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Running title. *Characterisation of L. monocytogenes controlling lactococci*

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, additives or ingredients, or they can be produced *in situ* by starter 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.

Keywords: bacteriocins, LAB, *Listeria monocytogenes*, 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 (Volgel *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 and Cogan, 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 characteristics considered beneficial in 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 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 regarded as safe (GRAS), they provide a more natural means of preservation in 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) or M17 agar supplemented with 0.5% glucose (GM17) broth and incubated for 16 hours 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) were inoculated (1% v/v) with revitalized strains and incubated at 30°C. The pH was measured after 6 and 24 hours with a pH meter (Microprocessor pH meter 213, Hanna instruments). 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 µl of revitalized strains were spotted onto the surface of an agar medium (SM) composed of 10% (w/v) skim milk powder and 2% (w/v) agar 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) at 30°C for 18 h according to Cogan *et al.* (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% NaCl. The ability of the strains to grow at each different salt concentration was evaluated after 24h at 30°C by measurement of optical density (OD_{600nm}) using a Spectophotometer (Beckman Coulter®

160 Ireland). Results were expressed as a ratio of growth in these media relative to that in standard
161 broth. All assays were performed in duplicate.
162 *2.2.5 Autolytic activity*
163 Autolysis of the cells was measured as described by Mora *et al*., (2003). The strains were
164 grown in M17 broth for 24 h at 30°C to reach an OD_{600nm} 0.8-1. The cells were washed in
165 potassium phosphate buffer (50 $mmol^{-1}$, pH 6.5) and resuspended in the same buffer to an
166 OD_{600nm} of 0.6-0.8 and incubated at 30°C. The degree of autolysis was expressed as the
167 percentage decrease in the OD_{600nm} after 4 and 24 h.
168 *2.2.6 Diacetyl production*
169 Diacetyl production was determined according to King (1948). Revitalized strains (1% v/v)
170 were inoculated in 10 ml of UHT milk and incubated at 30°C for 24 h. One ml of each cell
171 suspension was combined with 0.5 ml of α-naphthol (1% w/v) and KOH (16 % w/v) and
172 incubated at 30°C for 10 min. Diacetyl production is indicated by the formation of a red ring
173 at the top of the tubes.
174 *2.2.7 Antibiotic resistance profiles*
175 The strains were revitalized in M17 broth by growing overnight at 30°C. For antibiotic
176 resistant profile analysis, 20 ml of M17 agar was seeded with revitalized strains (1% v/v) and
177 allowed to solidify. Antibiotic disks containing the following different antibiotics, nalidixic
178 acid (30 µg ml^{-1}), rifampicin (30 µg ml^{-1}), novobiocin (5 µg ml^{-1}), vancomicin (30 µg ml^{-1}),
179 gentamicin (30 µg ml^{-1}) and chloramphenicol (10 µg ml^{-1}) were then placed onto each agar
180 plate. The plates were incubated at 30°C for 24 h. The occurrence of a clear zone of inhibition
181 around a disk indicated that the strain was susceptible to the antibiotic in question. The
182 analysis was performed in duplicate and the results expressed as diameter of clear zone (mm)
183 around the antibiotic disk.
184 *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; p335species: Tuc2009; 936 species: 645, P272, P113g, bIL66, bIL170, SKI, jj50, p2, 712). 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^{-1} agar) supplemented with 1% 1M $CaCl_2$ and poured onto 20 ml of an GM17 solid agar (8 g l^{-1} 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 Cottage cheese made with bacteriocin producing starter cultures

2.3.1 Microorganisms and culture conditions

Lact. lactis producers of nisin A (40FEL3), nisin Z producer (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 hours at 30°C. The indicator strain *L. monocytogenes* F2365 was provided by the UCC Culture Collection. *L. monocytogenes* F2365 was propagated in BHI broth (Oxoid, UK) and incubated for 16 hours 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.

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

Prior to the application of the *Lact. lactis* strains in cottage cheese making, the sensitivity of *L. monocytogenes* F2365 to cell-free supernatants derived from each of the *Lact. lactis* strains was examined. 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, Germany) at 5,000 x *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 pHs, i.e. 6.5, 5.5 and 4.5, with sterile 1M NaOH or 1M HCl. Ten ml of CFS was then inoculated with 10^4 CFU ml^{-1} of revitalized *L. monocytogenes* F2365 and incubated at 37°C. The CFS of *Lact. lactis* subsp. *cremoris* HP NCDO 607 was 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, UK) after 0, 2, 4, 6 and 24 hours. Analysis was performed in duplicate.

2.3.3 Manufacture of Cottage cheese

Commercially purchased low-fat pasteurized milk was heated to 32°C and subsequently inoculated with 1% of overnight cultures. Diluted rennet was added in milk (0.18 ml l^{-1}) 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, was added to the dressing at the

235 level of 10^3 cells ml^{-1}. Once the dressing was added, the cheese was left for 1 hour at room
236 temperature. Cottage cheese was stored at 4°C and enumeration of *L. monocytogenes* was
237 assessed at days 1, 2, 3, 5 and 7. At each time point the pH of the cheese was also measured
238 (Microprocessor pH meter 213, Hanna instruments).
239 *2.3.4 Enumeration of L. monocytogenes by direct plating method*
240 Samples of cottage cheese (1 g) were homogenized in ¼ Ringer's solution (Merck). Triplicate
241 dilutions were performed and plated on LSA. The plates were incubated at 37°C for 24 h,
242 after which *Listeria* were counted.
243 *2.3.5 Statistical analysis*
244 Statistical analysis of data was performed by using Statistica ver. 7.0 (StatSoft Inc., Tulsa,
245 USA) for one-way analysis of variance (ANOVA) and the Duncan mean comparison test.
246
247 **3. Results**
248 ***3.1 Technological characterization of bacteriocin producing strains***
249 *3.1.1 Acidification, extracellular proteolytic activity and exopolysaccharide (EPS) production*
250 Proteolytic activity, levels of lactic acid produced and the production of bacteriocins and
251 exopolysaccharide (EPS) are important attributes of starter bacteria used in commercial
252 cheese making. A test of the ability of each *Lact. lactis* strain to acidify skim milk showed
253 that all successfully reduced the pH over a 24 hour period of incubation at 30°C (Table 1). In
254 particular, seven strains (2 *Lact. lactis*, 4 *L .lactis* subsp. *lactis*, 1 *Lact. lactis* subsp. *cremoris*)
255 were found to be more efficient acidifiers than the *Lact. lactis* HP control strain. A similar
256 pattern was revealed when the strains where grown in UHT low-fat milk (1,5% fat) (Table 1).
257 In accordance with Bouton's classification (Bouton *et al*., 2002), the results obtained during
258 this study establish seven of the total *Lact. lactis* tested as being high acidifying strains
259 (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 low salt concentrations (4%) (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 hours. After 24 hours 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).

283 *3.1.3 Antibiotic and bacteriophage resistance*

284 The antibiotic resistance of the *Lact. lactis* strains relative to *Lact. lactis* subsp. *cremoris* HP,
285 a starter culture sensitive to all antibiotics, was also tested. The results obtained indicate that
286 all *Lact. lactis* strains tested were resistant to nalidixic acid (30 µg ml^{-1}) and were sensitive to
287 rifampicin, novobiocin, gentamycin, vancomycin and chloramphenicol (data not shown).
288 Bacteriophage sensitivity was established on the basis of the presence or absence of a typical
289 clear zone in a lawn of the test cells, due to cell lysis by phage. All *Lact. lactis* strains tested,
290 other than the positive control *Lact. lactis* MG1363 were resistant to the twelve phages tested
291 (data not shown).
292 Following completion of this array of biochemical and physical tests, a final evaluation of the
293 twenty strains that were initially selected for characterization determined that just four strains,
294 40FEL3 (nisin A), 29FL4 (nisin Z), 32FL3 (lacticin 481) and 32FL1 (lacticin 481) fulfilled all
295 the desired criteria and were further examined for their ability to control *Listeria* in the
296 manufacture and storage of cottage cheese.
297
298 TABLE 1
299
300 ***3.2 Evaluation of antilisterial activity of bacteriocin producing strains***

301 *3.2.1 Sensitivity of Listeria to bacteriocin cell-free supernatant at different pH values*
302 We wished to evaluate the inhibitory effect of cell-free supernatant (CFS) from the
303 bacteriocin producing strains 40FEL3, 29FL4, 32FL3 and 32FL1 on *L. monocytogenes*
304 F2365. Strain F2365 was inoculated at approximately 10^4 CFU ml^{-1} in CFS at three different
305 pHs (6.5, 5.5 and 4.5) and its growth was assessed after 0, 2, 4, 6 and 24 hours at 37°C. CFS
306 from the non-bacteriocinogenic HP culture was used as a negative control.

307 At pH 6.5, nisin A-containing CFS from strain 40FEL3 had a significant killing effect in that
308 *L. monocytogenes* F2365 numbers were reduced by ~ 3 log CFU ml^{-1} during the first 6 hours
309 of incubation (Figure 1). In comparison, an increase of 4 log CFU ml^{-1} in pathogen numbers
310 was detected at the same time point when non bacteriocin-containing CFS was used. In the
311 case of CFS from the nisin Z-producing *Lact. lactis* 29FL4, a decrease of just 0.5 log CFU ml^{-1}
312 was observed after 2 hours. Additionally, the CFS from the *Lact. lactis* subsp. *lactis* lacticin
313 481-producing strains 32FL3 and 32FL1 reduced *Listeria* counts relative to the non
314 bacteriocin-containing control, albeit only slightly. In all cases, the number of *Listeria*
315 increased after 6 hours as a result of renewed growth from the surviving *L. monocytogenes*
316 cells.
317
318 FIGURE 1
319
320 At pH 5.5, a dramatic decrease in cell counts of *Listeria* (to below detectable levels) was
321 observed following incubation in the nisin A containing CFS for 2 hours. In the case of the
322 nisin Z containing CFS, listerial cell numbers decreased by ~ 3 log CFU ml^{-1} after 4 hours and
323 after 6 hours the pathogen could not be detected (Figure 2).
324 In the case CFS from both lacticin 481 producers adjusted to pH 5.5, a slight increase in *L.*
325 *monocytogenes* F2365 numbers (1-2 log CFU ml^{-1}) was observed over the 24 hour period. In
326 comparison *Listeria* counts increased by ~ 3 log CFU ml^{-1} in non-bacteriocin CFS over the
327 same 24 hour period.
328
329 FIGURE 2
330

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 hours (Figure 3). In particular, after 24 hours the reduction in *Listeria* counts were 2.46 log CFU ml^{-1} for nisin A and 2.58 log CFU ml^{-1} for nisin Z. In contrast, no change in *Listeria* numbers was observed over 6 hours both in non-bacteriocin containing CFS and in CFS from both lacticin 481 producers.

FIGURE 3

From the results obtained, it can be seen that the growth of *Listeria* is greatly influenced by both low pH environments and the presence of bacteriocins, and by the two nisin variants in particular.

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 cell numbers over 24 hours. Interestingly, a slight decrease in *Listeria* numbers was noticeable after 24 hours in the case of lacticin 481-containing CFS at pH 4.5 (32FL1 and 32FL3 producers) when compared to the non-bacteriocin CFS control. Thus a lacticin 481 and low pH combination can at least partially retard the growth of *Listeria* in certain conditions, and can reduce cell numbers by approximately 0.5 log after 24 hours.

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

356 cheese fermentation. *L. monocytogenes* F2365 was added to reach an initial level of 10^3 CFU
357 g^{-1}. *Lact. lactis* subsp. *cremoris* HP NCDO 607, a non-bacteriocinogenic strain was used as
358 control starter. *Listeria* growth in the cheese was monitored in cheese by direct plating on
359 LSA medium at time 0 (inoculum of pathogen) and after 1, 2, 3, 5 and 7 days of storage at
360 4°C.

361 *Cheese pH*

362 Table 2 shows the pH values measured during the storage of cottage cheese. In curd (0d), the
363 initial pH range was ~ 4.65-4.80. No major differences in pH were detected between cheeses
364 made with the non-bacteriocin producing HP and those made with the bacteriocin positive
365 cultures after 7 days of storage, with the exception of *Lact. lactis* 29FL4 (nisin Z producer) in
366 which case the pH had reached a value of just 5.86. This pH is not within the necessary
367 parameters/requirements needed for good cottage cheese manufacturing (a final pH 4.65-4.75
368 is considered favourable) and thus *Lact. lactis* 29FL4 would be unsuitable for industrial
369 purposes unless assisted by the addition of acid or a nisin resistant acid producing strain.

370

371 TABLE 2

372

373 *3.2.3 Survival of L. monocytogenes in cheese*

374 Table 3 shows the inhibitory effect of the bacteriocin positive starter cultures against *L.*
375 *monocytogenes* F2365 in cottage cheese during 7 days of storage (expressed as mean log CFU
376 g^{-1}). Regular sampling of the cheeses established that the presence of *Lact. lactis* subsp.
377 *cremoris* 40FEL3 (nisin A) and *Lact. lactis* subsp. *lactis* 32FL1 or 32FL3 (lacticin 481)
378 resulted in a decrease in pathogen cell numbers ($P<0.001$) after 2 days of cottage cheese
379 storage. In contrast, there was no significant decrease in the levels of the pathogen in the
380 cheese containing the non-bacteriocin producing culture. A further decrease in *L.*

381 *monocytogenes* numbers was observed after day 3 in the cheeses containing the nisin A and
382 lacticin 481 producers. These levels were again lower than those present in the cheese
383 containing the bacteriocin-negative culture (P<0.001). On days 5 and 7 there was a slight
384 increase in *Listeria* numbers in the cheeses containing the nisin A and lacticin 481 producers
385 when compared to the counts taken on day 3. However counts of 0.30 log CFU g^{-1} for cheeses
386 containing the nisin A producer, and 0.14 and 0.12 log CFU g^{-1} for cheeses containing the
387 lacticin 481 producers were still below the initial inoculum levels. In contrast, the numbers of
388 *Listeria* had increased by 0.11 log CFU g^{-1} in cheese made with *Lact. lactis* HP after 7 days of
389 storage and an increase in *Listeria* counts (~ 3log CFU g^{-1}) was observed in cheese made with
390 the *Lact. lactis* nisin Z producer (29FL4). The latter result is most likely due to the relatively
391 poor acidification of the cheese and thus the provision of a less stressful environment for the
392 pathogen.
393
394 TABLE 3
395
396 **4. Discussions**
397 The growth of *Listeria* strains at temperatures ranging from 1° to 45°C, their high salt
398 tolerance, and their ability to initiate growth at a relatively low pH make these pathogens
399 particularly difficult to control in food (Vignolo *et al*., 2000). A promising means of
400 controlling, and even reducing, *Listeria* populations in foods is through the use of
401 bacteriocins, either produced *in situ* in fermented products or added to the product. Since
402 lactococci are the principal starters in a variety of fermented products, bacteriocinogenic
403 lactococci have been employed to improve product quality. Indeed, several studies have
404 demonstrated the ability of the broad spectrum bacteriocin, nisin, to inhibit the growth of *L.*
405 *monocytogenes* when applied in foods (Ryser, 1999; Rodriguez *et al.,* 2005; Samelis *et al*.,

406 2003). High levels of this bacteriocin have been shown to completely eliminate *L.*
407 *monocytogenes* in soft cheeses within periods as short as 24 h (Ryser, 1999*)*.
408 In this study, four different bacteriocinogenic cultures, producing either nisin A, nisin Z or
409 lacticin 481 (x 2), were selected as a result of displaying excellent starter culture attributes
410 including good acidifying activity, high extracellular proteolytic activity, bacteriophage
411 resistance and a lack of exopolysaccharide production as well as other favourable properties
412 from a larger collection of 20 strains. The selected cultures were then tested for their ability to
413 control *L. monocytogenes* growth both *in vitro* and in the manufacture of cottage cheese.
414 Importantly, these results highlighted that CFS from nisin bacteriocin producers were active
415 against *Listeria* at different pH levels (6.5, 5.5 and 4.5), but that significant variation is
416 evident. Specifically, nisin A CFS had an initial detrimental effect on *Listeria* at pH 6.5 and
417 5.5 but had much less effect over the first 6 hours at pH 4.5. It was also notable that nisin Z-
418 containing CFS exhibited considerable antilisterial activity at pH 5.5 but had less impact at
419 pH 6.5. At pH 4.5 the activity of nisin Z CFS was similar to that of nisin A CFS.
420 The greater inhibitory effect of nisin at mildly acidic, rather than neutral, pH is possibly due
421 to the greater solubility of nisin at acidic pH (Hurst and Hoover, 1983). Here, the activity of
422 nisin was greater at pH 5.5 than pH 4.5, which is in accordance with the findings of others
423 (Gross and Morell, 1971; Hurst, 1981; Matsusaki *et al.,* 1996, Amiali *et al*., 1998).
424 The target strain, *L. monocytogenes* F2365, was selected as a consequence of its association
425 with an epidemic outbreak of listeriosis (Linnan *et al.*, 1988, Mascola *et al.*, 1988) involving a
426 cheese product. Results obtained from the application of the selected strains in Cottage cheese
427 making led to establish that the combinatorial action of the high acidity reached during
428 cottage cheese manufacture and the production of bacteriocins was able to control and
429 partially reduce *L. monocytogenes* F2365 growth. Of the bacteriocinogenic cultures examined
430 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 *Listeria 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 (FSIS) 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 FDA reports that contamination by *L. monocytogenes* would be expected to be much lower (~ 20 CFU g^{-1}) than the levels used in this study (3 log CFU g^{-1}). The effect of bacteriocin-producing strains such as *Lact. lactis* subsp. *cremoris* 40FEL3 on typical background levels of *Listeria* could prevent manufactured and processed foods from breaching acceptable guidelines for *Listeria* contamination.

455 Although the activity of CFS from the nisin Z producing strain *Lact. lactis* 29FL4 yielded
456 promising results *in vitro* against *Listeria*, its inability to reduce the pH to desired levels
457 during cottage cheese production limits its use for this purpose. However, given the observed
458 antilisterial effect, its use in conjunction with other starter cultures or in pH adjusted products
459 cannot be ruled out.
460 Further studies regarding the influence of different environments and levels of *Listeria*
461 contamination on the antilisterial capacity of these nisin producing strains will be necessary to
462 fully explore their potential application for microbiological control in food manufacturing.
463 The two *Lact. lactis* subsp. *lactis* lacticin 481 producers (32FL3 and 32FL1) employed in this
464 study have demonstrated a weak ability to reduce *Listeria* counts both *in vitro* using cell-free
465 supernatant and *in situ* during cottage cheese making. The anti-listerial activity of lacticin 481
466 has been reported previously for *Lact. lactis* subsp. *cremoris* TAB 24 and some other isolates
467 from raw milk (Rodriguez *et al*., 2000), *Lact. lactis* subsp. *lactis* CNRZ 481 in milk and
468 Caprino cheese making (Piard *et al*. 1990) and lactococcal strains which co-produce the
469 lantibiotics lacticin 3147 and lacticin 481 (O'Sullivan *et al*., 2003). In a study by O'Sullivan
470 *et al*. (2002), production of lacticin 481 was responsible for the lysis of starter cultures and
471 consequently, the added benefit of acceleration in cheese ripening. Recently, this property of
472 lacticin 481 has been used successfully not only in the acceleration of cheese ripening but also
473 in flavor enhancement (Oumer *et al*., 2001; Garde *et al*., 2006). Lacticin 481 production has
474 also been combined with high pressure to reduce pathogen levels in cheese (Rodriguez *et al*.,
475 2005). Therefore, any applications involving the lacticin 481 producing strains as described
476 above will first require further investigation to ascertain the most advantageous setting for
477 future use. In conclusion, two *Lact. lactis* nisin producers (29FL4 and 40FEL3 strain) and two
478 *Lact. lactis* lacticin 481 producers (32FL1 and 32FL3 strain) should be considered for their
479 potential as starter cultures in novel food applications. Alternatively, they could be used as

480 strains for the production of bacteriocin preparations for food preservation; e.g. milk
481 fermentates for direct addition to food products. Further studies will be needed to fully
482 explore the potential application of these strains as bioprotective starter or co-starter cultures
483 en route to their use in the manufacture of safe and healthy food for human consumption.
484
485 Acknowledgements
486 We gratefully acknowledge Prof. Dowe van Sinderen and Dr. Jennifer Mahony for providing
487 the *Lact. lactis* phage utilized in this study. This research was also supported by the Irish
488 Government under the National Development Plan through a Science Foundation Ireland
489 Investigator award to C.H., R.P.R and P.D.C. (06/IN.1/B98) and by Piedmont Region, Italy
490 research program CIPE 2006.
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699 **Table 1.** Technological characterization of *Lact. lactis* bacteriocin producing strains

	Acidifying activity[a]						Extracellular proteolytic activity	EPS production	Autolysis [b] (%)		Diacetyl production
	pH drop in skim milk			pH drop in UHT milk							
Species	0	6h	24h	0	24h				4h	24h	
L. lactis nisin Z producers											
44SGL L1	6,71	6,57 ± 0,04	4,89 ± 0,01	6,75	5,47 ± 0,06		+	-	2	18	+
49SGL L1	6,71	6,50 ± 0,01	4,90 ± 0,03	6,75	5,40 ± 0,02		+	-	6	24	+
29F L4	6,71	5,96 ± 0,01	4,50 ± 0,02	6,75	4,67 ± 0,06		+++	-	2	20	-
30F L3	6,71	6,07 ± 0,09	4,54 ± 0,01	6,75	4,69 ± 0,01		++	-	0	27	-
44SGL L7	6,71	6,56 ± 0,03	4,98 ± 0,00	6,75	5,02 ± 0,04		+	-	3	22	+
44SGL L3	6,71	6,54 ± 0,01	4,98 ± 0,01	6,75	4,80 ± 0,03		++	-	2	22	-
44SGL L9	6,71	6,51 ± 0,00	5,03 ± 0,00	6,75	5,31 ± 0,03		++	-	2	21	+
L.lactis nisin A producers											
44SGL L8	6,71	6,54 ± 0,01	4,91 ± 0,03	6,75	5,19 ± 0,01		++	-	18	39	+
41F L5	6,71	6,56 ± 0,01	5,58 ± 0,01	6,75	4,90 ± 0,03		+	-	5	22	-
41F L8	6,71	6,40 ± 0,01	5,34 ± 0,01	6,75	4,19 ± 0,03		-	-	16	37	-
41F L13	6,71	6,41 ± 0,02	5,95 ± 0,03	6,75	5,96 ± 0,01		-	-	27	48	-
41F L15	6,71	6,39 ± 0,01	5,31 ± 0,01	6,75	5,39 ± 0,05		-	-	25	50	-
44SGL L2	6,71	6,60 ± 0,02	5,00 ± 0,02	6,75	5,40 ± 0,01		+	-			
L.lactis subsp. *lactis* nisin A producers											
41F LL8a	6,71	6,34 ± 0,01	5,04 ± 0,04	6,75	5,15 ± 0,04		-	-	3	37	+
41F LL2	6,71	6,09 ± 0,10	4,29 ± 0,01	6,75	4,20 ± 0,01		-	-	14	49	-
41F LL8b	6,71	6,40 ± 0,00	4,42 ± 0,03	6,75	4,20 ± 0,03		-	-	11	41	-
41F LL7	6,71	6,46 ± 0,00	5,61 ± 0,01	6,75	5,96 ± 0,00		-	-	11	44	-
L.lactis subsp. *cremoris* nisin A producer											
40F EL3	6,71	6,08 ± 0,03	4,20 ± 0	6,75	4,14 ± 0,02		+++	-	9	38	-
L.lactis subsp. *lactis* lct481 producers											
32F L3	6,71	6,08 ± 0,07	4,19 ± 0,01	6,75	4,14 ± 0,01		+++	-	5	31	-
32F L1	6,71	6,10 ± 0,05	4,14 ± 0,03	6,75	4,14 ± 0,01		+++	-	22	45	-
L.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	-

[a] Results are expressed as mean value ± SD of duplicate experiments

[b] Autolysis is express as (%) = 100- (OD_{t48h}/lowest value:/OD_{t48h}highest value* 100)

ND not evaluated

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701 **Table 2.** pH values during storage at 4°C of cottage cheese inoculated with *L. lactis*
702 bacteriocin-producing cultures.

		Storage time (days)					
***Lact. lactis* strain**	**Bacteriocin**	0	1d	2d	3d	5d	7d
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

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709 **Table 3**. *L. monocytogenes* count (mean log CFU g^{-1} ± SD) during the storage of cottage
710 cheese manufactured with bacteriocin-producing lactic acid bacteria and a non-bacteriocin
711 producing control lactic culture.

		Storage time (days)					
***Lact. lactis* strain**	**Bacteriocin**	0	1d	2d	3d	5d	7d
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	***	***	***	***	***

712 Mean data for the six batches of Cottage cheeses analysed in triplicate.

713 a, b, c, d, e : Different letters in the same column indicate significant statistical differences (Duncan
714 Test, $p < 0.05$).

715 ns = not significant.

716 *** $P < 0.001$.

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728 **Figure legends**

729 Fig. 1

730 Growth of *L. monocytogenes* F2365 strains in GM17 at pH 6.5 at 37°C in presence of CFS of
731 *Lact. lactis* 29FL4 nisin Z producer ( ); CFS of *Lact. lactis* subsp. *lactis* 32FL3 lacticin 481
732 producer ( ); CFS of *Lact. lactis* subsp. *lactis* 32FL1 lacticin 481 producer ( ); CFS of
733 *Lact. lactis* subsp. *cremoris* 40FEL3 nisin A producer ( ); CFS of *Lact. lactis* HP non-
734 bacteriocin producer ( ). The error bars indicate the mean standard deviations for the data
735 points. (P<0.001).

736

737 Fig. 2.

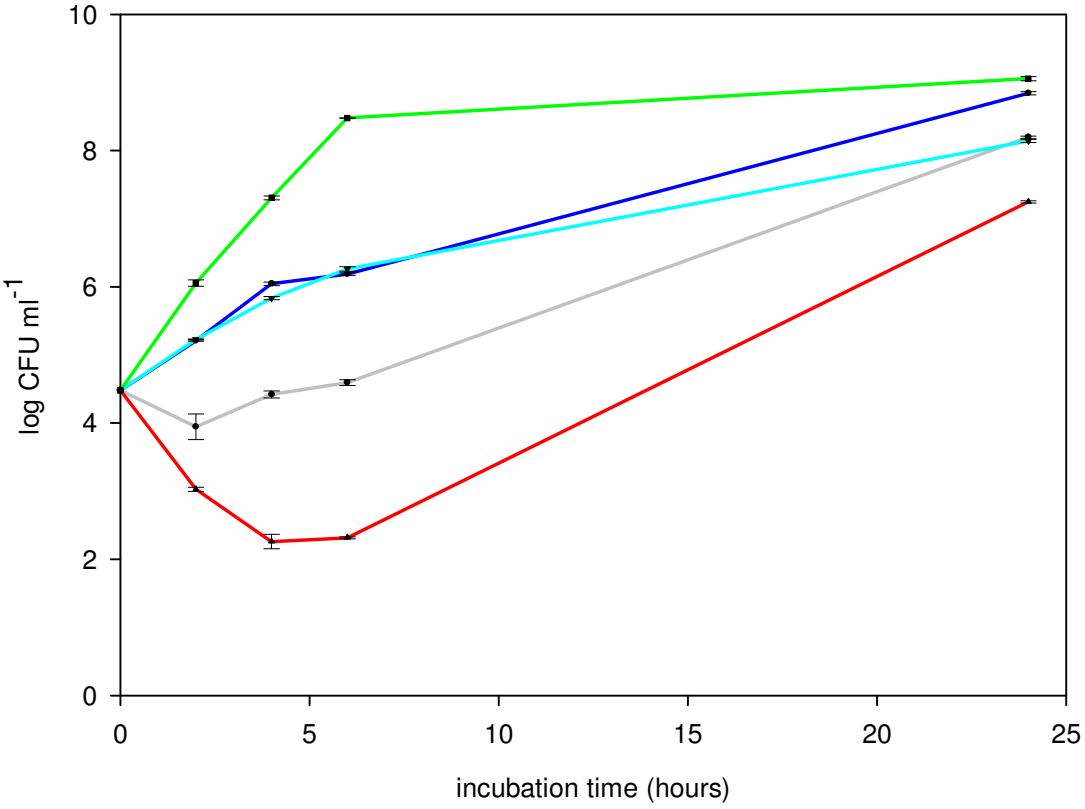
738 Growth of *L. monocytogenes* F2365 strains in GM17 at pH 5.5 at 37°C in presence of CFS of
739 *Lact. lactis* 29FL4 nisin Z producer ( ); CFS of *Lact. lactis* subsp. *lactis* 32FL3 lacticin
740 481 producer ( ); CFS of *Lact. lactis* subsp. *lactis* 32FL1 lacticin 481 producer ( ); CFS
741 of *Lact. lactis* subsp. *cremoris* 40FEL3 nisin A producer ( ); CFS of *Lact. lactis* HP strains
742 non- bacteriocin producer ( ). The error bars indicate the mean standard deviations for the
743 data points. (P<0.001).

744

745 Fig. 3.

746 Growth of *L. monocytogenes* F2365 strains on GM17 at pH 4.5 at 37°C in presence of CFS of
747 *Lact. lactis* 29FL4 nisin Z producer ( ); CFS of *Lact. lactis* subsp. *lactis* 32FL3 lacticin
748 481 producer ( ); CFS of *Lact. lactis* subsp. *lactis* 32FL1 lacticin 481 producer ( ); CFS
749 of *Lact. lactis* subsp. *cremoris* 40FEL3 nisin A producer ( ); CFS of the non-bacteriocin
750 producer *Lact. lactis* HP strain ( ). The error bars indicate the mean standard deviations for
751 the data points. (P<0.001).

752 **Fig.1**



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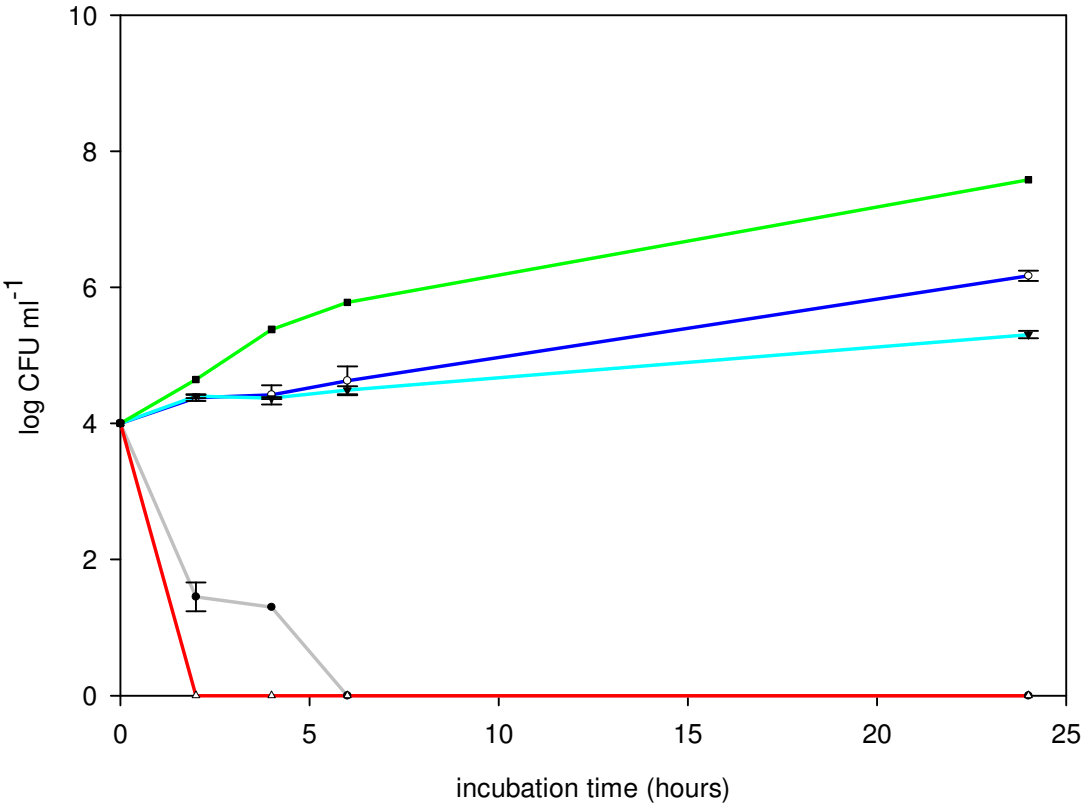
761 **Fig.2**



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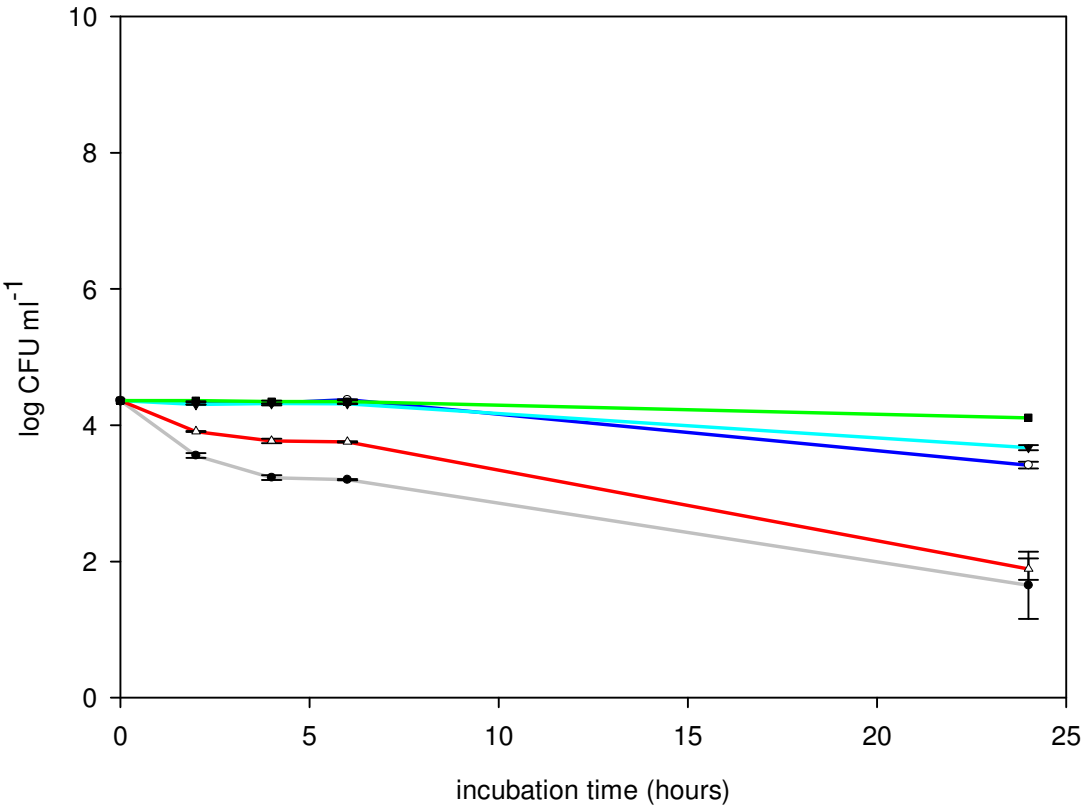
775 **Fig.3**



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