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Technological characterization of bacteriocin producing *Lactococcus lactis* strains employed to control *Listeria monocytogenes* in Cottage cheese

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ABSTRACT

In recent years, there has been a particular focus on the application of antimicrobial compounds produced by lactic acid bacteria (LAB) as natural preservatives to control the growth of spoilage and pathogenic bacteria in food. Bacteriocins are antimicrobial peptides which can be added to foods in concentrated forms as food preservatives, e.g. additives, or they can be produced *in situ* by starters or protective cultures.
In this study, twenty *Lactococcus lactis* bacteriocin producers previously isolated from Italian fermented foods were subjected to a variety of physical and biochemical tests in order to identify those with the greatest potential as starter cultures in cheese production. Of these, four strains isolated from cheese (one nisin Z producer, one nisin A producer and two lacticin 481 producers) which fulfilled the desired technological criteria were assessed for their ability to control *Listeria monocytogenes*. The subsequent application of these bacteriocinogenic strains as starter cultures in Cottage cheese established that the nisin A producing *Lact. lactis* 40FEL3, and to a lesser extent the lacticin 481 producers 32FL1 and 32FL3, successfully controlled the growth of the pathogen. This is the first study to directly compare the ability of nisin A, nisin Z and lacticin 481 producing strains to control listerial growth during the manufacture and storage of Cottage cheese.

1. Introduction

The development of starter cultures for food fermentations is a multidisciplinary endeavor, requiring not only an understanding of the food ecosystem (Vogel et al., 2002), but also the characterization of useful technological and physiological features of the predominant strains. In cheese production, the most important property of *Lactococcus* starter cultures is the ability to produce acid rapidly (Cogan et al., 1997). Other properties such as salt tolerance, proteolytic and peptidase activity (Kiernan et al., 2000; Hannon et al., 2003), production of biogenic amines (BA) (Law and Kolstad, 1983; Desmazeaud, 1996; Gardiner et al., 1999; Fernàndez-Garcia et al., 1999; Kimaryo et al., 2000), autolytic activity (El-Soda et al., 1993), exocellular proteolytic activity (Leroy and De Vuyst, 2004), diacetyl production (Beshkova et al., 2003), antibiotic resistance (Mathur and Singh, 2005; Franciosi et al., 2009; Nieto-Arribas et al., 2009) and phage-resistance (Garvey et al., 1995; Sing and Klaenhammer, 1993) are considered common technological features to determine the selection of potential strains for industrial applications. There has also been a significant focus on the ability of starter strains to produce bacteriocins (ribosomally synthesized antimicrobial peptides produced by bacteria; Cotter et al., 2005) which enhance their ability to control food-borne pathogens such as *Clostridium botulinum*, *Staphylococcus aureus* and *Listeria monocytogenes* (Nettles and Barefoot, 1993; Guinane et al., 2005; Deegan et al., 2006).

As with other minimally processed and refrigerated foods, many dairy products require additional strategies to control the growth and survival of *L. monocytogenes*. *L. monocytogenes* is the causative agent of listeriosis, one of the most significant foodborne diseases in industrialized countries (Schlech, 2000). The inclusion of additional hurdles to control this pathogen in food is particularly desirable given its widespread distribution in the environment, its ability to grow at refrigeration temperatures, and the fact that it can survive during the manufacture of Cottage cheese (Ryser et al., 1985), soft cheese (Morgan et al., 2001; Cataldo et al., 2007; Rogga et al., 2005) and Camembert (Back et al., 1993; Ryser and Marth, 1987). It is thus unsurprising that one of the novel approaches used to prevent the growth of *L. monocytogenes* in food is the use of bacteriocin-producing lactic acid bacteria (LAB) as starter cultures (Soomro et al., 2002), as well as the application of concentrated or purified LAB-derived bacteriocins (Muriana, 1996; Cintas et al., 1998). The extensive study of LAB bacteriocins over the last number of decades means that, in many cases, considerable knowledge has been accumulated with respect to their biosynthesis, structure, and mode of

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action (for reviews see Cintas et al., 2001; Chen and Hoover, 2003; Cotter et al., 2005; Bierbaum and Sahl, 2009). Given that traditional starters such as LAB are generally recognized as safe (GRAS), they provide a more natural means of preservation in food to allay consumer concerns over possible adverse health effects from the presence of chemical additives in foods. In this study, we assess 20 LAB bacteriocin producing strains from a technological perspective to determine their suitability for use as starter cultures in soft fresh cheese production. The ability of four strains which produce nisin or lacticin 481 (both Class I bacteriocins, also termed lantibiotics) to control *L. monocytogenes* growth during the manufacture and storage of Cottage cheese was then assessed. To our knowledge, this represents the first occasion involving the use of natural nisin and lacticin 481-producing starters in Cottage cheese with a view to the control of *L. monocytogenes*.

2. Materials and methods

2.1. Microorganisms and culture conditions

The ability of *Lact. lactis* nisin A producers (11 strains), *Lact. lactis* nisin Z producers (7 strains), *Lact. lactis* lacticin 481 producers (2 strains) isolated from Italian fermented foods (Dal Bello et al., 2010) to function as cheese starters were assessed. *Lact. lactis* subsp. *cremoris* HP NCDO 607 type strain and *Lact. lactis* strain MG1363 (phage sensitive) were used as positive controls (UCC Culture Collection). All lactococci were cultured in M17 broth (Oxoid, UK) or M17 agar supplemented with 0.5% glucose (GM17) broth and incubated for 16 h at 30 °C before analysis.

2.2. Technological characterization of bacteriocin producing strains

2.2.1. Acidifying activity

The strains were revitalized in M17 broth by growing overnight at 30 °C. For the acidifying activity test, tubes containing 10 mL of sterile skimmed milk (RSM 10% w/v) (Oxoid) were inoculated (1% v/v) with revitalized strains and incubated at 30 °C. The pH was measured after 6 and 24 h with a pH meter (Microprocessor pH meter 213, Hanna instruments, Ireland). The data are expressed as the mean of duplicate analysis.

2.2.2. Extracellular proteolytic activity

Extracellular proteolytic activity was determined following the method of Franciosi et al. (2009). Two microliters of revitalized strains were spotted onto the surface of an agar medium composed of 10% (w/v) skim milk powder (Oxoid) and 2% (w/v) agar (Oxoid) and incubated at 30 °C for 4 days. Proteolytic activity was indicated by a clear zone around the colonies.

2.2.3. Exopolysaccharide formation (EPS)

EPS production from lactose was determined by qualitatively measuring the degree of "stringiness" of cultures which had been grown in RSM (10% w/v) (Oxoid) at 30 °C for 18 h according to Cogan (1996). The culture was regarded as being EPS positive if the coagulated culture could be teased into a string with an inoculating loop.

2.2.4. Growth ability at different salt concentration

Strains were grown on M17 broth supplemented with 4%, 6% and 10% (w/v) NaCl. The ability of the strains to grow at each different salt concentration was evaluated after 24 h at 30 °C by measurement of optical density ($OD_{600\ nm}$) using a Spectophotometer DU® 640 UV/Vis (Beckman Coulter, CA, USA). Results were expressed as a ratio of growth in these media relative to that in standard broth. All assays were performed in duplicate.

2.2.5. Autolytic activity

Autolysis of the cells was measured as described by Mora et al. (2003). The strains were grown in M17 broth (Oxoid) for 24 h at 30 °C to reach an $OD_{600\ nm}$ 0.8–1. The cells were washed in potassium phosphate buffer (50 $mmol^{-1}$, pH 6.5) (Sigma-Aldrich, Ireland) and resuspended in the same buffer to an $OD_{600\ nm}$ of 0.6–0.8 and incubated at 30 °C. The degree of autolysis was expressed as the percentage decrease in the $OD_{600\ nm}$ after 4 and 24 h.

2.2.6. Diacetyl production

Diacetyl production was determined according to King (1948). Revitalized strains (1% v/v) were inoculated in 10 mL of UHT milk and incubated at 30 °C for 24 h. One milliliter of each cell suspension was combined with 0.5 mL of α-naphthol (1% w/v) and KOH (16% w/v) (Sigma-Aldrich) and incubated at 30 °C for 10 min. Diacetyl production is indicated by the formation of a red ring at the top of the tubes.

2.2.7. Antibiotic resistance profiles

The strains were revitalized in M17 broth by growing overnight at 30 °C. For antibiotic resistant profile analysis, 20 mL of M17 agar was seeded with revitalized strains (1% v/v) and allowed to solidify. Antibiotic disks (Oxoid) containing the following different antibiotics, nalidixic acid (30 μg/mL), rifampicin (30 μg/mL), novobiocin (5 μg/mL), vancomycin (30 μg/mL), gentamicin (30 μg/mL) and chloramphenicol (10 μg/mL) were then placed onto each agar plate. The plates were incubated at 30 °C for 24 h. The occurrence of a clear zone of inhibition around a disk indicated that the strain was susceptible to the antibiotic in question. The analysis was performed in duplicate and the results expressed as diameter of clear zone (mm) around the antibiotic disk.

2.2.8. Bacteriophage resistance

Phage resistance analysis was performed by using the phage plaque assay with twelve different *Lact. lactis* phages (C2 species: C2, 952; p335 species: Tuc2009; 936 species: 645, P272, P113g, bIL66, bIL170, SKI, jj50, p2, 712) obtained from the UCC phage collection. The spot assay for phage infection was performed as follows: 200 μL of each culture at the early exponential growth phase was mixed with 4 mL of GM17 media soft agar (4 g/L agar) (Oxoid) supplemented with 1% 1 M $CaCl_2$ (Sigma-Aldrich) and poured onto 20 mL of an GM17 solid agar (8 g/L agar) plate. After solidification of the media, 10 μL of each phage lysate was carefully pipetted onto the semi-solid agar layer and allowed to dry overnight at 30 °C. A phage infection was indicated by a clear lysis zone in the soft agar layer. The phage sensitive *Lact. lactis* strain MG1363 was used as a positive control.

2.3. Evaluation of *L. monocytogenes* growth in cell-free supernatant of bacteriocinogenic strains

The sensitivity of *L. monocytogenes* F2365 to cell-free supernatants derived from the *Lact. lactis* producers of nisin A (40FEL3), nisin Z producer (29FL4) and lacticin 481 (32FL1, 32FL3) was examined. *Lact. lactis* subsp. *cremoris* HP NCDO 607 non-bacteriocinogenic strain (UCC Culture Collection) was used as control. For cell-free supernatants (CFS), *Lact. lactis* bacteriocin-producing strains were grown in GM17 and incubated overnight at 37 °C. After incubation, the cells were then separated by centrifugation HERMLE Z 323 (HERMLE Labortechnik, Wehingen, Germany) at 5000×*g* for 10 min at 4 °C and the cell-free supernatant (CFS) was filter-sterilized through a 0.45 μm syringe-end filter system (Minisart Plus, Sartorius, Germany) to remove any remaining cells. CFS was then adjusted to three different pH values, i.e. 6.5, 5.5 and 4.5, with sterile 1 M NaOH or 1 M HCl (Sigma-Aldrich). Ten mL of CFS was then inoculated with 10^4 CFU/mL of revitalized *L. monocytogenes* F2365 and incubated at 37 °C. The CFS of *Lact. lactis* subsp. *cremoris* HP NCDO 607 was adjusted at the same pH values (6.5, 5.5

and 4.5) and used as a bacteriocin negative control. *Listeria* levels in the bacteriocin-containing CFS were evaluated by serial dilution and plating on LSA (Listeria selective medium) (Oxoid) after 0, 2, 4, 6 and 24 h. Analysis was performed in duplicate.

2.4. Evaluation of *L. monocytogenes* growth in Cottage cheese made with bacteriocin producing starter cultures

2.4.1. Microorganisms and culture conditions

Lact. lactis producers of nisin A (40FEL3), nisin Z (29FL4) and lacticin 481 (32FL1, 32FL3) were employed as starter cultures to manufacture Cottage cheese. *Lact. lactis* subsp. *cremoris* HP NCDO 607, which is a non bacteriocinogenic cheese-making strain (UCC Culture Collection), was used as control. Prior to Cottage cheese production, all revitalized lactococcal strains were grown in reconstituted skim milk (RSM 10%) and incubated for 16 h at 30 °C reaching a cell concentration of about 10^8 cells/mL. The indicator strain *L. monocytogenes* F2365 was provided by the UCC Culture Collection. *L. monocytogenes* F2365 was propagated in BHI broth (Oxoid) and incubated for 16 h at 37 °C. For inoculation in cheese, the revitalized cells of *L. monocytogenes* F2365 were pelleted by centrifugation, washed twice and resuspended in buffered peptone water and subsequently diluted to give the desired cell number (10^3 cells/mL).

2.4.2. Manufacture of Cottage cheese

Commercially purchased low-fat pasteurized milk was heated to 32 °C and subsequently inoculated with 1% of overnight cultures. All cultures were individually inoculated in separate vats. Cheese rennet (92% chymosin–8% pepsin, 1 mL/100 L) (CHR Hansen, Hørsholm, Denmark) was added in milk 30 min after starter addition and the milk incubated at 21 °C for 16 h until a pH of 4.65–4.75 was reached. The coagulum was cut into 2 cm cubes and allowed to stand for 15 min. The temperature of the curd was gradually increased to 50–52 °C over a period of 90 min. The whey was drained to curd level and the curd was washed three times at 20 min intervals using water at 22 °C, 10 °C and 4 °C, respectively. The curd was drained of whey and left to stand overnight at 4 °C. Cream dressing was then added at the ratio of 3 parts curd to 1 part cream. The dressing was composed of 54% (w/v) commercially-pasteurized cream (about 33% fat), 42% (w/v) non-fat milk and 4% (w/v) NaCl. The final composition of cream was 18% fat. *L. monocytogenes* F2365, previously subcultured in BHI broth (Oxoid), was added to the dressing at the level of 10^3 cells/mL. Once the dressing was added, the cheese was left for 1 h at room temperature. Cottage cheese was stored at 4 °C and enumeration of *L. monocytogenes* was assessed at days 0, 1, 2, 3, 5 and 7. At each time point the pH of the cheese was also measured (Microprocessor pH meter 213, Hanna instruments, Ireland).

For enumeration of *L. monocytogenes*, samples of Cottage cheese (1 g) were homogenized in ¼ Ringer's solution (Merck, Ireland). Triplicate dilutions were performed and plated on LSA. The plates were incubated at 37 °C for 24 h, after which *Listeria* were counted.

2.4.3. Statistical analysis

One-way analysis of variance (ANOVA) and the Duncan test for mean comparison were performed by using Statistica ver. 7.0 (StatSoft Inc., Tulsa, USA).

3. Results

3.1. Technological characterization of bacteriocin producing strains

3.1.1. Acidification, extracellular proteolytic activity and exopolysaccharide (EPS) production

A test of the ability of each *Lact. lactis* strain to acidify skim milk showed that all successfully reduced the pH over a 24 hour period of incubation at 30 °C (Table 1). In particular, seven strains (2 *Lact. lactis*, 4 *Lact. lactis* subsp. *lactis*, 1 *Lact. lactis* subsp. *cremoris*) were found to be more efficient acidifiers than the *Lact. lactis* HP control strain. A similar pattern was revealed when the strains where grown in UHT low-fat milk (1.5% fat) (Table 1). In accordance with Bouton's classification (Bouton et al., 2002), the results obtained during this study establish seven of the total *Lact. lactis* tested as being high acidifying strains (29FL4, 30FL3, 41FLL2, 41FLL8b, 40FEL3, 32FL1, 32FL3), eight as intermediate (44SGLL1, 49SGLL1, 44SGLL7, 44SGLL3, 44SGLL9, 44SGLL8, 44SGLL2, 41FLL8a) and five as low acidifying strains (41FL5, 41FL8, 41FL15, 41FL13, 41FLL7).

In this study, proteolytic activity was greatest in eight *Lact. lactis* strains (29FL4, 30FL3, 44SGLL3, 44SGLL9, 44SGLL8, 40FEL3, 32FL3, 32FL1) as well as the HP control, while five strains showed medium proteolytic activity. Seven *Lact. lactis* strains appeared to lack proteolytic activity (Table 1).

Exopolysaccharide (EPS) production from lactose was determined qualitatively and all strains proved to be EPS negative (Table 1).

3.1.2. Effect of NaCl, autolysis, diacetyl production

Sodium chloride (NaCl) tolerance tests of the lactococci revealed that all strains were able to grow at the lowest salt concentrations used (4% w/v) (data not shown). Two *Lact. lactis* subsp. *lactis* strains, 41FLL8b and 41FLL7, grew poorly at this salt concentration. None of the strains were able to grow in the presence of salt concentrations above 6% NaCl (data not shown).

All of the *Lact. lactis* strains assessed exhibited good autolytic ability in M17 broth at 30 °C. At least 15% autolysis was noted for five strains after incubation for 4 h. After 24 h high levels of autolysis, ranging from between 20% and 40%, were attained for all test strains. Six strains (41FL15, 44SGLL2, 41FLL2, 41FLL8b, 41FLL7, 32FL3) were particularly notable as levels of autolysis ranged from 41% to 50% (Table 1). Instead twelve of the 20 strains tested fell within the desired range of 25% to 50%, as proposed by Ayad et al. (2004) (Table 1). Among the twenty *Lact. lactis* tested, high levels of diacetyl production were found in seven strains (44SGLL1, 49SGLL1, 44SGLL7, 44SGLL9, 44SGLL8, 41FL5, 41FLL8a). The remaining thirteen strains tested negative as did the *Lact. lactis* HP control strain (Table 1).

3.1.3. Antibiotic and bacteriophage resistance

The antibiotic resistance of the *Lact. lactis* strains relative to *Lact. lactis* subsp. *cremoris* HP, a starter culture sensitive to all antibiotics, was also tested. The results obtained indicate that all *Lact. lactis* strains tested were resistant to nalidixic acid (30 μg/mL) and were sensitive to rifampicin, novobiocin, gentamicin, vancomycin and chloramphenicol (data not shown).

Bacteriophage sensitivity was established on the basis of the presence or absence of a typical clear zone in a lawn of the test cells, due to cell lysis by phage. All *Lact. lactis* strains tested, other than the positive control *Lact. lactis* MG1363 were resistant to the twelve phages tested (data not shown).

Following completion of this array of biochemical and physical tests, a final evaluation of the twenty strains that were initially selected for characterization determined that just four strains, 40FEL3 (nisin A), 29FL4 (nisin Z), 32FL3 (lacticin 481) and 32FL1 (lacticin 481) fulfilled all the desired criteria and were further examined for their ability to control *L. monocytogenes* in the manufacture and storage of Cottage cheese.

3.2. Evaluation of antilisterial activity of bacteriocin producing strains

3.2.1. Sensitivity of *L. monocytogenes* to bacteriocin cell-free supernatant at different pH values

We wished to evaluate the inhibitory effect of cell-free supernatant (CFS) from the bacteriocin producing strains 40FEL3, 29FL4, 32FL3 and 32FL1 on *L. monocytogenes* F2365. Strain F2365 was inoculated at approximately 10^4 CFU/mL in CFS at three different pH values

Table 1
Technological characterization of *Lact. lactis* bacteriocin producing strains.

Species	Acidifying activity[a]					Extracellular proteolytic activity	EPS production	Autolysis[b] (%)		Diacetyl production
	pH drop in skim milk			pH drop in UHT milk						
	0	6 h	24 h	0	24 h			4 h	24 h	
Lact. lactis nisin Z producers										
44SGLL1	6.71	6.57 ± 0.04	4.89 ± 0.01	6.75	5.47 ± 0.06	+	−	2	18	+
49SGLL1	6.71	6.50 ± 0.01	4.90 ± 0.03	6.75	5.40 ± 0.02	+	−	6	24	+
29FL4	6.71	5.96 ± 0.01	4.50 ± 0.02	6.75	4.67 ± 0.06	+++	−	2	20	
30FL3	6.71	6.07 ± 0.09	4.54 ± 0.01	6.75	4.69 ± 0.01	++	−	0	27	−
44SGLL7	6.71	6.56 ± 0.03	4.98 ± 0.00	6.75	5.02 ± 0.04	+	−	3	22	+
44SGLL3	6.71	6.54 ± 0.01	4.98 ± 0.01	6.75	4.80 ± 0.03	++	−	2	22	−
44SGLL9	6.71	6.51 ± 0.00	5.03 ± 0.00	6.75	5.31 ± 0.03	++	−	2	21	+
Lact. lactis nisin A producers										
44SGLL8	6.71	6.54 ± 0.01	4.91 ± 0.03	6.75	5.19 ± 0.01	++	−	1	24	+
41FL5	6.71	6.56 ± 0.01	5.58 ± 0.01	6.75	4.90 ± 0.03	+	−	18	39	+
41FL8	6.71	6.40 ± 0.01	5.34 ± 0.01	6.75	4.19 ± 0.03	−	−	5	22	−
41FL13	6.71	6.41 ± 0.02	5.95 ± 0.03	6.75	5.96 ± 0.01	−	−	16	37	−
41FL15	6.71	6.39 ± 0.01	5.31 ± 0.01	6.75	5.39 ± 0.05	−	−	27	48	−
44SGLL2	6.71	6.60 ± 0.02	5.00 ± 0.02	6.75	5.40 ± 0.01	+	−	25	50	−
Lact. lactis subsp, *lactis* nisin A producers										
41FLL8a	6.71	6.34 ± 0.01	5.04 ± 0.04	6.75	5.15 ± 0.04	−	−	3	37	+
41FLL2	6.71	6.09 ± 0.10	4.29 ± 0.01	6.75	4.20 ± 0.01	−	−	14	49	−
41FLL8b	6.71	6.40 ± 0.00	4.42 ± 0.03	6.75	4.20 ± 0.03	−	−	11	41	−
41FLL7	6.71	6.46 ± 0.00	5.61 ± 0.01	6.75	5.96 ± 0.00	−	−	11	44	−
Lact. lactis subsp, *cremoris* nisin A producer										
40FEL3	6.71	6.08 ± 0.03	4.20 ± 0.03	6.75	4.14 ± 0.02	+++	−	9	38	−
Lact. lactis subsp, *lactis* lct481 producers										
32FL3	6.71	6.08 ± 0.07	4.19 ± 0.01	6.75	4.14 ± 0.01	+++	−	5	31	−
32FL1	6.71	6.10 ± 0.05	4.14 ± 0.03	6.75	4.14 ± 0.01	+++	−	22	45	−
Lact. lactis subsp, *cremoris* no bacteriocin producer										
HP	6.71	5.50 ± 0.03	4.16 ± 0.01	6.75	4.18 ± 0.01	++	−	8	34	−

ND not evaluated.
[a] Results are expressed as mean value ± SD of duplicate experiment.
[b] Autolysis is expressed as (%) = 100 − ($OD_{650\ nm}$ lowest value / $OD_{650\ nm}$ highest value * 100).

(6.5, 5.5 and 4.5) and its growth was assessed after 0, 2, 4, 6 and 24 h at 37 °C. CFS from the non-bacteriocinogenic HP culture adjusted at pH 6.5, 5.5 and 4.5 was used as a negative control.

At pH 6.5, nisin A-containing CFS from strain 40FEL3 had a considerable killing effect in that *L. monocytogenes* F2365 numbers were reduced by ~3 log CFU/mL during the first 6 h of incubation (Fig. 1). In comparison, an increase of 4 log CFU/mL in pathogen numbers was detected at the same time point when non bacteriocin-containing CFS was used. In the case of CFS from the nisin Z-producing *Lact. lactis* 29FL4, a decrease of just 0.5 log CFU/mL was

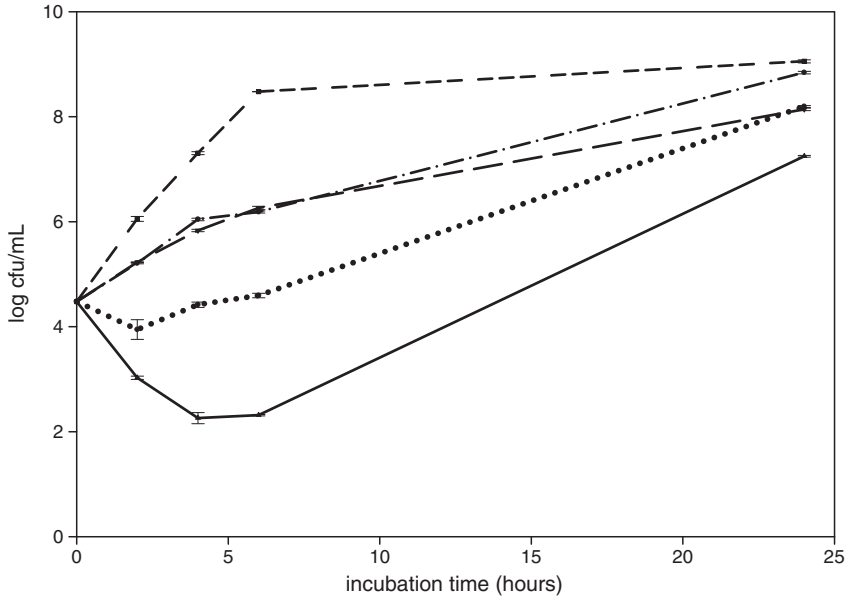

Fig. 1. Growth of *L. monocytogenes* F2365 strains in GM17 at pH 6.5 at 37 °C in presence of CFS of *Lact. lactis* 29FL4 nisin Z producer (▪▪▪▪); CFS of *Lact. lactis* subsp. *lactis* 32FL3 lacticin 481 producer (—•—•); CFS of *Lact. lactis* subsp. *lactis* 32FL1 lacticin 481 producer (— —) CFS of *Lact. lactis* subsp. *cremoris* 40FEL3 nisin A producer (———); CFS of *Lact. lactis* HP non-bacteriocin producer (— — —). The bars indicate the standard deviations for the data points.

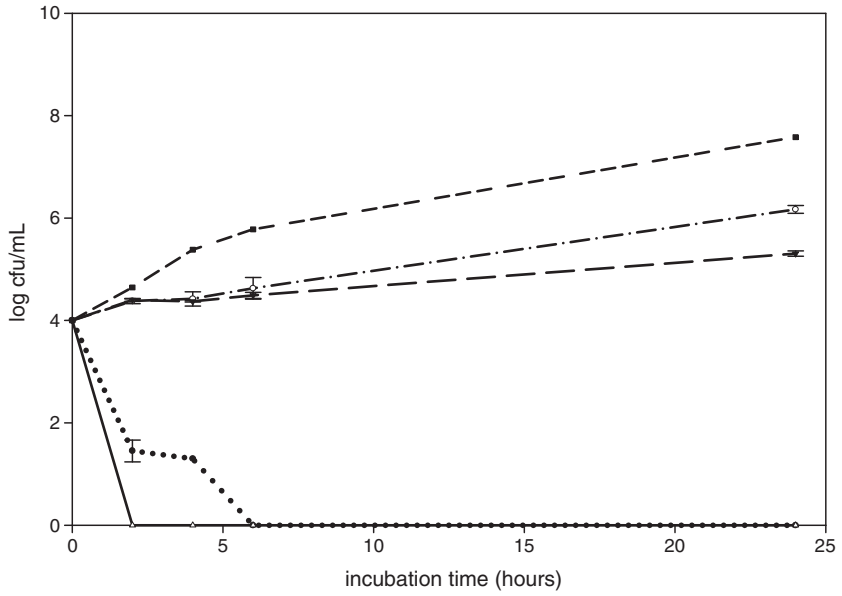

Fig. 2. Growth of *L. monocytogenes* F2365 strains in GM17 at pH 5.5 at 37 °C in presence of CFS of *Lact. lactis* 29FL4 nisin Z producer (▪▪▪▪); CFS of *Lact. lactis* subsp. *lactis* 32FL3 lacticin 481 producer (—•—•); CFS of *Lact. lactis* subsp. *lactis* 32FL1 lacticin 481 producer (— —); CFS of *Lact. lactis* subsp. *cremoris* 40FEL3 nisin A producer (———); CFS of *Lact. lactis* HP strains non-bacteriocin producer (— — —). The bars indicate the standard deviations for the data points.

observed after 2 h. Additionally, the CFS from the *Lact. lactis* subsp. *lactis* lacticin 481-producing strains 32FL3 and 32FL1 reduced *Listeria* counts relative to the non bacteriocin-containing control, albeit only slightly. In all cases, the number of *Listeria* increased after 6 h as a result of renewed growth from the surviving *L. monocytogenes* cells.

At pH 5.5, a dramatic decrease in cell counts of *Listeria* (to below detectable levels) was observed following incubation in the nisin A containing CFS for 2 h. In the case of the nisin Z containing CFS, listerial cell numbers decreased by ~3 log CFU/mL after 4 h and after 6 h the pathogen could not be detected (Fig. 2).

In the case CFS from both lacticin 481 producers adjusted to pH 5.5, a slight increase in *L. monocytogenes* F2365 numbers (1–2 log CFU/mL) was observed over the 24 hour period. In comparison *Listeria* counts increased by ~3 log CFU/mL in non-bacteriocin CFS over the same 24 hour period.

Bacteriocin-containing CFS of each nisin variant (nisin A and Z), adjusted to pH 4.5, caused a reduction in *Listeria* numbers as observed at each sampling point up until the final measurement at 24 h (Fig. 3). In particular, after 24 h the reduction in *Listeria* counts were 2.46 log CFU/mL for nisin A and 2.58 log CFU/mL for nisin Z. In contrast, no change in *Listeria* numbers was observed over 6 h both in non-bacteriocin containing CFS and in CFS from both lacticin 481 producers.

In the case of lacticin 481 CFS, *L. monocytogenes* F2365 was able to grow quite well at pH 6.5, but at a slightly lower rate than the non-bacteriocin CFS control. At pH 5.5, an almost bacteriostatic effect was observed for both lacticin 481 CFSs, with a 1–1.5 log increase in

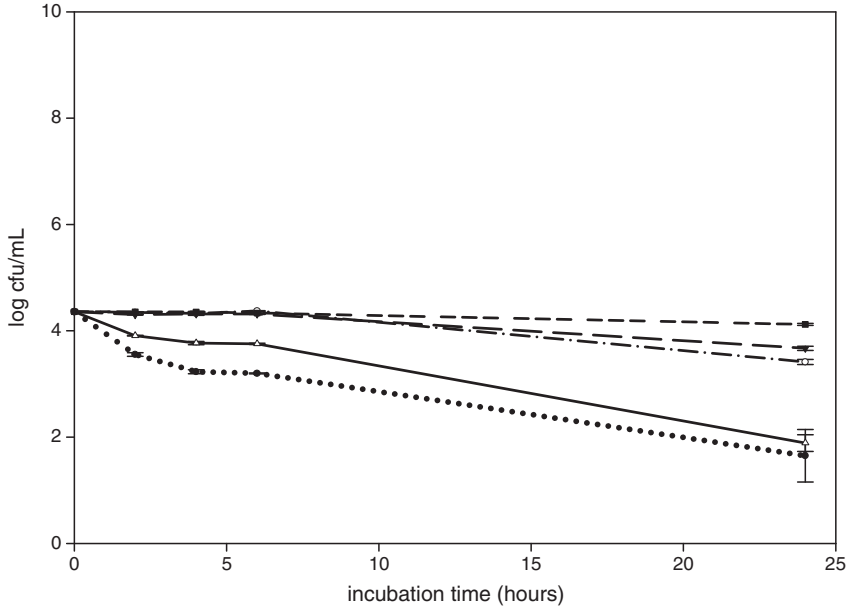

Fig. 3. Growth of *L. monocytogenes* F2365 strains on GM17 at pH 4.5 at 37 °C in presence of CFS of *Lact. lactis* 29FL4 nisin Z producer (▪▪▪▪); CFS of *Lact. lactis* subsp. *lactis* 32FL3 lacticin 481 producer (—•—•); CFS of *Lact. lactis* subsp. *lactis* 32FL1 lacticin 481 producer (— —); CFS of *Lact. lactis* subsp. *cremoris* 40FEL3 nisin A producer (———); CFS of the non-bacteriocin producer *Lact. lactis* HP strain (— — —). The bars indicate the standard deviations for the data points.

Table 2
pH values during storage at 4 °C of Cottage cheese inoculated with *Lact. lactis* bacteriocin-producing cultures.

Lact. lactis strain	Bacteriocin	Storage time (days)					
		0	1	2	3	5	7
29FL4	Nisin Z	5.86	5.84	5.90	5.71	5.76	6.00
32FL3	Lacticin 481	4.80	4.60	4.64	4.67	4.56	4.74
32FL1	Lacticin 481	4.70	4.60	4.58	4.62	4.50	4.68
40FEL3	Nisin A	4.76	4.65	4.64	4.68	4.58	4.74
HP	Bacteriocin negative	4.65	5.43	4.55	4.58	4.46	4.59

cell numbers over 24 h. Interestingly, a slight decrease in *Listeria* numbers was noticeable after 24 h in the case of lacticin 481-containing CFS at pH 4.5 (32FL1 and 32FL3 producers) when compared to the non-bacteriocin CFS control.

3.2.2. Impact of bacteriocin producing Lact. lactis on the survival of L. monocytogenes in Cottage cheese

Lact. lactis subsp. *cremoris* 40FEL3 (nisin A), *Lact. lactis* 29FL4 (nisin Z) and *Lact. lactis* subsp. *lactis* 31FL1, 32FL3 (lacticin 481) were inoculated as starter cultures for a Cottage cheese fermentation. *L. monocytogenes* F2365 was added to reach an initial level of 10^3 CFU/g. *Lact. lactis* subsp. *cremoris* HP NCDO 607, a non-bacteriocinogenic strain was used as control starter. *Listeria* growth in the cheese was monitored in cheese by direct plating on LSA medium at time 0 (inoculum of pathogen) and after 1, 2, 3, 5 and 7 days of storage at 4 °C.

3.2.3. Cheese pH

Table 2 shows the pH values measured during the storage of Cottage cheese. In curd (0 d), the initial pH range was ~4.65–4.80. No major differences in pH were detected between cheeses made with the non-bacteriocin producing HP and those made with the bacteriocin positive cultures after 7 days of storage, with the exception of *Lact. lactis* 29FL4 (nisin Z producer) in which case the pH had reached a value of just 5.86.

3.2.4. Survival of L. monocytogenes in cheese

Inhibitory effect of the bacteriocin positive starter cultures against *L. monocytogenes* F2365 in Cottage cheese during 7 days of storage was evaluated by one-way analysis of variance performed among the different treatments (Table 3). After 2 days of Cottage cheese storage a decrease of *L. monocytogenes* numbers ($P<0.001$) was observed in cheese inoculated with *Lact. lactis* subsp. *cremoris* 40FEL3 (nisin A), *Lact. lactis* subsp. *lactis* 32FL1 and 32FL3 (lacticin 481). In contrast, there was no significant decrease in the levels of the pathogen in the cheese containing the non-bacteriocin producing culture. A further decrease in *L. monocytogenes* numbers was observed after day 3 in the cheeses containing the nisin A and lacticin 481 producers. These levels were again lower than those present in the cheese containing the bacteriocin-negative culture. On days 5 and 7 there was a slight increase in *Listeria* numbers in the cheeses containing the nisin A and lacticin 481 producers when compared to the counts taken on day 3. However increases of 0.30 log CFU/g for cheeses containing the nisin A producer, and 0.14 and 0.12 Log CFU/g for cheeses containing the lacticin 481 producers were still below the initial inoculum levels. In contrast, the numbers of *Listeria* had increased by 0.11 log CFU/g in cheese made with *Lact. lactis* HP after 7 days of storage and an increase in *Listeria* counts (~3 log CFU/g) was observed in cheese made with the *Lact. lactis* nisin Z producer (29FL4). The latter result is most likely due to the relatively poor acidification of the cheese and thus the provision of a less stressful environment for the pathogen.

4. Discussions

The growth of *Listeria* strains at temperatures ranging from 1 to 45 °C, their high salt tolerance, and their ability to initiate growth at a relatively low pH make these pathogens particularly difficult to control in food (Vignolo et al., 2000). A promising means of controlling, and even reducing, *Listeria* populations in foods is through the use of bacteriocins, either produced *in situ* in fermented products or added to the product. Since lactococci are the principal starters in a variety of fermented products, bacteriocinogenic lactococci have been employed to improve product quality. Indeed, several studies have demonstrated the ability of the broad spectrum bacteriocin, nisin, to inhibit the growth of *L. monocytogenes* when applied in foods (Ryser, 1999; Rodriguez et al., 2005; Samelis et al., 2003). High levels of this bacteriocin have been shown to completely eliminate *L. monocytogenes* in soft cheeses within periods as short as 24 h (Ryser, 1999).

In this study, four different bacteriocinogenic cultures, producing either nisin A, nisin Z or lacticin 481 (two strains), were selected as a result of displaying excellent starter culture attributes including good acidifying activity, high extracellular proteolytic activity and bacteriophage resistance as well as other favorable properties from a larger collection of 20 strains. The selected cultures were then tested for their ability to control *L. monocytogenes* growth both *in vitro* and in the manufacture of Cottage cheese. Importantly, these results highlighted that CFS from nisin bacteriocin producers were active against *Listeria* at different pH levels (6.5, 5.5 and 4.5), but that significant variation is evident. Specifically, nisin A CFS had an initial detrimental effect on *Listeria* at pH 6.5 and 5.5 but had much less effect over the first 6 h at pH 4.5. It was also notable that nisin Z-containing CFS exhibited considerable antilisterial activity at pH 5.5 but had less impact at pH 6.5. At pH 4.5 the activity of nisin Z CFS was similar to that of nisin A CFS.

The greater inhibitory effect of nisin at mildly acidic, rather than neutral, pH is possibly due to the greater solubility of nisin at acidic pH (Hurst and Hoover, 1993). Here, the activity of nisin was greater at pH 5.5 than pH 4.5, which is in accordance with the findings of

Table 3
L. monocytogenes count (mean log CFU/g ± SD) during the storage of Cottage cheese manufactured with bacteriocin-producing lactic acid bacteria and a non-bacteriocin producing control lactic culture. Results of one-way analysis of variance performed among the different treatments for each storage day are also reported.

Lact. lactis strain	Bacteriocin	Storage time (days)					
		0	1	2	3	5	7
29FL4	Nisin Z	3.04 ± 0.01	3.41 ± 0.12^{b}	4.03 ± 0.12^{b}	4.48 ± 0.14^{d}	5.10 ± 0.14^{e}	5.80 ± 0.11^{d}
32FL3	Lacticin 481	3.15 ± 0.15	2.93 ± 0.18^{a}	3.05 ± 0.18^{ab}	3.03 ± 0.02^{c}	3.23 ± 0.02^{d}	3.02 ± 0.12^{b}
32FL1	Lacticin 481	3.11 ± 0.02	2.90 ± 0.18^{a}	2.69 ± 0.05^{a}	2.89 ± 0.09^{b}	2.92 ± 0.09^{b}	2.97 ± 0.08^{b}
40FEL3	Nisin A	3.00 ± 0.03	2.88 ± 0.14^{a}	2.75 ± 0.14^{a}	2.58 ± 0.04^{a}	2.77 ± 0.04^{a}	2.70 ± 0.08^{a}
HP	Bacteriocin negative	3.08 ± 0.20	3.03 ± 0.03^{a}	3.37 ± 0.03^{c}	3.03 ± 0.02^{c}	3.09 ± 0.02^{c}	3.19 ± 0.03^{c}
Statistical significance		ns	***	***	***	***	***

Mean data for the five batches of Cottage cheeses analyzed in triplicate.
Different letters in the same column indicate significant statistical differences ($p<0.05$) as analyzed by Duncan test.
ns = not significant.
***$P<0.001$.

others (Gross and Morell, 1971; Hurst, 1981; Matsusaki et al., 1996; Amiali et al., 1998).

The target strain, *L. monocytogenes* F2365, was selected as a consequence of its association with an epidemic outbreak of listeriosis (Linnan et al., 1988; Mascola et al., 1988) involving a cheese product. Results obtained from the application of the selected strains in Cottage cheese making led to establish that the combinatorial action of the high acidity reached during Cottage cheese manufacture and the production of bacteriocins was able to control and partially reduce *L. monocytogenes* F2365 growth. Of the bacteriocinogenic cultures examined here, the nisin A producing strain *Lact. lactis* subsp. *cremoris* 40FEL3 most efficiently controlled *L. monocytogenes* F2365 growth. However, while this antilisterial activity was dramatic when assessed in culture media, it was less substantial when assessed in the context of cheese manufacture. The differences with respect to inhibition could be due to many factors related to the composition of the cheese. Among these factors, fat content (Jung et al., 1992; Davies et al., 1999) proteolytic degradation (Murray and Richard, 1997), partitioning into polar or non-polar food components (Murray and Richard, 1997) and sodium chloride concentrations (Chollet et al., 2008) can influence the effectiveness of nisin. Also, the ability of the strains to produce high levels of nisin (approx 10 mg/L) must be considered (data not shown). In the study by Field et al. (2010), the two most nisin A resistant *L. monocytogenes* strains had nisin A minimum inhibitory concentrations of 12.57 mg/L. Thus, the issue of low bacteriocin production *in situ* may be a factor in the inability of these nisin producers to completely eradicate *L. monocytogenes* F2365 in Cottage cheese. As reported by Bhatti et al. (2004), the chemical composition and treatment of foods as well as the initial level of *L. monocytogenes* contamination are all of crucial importance.

Notably, in 2003 the Food Safety and Inspection Services (http://www.fsis.usda.gov/) announced a ruling requiring manufacturers of ready-to-eat foods to take further steps to address the problem posed by the presence of *L. monocytogenes*. The rule encourages all establishments to employ additional *Listeria* control measures and to incorporate technologies that can kill the bacteria/prevent its growth after cooking or packaging. In the processing environment, the Food and Drug Administration reports that contamination by *L. monocytogenes* would be expected to be much lower (~20 CFU/g) than the levels used in this study (3 log CFU/g) (Food and Drug Administration, 2003). The effect of bacteriocin-producing strains such as *Lact. lactis* subsp. *cremoris* 40FEL3 on typical background levels of *Listeria* could be a promising tool, although it should be considered only as an additional measure to implement to control *Listeria* contamination.

Although the activity of CFS from the nisin Z producing strain *Lact. lactis* 29FL4 yielded promising results *in vitro* against *Listeria*, its inability to reduce the pH to desired levels during Cottage cheese production limits its use for this purpose. However, given the observed antilisterial effect, its use in conjunction with other starter cultures or in pH adjusted products cannot be ruled out.

Further studies regarding the influence of different environments and levels of *Listeria* contamination on the antilisterial capacity of these nisin producing strains will be necessary to fully explore their potential application for microbiological control in food manufacturing.

The two *Lact. lactis* subsp. *lactis* lacticin 481 producers (32FL3 and 32FL1) employed in this study have demonstrated a weak ability to reduce *Listeria* counts both *in vitro* using cell-free supernatant and *in situ* during Cottage cheese making. The anti-listerial activity of lacticin 481 has been reported previously for *Lact. lactis* subsp. *cremoris* TAB 24 and some other isolates from raw milk (Rodriguez et al., 2000), *Lact. lactis* subsp. *lactis* CNRZ 481 in milk and Caprino cheese making (Piard et al., 1990) and lactococcal strains which co-produce the lantibiotics lacticin 3147 and lacticin 481 (O'Sullivan et al., 2003). In a study by O'Sullivan et al. (2002), production of lacticin 481 was responsible for the lysis of starter cultures and consequently, the added benefit of acceleration in cheese ripening. Recently, this property of lacticin 481 has been used successfully not only in the acceleration of cheese ripening but also in flavor enhancement (Oumer et al., 2001; Garde et al., 2006). Lacticin 481 production has also been combined with high pressure to reduce pathogen levels in cheese (Rodriguez et al., 2005). Therefore, any applications involving the lacticin 481 producing strains as described above will first require further investigation to ascertain the most advantageous setting for future use. In conclusion, two *Lact. lactis* nisin producers (29FL4 and 40FEL3 strain) and two *Lact. lactis* lacticin 481 producers (32FL1 and 32FL3 strain) should be considered for their potential as starter cultures in novel food applications. Alternatively, they could be used as strains for the production of bacteriocin preparations for food preservation; e.g. milk fermentates for direct addition to food products. Further studies will be needed to fully explore the potential application of these strains as bioprotective starter or co-starter cultures en route to their use in the manufacture of safe and healthy food for human consumption.

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