pm 0.71 \log_{10} \text{ cfu g}^{-1}$ at T_0 and gradually increased during refrigerated storage up to $6.34 \pm 0.87 \log_{10} \text{ cfu g}^{-1}$ at T_{21} . The mesophilic lactic acid bacteria ranged between $2.25 \pm 0.06 \log_{10} \text{ cfu g}^{-1}$ at T_0 and $3.83 \pm 1.06 \log_{10} \text{ cfu g}^{-1}$ at T_{21} . *Enterobacteriaceae* were observed in one samples both at T_0 and at T_7 with counts of ca. $2 \log_{10} \text{ cfu g}^{-1}$; their concentration increased to $3.48 \pm 0.58 \log_{10} \text{ cfu g}^{-1}$ 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 $2.15 \pm 0.21 \log_{10} \text{ cfu g}^{-1}$ at T_0 and increased to $6.48 \pm 0.78 \log_{10} \text{ cfu g}^{-1}$ at T_{21} . In *ricotta fresca* samples inoculated with the protective culture, *Carnobacterium* spp. grew from the initial concentration of $6.36 \pm 0.32 \log_{10} \text{ cfu g}^{-1}$ at T_0 to $8.78 \pm 0.25 \log_{10} \text{ cfu g}^{-1}$ 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. $2.30 \log_{10} \text{ cfu g}^{-1}$. After 14 and 21 days of refrigerated storage *Pseudomonas* concentration was respectively 1.28 and $0.83 \log_{10} \text{ cfu g}^{-1}$ 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 ($\log_{10} \text{ cfu g}^{-1}$; $\bar{x} \pm \text{SD}$) 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 $8.40 \pm 0.63 \log_{10} \text{ cfu g}^{-1}$ (n = 9/9), mesophilic lactic acid bacteria $3.12 \pm 0.41 \log_{10} \text{ cfu g}^{-1}$ (n = 6/9), *Carnobacterium* spp $6.90 \pm 1.89 \log_{10} \text{ cfu g}^{-1}$ (n = 9/9), *Enterobacteriaceae* $2.15 \log_{10} \text{ cfu g}^{-1}$ (n = 1/9), *Pseudomonas* spp $7.65 \pm 0.51 \log_{10} \text{ cfu g}^{-1}$ (n = 9/9), yeast and molds $2.86 \pm 0.43 \log_{10} \text{ cfu g}^{-1}$ (n = 5/9), while *L. monocytogenes* was never detected.

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249 3.2. Physico-chemical characteristics and MAP gas composition

250 A moderate decrease of pH was observed over time in all *ricotta fresca* samples. However,
251 significant difference between blank samples and in samples inoculated with *Carnobacterium spp*
252 were observed only at T₂₁, showing respectively values of 6.60±0.04 and 6.48±0.07 (P<0.05). The
253 a_w was stable over time and between sample types ranging from 0.984±0.001 and 0.987±0.001. No
254 significant difference was observed among blank samples and ricotta inoculated with
255 biopreservatives in moisture, fat and protein composition, showing respectively a mean content of
256 72.38±3.15, 15.67±3.99 and 10.70±0.93. The O₂ content in the headspace increased from the initial
257 level of 0.52±0.24% up to 1.30±1.28% at T₇, to decrease again as low as 0.03±0.05 at T₂₁. Instead,
258 the CO₂ content decreased from T₀ to T₂₁ respectively from 16.71±0.61% to 7.16%. No significant
259 difference in gas composition were observed between blank and inoculated *ricotta fresca* samples.
260 Intrinsic properties, composition and gas composition in the headspace ($\bar{x} \pm SD$) during the
261 refrigerated storage are reported in table 3.

262 3.3. *Pseudomonas spp* natural and artificial contamination

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

269 4. Discussion

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

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652 274 protection against the growth of unwanted bacteria. A possible strategy is the use of microbiological
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654 275 cultures aimed to control the multiplication of contaminants. Previous investigation has been
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657 276 conducted to assess the feasibility of using different biopreservatives in *ricotta fresca* (Spanu et al.,
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659 277 2017). The study, conducted on naturally contaminated ricotta, indicated *Carnobacterium* spp as a
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661 278 possible protective culture to be used to control the growth of *Pseudomonas* spp. The present study
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663 279 was aimed to validate the efficacy of *Carnobacterium* spp inoculated on the surface of ricotta fresca
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665 280 before packaging to control the growth of *Pseudomonas* spp in artificially contaminated samples.
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667 281 The microbiological profile of controls (not inoculated) samples showed a natural initial
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669 282 contamination level of *Pseudomonas* spp of ca. 2 log cfu g⁻¹. *Pseudomonas* spp grew to level as
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671 283 high as 7 log cfu g⁻¹ after 21 days of refrigerated storage. *Carnobacterium* spp showed a good
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673 284 adaptation to the substrate showing an increase in mean counts of ca. 2 log cfu g⁻¹ during
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676 285 refrigerated storage. The use of *Carnobacterium* spp was effective in reducing *Pseudomonas* spp
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678 286 concentration respectively of 1.28 log cfu g⁻¹ and 0.83 log cfu g⁻¹ after 14 and 21 days of storage.
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680 287 These results are in agreement with previous studies conducted on naturally contaminated sheep
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682 288 *ricotta fresca* (Spanu et al., 2017). This is explained by the level of artificial contamination used in
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684 289 the present study, which was comparable with the natural level of *Pseudomonas* contamination. In
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686 290 turn, this finding highlight the need of considering *ricotta fresca* as a “high risk” production and of
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688 291 improving the procedures based on the HACCP principles put in place in Sardinian cheesemaking
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691 292 plants. Despite the growth of *Carnobacterium* spp, little difference was observed in pH value
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693 293 between ricotta fresca inoculated with the protective culture and control samples. Variation of pH
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695 294 over time was comparable with previous observations (Pala et al., 2016; Spanu et al., 2017). No
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697 295 significant differences were observed in a_w and composition between blank samples and samples
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699 296 inoculated with *Carnobacterium* spp. However, the possible impact on sensory properties of the
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701 297 protective culture should be further investigated. In the present study sensory analysis were not
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703 298 performed because the level of *Pseudomonas* spp contamination at T₁₄ was already as high as 6
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711 299 \log_{10} , compatible with possible alteration of the product. Before performing sensory analysis it is
712
713 300 necessary first to guarantee the safety of the product and the absence of any alteration that could
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716 301 compromise acceptability of consumers.
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718 302 The evolution of the gas composition in the headspace followed a trend which was expected. After
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720 303 MAP packaging a residual concentration of ca. 0,52% O₂ persist in the headspace, which increases
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722 304 during the first week of storage to above 1.0% and successively decrease until ca. 0.03% at T₂₁.
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724 305 This could be explained with the release into the headspace of O₂ incorporated in the food matrix at
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726 306 first, than the successive reduction is attributable to the consumption of O₂ during the successive
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728 307 growth of the microorganisms. The differences in the CO₂ content in the headspace with respect to
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730 308 the concentration used in the gas mixture was the result of prompt gas solving in the product, while
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733 309 the successive CO₂ reduction could be related to permeability of the plastic lidding film.
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735 310 To mimic foreseeable condition during retail selling after 7 days of refrigerated storage *Ricotta*
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737 311 *fresca* samples were opened by removal of the film lid and exposing the product to atmospheric air.
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739 312 Samples were then submitted to three days of thermal abuse (T₁₀) at 8±2°C. Abused samples
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741 313 showed a significant increase in aerobic mesophilic bacteria count and of *Pseudomonas* spp that
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743 314 reached respectively, level as high as 8 log cfu g⁻¹ and 7.65 log cfu g⁻¹. This further confirms the
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745 315 importance of the cold chain in preserving the microbiological durability of *ricotta fresca*.
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749 317 **5. Conclusion**

751
752 318 The use of *Carnobacterium* spp as a protective culture, sprayed on the surface of *ricotta fresca*
753
754 319 during refrigerated storage, demonstrated to be effective to obtain a reduction of ca 1 log of
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756 320 *Pseudomonas* spp growth. Despite *Carnobacterium* spp does not serve a single step against
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758 321 *Pseudomonas*, it may be part of an integrated approach where several combined control measures
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760 322 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					Total
		T ₀	T ₇	T ₁₀	T ₁₄	T ₂₁	
- Microbiological profile: aerobic mesophilic bacteria; mesophilic lactic acid bacteria; <i>Enterobacteriaceae</i> ; <i>Pseudomonas</i> spp; L. monocytogenes; <i>Carnobacterium</i> 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;	Ph-Ch	9	9	9	9	9	45
- Headspace composition (%): CO ₂ ; O ₂							
Total		36	36	18	36	36	162

T₀ = day of inoculum; T₇, T₁₀, T₁₄, and T₂₁= 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} ; $\bar{x} \pm SD$) 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)
<i>Enterobacteriaceae</i>	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)
<i>Pseudomonas</i> 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)
<i>Carnobacterium</i> 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)

T₀ = day of inoculum; T₇, T₁₀, T₁₄, and T₂₁ = 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 ($\bar{x} \pm SD$) of ricotta during refrigerated storage by sample type.

Parameters	Type of samples	Day of storage			
		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}
CO 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}

T₀ = day of inoculum; T₇, T₁₀, T₁₄, and T₂₁= 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).