A Multi-Bacteriocin Cheese Starter System comprising Nisin and Lacticin 3147 in *Lactococcus lactis*, in Combination with Plantaricin from *Lactobacillus plantarum*

Running Title: *Multi-bacteriocin producing starter system*

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ABSTRACT

Functional starter cultures demonstrating superior technological and food safety properties are advantageous to the food fermentation industry. We evaluated the efficacy of single and double bacteriocin-producing starters of *Lactococcus lactis* capable of producing the Class I bacteriocins, nisin A and/or lacticin 3147 in terms of starter performance. Single producers were generated by mobilising the conjugative, bacteriophage resistance plasmid pMRC01, encoding lacticin genetic determinants, or the conjugative transposon Tn5276, encoding nisin genetic determinants, to the commercial starter *L. lactis* CSK2775. The effect of bacteriocin co-production was examined by superimposing pMRC01 into the newly constructed nisin transconjugant. Transconjugants were improved with regard to antimicrobial activity and bacteriophage insensitivity when compared to the recipient strain and the double producer was immune to both bacteriocins. Bacteriocin production in the starter was stable, although the recipient strain proved to be a more efficient acidifier than transconjugant derivatives. Overall, combining Class I bacteriocins (the double-producer or a combination of single producers) proved as effective as individual bacteriocins for controlling *Listeria innocua* growth in laboratory-scale cheeses. However, using the double producer in combination with the Class II bacteriocin producer *Lactobacillus plantarum*, or the lacticin producer with the Class II producer, proved most effective for reducing bacterial load. As emergence of bacteriocin tolerance was reduced 10-fold in the presence of nisin and lacticin, we suggest that the double producer in conjunction with the Class II producer could serve as a protective culture providing a food-grade, multi-hurdle approach to control pathogenic growth in a variety of industrial applications.

53 **IMPORTANCE**

54 We generated a suite of single and double-bacteriocin producing starter cultures capable
55 of generating the Class I bacteriocins lacticin 3147 or nisin or both bacteriocins
56 simultaneously via conjugation. The transconjugants exhibited improved bacteriophage
57 resistance and antimicrobial activity. The single producers proved as effective as the
58 double-bacteriocin producer at reducing *Listeria* numbers in laboratory-scale cheese.
59 However, combining the double producer or the lacticin producing starter with a Class
60 II bacteriocin producer, *Lactobacillus plantarum* LMG P-26358, proved most effective
61 at reducing *Listeria* numbers, and was significantly better than a combination of the
62 three bacteriocin producing strains, as the double producer is not inhibited by either of
63 the Class I bacteriocins. Since the simultaneous use of lacticin and nisin should reduce
64 the emergence of bacteriocin tolerant derivatives this study suggests that a protective
65 starter system produced by bacteriocin stacking is a worthwhile multi-hurdle approach
66 for food safety applications.

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78 **INTRODUCTION**

79 The development and characterisation of starter cultures that demonstrate superior
80 technological properties such as improved proteolytic activity and flavour production,
81 exopolysaccharide production or bacteriophage resistance are considered highly
82 advantageous within the food fermentation industry (1). The ability of starter strains to
83 produce bacteriocins is also considered an important technological trait for controlling
84 undesirable and/or pathogenic growth in situ and for improving sensory characteristics
85 (1-3). Bacteriocins are ribosomally synthesised, heat stable antimicrobial peptides that
86 generally act by depolarising the target cell membrane and/or through inhibiting cell
87 wall synthesis where the producing strain is immune to the antimicrobial effect (4).
88 They comprise a highly heterogeneous group that have recently been divided into three
89 distinct Classes (5).

90 The exploitation of bacteriocin-producing cultures is a particularly attractive
91 option for the food industry owing to the generally recognized as safe (GRAS) status of
92 the cultures, immediately fulfilling the consumers' demand for minimally processed
93 foods lacking artificial food additives. The bacteriocin producer can serve as the starter
94 culture or be added as an additional protective culture. Several studies have highlighted
95 the efficacy of such approaches where bacteriocin-producing cultures have proven
96 effective for inhibiting the growth and proliferation of pathogenic and food spoilage
97 microorganisms (6-9). Despite this, the use of bacteriocins in the food industry remains
98 limited possibly owing to the fact that a bacteriocin alone may not be capable of
99 providing sufficient protection against contamination (10). The use of bacteriocin
100 combinations or bacteriocin stacking may represent an alternative approach. Indeed,
101 improved antimicrobial activity of bacteriocin combinations has been reported
102 previously (11, 12, 13). However, when using multiple bacteriocins, it is essential that

other important cultures are not inhibited. This can be overcome to some degree by developing a multi-bacteriocinogenic culture which is immune to the bacteriocins it produces.

In the present study, we generated single and double bacteriocin-producing cultures of *L. lactis* CSK2775 with the capacity to produce Class I bacteriocins, lacticin 3147 (hereafter lacticin), nisin A (hereafter nisin) or lacticin and nisin. Both bacteriocins target lipid II to generate pores in the cell membrane causing proton-motive force dissipation and subsequent cell death (14-17). Resulting transconjugants were assessed for bacteriocin production, bacteriophage resistance properties, acidification efficiency and antimicrobial activity against a spectrum of indicator strains including food pathogens and other lactic acid bacteria (LAB). The ability of the transconjugants (single and double) to produce bacteriocins in laboratory-scale cheese was assessed and we also evaluated the anti-listerial potential of the Class I producers alone and in combination with the Class IIa bacteriocin producer *Lactobacillus plantarum* LMG P-26358 (18). Class IIa bacteriocins cause pore formation by binding to and irreversibly opening the sugar transporter mannose phosphotransferase (Man-PTS) system in the target cell (19). In this study, *Listeria innocua* served as a surrogate for *Listeria monocytogenes* for reasons of safety and efficiency (as in many other studies) and because *L. innocua* has been successfully used in previous studies investigating the anti-listerial potential of nisin (20-24), lacticin (25-28) and plantaricin (18) in food systems.

MATERIALS AND METHODS

Bacterial strains and media

Bacterial strains used in this study are listed in Tables 1 and 2. *L. lactis* strains were routinely propagated at 30°C in M17 medium (Difco Laboratories, Detroit, MI, USA) supplemented with 0.5% (w/v) lactose (LM17) or glucose (GM17). *Lb. plantarum* was grown in MRS medium (29) (Difco Laboratories) at 30°C. *L. innocua* was routinely propagated in GM17 broth at 37°C containing 500 µg/ml streptomycin (Sigma Aldrich, Ireland). Other media used in this study include BHI (Brain-Heart Infusion) broth (Oxoid Ltd., Basingstoke, Hampshire, England) and RCM (Re-inforced Clostridial Medium) (Merck, Darmstadt, Germany). All strains were stored in 50% glycerol at -20°C.

Strain construction and analytical tests

Strain construction

The conjugation method of Coakley et al. (30) was used with slight modifications to generate lacticin transconjugants. Inocula (2%) of both donor and recipient were grown for 4 h in GM17 broth at 30°C. After the growth period, 1 ml of recipient and 1 ml of donor were harvested by centrifugation (16,000 x g for 1 min) and rinsed twice with GM17 broth. After the final rinse, each strain was resuspended in 50 µl of GM17 broth. The concentrated recipient and donor (20x) were then mixed with each other at the following ratios, 1:1, 2:1 and 20:1. Each mixture was spotted onto the centre of a GM17 agar plate and incubated for 18 h at 30°C. The following day, spots were harvested in 1 ml of maximum recovery diluent (MRD; Oxoid) and serially diluted before plating on lactose indicator agar (LIA) containing lacticin (400 arbitrary units (AU)/ml) as described previously (30). Following 48 h of incubation at 30°C the lacticin-containing

153 LIA plates were examined for lactose-positive colonies (yellow) against a background
154 of lactose-negative colonies (white), and lactose-positive colonies were selected and
155 grown in LM17 broth for further analysis.
156 Nisin transconjugants were generated according to the method of Gireesh et al. (31)
157 with modifications: inocula of donor (1.5%) and recipient (2%) were grown for 4 h in
158 GM17 broth at 30°C. Donor and recipient were then mixed at the following ratios, 1:10
159 and 1:100 in the presence of 400 µg/ml α-chymotrypsin (Sigma Aldrich). The cells
160 were collected onto membrane filters (0.45 µm pore size, Merck, Millipore, Darmstadt,
161 Germany) after which the filters were placed on GM17 agar plates (cell side down).
162 Following 18 h of incubation at 30°C, cells were harvested from the filter and added to
163 10% reconstituted skimmed milk (RSM) containing 400 AU/ml nisin (Sigma Aldrich)
164 and incubated at 30°C for 24-48 h. Clotted samples were serially diluted, plated on LIA
165 and following 48 h of incubation at 30°C, yellow colonies were selected for further
166 analysis.
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168 *Bacteriocin production and immunity*
169 Bacteriocin production and immunity was assessed by performing the agar well
170 diffusion assay as described by Ryan et al. (32). Indicator organisms are listed in Table
171 2. Bacteriocin sensitivity was scored according to the diameter of the zone of inhibition
172 surrounding the well which contained cell free supernatant from the bacteriocin
173 producer. The concentration of bacteriocin produced by the double producer was
174 measured by agar well diffusion assay using a serial two-fold dilution of the filtered
175 culture supernatant and bacteriocin activity was calculated as the inverse of the last
176 dilution that gave a definite zone of clearance after overnight incubation where AU
177 were expressed per ml.

178 *Colony mass spectrometry*

179 Colony mass spectrometry was performed according to the method described by Field
180 et al. (33).

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182 *PCR scan*

183 Genomic DNA was extracted from 1.5 ml of 18 h cultures according to the method of
184 Hoffman and Winston (34) slightly modified as described previously (35). Primer pairs
185 used to scan strains for the presence of pMRC01 as well as the genes associated with
186 nisin production are listed in Table 3. PCR was performed in a Hybaid PCR express
187 unit (Hybaid Ltd., Middlesex, UK) using MyTaq^{TM} Red Mix polymerase (Bioline Ltd.,
188 London, U.K.) according to manufacturers' specifications combined with an annealing
189 temperature of 55^{o}C.

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191 *Pulsed field gel electrophoresis*

192 Pulsed field gel electrophoresis (PFGE) was performed according to Mills et al. (35)
193 using the restriction enzyme SmaI (New England Biolabs, Hertfordshire, U.K.). DNA
194 fragments were run on a CHEF-DR III pulsed-field system (Bio-Rad laboratories,
195 California, USA) at 6V/cm for 22 h with a 1-30 s linear ramp pulse time. Molecular size
196 markers (N0340S, N0350S) were purchased from New England BioLabs.

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198 *Bacteriophage assays*

199 Bacteriophages were propagated according to the method outlined previously (36).
200 Sensitivity to bacteriophage infection was performed by the double agar layer plaque
201 assay as described previously (30).

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Characterisation of acid production

Acid production was monitored in 10% RSM in the presence and absence of 0.1% yeast extract according to the method of Harrington and Hill (37).

Laboratory-scale cheese manufacture

Cultures were grown from frozen stocks in their respective media for 18 h (Table 1). The cultures were then inoculated at 1% (v/v) into 10% (w/v) RSM and incubated for a further 18 h at 30°C. In the case of *Lb. plantarum* LMG P-26358, the culture was grown in 10% RSM containing 0.1% (v/v) yeast extract and 0.2 g/l $MnSO_4 4H_2O$ as previously reported (18).

One litre vats of whole milk heated to 31°C were inoculated with the 18 h RSM cultures as follows:

-Vat 1 = 0.75% (v/v) *L. lactis* DPC4268, 0.75% (v/v) *L. lactis* CSK2775 (no bacteriocin)

-Vat 2 = 0.75% (v/v) *L. lactis* DPC4268, 0.75% (v/v) *L. lactis* CSK3281 (nisin producer)

-Vat 3 = 0.75% (v/v) *L. lactis* DPC4268, 0.75% (v/v) *L. lactis* CSK3594 (lacticin producer)

-Vat 4 = 0.75% (v/v) *L. lactis* DPC4268, 0.75% (v/v) *L. lactis* CSK3533 (nisin-lacticin double producer)

-Vat 5 = 0.75% (v/v) *L. lactis* DPC4268, 0.5% (v/v) *L. lactis* CSK3281 (nisin producer), 0.5% (v/v) *Lb. plantarum* LMG P-26358 (plantaricin producer)

-Vat 6 = 0.75% (v/v) *L. lactis* DPC4268, 0.5% (v/v) *L. lactis* CSK3594 (lacticin producer), 0.5% (v/v) *Lb. plantarum* LMG P-26358 (plantaricin producer)

-Vat 7 = 0.75% (v/v) *L. lactis* DPC4268, 0.5% (v/v) *L. lactis* CSK3533 (nisin–lacticin double producer), 0.5% (v/v) *Lb. plantarum* LMG P-26358 (plantaricin producer)

-Vat 8 = 0.75% (v/v) *L. lactis* DPC4268, 0.5% (v/v) CSK3594 (lacticin producer), 0.5% (v/v) CSK3281 (nisin producer)

-Vat 9 = 0.75% (v/v) *L. lactis* DPC4268, 0.5% (v/v) CSK3594 (lacticin producer), 0.5% (v/v) CSK3281 (nisin producer), 0.5% (v/v) *Lb. plantarum* LMG P-26358 (plantaricin producer)

A streptomycin resistant derivative of *L. innocua* (DPC6578) grown for 18 h was added to each vat at a level of 10^4 cfu/ml. Thirty min after inoculation, 150 international milk clotting units/ml Kalase rennet (CSK Food Enrichment, The Netherlands) was added according to manufacturer's specifications and after a further 15 min the curd was cut into cubes. Following a 10 min stirring step, approximately 35% of the whey was removed and the curd was stirred for a further 5 min. The temperature was then elevated to 36°C over a 5 min period and the curd was stirred for a further 20 min. The curd was further drained and lightly pressed into moulds for 20 min before pressing overnight. After 24 h the cheeses were submerged in a brine bath (23% NaCl [w/v], 0.22% phosphoric acid [v/v], 0.1% NaOH [w/v], 0.6% $CaCl_2$ [w/v]) at 10-12°C for 5 h after which they were vacuum-packed and ripened at 7°C for 4 weeks. *L. innocua* DPC6578 was enumerated in each cheese on a weekly basis by homogenising 1 g of cheese in 2% sterile tri-sodium citrate and plating serial dilutions on selective medium (GM17 agar with 500 µg/ml of streptomycin). The cheese trial was performed in triplicate and sampling for each trial was performed in duplicate.

Nisin (3352 ± 3 Da), lacticin (Ltnβ: 2847 ± 4 Da) and plantaricin (3928 ± 3 Da) present within cheese samples from Vats 4, 5, 6, and 7 were verified by MALDI-TOF mass spectrometry (MALDI-TOF MS) as described previously (18). In the case of lacticin,

the presence of the correct mass for Ltnβ was indicative of lacticin since Ltnα can be difficult to detect in a complex fraction. All fractions were tested for antimicrobial activity by agar well diffusion assays against the appropriate indicator strains (lacticin and nisin against *L. lactis* HP; plantaricin against *L. innocua*) where mass and concomitant activity were indicative of bacteriocin presence. Fractions expected to contain the lacticin peptides (23/24: Ltnα and 37: Ltnβ) were combined or wells were positioned near each other to assess lacticin activity.

Frequency of bacteriocin resistance/tolerance

To determine the frequency of bacteriocin resistance/tolerance development in *L. innocua*, freshly prepared 18 h cultures were serially diluted in MRD and spread plated on to GM17 or GM17 containing either 1000 AU/ml or 320 AU/ml of the appropriate bacteriocin or bacteriocin combination, the latter concentration representing the arbitrary in situ concentration of the bacteriocins in the cheeses. Plates were incubated aerobically at 37°C for up to 48h, at which time, the frequency of bacteriocin resistance/tolerance was calculated as described previously (38). All experiments were performed in triplicate.

Statistical Analysis

Listeria counts in laboratory-scale cheeses were statistically analyzed using one-way ANOVA. Post hoc multiple comparisons were determined by Tukey's test and differences were considered to be statistically significant at $P<0.05$. Statistical tests were performed using XLSTAT statistical software.

277 **RESULTS**

278 *Transconjugant validation*

279 The presence of the plasmid, pMRC01, and the nisin transposon, Tn5276, in *L. lactis*
280 CSK2775 transconjugants was validated by PCR using plasmid- and transposon-
281 specific primers, respectively (Fig. 1). PCR analysis confirmed that the genetic
282 determinants responsible for lacticin production were present in 2775 (pMRC01)
283 creating the lacticin transconjugant *L. lactis* CSK3594, and confirmed the presence of
284 the nisin genetic determinants in 2775 (Tn5276) creating the nisin transconjugant
285 CSK3281. The presence of the lacticin and nisin genetic determinants was confirmed in
286 the double producer resulting in the nisin-lacticin transconjugant *L. lactis* CSK3533. To
287 confirm the identity of each transconjugant, genomic fingerprints were generated by
288 PFGE with the restriction endonuclease, SmaI. All transconjugants analysed generated
289 the same restriction pattern as the recipient strain, CSK2775 (results not shown). Well
290 diffusion assays confirmed that CSK3594 was sensitive to nisin and that CSK3281was
291 sensitive to lacticin but the double producer was immune to both bacteriocins.

292 Colony mass spectrometry (CMS) confirmed that CSK3594 and CSK3533 each
293 produced a peptide with a mass of approximately 2847 ± 4 Da corresponding to the
294 lacticin peptide, Ltnβ (Fig. 2). However, lacticin peptides (Ltnα or Ltnβ) could not be
295 detected in the recipient strain, CSK2775 (Fig. 2). CMS also detected a peptide with a
296 mass of 3352 ± 3 Da corresponding to nisin in strains CSK3281 and CSK3533; this
297 peptide was absent in the recipient strain. These data confirm that lacticin is produced
298 by CSK3594, nisin is produced by CSK3281, and that both nisin and lacticin are
299 produced by CSK3533 (Fig. 2).

300 The level of inhibitory activity in the culture supernatant of the double producer,
301 CSK3533, against *L. lactis* HP was determined to be 1000 AU/ml when measured by

agar well diffusion assays, corresponding to a zone size of 4.5 mm which is equivalent to the zone size produced by the nisin transconjugant *L. lactis* CSK3281. The lacticin transconjugant, CSK3594 produced a 2.5 mm zone against *L. lactis* HP. To our knowledge, this is the first report of the successful construction of a food-grade commercial *L. lactis* starter strain capable of producing both nisin and lacticin 3147, two potent Class I bacteriocins.

Strain performance and stability

The stability of the bacteriocin/lactose positive phenotype in each transconjugant was confirmed via repeated "passaging" in GM17 followed by bacteriocin activity assays against the indicator, *L. lactis* HP. Bacteriocin production and immunity in CSK3281, CSK3594 and CSK3533 proved to be stable over time. However, upon passaging of the double producer, CSK3533, in GM17, a mixed culture containing lactose fermenting and non-fermenting colonies could be observed when plated on LIA. This mixed culture was subsequently attributed to the loss of a large plasmid (>50 kb) present in CSK3533 (confirmed by plasmid profile analysis; results not shown) and is presumed to be involved in lactose metabolism. The lactose fermenting phenotype could be preserved through the supplementation of lactose to the growth medium.

Comparative analyses of acidification profiles of each transconjugant and the recipient strain revealed that the bacteriocin-free recipient, CSK2775, proved to be the most efficient acidifier (Fig. 3). Although the lacticin single producer (CSK3594) was more efficient than the nisin single producer (CSK3281), both proved to be more efficient than the double producer, CSK3533 (Fig. 3). The addition of 0.1% yeast extract improved lactic acid production in the transconjugants.

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Spectrum of inhibition and bacteriophage resistance

The activity of the single bacteriocin producers CSK3594 and CSK3281 as well as the double producer, CSK3533, were assayed against a range of indicator strains including food spoilage, pathogenic bacteria, as well as LAB, and non starter LAB (NSLAB) (Table 2). The single lacticin producer was found to inhibit primarily lactococci, lactobacilli, and clostridia while a wider spectrum of inhibition was observed for both the nisin producer and the double producer. The double producer proved to be more effective than either lacticin or nisin single producers with regard to *Clostridium tyrobutyricum* inhibition producing a 6 mm zone while the lacticin producer, CSK3594, and nisin producer, CSK3281, each produced zones of 4 mm and 3 mm, respectively. Interestingly, the recipient strain, CSK2775, also produced a 1 mm zone against *Clostridium tyrobutyricum* suggesting that some other antimicrobial effect is potentially working in conjunction with the bacteriocins in the transconjugants. In addition, the double producer generated a 4 mm zone against CSK3281 in comparison to a 2.5 mm zone produced by CSK3594. This increase in zone size is surprising given that CSK3281 harbours the genetic machinery for nisin immunity and indeed was proven to be immune to nisin in the antimicrobial assays. However, the increased susceptibility of the nisin transconjugant to lacticin may be due to a lower cell density in the seeded plate as a consequence of a slower growth rate although this has not been confirmed.

Bacteriophage sensitivity assays confirmed that CSK2775, the non-bacteriocinogenic recipient, was sensitive to all bacteriophages analysed (Table 4). The nisin producer, CSK3281 was resistant to 50% of the bacteriophages analysed, while the lacticin single producer and the double producer were each resistant to 80% of bacteriophages analysed (Table 4).

352 *Laboratory-scale cheese production*

353 To analyse the in situ inhibitory activity of the bacteriocin producers (single, double and
354 in combination with the plantaricin producer, *Lb. plantarum* LMG P-26358) laboratory-
355 scale cheeses were manufactured with the fast acidifier *L. lactis* DPC4268 and the
356 bacteriocin producers served as protective cultures. The cheeses were spiked with 10^4
357 cfu/ml of *L. innocua*. Each cheese was ripened for 4 weeks at 7°C; *Listeria* was
358 enumerated weekly during the ripening period. Fig. 4 shows *Listeria* viable cell counts
359 over the 4 week period where bacteriocin containing vats were compared with Vat 1 (no
360 bacteriocin) at each week. At week 0, *Listeria* numbers were significantly different
361 between Vat 1 (5.6 log cfu/g) and all other vats with lowest *Listeria* numbers recorded
362 for vat 7 (CSK3533; *Lb. plantarum*) at 3.5 log cfu/g ($P<0.001$), followed by Vat 2
363 (CSK3281) at 3.8 log cfu/g ($P<0.001$). *Listeria* numbers in the remaining vats were
364 reduced by 0.9 to 1.5 logs when compared to Vat 1. By week 1, *Listeria* numbers in Vat
365 7 continued to decrease significantly compared to Vat 1 with a 3 log reduction recorded
366 ($P<0.001$). *Listeria* numbers in Vat 6 (CSK3594; *Lb. plantarum*) were also significantly
367 different to Vat 1 with a 2.7 log reduction ($P<0.001$). Significant reductions were also
368 observed for Vat 9 (CSK3281; CSK3594; *Lb. plantarum*) (1.6 log reduction; $P<0.01$)
369 and Vat 5 (CSK3281; *Lb. plantarum*) (0.8 log reduction; $P<0.05$). At week 2 lowest
370 *Listeria* numbers were recorded for Vat 6 (0.4 log cfu/g) and Vat 7 (0.7 log cfu/g)
371 representing 2.6 and 2.3 log reductions compared to Vat 1 (3 log cfu/g) ($P<0.01$). Vat 5
372 (CSK3281; *Lb. plantarum*) was also significantly different to Vat 1 (1.8 log reduction;
373 $P<0.01$). By week 3, Vat 7 exhibited lowest *Listeria* numbers (0.4 log cfu/g) followed
374 by Vat 9 (0.8 log cfu/g), which were both significantly different to Vat 1 (2.5 log cfu/g)
375 ($P<0.05$). By the week 4, *Listeria* numbers for Vat 1 (2.9 log cfu/g) were significantly
376 different to most other Vats, with no *Listeria* detected in Vat 6 ($P<0.01$) and numbers

377 reduced to 0.3 log cfu/g for Vat 7 (P<0.01). *Listeria* numbers in the remaining vats (2,
378 3, 4, 8, 9) ranged from 0.8 to 1 log cfu/g representing significant differences compared
379 to Vat 1 (P<0.05). While, *Listeria* numbers in Vat 5 were not deemed significantly
380 different to Vat 1, they approached a significant reduction (P=0.07).
381 In terms of the lacticin and nisin transconjugants (without *Lb. plantarum*), no
382 significant differences were observed between the double producer (Vat 4) and either of
383 the single producers (Vats 2 and 3) with regards to *Listeria* numbers at any week. We
384 then compared Vat 8 which consists of the two single producers (CSK3281; CSK3594)
385 (4.4 log cfu/g; week 0) with Vats 2, 3 and 4. While Vat 8 was found to be significantly
386 different to Vat 2 (3.7 log cfu/g) at week 0 (P<0.05) whereby the nisin producer
387 generated greater *Listeria* reductions than the combination of nisin and lacticin single
388 producers, no significant differences were observed for weeks 1-4. Likewise, no
389 significant differences were observed between Vat 8 and Vats 3 or 4 over the ripening
390 period.
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392 *Bacteriocin detection in Vats 4, 5, 6 and 7*
393 The correct masses for nisin (fraction 21), Ltnβ (fraction 37) and plantaricin (fraction
394 19) were detected by MALDI-TOF MS in Vat 7 (double producer and plantaricin) at
395 week 0 (Fig 5A). Antimicrobial assays also revealed that these fractions contained
396 activity (lacticin activity was restored by combining fractions 23 (Ltnα) and 37).
397 At week 4, a mass corresponding to plantaricin was detected in fraction 19 but there
398 was no antimicrobial activity. Nisin was detected in fraction 21 at week 4 and activity
399 was also confirmed. The correct mass for Ltnβ could not be detected at week 4. Despite
400 this, combining fractions 23 and 37 did yield a zone of inhibition against the indicator
401 strain, suggesting the bacteriocin is present.

The correct mass for nisin was not detected in Vat 4 (double producer) at weeks 0 or 4, however, fraction 21, which is generally expected to contain nisin, yielded a zone of inhibition against the indicator strain on both weeks, suggesting the bacteriocin is present (Fig 5B). The correct mass for Ltnβ was detected in Vat 4 (double producer) at weeks 0 and 4 (Fig 5B). Lacticin activity was confirmed when fractions 23 and 37 were positioned beside each other in the agar well diffusion assays.

Vat 5 (nisin and plantaricin) was found to contain the nisin mass at weeks 0 and 4 although the correct mass was found in fraction 22 at week 0 and in fraction 23 at week 4 (Fig 5C). This is presumably due to slight variations in the times the peptide eluted from the HPLC. These fractions exhibited antimicrobial activity against the indicator strain but the zones were smaller than previously observed. The plantaricin mass was not detected in Vat 5 at week 0 but a zone of inhibition against *Listeria* was observed for fraction 19. However, the plantaricin mass was detected in Vat 5 at week 4 and antimicrobial activity was confirmed.

Vat 6 (lacticin and plantaricin) was shown to contain the correct Ltnβ and plantaricin masses at weeks 0 and 4 in fractions 37 and 19, respectively (Fig 5D). Antimicrobial activity was confirmed for fraction 19 although the zone was smaller at week 0 when compared to week 4. In the case of lacticin, combining fractions 24 (Ltnα) and 37 confirmed antimicrobial activity.

Development of bacteriocin tolerance

The frequency of tolerance/resistance development in *L. innocua* was assessed using 1000 AU/ml of each bacteriocin. The frequency of resistance against 1000 AU/ml of nisin was calculated to be 6.56×10^{-4}. Resistance development could not be observed when *Listeria* was exposed to 1000 AU/ml lacticin or 1000 AU/ml nisin and lacticin.

427 On the other hand, the frequency of resistance development against 320 AU/ml nisin or
428 320 AU/ml lacticin (representing arbitrary concentrations in cheese) was much lower at
429 4.9×10^{-1} and 3.02×10^{-1}, respectively. Simultaneous exposure to lacticin and nisin at
430 320 AU/ml decreased the frequency of resistance to 3.18×10^{-2}. However, bacteriocin
431 resistant colonies remained sensitive to 1000 AU/ml indicating that *Listeria* cells were
432 tolerant rather than completely resistant.
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DISCUSSION

In this study, an isogenic family of nisin and lacticin transconjugants was developed with a view to better understand the impact of multiple bacteriocin production and genetic load on starter culture functionality. Genotypic and phenotypic analyses including PCR, well diffusion assays and CMS confirmed the acquisition of lacticin and/or nisin in each transconjugant.

In agreement with previous findings (39) which revealed that pMRC01 imposes a burden on lactococcal metabolism affecting growth and acidification rates, the presence of pMRC01 was shown to influence lactococcal acidification in the lacticin transconjugant, as did the presence of the nisin transposon in the nisin transconjugant. However, bacteriocin stacking resulted in slowest acidification rates but which can be overcome with the addition of 0.1% yeast extract to the double producer. Yeast extract presumably lessens the burden of plasmid and transposon acquisition as it provides amino acids along with purine and pyrimidine bases and inorganic constituents which have been shown to stimulate lactococcal growth (40). We therefore suggest that the double producer has potential to serve as a protective culture when used in conjunction with a suitable acidifier. Despite this, the lactose utilization phenotype in the double producer, CSK3533, was found to be unstable in GM17 apparently due to the loss of a large (>50kb) plasmid likely involved in lactose utilization. Both the plasmid instability and slower acidification profiles observed in the double producer (CSK3533) may be attributed to the metabolic burden imposed by the presence of pMRC01 and the nisin transposon. In an effort to ease the metabolic load, it is possible that as the energy demand of the cell increases and metabolites are exhausted, a reduction in growth rate and perhaps the loss of nonessential plasmids may occur (39, 41). This is supported by

476 the fact that the addition of lactose to M17 broth maintains the lactose utilization
477 phenotype in the double producer.

478 Antimicrobial activity assays confirmed that co-production of nisin and lacticin
479 by CSK3533 was as effective as its single producing counterparts against most of the
480 indicator strains tested. This indicates that bacteriocin production is not affected by the
481 slower growth rate observed in the double producer. Furthermore, with the exception of
482 *C. tyrobutyricum*, the co-production of the two potent bacteriocins did not result in an
483 increase in antimicrobial activity. Studies suggest that bacteriocin production and
484 subsequent inhibition may be influenced by growth medium (42, 43). The multi-
485 bacteriocinogenic strain *L. lactis* INIA 415 was capable of producing the Class I
486 bacteriocin lacticin 481 in M17 broth only and produced nisin in milk only (42).
487 Therefore, it is possible that growth of CSK3533 under different conditions could result
488 in higher levels of nisin or lacticin being produced.

489 In terms of bacteriophage resistance, transconjugants were shown to be more
490 resistant to bacteriophage attack than the recipient strain with superior resistance
491 properties observed in pMRC01 derivatives, which is known to harbour an abortive
492 infection mechanism (30).

493 Laboratory-scale cheese inoculated with *Listeria* was used to assess the efficacy
494 of single and double bacteriocin producers alone and in combination with the
495 plantaricin producer, *Lb. plantarum* LMG P-26358 in situ. The latter strain was
496 previously shown to have a narrow spectrum of inhibition, inhibiting *Listeria* and
497 enterococcal strains but not clostridia, *E. coli*, *Bacillus* species, *Salmonella*, or members
498 of the LAB (18). The strain proved to be an effective adjunct for controlling *Listeria*
499 growth in a cheese model (18). In the present study, CSK3594, CSK3281 and CSK3533
500 failed to inhibit *L. innocua* by agar well diffusion assay, however, by the end of the

ripening period, *Listeria* numbers from cheeses prepared with these starters were significantly reduced when compared to the control, Vat 1 (no bacteriocin). Interestingly, the double producer combined with the plantaricin producer exhibited the greatest reduction in *Listeria* numbers at week 0, a trend which continued to Week 1 suggesting the effectiveness of this combination for reducing initial bacterial load. The inhibitory effect of this combination was on the whole significantly better than using both single producers with the plantaricin producer. This can be explained by the fact that the nisin producing transconjugant inhibits the lacticin transconjugant and vice versa whereas the double producer is immune to both bacteriocins. The combination of the lacticin producer with *Lb. plantarum* LMG P-26358 also significantly reduced *Listeria* numbers by week 1 and indeed by week 4, *Listeria* could not be detected in this vat. Overall, the double producer combined with the plantaricin producer followed by the lacticin producer combined with plantaricin exhibited the most significant reductions in *Listeria* numbers over the ripening period.

In general, a similar inhibitory trend was observed amongst the single Class I producers, the double producer or the combined single producers which were significantly different to Vat 1 at week 0 and week 4. While the double producer did not alter *Listeria* numbers significantly when compared to the single producers alone, a 10-fold reduction in the emergence of bacteriocin tolerance was observed when *Listeria* was exposed to both nisin and lacticin, suggesting that bacteriocin stacking could be an effective method to prevent pathogen growth in food applications. However, combining bacteriocins from different Classes or sub-classes is considered most effective for reducing the emergence of resistance (44) which explains the increased antimicrobial efficacy for vats containing the Class I and Class II bacteriocins in this study.

525 Interestingly, cheese prepared with non-bacteriocinogenic CSK2775 also
526 resulted in reduced *Listeria* numbers over the four week period although to a lesser
527 extent than the bacteriocin containing cheeses. Therefore, it is probable that bacterial
528 competition coupled with unfavourable conditions relating to cheese manufacture
529 including lactic acid and high salt concentrations have provided a hurdle-effect to cause
530 the observed reductions. Indeed, several intrinsic factors including moisture content,
531 acidity and competitive flora are known to dictate pathogen survival in cheese (45, 46).
532 MALDI-TOF MS of cheeses from vats 4, 5, 6 and 7 indicated that nisin and
533 lacticin were present in the appropriate cheeses implying that bacteriocin integrity was
534 not compromised in the cheese environment. The presence of plantaricin could not be
535 confirmed in Vat 7 cheese (double producer and plantaricin) at week 4 but it was
536 present in vats 5 and 6 at both times as expected. MALDI-TOF MS is not quantitative
537 and is also subject to preferential ionisation in that some peptides ionise better than
538 others. The peptide content in a cheese increases during ripening due to the breakdown
539 of casein so a number of bacteriocin purification steps were performed to increase the
540 chances of detecting bacteriocin masses. Cheeses were passed through C18 SPE
541 columns and peptides were further separated using RP-HPLC. Each HPLC fraction
542 potentially contains numerous peptides making it difficult to detect the bacteriocin
543 masses which are present at low concentrations. Usually the bacteriocin mass and a
544 concomitant zone of inhibition is taken as proof of the presence of bacteriocin but in the
545 case of a cheese fraction the presence of a zone of inhibition alone may be taken as
546 indicative of bacteriocin presence.
547 Natural isolates capable of producing multiple bacteriocins have been reported
548 in the literature (47-51). Most recently, *L. lactis* LMG2081 was shown to produce two
549 different Classes of bacteriocins, a novel lantibiotic and the Class IIb bacteriocin,

lactococcin G (52). However, the ability to generate a multi-bacteriocin producer from an already established culture through food-grade enabling technologies poses several benefits. Firstly, the technological properties of the culture are known. The number of cultures required to produce multiple bacteriocins is reduced. The risk of bacteriocin inhibition is removed since the multibacteriocin-producing starter will also harbour the genetic machinery for bacteriocin immunity. As conjugation is a natural process, the resulting transconjugants do not fall under current European regulations governing the use of genetically modified microorganisms (53, 54). Therefore, transconjugants can be used in food applications in a similar manner to the recipient strain (55). While the double bacteriocin producer generated in this study proved to be a slower acidifier than the recipient strain, it has potential to serve as a protective culture. However, studies generating multiple bacteriocin producers have been rare (13). This can most likely be attributed to the complex biosynthetic process required for bacteriocin production and secretion. Indeed, previous attempts to construct nisin-lacticin transconjugants were unsuccessful, often attributed to the incompatibility of bacteriocin modification machinery or bacteriocin sensitivity (13, 30). Traditionally, the discovery of technologically valuable industrial strains has focused on large-scale screening strategies from a variety of sources (56). However, the successful transfer of lacticin and nisin to commercial starter cultures as reported in this study may provide additional avenues for the development of multi-hurdle protective cultures using food-grade methods.

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826 FIG 1 PCR amplification using pMRC01-specific primers (*orf27*, *orf49*, *orf51*, and
827 *orf52*) to detect the presence of pMRC01 in *L. lactis* CSK3594 and CSK3533. PCR
828 amplification using primers designed to regions of the nisin operon (*nisA*, *nisFEG*) to
829 confirm the presence of nisin genetic determinants in *L. lactis* CSK3281 and *L. lactis*
830 CSK3533. (M: 100 bp DNA ladder; New England BioLabs).
831 FIG 2 Colony mass spectrometry analysis of *L. lactis* CSK2775, *L. lactis* CSK3594
832 (lacticin transconjugant, Ltn+), *L. lactis* CSK3281 (nisin transconjugant, Nis+) and *L.*
833 *lactis* CSK3533 (nisin and lacticin double producer, Ltn+, Nis+). Masses corresponding
834 to the bacteriocins are indicated. Inset photos show inhibition zones produced by each
835 strain against the indicator strain *L. lactis* HP.
836 FIG 3 Acidification profiles of *L. lactis* CSK2775 (□); *L. lactis* CSK3594 (lacticin)
837 (■); *L. lactis* CSK3281 (nisin) (○); and *L. lactis* CSK3533 (nisin, lacticin) (●) grown
838 in 10% RSM.
839 FIG 4 Counts of viable *L. innocua* cells in laboratory-scale cheeses. Bacteriocin-
840 containing vats were compared to Vat 1 (no bacteriocin) at each week (* $P<0.05$; **
841 $P<0.01$; *** $P<0.001$).
842 FIG 5 MALDI-TOF MS analysis of Vat 7 (lacticin, nisin and plantaricin) (A), Vat 4
843 (lacticin and nisin) (B), Vat 5 (nisin and plantaricin) (C) and Vat 6 (lacticin and
844 plantaricin) (D). Masses corresponding to the bacteriocins are indicated. Inset photos
845 show inhibition zones produced by correct mass-containing fractions against the
846 indicator strains *L. lactis* HP (nisin and lacticin) or *L. innocua* (plantaricin) where F
847 denotes Fraction.
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854 TABLE 1 Bacterial strains used in this study

Bacterial strain	Relevant Detail	Relevant genotype and phenotype[a]	Source or reference
L. lactis HP	Bacteriocin sensitive indicator strain	*Ltn*$^-$; *Nis*$^-$	TFRC[b]
L. lactis MG1363 (pMRC01)	Donor strain harbouring pMRC01, lacticin producer	Lac$^-$; *Ltn*$^+$; *Nis*$^-$	TFRC
L. lactis CSK2583	Donor strain harbouring Tn5276, nisin producer	Lac$^-$; *Nis*$^+$; *Ltn*$^-$	CSK, The Netherlands
L. lactis CSK2775	Recipient strain	Lac$^+$; *Nis*$^-$; *Ltn*$^-$	CSK, The Netherlands
L. lactis CSK3281	CSK2775 derivative, nisin producer	Lac$^+$; *Nis*$^+$	This study
L. lactis CSK3594	CSK2775 transconjugant harbouring pMRC01, lacticin producer	Lac$^+$; *Ltn*$^+$	This study
L. lactis CSK3533	CSK3281 transconjugant harbouring pMRC01, nisin-lacticin double producer	Lac$^+$; *Nis*$^+$, *Ltn*$^+$	This study
Lb. plantarum LMG P-2658	Plantaricin 423 producer	*Pln*$^+$	(18)
L. lactis DPC4268	Starter culture for cheese manufacture	*Lac*$^+$	TFRC

855 [a]Lac, lactose utlization; *Ltn*, lacticin genetic determinants; *Nis*, nisin genetic determinants; *Pln*,
856 plantaricin genetic determinants.
857 [b]TFRC, Teagasc Food Research Centre, Moorepark, Fermoy, County Cork, Ireland.

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874 TABLE 2 Antimicrobial spectrum of the *L. lactis* isogenic family of nisin and lacticin transconjugants

Indicator Strain or Species	**Growth Medium**	**Strains Tested for Antimicrobial Activity**				**Source**
		CSK2275	CSK3594 (Ltn)	CSK3281 (Nis)	CSK3533 (Ltn, Nis)	
Bacillus cereus DPC6085/6086	BHI[a]	No zone	No zone	1mm zone	1mm zone	TFRC
Bacillus subtilus DPC6511	BHI[a]	No zone	No zone	No zone	No zone	TFRC
Enterococcus faecalis DPC5055/LMG 7973	BHI[a]	No zone	No zone	3mm zone	3mm zone	TFRC
Enterococcus faecium DPC5056[*]	BHI[a]	No zone	No zone	6mm zone	6mm zone	TFRC
Escherichia coli P1432- DPC6054	BHI[a]	No zone	No zone	No zone	No zone	TFRC
Clostridium sporogenes DPC6341[*]	RCM[a]	No zone	1.5 mm zone	1.5mm zone	1mm zone	TFRC
Clostridium tyrobutyricum DPC6342[*]	RCM[a]	1mm zone	4mm zone	3mm zone	6mm zone	TFRC
Lactobacillus casei DPC6125	MRS[c]	No zone	No zone	7mm zone	7mm zone	TFRC
Lactobacillus acidophilus DPC5378	MRS[a]	No zone	1mm zone	3mm zone	3mm zone	TFRC
Lactobacillus delbreukii subsp. *delbreukii* DPC5385	MRS[a]	No zone	No zone	6mm zone	6mm zone	TFRC
Lb. delbreukii subsp. *lactis* DPC5387	MRS[c]	No zone	1.5mm zone	6mm zone	6mm zone	TFRC
Lb delbreukii subsp. *bulgaricus* DPC5383	MRS[c]	No zone	2mm zone	9.5mm zone	9.5mm zone	TFRC
Lactobacillus helveticus DPC4571	MRS[a]	No zone	1.5mm zone	8mm zone	8mm zone	TFRC
L .lactis subsp. *lactis* biovar *diacetyllactis* CSK1411	LM17[b]	No zone	1.5mm zone	4mm zone	4mm zone	CSK
L. lactis subsp. *cremoris* HP DPC5718	LM17[c]	No zone	2.5mm zone	4.5mm zone	4.5mm zone	TFRC
L. lactis subsp. *lactis* DPC4268/ 303	LM17[c]	No zone	No zone	0.5mm zone	0.25mm zone	TFRC
L. lactis subsp. *lactis* CSK2775	LM17[c]	No zone	2.5 mm	4.5 mm	4.5 mm	CSK
L. lactis subsp. *lactis* CSK3594	LM17[c]	No zone	No zone	4.5 mm	4.5 mm	CSK
L. lactis subsp. *lactis* CSK3281	LM17[c]	No zone	2.5 mm	No zone	4 mm	CSK
L. lactis subsp. *lactis* CSK3533	LM17[c]	No zone	No zone	No zone	No zone	CSK
Leuconoctos lactis DPC3838	MRS[c]	No zone	No zone	1.5mm zone	1.5mm zone	TFRC
L. innocua DPC6578	GM17[a]	No zone	No zone	No zone	No zone	TFRC

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876 [*]Cultures grown anaerobically for up to 48h. [a]Cultures grown at 37°C for up to 48h. [b]Cultures grown at 35°C for up to 48h.[c]Cultures
877 grown at 30°C for up to 48h.

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885 TABLE 3 Primer pairs used in this study

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Primer	Sequence	Target gene(s)	Size (bp)
27-F 5'-GGGGAACAATCTTACCTA **27-R** 5'-ATTATTTTTATTGCATTCTACTA		*orf 27*	326
49-F 5'-CCAATACCCGCCAAAATAAAGT **49-R** 5'-CTAAGCGCAGAGGAAATACAACC		*orf 49*	347
51-F 5'-TTCTCAAAATCATCAAAATCAAGT **51-R** 5'-GTACGAACAGGAGCGAAAAA		*orf51*	293
52-F 5'- CCTAAGTTGTCTATTCGTGTCCA **52-R** 5'- ATTAGGTGAGTGCTCTGATTTTTC		*orf52*	210
nisA-F 5'- CAAAAGATTTTAACTTGGATTTG **nisA-R** 5'- ACGTGAATACTACAATGACAAG		*nisA*	163
nisFG-F 5'- GGTTTAATTTCTGCAGATACTG **nisFG-R** 5'- GTAATTATCCAGATCATTGCTG		*nisFEG*	1573

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904 TABLE 4 *L. lactis* transconjugants surveyed for bacteriophage sensitivity

Bacteriophage	***L. lactis* CSK2775**	***L. lactis* CSK3281 (Nis$^+$)**	***L. lactis* CSK3594 (Ltn$^+$)**	***L. lactis* CSK3533 (Nis$^+$Ltn$^+$)**
5410F	+	+	-	-
5163F	+	-	-	-
5210 F	+	+	+	+
5167F	+	-	-	-
5385F (Bacteriophage cocktail)	+	-	-	-
5386F (Bacteriophage cocktail)	+	+	-	-

905 + indicates bacteriophage sensitivity observed by a clearing of the bacterial population.
906 - indicates bacteriophage insensitivity observed as growth (turbidity) of the bacterial population.
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FIG 1

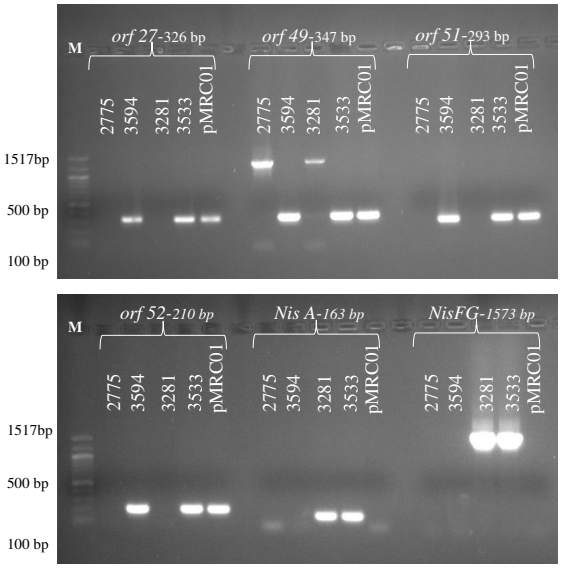


FIG 2

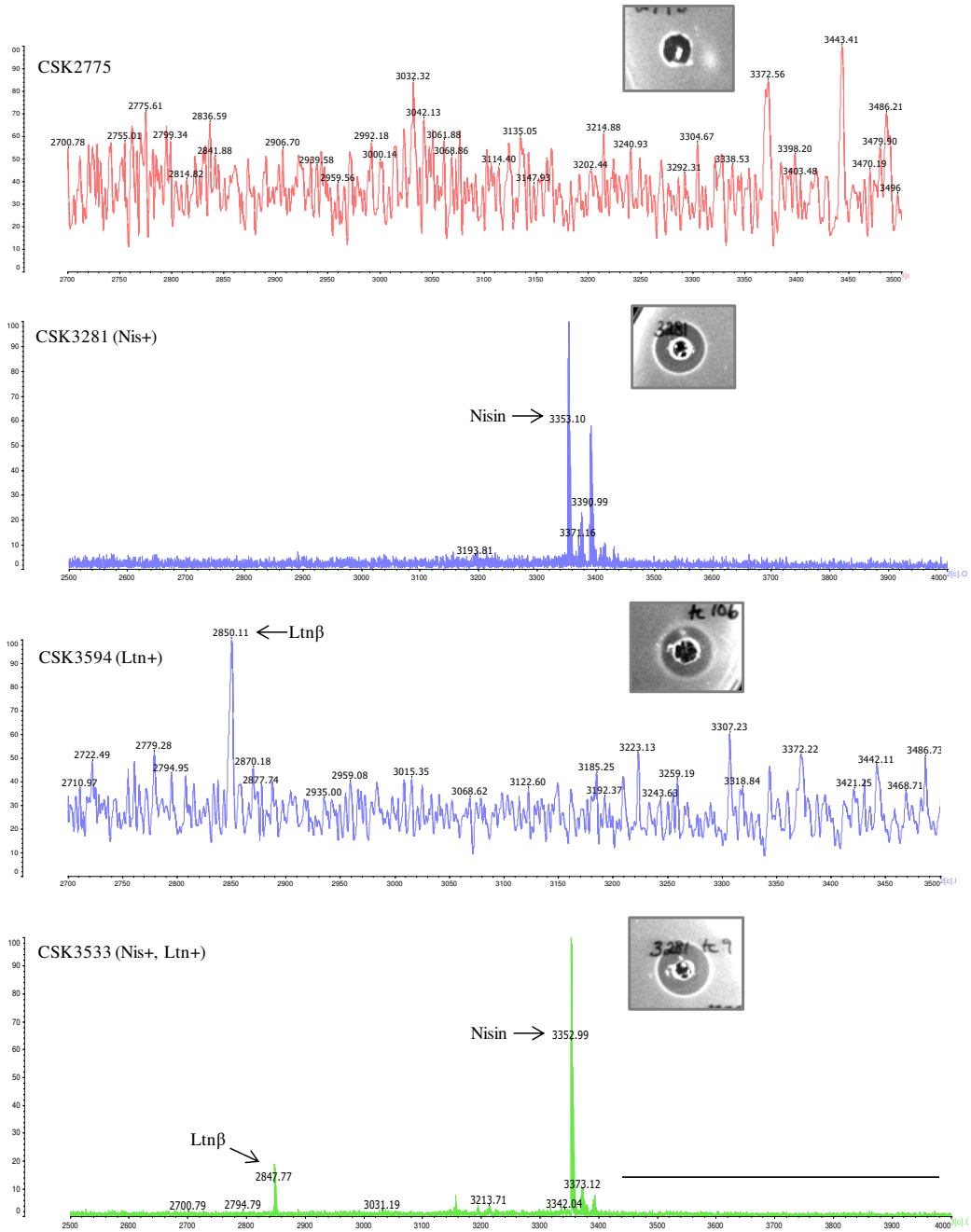


FIG 3

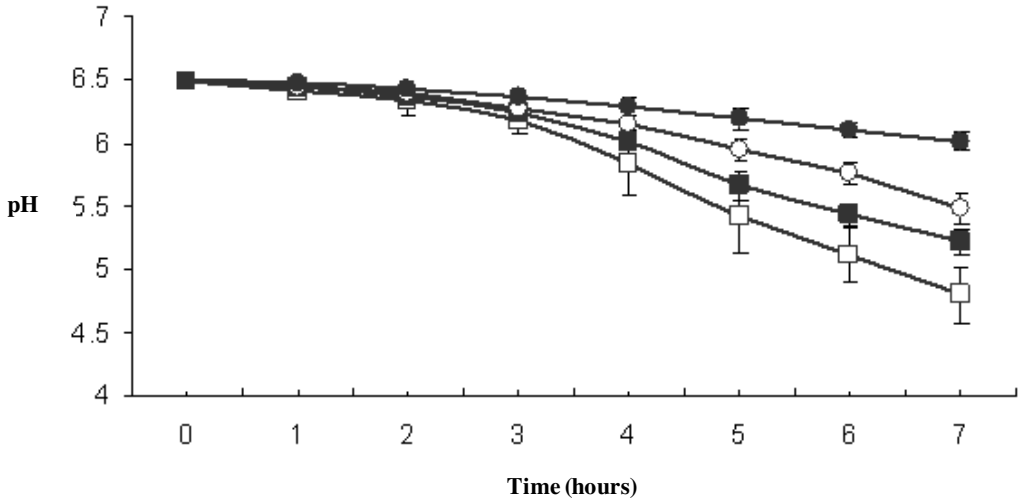


FIG 4

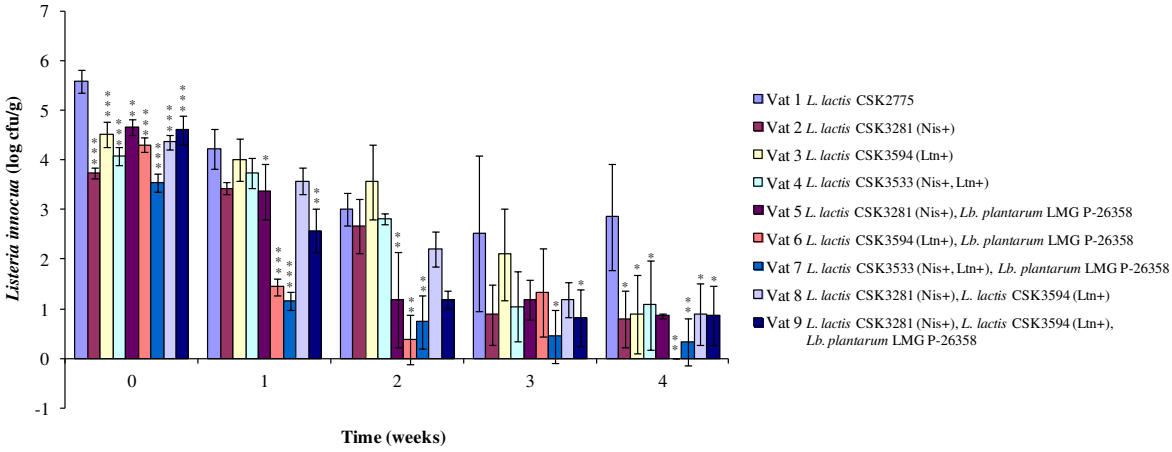


FIG 5 (A)

Vat 7 *L. lactis* CSK3533, *Lb. plantarum* LMG P-26358

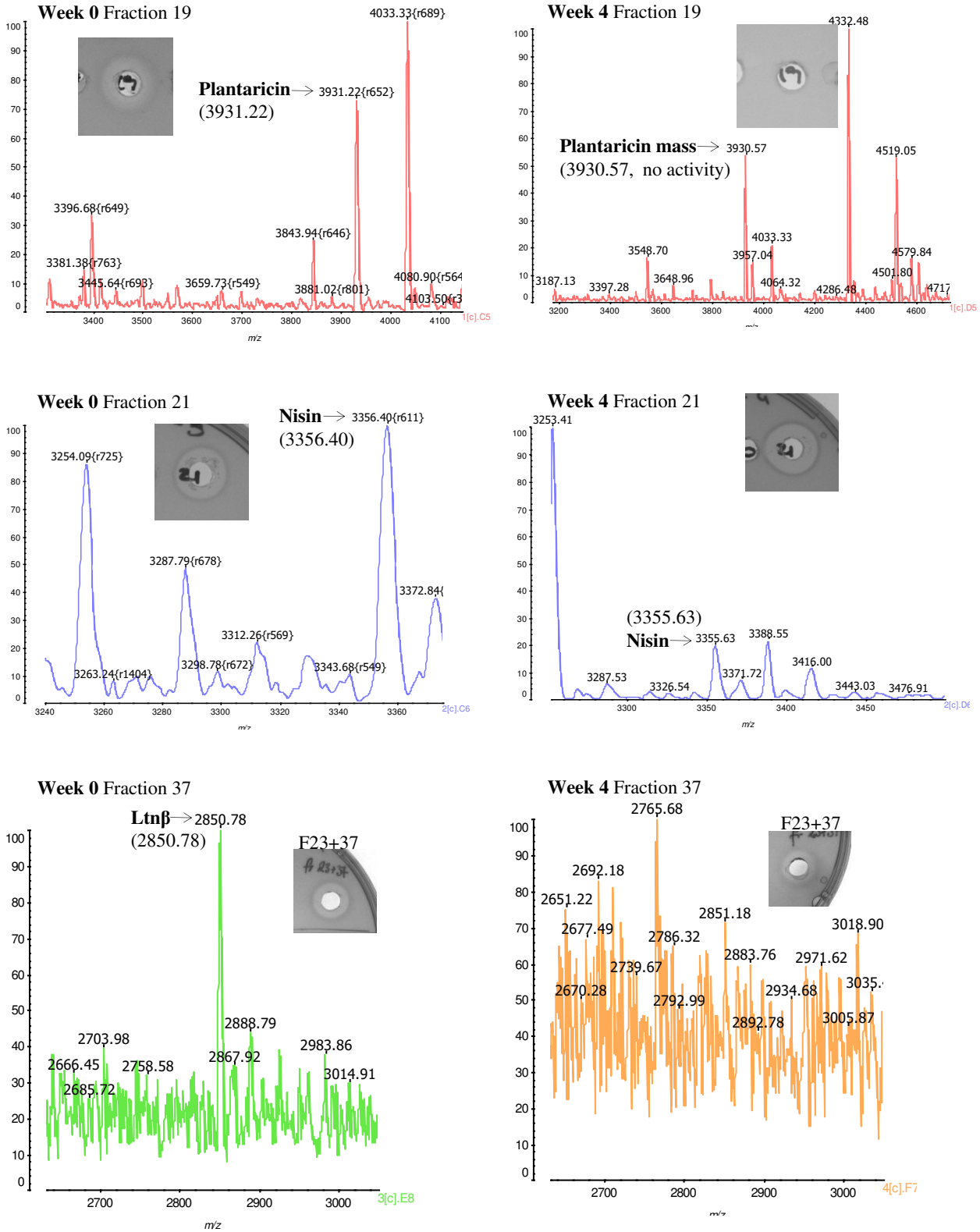
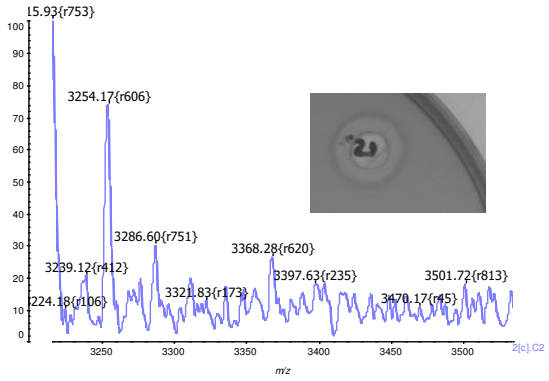


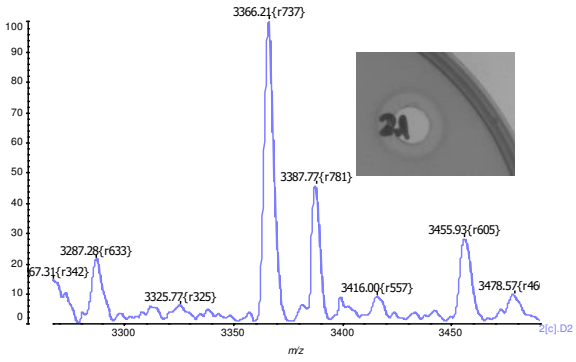
FIG 5 (B)

Vat 4 *L. lactis* CSK3533

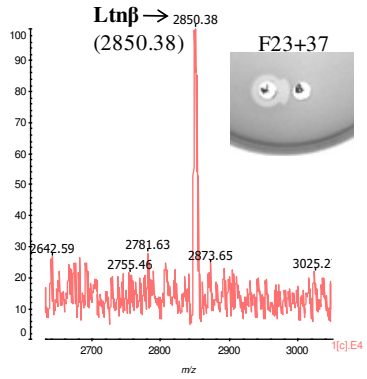
Week 0 Fraction 21



Week 4 Fraction 21



Week 0 Fraction 37



Week 4 Fraction 37

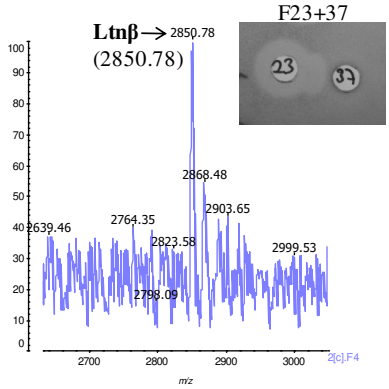


FIG 5 (C)

Vat 5 *L. lactis* CSK3281, *Lb. plantarum* LMG P-26358

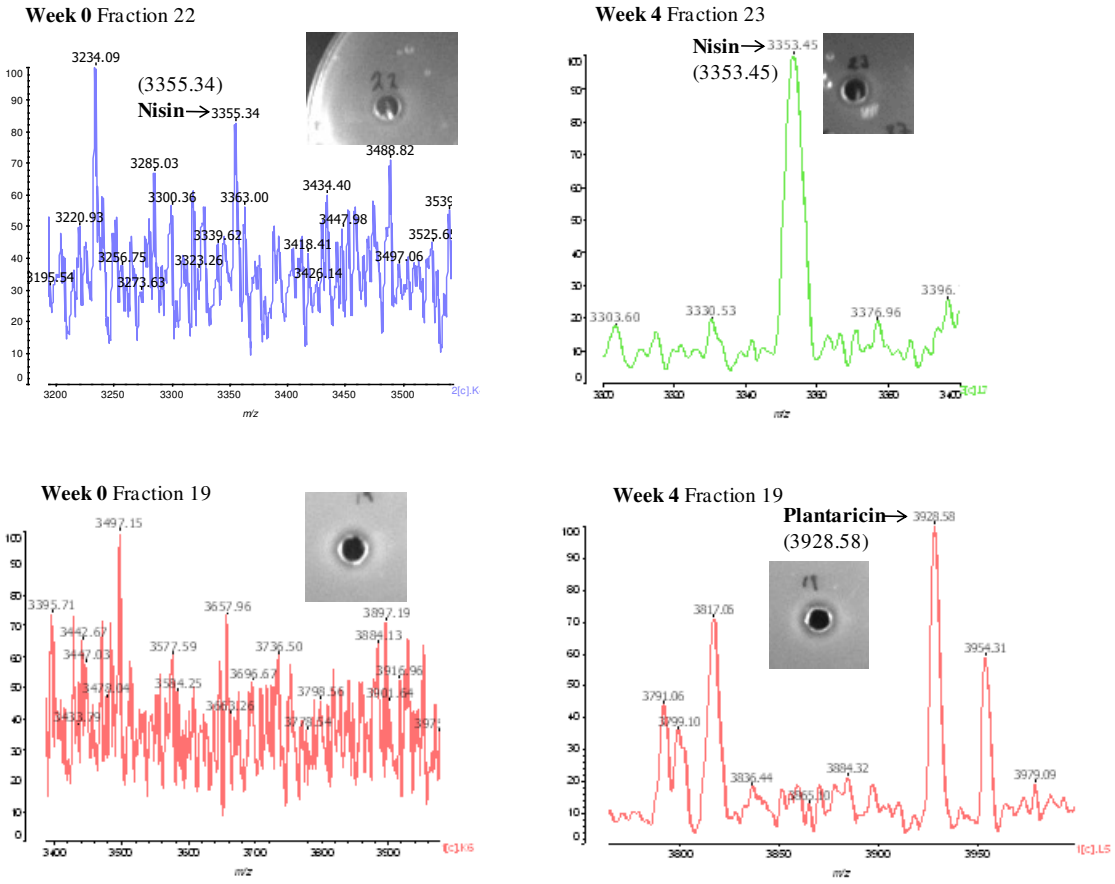
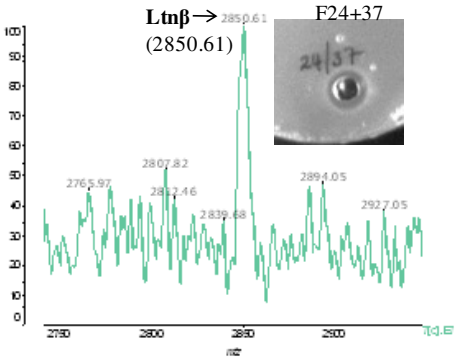


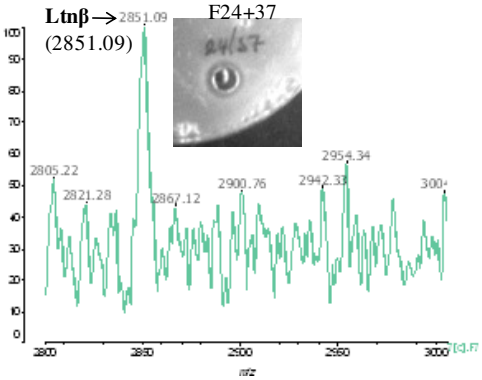
FIG 5 (D)

Vat 6 *L. lactis* CSK3594, *Lb. plantarum* LMG P-26358

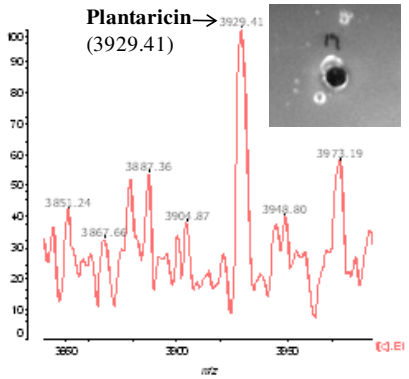
Week 0 Fraction 37



Week 4 Fraction 37



Week 0 Fraction 19



Week 4 Fraction 19