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> 1 A Multi-Bacteriocin Cheese Starter System comprising Nisin and Lacticin 3147 in 2 Lactococcus lactis, in Combination with Plantaricin from Lactobacillus plantarum 3 4 5 Running Title: Multi-bacteriocin producing starter system 6 S. Mills,<sup>c,d</sup> C. Griffin,<sup>b,c</sup> P. M. O'Connor,<sup>b</sup> L. M. Serrano,<sup>c</sup> W. C. Meijer,<sup>c</sup> C. Hill,<sup>a,d</sup> R. 7 P. Ross<sup>d,e</sup># 8 9 School of Microbiology, University College Cork, Ireland<sup>a</sup>; Teagasc, Moorepark Food Research Centre, Fermoy, Co. Cork, Ireland<sup>b</sup>; CSK Food Enrichment, Ede, The 10 Netherlands<sup>c</sup>; APC Microbiome Institute, University College Cork, Ireland<sup>d</sup>; College of 11 12 Science, Engineering and Food Science, University College Cork, Ireland<sup>e</sup> 13 14 15 #Address correspondence to R. P. Ross, p.ross@ucc.ie 16 17 18 19 20 21 22 23 24 25 26 27

#### 28 ABSTRACT

29 Functional starter cultures demonstrating superior technological and food safety 30 properties are advantageous to the food fermentation industry. We evaluated the 31 efficacy of single and double bacteriocin-producing starters of Lactococcus lactis 32 capable of producing the Class I bacteriocins, nisin A and/or lacticin 3147 in terms of 33 starter performance. Single producers were generated by mobilising the conjugative, 34 bacteriophage resistance plasmid pMRC01, encoding lacticin genetic determinants, or 35 the conjugative transposon Tn5276, encoding nisin genetic determinants, to the 36 commercial starter L. lactis CSK2775. The effect of bacteriocin co-production was 37 examined by superimposing pMRC01 into the newly constructed nisin transconjugant. 38 Transconjugants were improved with regard to antimicrobial activity and bacteriophage 39 insensitivity when compared to the recipient strain and the double producer was 40 immune to both bacteriocins. Bacteriocin production in the starter was stable, although 41 the recipient strain proved to be a more efficient acidifier than transconjugant 42 derivatives. Overall, combining Class I bacteriocins (the double-producer or a 43 combination of single producers) proved as effective as individual bacteriocins for 44 controlling Listeria innocua growth in laboratory-scale cheeses. However, using the 45 double producer in combination with the Class II bacteriocin producer Lactobacillus 46 plantarum, or the lacticin producer with the Class II producer, proved most effective for 47 reducing bacterial load. As emergence of bacteriocin tolerance was reduced 10-fold in 48 the presence of nisin and lacticin, we suggest that the double producer in conjunction 49 with the Class II producer could serve as a protective culture providing a food-grade, 50 multi-hurdle approach to control pathogenic growth in a variety of industrial 51 applications.

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#### 53 IMPORTANCE

We generated a suite of single and double-bacteriocin producing starter cultures capable 54 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. However, combining the double producer or the lacticin producing starter with a Class 59 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 (1-3). Bacteriocins are ribosomally synthesised, heat stable antimicrobial peptides that 85 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

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Applied and Environmental Microbioloav 103 other important cultures are not inhibited. This can be overcome to some degree by 104 developing a multi-bacteriocinogenic culture which is immune to the bacteriocins it 105 produces.

106 In the present study, we generated single and double bacteriocin-producing 107 cultures of L. lactis CSK2775 with the capacity to produce Class I bacteriocins, lacticin 108 3147 (hereafter lacticin), nisin A (hereafter nisin) or lacticin and nisin. Both 109 bacteriocins target lipid II to generate pores in the cell membrane causing proton-110 motive force dissipation and subsequent cell death (14-17). Resulting transconjugants 111 were assessed for bacteriocin production, bacteriophage resistance properties, 112 acidification efficiency and antimicrobial activity against a spectrum of indicator strains 113 including food pathogens and other lactic acid bacteria (LAB). The ability of the 114 transconjugants (single and double) to produce bacteriocins in laboratory-scale cheese 115 was assessed and we also evaluated the anti-listerial potential of the Class I producers 116 alone and in combination with the Class IIa bacteriocin producer Lactobacillus 117 plantarum LMG P-26358 (18). Class IIa bacteriocins cause pore formation by binding 118 to and irreversibly opening the sugar transporter mannose phosphotransferase (Man-119 PTS) system in the target cell (19). In this study, Listeria innocua served as a surrogate 120 for Listeria monocytogenes for reasons of safety and efficiency (as in many other 121 studies) and because L. innocua has been successfully used in previous studies 122 investigating the anti-listerial potential of nisin (20-24), lacticin (25-28) and plantaricin 123 (18) in food systems.

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#### 128 MATERIALS AND METHODS

#### 129 Bacterial strains and media

130 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) 131 132 supplemented with 0.5% (w/v) lactose (LM17) or glucose (GM17). Lb. plantarum was 133 grown in MRS medium (29) (Difco Laboratories) at 30°C. L. innocua was routinely 134 propagated in GM17 broth at 37°C containing 500 µg/ml streptomycin (Sigma Aldrich, 135 Ireland). Other media used in this study include BHI (Brain-Heart Infusion) broth 136 (Oxoid Ltd., Basingstoke, Hampshire, England) and RCM (Re-inforced Clostridial 137 Medium) (Merck, Darmstadt, Germany). All strains were stored in 50% glycerol at -138 20°C.

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#### 140 Strain construction and analytical tests

#### 141 Strain construction

142 The conjugation method of Coakley et al. (30) was used with slight modifications to 143 generate lacticin transconjugants. Inocula (2%) of both donor and recipient were grown 144 for 4 h in GM17 broth at 30°C. After the growth period, 1 ml of recipient and 1 ml of 145 donor were harvested by centrifugation (16,000 x g for 1 min) and rinsed twice with 146 GM17 broth. After the final rinse, each strain was resuspended in 50 µl of GM17 broth. 147 The concentrated recipient and donor (20x) were then mixed with each other at the 148 following ratios, 1:1, 2:1 and 20:1. Each mixture was spotted onto the centre of a GM17 149 agar plate and incubated for 18 h at 30°C. The following day, spots were harvested in 1 150 ml of maximum recovery diluent (MRD; Oxoid) and serially diluted before plating on 151 lactose indicator agar (LIA) containing lacticin (400 arbitrary units (AU)/ml) as 152 described previously (30). Following 48 h of incubation at 30°C the lacticin-containing LIA plates were examined for lactose-positive colonies (yellow) against a background
of lactose-negative colonies (white), and lactose-positive colonies were selected and
grown in LM17 broth for further analysis.

Nisin transconjugants were generated according to the method of Gireesh et al. (31) 156 157 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.

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#### 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

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

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

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#### 203 Characterisation of acid production

204 Acid production was monitored in 10% RSM in the presence and absence of 0.1% yeast

- 205 extract according to the method of Harrington and Hill (37).
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#### 207 Laboratory-scale cheese manufacture

208 Cultures were grown from frozen stocks in their respective media for 18 h (Table 1).

- 209 The cultures were then inoculated at 1% (v/v) into 10% (w/v) RSM and incubated for a
- 210 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<sub>4</sub>4H<sub>2</sub>O as previously
  reported (18).
- One litre vats of whole milk heated to 31°C were inoculated with the 18 h RSM cultures
  as follows:
- 215 -Vat 1 = 0.75% (v/v) L. lactis DPC4268, 0.75% (v/v) L. lactis CSK2775 (no
  216 bacteriocin)
- 217 -Vat 2 = 0.75% (v/v) L. lactis DPC4268, 0.75% (v/v) L. lactis CSK3281 (nisin
  218 producer)
- 219 -Vat 3 = 0.75% (v/v) L. lactis DPC4268, 0.75% (v/v) L. lactis CSK3594 (lacticin
  220 producer)
- -Vat 4 = 0.75% (v/v) *L. lactis* DPC4268, 0.75% (v/v) *L. lactis* CSK3533 (nisin-lacticin
  double producer)
- 223 -Vat 5 = 0.75% (v/v) L. lactis DPC4268, 0.5% (v/v) L. lactis CSK3281 (nisin
- 224 producer), 0.5% (v/v) *Lb. plantarum* LMG P-26358 (plantaricin producer)
- 225 -Vat 6 = 0.75% (v/v) L. lactis DPC4268, 0.5% (v/v) L. lactis CSK3594 (lacticin
- 226 producer), 0.5% (v/v) Lb. plantarum LMG P-26358 (plantaricin producer)

227	-Vat 7 = $0.75\%$ (v/v) L. lactis DPC4268, $0.5\%$ (v/v) L. lactis CSK3533 (nisin–lacticin
228	double producer), 0.5% (v/v) Lb. plantarum LMG P-26358 (plantaricin producer)
229	-Vat 8 = 0.75% (v/v) <i>L. lactis</i> DPC4268, 0.5% (v/v) CSK3594 (lacticin producer), 0.5%
230	(v/v) CSK3281 (nisin producer)

230 (v/v) CSK3281 (nisin producer)

231 -Vat 9 = 0.75% (v/v) *L. lactis* DPC4268, 0.5% (v/v) CSK3594 (lacticin producer), 0.5%

232 (v/v) CSK3281 (nisin producer), 0.5% (v/v) *Lb. plantarum* LMG P-26358 (plantaricin
233 producer)

234 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 235 236 clotting units/ml Kalase rennet (CSK Food Enrichment, The Netherlands) was added 237 according to manufacturer's specifications and after a further 15 min the curd was cut 238 into cubes. Following a 10 min stirring step, approximately 35% of the whey was 239 removed and the curd was stirred for a further 5 min. The temperature was then 240 elevated to 36°C over a 5 min period and the curd was stirred for a further 20 min. The 241 curd was further drained and lightly pressed into moulds for 20 min before pressing 242 overnight. After 24 h the cheeses were submerged in a brine bath (23% NaCl [w/v], 243 0.22% phosphoric acid [v/v], 0.1% NaOH [w/v], 0.6% CaCl<sub>2</sub> [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 244 245 DPC6578 was enumerated in each cheese on a weekly basis by homogenising 1 g of 246 cheese in 2% sterile tri-sodium citrate and plating serial dilutions on selective medium 247 (GM17 agar with 500 µg/ml of streptomycin). The cheese trial was performed in 248 triplicate and sampling for each trial was performed in duplicate.

Nisin  $(3352 \pm 3 \text{ Da})$ , lacticin (Ltn $\beta$ : 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, Downloaded from http://aem.asm.org/ on May 11, 2017 by UNIV COLLEGE CORK

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the presence of the correct mass for Ltn $\beta$  was indicative of lacticin since Ltn $\alpha$  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: Ltn $\alpha$  and 37: Ltn $\beta$ ) were combined or wells were positioned near each other to assess lacticin activity.

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#### 260 Frequency of bacteriocin resistance/tolerance

261 To determine the frequency of bacteriocin resistance/tolerance development in L. 262 innocua, freshly prepared 18 h cultures were serially diluted in MRD and spread plated 263 on to GM17 or GM17 containing either 1000 AU/ml or 320 AU/ml of the appropriate 264 bacteriocin or bacteriocin combination, the latter concentration representing the 265 arbitrary in situ concentration of the bacteriocins in the cheeses. Plates were incubated 266 aerobically at 37°C for up to 48h, at which time, the frequency of bacteriocin 267 resistance/tolerance was calculated as described previously (38). All experiments were 268 performed in triplicate.

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#### 270 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</li>
were performed using XLSTAT statistical software.

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#### 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  $2847 \pm 4$  Da corresponding to the 294 lacticin peptide, Ltn $\beta$  (Fig. 2). However, lacticin peptides (Ltn $\alpha$  or Ltn $\beta$ ) could not be 295 detected in the recipient strain, CSK2775 (Fig. 2). CMS also detected a peptide with a 296 mass of  $3352 \pm 3$  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).

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

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302 agar well diffusion assays, corresponding to a zone size of 4.5 mm which is equivalent 303 to the zone size produced by the nisin transconjugant *L. lactis* CSK3281. The lacticin 304 transconjugant, CSK3594 produced a 2.5 mm zone against *L. lactis* HP. To our 305 knowledge, this is the first report of the successful construction of a food-grade 306 commercial *L. lactis* starter strain capable of producing both nisin and lacticin 3147, 307 two potent Class I bacteriocins.

308

309 Strain performance and stability

310 The stability of the bacteriocin/lactose positive phenotype in each transconjugant was 311 confirmed via repeated "passaging" in GM17 followed by bacteriocin activity assays 312 against the indicator, L. lactis HP. Bacteriocin production and immunity in CSK3281, 313 CSK3594 and CSK3533 proved to be stable over time. However, upon passaging of the 314 double producer, CSK3533, in GM17, a mixed culture containing lactose fermenting 315 and non-fermenting colonies could be observed when plated on LIA. This mixed culture 316 was subsequently attributed to the loss of a large plasmid (>50 kb) present in CSK3533 317 (confirmed by plasmid profile analysis; results not shown) and is presumed to be 318 involved in lactose metabolism. The lactose fermenting phenotype could be preserved 319 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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#### 327 Spectrum of inhibition and bacteriophage resistance

328 The activity of the single bacteriocin producers CSK3594 and CSK3281 as well as the 329 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) 330 331 (Table 2). The single lacticin producer was found to inhibit primarily lactococci, 332 lactobacilli, and clostridia while a wider spectrum of inhibition was observed for both 333 the nisin producer and the double producer. The double producer proved to be more 334 effective than either lacticin or nisin single producers with regard to Clostridium 335 tyrobutyricum inhibition producing a 6 mm zone while the lacticin producer, CSK3594, 336 and nisin producer, CSK3281, each produced zones of 4 mm and 3 mm, respectively. 337 Interestingly, the recipient strain, CSK2775, also produced a 1 mm zone against 338 *Clostridium tyrobutyricum* suggesting that some other antimicrobial effect is potentially 339 working in conjunction with the bacteriocins in the transconjugants. In addition, the 340 double producer generated a 4 mm zone against CSK3281 in comparison to a 2.5 mm 341 zone produced by CSK3594. This increase in zone size is surprising given that 342 CSK3281 harbours the genetic machinery for nisin immunity and indeed was proven to 343 be immune to nisin in the antimicrobial assays. However, the increased susceptibility of 344 the nisin transconjugant to lacticin may be due to a lower cell density in the seeded 345 plate as a consequence of a slower growth rate although this has not been confirmed.

Bacteriophage sensitivity assays confirmed that CSK2775, the nonbacteriocinogenic 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).

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#### 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<sup>4</sup> 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; P<0.01). By week 3, Vat 7 exhibited lowest Listeria numbers (0.4 log cfu/g) followed 373 374 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),  $Ltn\beta$  (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 ( $Ltn\alpha$ ) 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 Ltn $\beta$  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. Downloaded from http://aem.asm.org/ on May 11, 2017 by UNIV COLLEGE CORK

402 The correct mass for nisin was not detected in Vat 4 (double producer) at weeks 403 0 or 4, however, fraction 21, which is generally expected to contain nisin, yielded a 404 zone of inhibition against the indicator strain on both weeks, suggesting the bacteriocin 405 is present (Fig 5B). The correct mass for Ltn $\beta$  was detected in Vat 4 (double producer) 406 at weeks 0 and 4 (Fig 5B). Lacticin activity was confirmed when fractions 23 and 37 407 were positioned beside each other in the agar well diffusion assays.

408 Vat 5 (nisin and plantaricin) was found to contain the nisin mass at weeks 0 and 409 4 although the correct mass was found in fraction 22 at week 0 and in fraction 23 at 410 week 4 (Fig 5C). This is presumably due to slight variations in the times the peptide 411 eluted from the HPLC. These fractions exhibited antimicrobial activity against the indicator strain but the zones were smaller than previously observed. The plantaricin 412 413 mass was not detected in Vat 5 at week 0 but a zone of inhibition against Listeria was 414 observed for fraction 19. However, the plantaricin mass was detected in Vat 5 at week 4 415 and antimicrobial activity was confirmed.

416 Vat 6 (lacticin and plantaricin) was shown to contain the correct Ltn $\beta$  and plantaricin 417 masses at weeks 0 and 4 in fractions 37 and 19, respectively (Fig 5D). Antimicrobial 418 activity was confirmed for fraction 19 although the zone was smaller at week 0 when 419 compared to week 4. In the case of lacticin, combining fractions 24 (Ltn $\alpha$ ) and 37 420 confirmed antimicrobial activity.

421

#### 422 Development of bacteriocin tolerance

423 The frequency of tolerance/resistance development in *L. innocua* was assessed using 424 1000 AU/ml of each bacteriocin. The frequency of resistance against 1000 AU/ml of 425 nisin was calculated to be  $6.56 \times 10^{-4}$ . Resistance development could not be observed 426 when *Listeria* was exposed to 1000 AU/ml lacticin or 1000 AU/ml nisin and lacticin.

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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 x $10^{-1}$ and 3.02 x $10^{-1}$ , respectively. Simultaneous exposure to lacticin and nisin at
430	320 AU/ml decreased the frequency of resistance to $3.18 \times 10^{-2}$ . However, bacteriocin
431	resistant colonies remained sensitive to 1000 AU/ml indicating that Listeria cells were
432	tolerant rather than completely resistant.
433	

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#### 452 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.

458 In agreement with previous findings (39) which revealed that pMRC01 imposes 459 a burden on lactococcal metabolism affecting growth and acidification rates, the 460 presence of pMRC01 was shown to influence lactococcal acidification in the lacticin 461 transconjugant, as did the presence of the nisin transposon in the nisin transconjugant. 462 However, bacteriocin stacking resulted in slowest acidification rates but which can be 463 overcome with the addition of 0.1% yeast extract to the double producer. Yeast extract 464 presumably lessens the burden of plasmid and transposon acquisition as it provides 465 amino acids along with purine and pyrimidine bases and inorganic constituents which 466 have been shown to stimulate lactococcal growth (40). We therefore suggest that the 467 double producer has potential to serve as a protective culture when used in conjunction 468 with a suitable acidifier. Despite this, the lactose utilization phenotype in the double 469 producer, CSK3533, was found to be unstable in GM17 apparently due to the loss of a 470 large (>50kb) plasmid likely involved in lactose utilization. Both the plasmid instability 471 and slower acidification profiles observed in the double producer (CSK3533) may be 472 attributed to the metabolic burden imposed by the presence of pMRC01 and the nisin 473 transposon. In an effort to ease the metabolic load, it is possible that as the energy 474 demand of the cell increases and metabolites are exhausted, a reduction in growth rate 475 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 utilization477 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 increase in antimicrobial activity. Studies suggest that bacteriocin production and 483 484 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.

In terms of bacteriophage resistance, transconjugants were shown to be more resistant to bacteriophage attack than the recipient strain with superior resistance properties observed in pMRC01 derivatives, which is known to harbour an abortive 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

501 ripening period, Listeria numbers from cheeses prepared with these starters were 502 significantly reduced when compared to the control, Vat 1 (no bacteriocin). 503 Interestingly, the double producer combined with the plantaricin producer exhibited the 504 greatest reduction in *Listeria* numbers at week 0, a trend which continued to Week 1 505 suggesting the effectiveness of this combination for reducing initial bacterial load. The 506 inhibitory effect of this combination was on the whole significantly better than using 507 both single producers with the plantaricin producer. This can be explained by the fact 508 that the nisin producing transconjugant inhibits the lacticin transconjugant and vice 509 versa whereas the double producer is immune to both bacteriocins. The combination of 510 the lacticin producer with Lb. plantarum LMG P-26358 also significantly reduced 511 Listeria numbers by week 1 and indeed by week 4, Listeria could not be detected in this 512 vat. Overall, the double producer combined with the plantaricin producer followed by 513 the lacticin producer combined with plantaricin exhibited the most significant 514 reductions in Listeria numbers over the ripening period.

515 In general, a similar inhibitory trend was observed amongst the single Class I 516 producers, the double producer or the combined single producers which were 517 significantly different to Vat 1 at week 0 and week 4. While the double producer did not 518 alter Listeria numbers significantly when compared to the single producers alone, a 10-519 fold reduction in the emergence of bacteriocin tolerance was observed when Listeria 520 was exposed to both nisin and lacticin, suggesting that bacteriocin stacking could be an 521 effective method to prevent pathogen growth in food applications. However, combining 522 bacteriocins from different Classes or sub-classes is considered most effective for 523 reducing the emergence of resistance (44) which explains the increased antimicrobial 524 efficacy for vats containing the Class I and Class II bacteriocins in this study.

Applied and Environmental Microbioloav Interestingly, cheese prepared with non-bacteriocinogenic CSK2775 also resulted in reduced *Listeria* numbers over the four week period although to a lesser extent than the bacteriocin containing cheeses. Therefore, it is probable that bacterial competition coupled with unfavourable conditions relating to cheese manufacture including lactic acid and high salt concentrations have provided a hurdle-effect to cause the observed reductions. Indeed, several intrinsic factors including moisture content, 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,

550 lactococcin G (52). However, the ability to generate a multi-bacteriocin producer from 551 an already established culture through food-grade enabling technologies poses several 552 benefits. Firstly, the technological properties of the culture are known. The number of 553 cultures required to produce multiple bacteriocins is reduced. The risk of bacteriocin 554 inhibition is removed since the multibacteriocin-producing starter will also harbour the 555 genetic machinery for bacteriocin immunity. As conjugation is a natural process, the 556 resulting transconjugants do not fall under current European regulations governing the 557 use of genetically modified microorganisms (53, 54). Therefore, transconjugants can be 558 used in food applications in a similar manner to the recipient strain (55). While the 559 double bacteriocin producer generated in this study proved to be a slower acidifier than 560 the recipient strain, it has potential to serve as a protective culture. However, studies 561 generating multiple bacteriocin producers have been rare (13). This can most likely be 562 attributed to the complex biosynthetic process required for bacteriocin production and 563 secretion. Indeed, previous attempts to construct nisin-lacticin transconjugants were 564 unsuccessful, often attributed to the incompatibility of bacteriocin modification 565 machinery or bacteriocin sensitivity (13, 30). Traditionally, the discovery of 566 technologically valuable industrial strains has focused on large-scale screening 567 strategies from a variety of sources (56). However, the successful transfer of lacticin 568 and nisin to commercial starter cultures as reported in this study may provide additional 569 avenues for the development of multi-hurdle protective cultures using food-grade 570 methods.

571 572

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FIG 1 PCR amplification using pMRC01-specific primers (*orf27*, *orf49*, *orf51*, and *orf52*) to detect the presence of pMRC01 in *L. lactis* CSK3594 and CSK3533. PCR
amplification using primers designed to regions of the nisin operon (*nisA*, *nisFEG*) to
confirm the presence of nisin genetic determinants in *L. lactis* CSK3281 and *L. lactis*CSK3533. (M: 100 bp DNA ladder; New England BioLabs).
FIG 2 Colony mass spectrometry analysis of *L. lactis* CSK2775, *L. lactis* CSK3594
(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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Bacterial strain	Relevant Detail	Relevant genotype	Source or reference
		and phenotype <sup>a</sup>	
L. lactis HP	Bacteriocin sensitive indicator strain	Ltn; Nis	TFRC <sup>b</sup>
L. lactis MG1363	Donor strain harbouring pMRC01,	Lac <sup>-</sup> ; Ltn <sup>+</sup> ; Nis <sup>-</sup>	TFRC
(pMRC01)	lacticin producer		
L. lactis CSK2583	Donor strain harbouring Tn5276, nisin	Lac <sup>-</sup> ; Nis <sup>+</sup> ; Ltn <sup>-</sup>	CSK, The Netherlands
	producer		
L. lactis CSK2775	Recipient strain	Lac <sup>+</sup> ; Nis <sup>-</sup> ; Ltn <sup>-</sup>	CSK, The Netherlands
L. lactis CSK3281	CSK2775 derivative, nisin producer	$Lac^+; Nis^+$	This study
L. lactis CSK3594	CSK2775 transconjugant harbouring	$Lac^+; Ltn^+$	This study
	pMRC01, lacticin producer		
L. lactis CSK3533	CSK3281 transconjugant harbouring	$Lac^+$ ; $Nis^+$ , $Ltn^+$	This study
	pMRC01, nisin-lacticin double producer		
Lb. plantarum LMG P-	Plantaricin 423 producer	$Pln^+$	(18)
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L. lactis DPC4268	Starter culture for cheese manufacture	$Lac^+$	TFRC

<sup>a</sup>Lac, lactose utilization; Ltn, lacticin genetic determinants; Nis, nisin genetic determinants; Pln,

856 857 plantaricin genetic determinants. <sup>b</sup> 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	Strains Tested for Antimicrobial Activity			Source	
	Medium	CSK2275	CSK3594 (Ltn)	CSK3281 (Nis)	CSK3533 (Ltn, Nis)	
Bacillus cereus DPC6085/6086	BHI <sup>a</sup>	No zone	No zone	1mm zone	1mm zone	TFRC
Bacillus subtilus DPC6511	BHI <sup>a</sup>	No zone	No zone	No zone	No zone	TFRC
Enterococcus faecalis DPC5055/LMG 7973	BHI <sup>a</sup>	No zone	No zone	3mm zone	3mm zone	TFRC
Enterococcus faecium DPC5056*	BHI <sup>a</sup>	No zone	No zone	6mm zone	6mm zone	TFRC
Escherichia coli P1432- DPC6054	BHI <sup>a</sup>	No zone	No zone	No zone	No zone	TFRC
Clostridium sporogenes DPC6341 <sup>*</sup>	RCM <sup>a</sup>	No zone	1.5 mm zone	1.5mm zone	1mm zone	TFRC
Clostridium tyrobutyricum DPC6342*	RCM <sup>a</sup>	1mm zone	4mm zone	3mm zone	6mm zone	TFRC
Lactobacillus casei DPC6125	MRS <sup>c</sup>	No zone	No zone	7mm zone	7mm zone	TFRC
Lactobacillus acidophilus DPC5378	MRS <sup>a</sup>	No zone	1mm zone	3mm zone	3mm zone	TFRC
Lactobacillus delbreukii subsp. delbreukii DPC5385	MRS <sup>a</sup>	No zone	No zone	6mm zone	6mm zone	TFRC
Lb. delbreukii subsp. lactis DPC5387	MRS <sup>c</sup>	No zone	1.5mm zone	6mm zone	6mm zone	TFRC
Lb delbreukii subsp. bulgaricus DPC5383	MRS <sup>c</sup>	No zone	2mm zone	9.5mm zone	9.5mm zone	TFRC
Lactobacillus helveticus DPC4571	MRS <sup>a</sup>	No zone	1.5mm zone	8mm zone	8mm zone	TFRC
L.lactis subsp. lactis biovar diacetyllactis CSK1411	LM17 <sup>b</sup>	No zone	1.5mm zone	4mm zone	4mm zone	CSK
L. lactis subsp. cremoris HP DPC5718	LM17 <sup>c</sup>	No zone	2.5mm zone	4.5mm zone	4.5mm zone	TFRC
L. lactis subsp. lactis DPC4268/ 303	LM17 <sup>c</sup>	No zone	No zone	0.5mm zone	0.25mm zone	TFRC
L. lactis subsp. lactis CSK2775	LM17 <sup>c</sup>	No zone	2.5 mm	4.5 mm	4.5 mm	CSK
L. lactis subsp. lactis CSK3594	LM17 <sup>c</sup>	No zone	No zone	4.5 mm	4.5 mm	CSK
L. lactis subsp. lactis CSK3281	LM17 <sup>c</sup>	No zone	2.5 mm	No zone	4 mm	CSK
L. lactis subsp. lactis CSK3533	LM17 <sup>c</sup>	No zone	No zone	No zone	No zone	CSK
Leuconoctos lactis DPC3838	MRS <sup>c</sup>	No zone	No zone	1.5mm zone	1.5mm zone	TFRC
L. innocua DPC6578	GM17 <sup>a</sup>	No zone	No zone	No zone	No zone	TFRC

\*Cultures grown anaerobically for up to 48h. <sup>a</sup>Cultures grown at 37°C for up to 48h. <sup>b</sup>Cultures grown at 35°C for up to 48h.<sup>c</sup>Cultures

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grown at 30°C for up to 48h.

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## 886 TABLE 3 Primer pairs used in this study

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Primer	Sequence	Target gene(s)	Size (bp)
27-F 5'-GGGGAACAATCTTACCTA		orf 27	326
27-R 5'-ATTATTTT	-		
<b>49-F</b> 5'-CCAATACC	CGCCAAAATAAAGT	orf 49	347
49-R 5'-CTAAGCGC			
51-F 5'-TTCTCAAA	ATCATCAAAATCAAGT	orf51	293
51-R 5'-GTACGAAC	CAGGAGCGAAAAA		
52-F 5'- CCTAAGTT	GTCTATTCGTGTCCA	orf52	210
52-R 5'- ATTAGGTO	GAGTGCTCTGATTTTTC		
nisA-F 5'- CAAAAG	ATTTTAACTTGGATTTG	nisA	163
nisA-R 5'- ACGTGA	ATACTACAATGACAAG		
nisFG-F 5'- GGTTTA	ATTTCTGCAGATACTG	nisFEG	1573
nisFG-R 5'- GTAAT	TATCCAGATCATTGCTG		

Bacteriophage	L. lactis CSK2775	<i>L. lactis</i> CSK3281 (Nis⁺)	L. lactis CSK3594 (Ltn <sup>+</sup> )	<i>L. lactis</i> CSK3533 (Nis <sup>+</sup> Ltn <sup>+</sup> )
5410F	+	+	-	-
5163F	+	-	-	-
5210 F	+	+	+	+
5167F	+	-	-	-
5385F	+	-	-	-
(Bacteriophage				
cocktail)				
5386F	+	+	-	-
(Bacteriophage				
cocktail)				

904 TABLE 4 L. lactis transconjugants surveyed for bacteriophage sensitivity

905 906 + indicates bacteriophage sensitivity observed by a clearing of the bacterial population.

- indicates bacteriophage insensitivity observed as growth (turbidity) of the bacterial population. 907

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FIG 2

FIG 3



Time (hours)



Time (weeks)

Vat 1 L. lactis CSK2775
 Vat 2 L. lactis CSK3281 (Nis+)
 Vat 3 L. lactis CSK3594 (Lm+)
 Vat 4 L. lactis CSK3533 (Nis+, Lm+)
 Vat 5 L. lactis CSK3581 (Nis+), Lb. plantarum LMG P-26358
 Vat 6 L. lactis CSK3594 (Lm+), Lb. plantarum LMG P-26358
 Vat 7 L. lactis CSK3533 (Nis+, Lm+), Lb. plantarum LMG P-26358
 Vat 8 L. lactis CSK3281 (Nis+), L. lactis CSK3594 (Lm+)
 Vat 9 L. lactis CSK3281 (Nis+), L. lactis CSK3594 (Lm+), Lb. plantarum LMG P-26358

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FIG 5 (B)

#### Vat 4 L. lactis CSK3533

Week 4 Fraction 21



Week 0 Fraction 37





Week 4 Fraction 37





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Week 4 Fraction 23

Nisin→<sup>335</sup>

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



Week 0 Fraction 19





