Short Communication

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 FERM 430D. Not inoculated control and experimental ricotta were MAP packed 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. *Carnobacterium* spp reduced the concentration of *Pseudomonas* spp and *Enterobacteriaceae* of at least 1 $\log_{10}$ at the end of the shelf-life. Therefore, *Carnobacterium* spp was selected as the culture of choice to conduct a challenge study against *Pseudomonas* spp.

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

1. 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 by Food Business Operators 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 (De Santis and Mazzette, 2002). The use of bio preservatives (i.e. nisin, other bacteriocins, fermentates or bioprotective cultures) alone or combined with other treatments, have been proposed 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 during refrigerated storage of MAP *ricotta fresca*. The main objective was to select which among two commercial bioprotective cultures and a fermentate was suitable to be used for a successive validation study. The selection of the biopreservative to be used was based on the adaptation to *ricotta fresca* substrate and on the reduction of psychotropic microorganism's growth.

2. Materials and methods

2.1. 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 *Enterococcus faecium*, *Lactobacillus plantarum* and *Lactobacillus rhamnosus* in the ratio 1:1:1 with an optimum growth temperature of 37 °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. The fermentate, the microbial fermentation complex FERM 430D (Danisco), like other fermented products has a complex and undefined composition.

2.2. Samples

144 Ricotta *fresca* cheese samples were randomly selected from 3 different batches (48 from each batch), manufactured in a local industrial sheep cheese making plant. The day after production ricotta fresca samples were packed in rigid polypropylene trays sealed with lidding films and 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 FERM 430D. Blank samples (BS) were untreated ricotta *fresca*. According to manufactures instruction protective cultures were individually diluted in distilled water to a final concentration of 10^6 cfu mL^{-1} while the fermentate was resuspended in distilled water in order of 0.5-1% of the samples weight. Then 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 FERM 430D final suspension distributed on the surface of FERM samples. After the inoculum all *Ricotta fresca cheese* samples were repacked in MAP (30% CO_2 and 70% N_2) using the FP Basic Sec tray sealer (Ilpra, Vigevano, Italy).

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. Microbiological analysis were conducted for the enumeration of aerobic mesophilic bacteria (ISO 4833:2003), for the enumeration of mesophilic lactic acid bacteria (ISO 15214: 1998), for the enumeration of *Pseudomonas* spp (ISO/TS 11059:2009), for the detection and enumeration of *Enterobacteriaceae* (ISO 21528-1:2004) and for the enumeration of yeast and molds (ISO 6611/IDF094:2004). Detection and enumeration of *Listeria monocytogenes* was also conducted (ISO 11290-1: 1996, ISO 11290-2:1998). 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 using a near infrared transmittance (NIT) compositional analyzer (FOSS, Eden Prairie, MN, USA). 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).

3. Results and discussion

3.1. 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 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 1. *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).

3.2. *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%. Intrinsic properties, composition and gas composition in the headspace ($\bar{x} \pm$ SD) during the refrigerated storage are reported in table 2.

4. Discussion

Ricotta fresca cheese as consequence of high temperature applied during manufacturing has poor competing microbiota, which reflects on the growth of psychotropic 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 could exert a competitive activity against other species, including pathogens (Pala *et al.*, 2016). As the improvement of the hygiene management procedures is a measure that could only reduce the level of initial contamination, the use of bio preservatives to compete with contaminants is an interesting perspective in Ricotta cheese. 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 demonstrated, despite the refrigeration, a slight increase during storage (ca 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 2 $\log_{10}$ for *Pseudomonas* and almost 3 log10 for *Enterobacteriaceae*. It is worth to note that the growth of *Carnobacterium* spp did not lowered Ricotta fresca pH, which may have had an impact of the sensory characteristics. The possible adoption of CLAB as protective culture requires the determination of the changes in the sensory profile of *Ricotta fresca*. However, sensory analysis could not be performed in the present research since the level of *Pseudomonas* spp contamination at T_{14} was already as high as 6 log_{10}, compatible with alteration of the product, and yet beyond the acceptability of consumers.

The gas mixture chosen for MAP packaging of ricotta *fresca* (30% CO_2 and 70% O_2) is the composition generally used in sardinian industrial cheesemaking plants. As previously demonstrated (Pala *et al.*, 2016), the concentration of CO_2 in the head space at T_0 differs from the level used during packaging, as a result of gas solving in the product, while the further reduction during the successive storage is attributable to gas permeability of packaging materials used. Instead, the reduction of O_2% during storage is associated with the growth of aerobic mesophilic microorganisms.

5. 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 finished product showed a good adaptation to grow in ricotta *fresca* and promising results in controlling 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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Table 1. Microbiological profile (log_{10} cfu g^{-1}; $\bar{x}$ ± SD) of ricotta fresca by time (days after) and sample type.

Parameters	sample unit	T_0	T_7	T_{14}	T_{21}
Aerobic mesophilic bacteria	BS	2.72 ± 0.44^{a} (*n* = 9/9)	4.90 ± 1.36^{b} (*n* = 9/9)	6.01 ± 0.56^{c} (*n* = 9/9)	6.90 ± 0.86^{d} (*n* = 9/9)
	CNBAL	3.18 ± 1.95^{a} (*n* = 9/9)	6.53 ± 0.85^{b} (*n* = 9/9)	6.87 ± 1.13^{b} (*n* = 9/9)	8.87 ± 0.38^{c} (*n* = 9/9)
	FRP2	5.11 ± 0.62^{a} (*n* = 9/9)	6.63 ± 0.91^{b} (*n* = 9/9)	7.35 ± 0.51^{c} (*n* = 9/9)	7.85 ± 0.22^{c} (*n* = 9/9)
	FERM	2.91 ± 0.51^{a} (*n* = 9/9)	5.21 ± 1.05^{b} (*n* = 9/9)	6.11 ± 0.82^{c} (*n* = 9/9)	6.92 ± 0.51^{d} (*n* = 9/9)
mesophilic lactic acid bacteria	BS	ND	3.55 ± 0.49^{a} (*n* = 9/9)	4.33 ± 0.71^{b} (*n* = 9/9)	4.92 ± 0.67^{b} (*n* = 9/9)
	CNBAL	2.13 ± 0.76^{a} (*n* = 4/9)	3.30 ± 0.93^{b} (*n* = 9/9)	3.76 ± 0.62^{b} (*n* = 9/9)	3.32 ± 0.60^{b} (*n* = 9/9)
	FRP2	5.01 ± 0.72^{a} (*n* = 9/9)	5.35 ± 0.78^{ab} (*n* = 9/9)	5.16 ± 0.44^{a} (*n* = 9/9)	5.77 ± 0.40^{b} (*n* = 9/9)
	FERM	1.52 ± 0.24^{a} (*n* = 3/9)	2.86 ± 0.05^{ab} (*n* = 2/9)	4.05 ± 0.68^{b} (*n* = 7/9)	4.58 ± 1.68^{b} (*n* = 7/9)
Enterobacteriacae	BS	2.20 ± 1.02^{a} (*n* = 4/9)	4.05 ± 0.86^{b} (*n* = 5/9)	4.43 ± 0.99^{b} (*n* = 7/9)	5.34 ± 0.97^{b} (*n* = 8/9)
	CNBAL	ND	1.95 ± 0.00^{ab} (*n* = 1/9)	1.77 ± 1.15^{a} (*n* = 6/9)	3.90 ± 0.42^{b} (*n* = 5/9)
	FRP2	2.03 ± 0.00^{a} (*n* = 2/9)	3.79 ± 0.67^{b} (*n* = 9/9)	5.41 ± 0.75^{c} (*n* = 9/9)	6.29 ± 0.47^{d} (*n* = 9/9)
	FERM	3.79 ± 1.24^{a} (*n* = 4/9)	3.21 ± 0.82^{a} (*n* = 6/9)	4.24 ± 0.91^{a} (*n* = 6/9)	5.84 ± 0.59^{b} (*n* = 7/9)
Pseudomonas spp	BS	2.64 ± 0.59^{a} (*n* = 5/9)	4.89 ± 1.21^{b} (*n* = 9/9)	6.52 ± 0.99^{c} (*n* = 9/9)	6.83 ± 0.91^{c} (*n* = 9/9)
	CNBAL	2.43 ± 0.18^{a} (*n* = 4/9)	2.59 ± 0.67^{a} (*n* = 9/9)	4.59 ± 0.65^{b} (*n* = 9/9)	5.27 ± 0.64^{b} (*n* = 9/9)
	FRP2	2.53 ± 0.51^{a} (*n* = 5/9)	5.89 ± 0.64^{b} (*n* = 9/9)	6.81 ± 0.82^{c} (*n* = 9/9)	7.01 ± 0.53^{c} (*n* = 9/9)
	FERM	2.69 ± 0.27^{a} (*n* = 6/9)	5.02 ± 0.81^{b} (*n* = 9/9)	6.33 ± 0.94^{c} (*n* = 9/9)	7.26 ± 0.31^{d} (*n* = 9/9)
Yeast and molds	BS	ND	2.78 ± 0.40^{a} (*n* = 4/9)	3.62 ± 0.33^{b} (*n* = 3/9)	3.43 ± 0.76^{ab} (*n* = 5/9)
	CNBAL	2.36 ± 0.10^{a} (*n* = 3/9)	2.15 ± 0.21^{a} (*n* = 2/9)	3.00 ± 0.00^{b} (*n* = 1/9)	ND
	FRP2	2.00 ± 0.00^{a} (*n* = 1/9)	3.01 ± 0.49^{ab} (*n* = 3/9)	3.52 ± 0.38^{b} (*n* = 5/9)	3.64 ± 0.73^{b} (*n* = 3/9)
	FERM	ND	3.97 ± 0.42^{a} (*n* = 3/9)	3.88 ± 0.68^{a} (*n* = 9/9)	3.19 ± 1.14^{a} (*n* = 8/9)

BS indicates blank sample units used as negative control; C1, C2 and FERM indicates samples units inoculated respectively with protective culture CNBAL and FRP2 and the fermentate. Means in the same row with different superscript were significantly different ($P<0.05$); Values within brackets indicate the prevalence of positive samples.

Table 2. Intrinsic properties and composition ($\bar{x}$ ±SD) of Ricotta *fresca* cheese at different testing times.

Parameters	sample unit	T_0	T_7	T_{14}	T_{21}
pH	BS	6.67 ± 0.1^{a}	6.58 ± 0.05^{bc}	6.61 ± 0.07^{ab}	6.52 ± 0.11^{c}
	CNBAL	6.66 ± 0.12^{a}	6.66 ± 0.11^{a}	6.66 ± 0.09^{ab}	6.54 ± 0.04^{c}
	FRP2	6.67 ± 0.06^{a}	6.59 ± 0.05^{b}	6.55 ± 0.07^{b}	6.31 ± 0.07^{c}
	FERM	6.68 ± 0.10^{a}	6.56 ± 0.04^{bc}	6.60 ± 0.08^{ab}	6.49 ± 0.14^{c}
a_w	BS	0.990 ± 0.003^{a}	0.996 ± 0.006^{a}	0.993 ± 0.006^{a}	0.993 ± 0.006^{a}
	CNBAL	0.991 ± 0.005^{a}	0.989 ± 0.008^{a}	0.986 ± 0.001^{a}	0.985 ± 0.001^{a}
	FRP2	0.995 ± 0.004^{a}	0.995 ± 0.001^{a}	0.997 ± 0.001^{a}	0.997 ± 0.002^{a}
	FERM	0.994 ± 0.003^{a}	0.994 ± 0.001^{a}	0.992 ± 0.005^{a}	0.993 ± 0.008^{a}
Moisture (%)	BS	71.23 ± 3.52^{a}	73.37 ± 2.10^{a}	73.56 ± 2.08^{a}	74.70 ± 0.91^{a}
	CNBAL	72.02 ± 3.38^{a}	71.97 ± 3.82^{a}	71.20 ± 3.92^{a}	71.68 ± 2.91^{a}
	FRP2	77.43 ± 3.11^{a}	73.27 ± 2.83^{a}	74.35 ± 3.54^{a}	72.22 ± 2.09^{a}
	FERM	74.26 ± 2.74^{a}	74.61 ± 0.83^{a}	74.13 ± 2.01^{a}	73.46 ± 1.29^{a}
Fat (%)	BS	18.13 ± 5.80^{a}	14.31 ± 2.31^{a}	13.30 ± 2.60^{a}	14.66 ± 2.96^{a}
	CNBAL	18.03 ± 4.48^{a}	17.48 ± 4.46^{a}	17.15 ± 3.95^{a}	17.49 ± 4.46^{a}
	FRP2	11.03 ± 2.59^{a}	17.15 ± 1.16^{c}	13.09 ± 2.98^{ab}	15.11 ± 1.08^{bc}
	FERM	13.78 ± 1.62^{a}	12.43 ± 2.10^{a}	13.59 ± 1.88^{a}	14.67 ± 2.53^{a}
Protein (%)	BS	9.81 ± 0.78^{a}	9.97 ± 1.04^{a}	10.23 ± 1.36^{a}	8.94 ± 2.61^{a}
	CNBAL	9.38 ± 0.45^{a}	9.33 ± 0.75^{a}	9.46 ± 0.39^{ab}	10.46 ± 0.48^{b}
	FRP2	10.05 ± 0.02^{a}	11.15 ± 1.07^{a}	9.99 ± 0.73^{a}	10.86 ± 0.24^{a}
	FERM	9.77 ± 1.50^{a}	10.32 ± 1.37^{a}	9.87 ± 0.76^{a}	10.19 ± 0.81^{a}
O_2%	BS	0.87 ± 0.49^{a}	1.80 ± 1.18^{b}	1.05 ± 0.82^{a}	0.42 ± 0.78^{a}
	CNBAL	0.99 ± 0.58^{ab}	1.12 ± 1.65^{b}	0.14 ± 0.17^{ab}	0.02 ± 0.01^{b}
	FRP2	0.99 ± 0.54^{a}	1.89 ± 1.47^{b}	0.31 ± 0.25^{ac}	0.004 ± 0.01^{c}
	FERM	1.01 ± 0.63^{a}	1.66 ± 0.32^{b}	0.75 ± 0.91^{a}	0.51 ± 0.84^{a}
$C0_2$%	BS	13.05 ± 2.88^{a}	6.20 ± 2.09^{b}	5.50 ± 2.96^{b}	6.78 ± 2.89^{b}
	CNBAL	13.55 ± 2.42^{a}	5.50 ± 2.02^{b}	5.00 ± 2.08^{b}	5.18 ± 1.91^{b}
	FRP2	13.68 ± 2.23^{a}	6.96 ± 1.55^{c}	7.50 ± 1.73^{c}	10.22 ± 1.72^{b}
	FERM	13.24 ± 2.21^{a}	6.47 ± 2.10^{b}	5.79 ± 2.67^{b}	6.98 ± 4.15^{b}

BS indicates blank sample units used as negative control; C1, C2 and FERM indicates samples units inoculated respectively with protective culture CNBAL and FRP2 and the fermentate. Means in the same row with different superscripts were significantly different ($P < 0.05$).

Highlights

- Ricotta fresca support psychotrophic microorganism growth during refrigerated storage
- 3 Commercial biopreservatives were tested against spoilage bacteria in ricotta fresca
- Cultures Lyofast FPR 2, Lyofast CNBAL and the fermentate FERM 430D were tested
- Inoculation of Carnobacterium spp protective culture reduced *Pseudomonas* spp growth