



# A Multibacteriocin Cheese Starter System, Comprising Nisin and Lacticin 3147 in *Lactococcus lactis*, in Combination with Plantaricin from *Lactobacillus plantarum*

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. P. Ross<sup>b,d,e</sup>

School of Microbiology, University College Cork, Cork, Ireland<sup>a</sup>; Teagasc Food Research Centre, Moorepark, Fermoy, County Cork, Ireland<sup>b</sup>; CSK Food Enrichment, Ede, The Netherlands<sup>c</sup>; APC Microbiome Institute, University College Cork, Cork, Ireland<sup>d</sup>; College of Science, Engineering and Food Science, University College Cork, Cork, Ireland<sup>e</sup>

**ABSTRACT** Functional starter cultures demonstrating superior technological and food safety properties are advantageous to the food fermentation industry. We evaluated the efficacies 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 mobilizing the conjugative bacteriophage resistance plasmid pMRC01, carrying lacticin genetic determinants, or the conjugative transposon Tn5276, carrying nisin genetic determinants, to the commercial starter *L. lactis* CSK2775. The effect of bacteriocin coproduction was examined by superimposing pMRC01 into the newly constructed nisin transconjugant. Transconjugants were improved with regard to antimicrobial activity and bacteriophage insensitivity 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, combinations of class I bacteriocins (the double producer or a combination of single producers) proved to be 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 using the lacticin producer with the class II producer proved to be 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, multihurdle approach to control pathogenic growth in a variety of industrial applications.

**IMPORTANCE** We generated a suite of single- and double-bacteriocin-producing starter cultures capable of generating the class I bacteriocin lacticin 3147 or nisin or both bacteriocins simultaneously via conjugation. The transconjugants exhibited improved bacteriophage resistance and antimicrobial activity. The single producers proved to be as effective as the double-bacteriocin producer at reducing *Listeria* numbers in laboratory-scale cheese. However, combining the double producer or the lacticin-producing starter with a class II bacteriocin producer, *Lactobacillus plantarum* LMG P-26358, proved to be most effective at reducing *Listeria* numbers and was significantly better than a combination of the three bacteriocin-producing strains, as the double producer is not inhibited by either of the class I bacteriocins. Since the simultaneous use of lacticin and nisin should reduce the emergence of

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Address correspondence to R. P. Ross, [p.ross@ucc.ie](mailto:p.ross@ucc.ie).

bacteriocin-tolerant derivatives, this study suggests that a protective starter system produced by bacteriocin stacking is a worthwhile multihurdle approach for food safety applications.

**KEYWORDS** food safety, protective culture, bacteriocins

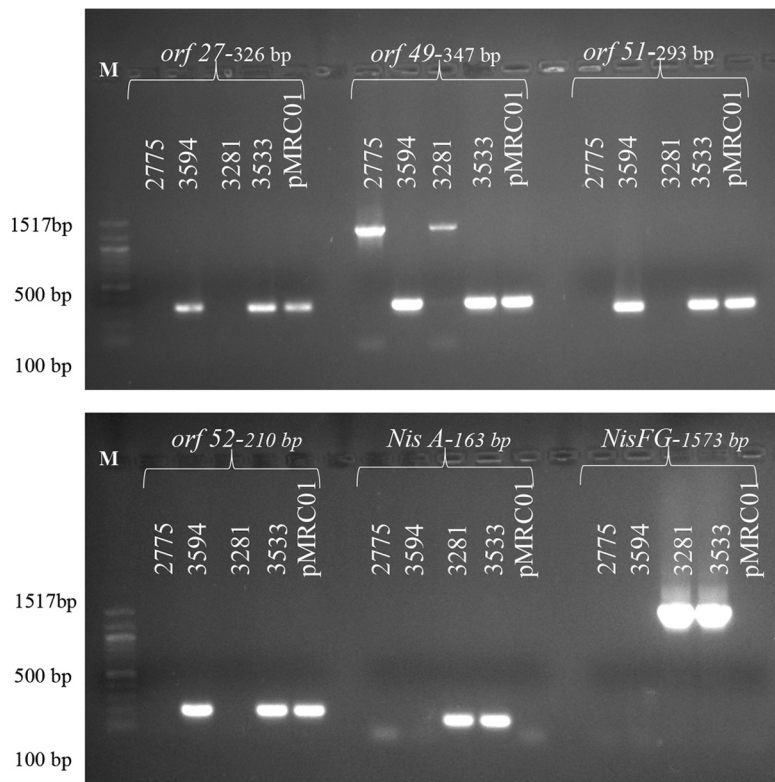
The development and characterization of starter cultures that demonstrate superior technological properties, such as improved proteolytic activity and flavor production, exopolysaccharide production, or bacteriophage resistance, are considered highly advantageous within the food fermentation industry (1). The ability of starter strains to produce bacteriocins is also considered an important technological trait for controlling undesirable and/or pathogenic growth *in situ* and for improving sensory characteristics (1–3). Bacteriocins are ribosomally synthesized, heat-stable antimicrobial peptides that generally act by depolarizing the target cell membrane and/or by inhibiting cell wall synthesis where the producing strain is immune to the antimicrobial effect (4). They comprise a highly heterogeneous group that has recently been divided into three distinct classes (5).

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

In the present study, we generated single- and double-bacteriocin-producing cultures of *Lactococcus lactis* CSK2775 with the capacity to produce the class I bacteriocins lactacin 3147 (lactacin), nisin A (nisin), or lactacin 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). The 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 antilisterial 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 antilisterial potential of nisin (20–24), lactacin (25–28), and plantaricin (18) in food systems.

## RESULTS

**Transconjugant validation.** The presence of the plasmid pMRC01 and the nisin transposon Tn5276 in *L. lactis* CSK2775 transconjugants was validated by PCR using plasmid- and transposon-specific primers, respectively (Fig. 1). PCR analysis confirmed that the genetic determinants responsible for lactacin production were present in

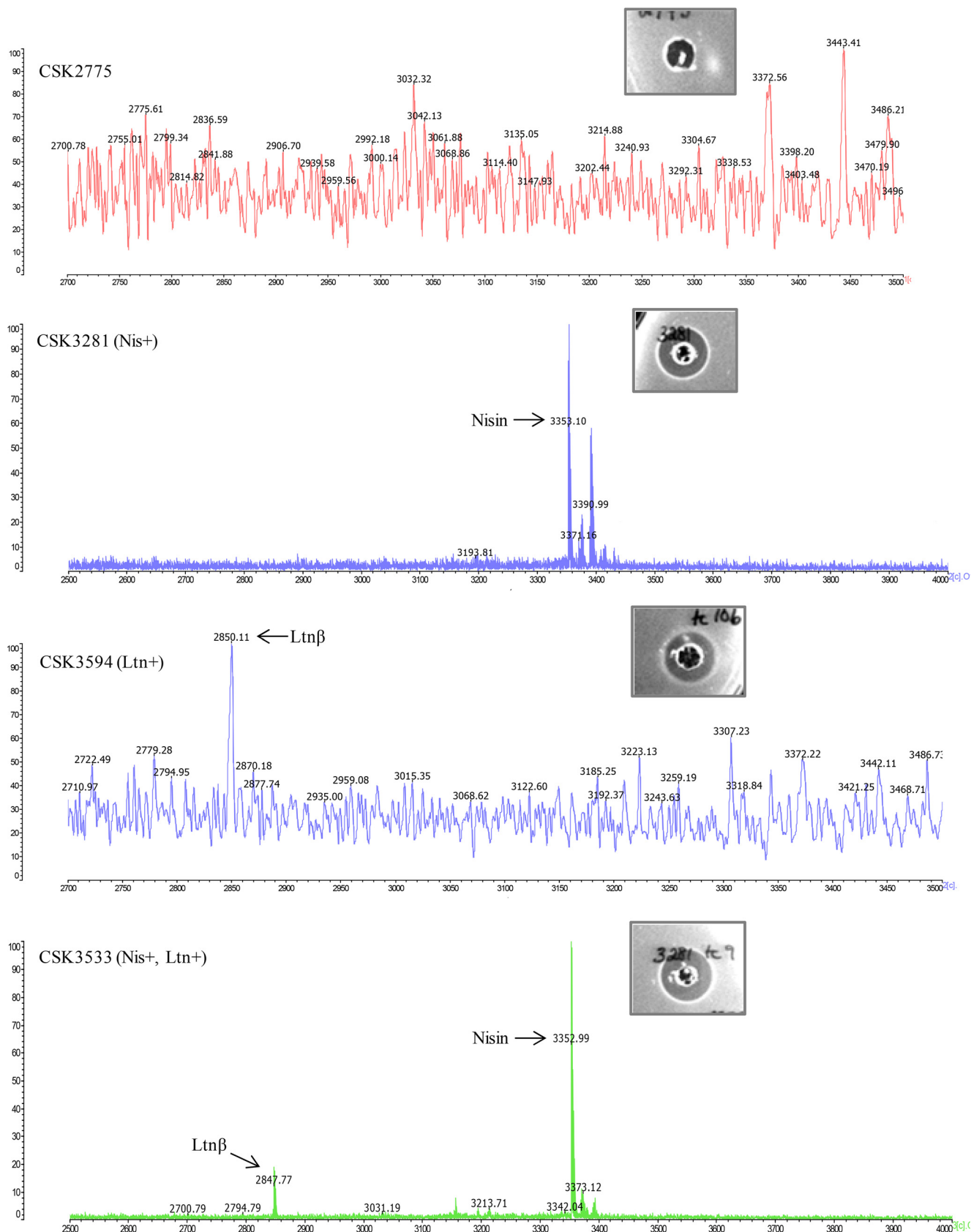


**FIG 1** PCR amplification using pMRC01-specific primers (*orf27*, *orf49*, *orf51*, and *orf52*) to detect the presence of pMRC01 in *L. lactis* CSK3594 and CSK3533 and using primers designed to regions of the nisin operon (*nisA* and *nisFEG*) to confirm the presence of nisin genetic determinants in *L. lactis* CSK3281 and *L. lactis* CSK3533. Lane M, 100-bp DNA ladder (New England BioLabs).

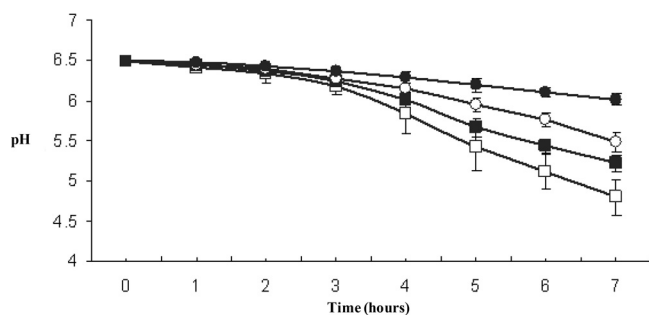
CSK2775(pMRC01), creating the lactacin transconjugant *L. lactis* CSK3594, and confirmed the presence of the nisin genetic determinants in CSK2775(Tn5276), creating the nisin transconjugant CSK3281. The presence of the lactacin and nisin genetic determinants was confirmed in the double producer, resulting in the nisin-lactacin transconjugant *L. lactis* CSK3533. To confirm the identity of each transconjugant, genomic fingerprints were generated by pulsed-field gel electrophoresis (PFGE) with the restriction endonuclease *Sma*I. All transconjugants analyzed generated the same restriction pattern as the recipient strain, CSK2775 (results not shown). Well diffusion assays confirmed that CSK3594 was sensitive to nisin and that CSK3281 was sensitive to lactacin but that the double producer was immune to both bacteriocins.

Colony mass spectrometry (CMS) confirmed that CSK3594 and CSK3533 each produced a peptide with a mass of approximately  $2,847 \pm 4$  Da, corresponding to the lactacin peptide Ltn $\beta$  (Fig. 2). However, lactacin peptides (Ltn $\alpha$  or Ltn $\beta$ ) could not be detected in the recipient strain, CSK2775 (Fig. 2). CMS also detected a peptide with a mass of  $3,352 \pm 3$  Da, corresponding to nisin, in strains CSK3281 and CSK3533; this peptide was absent in the recipient strain. These data confirm that lactacin is produced by CSK3594, nisin is produced by CSK3281, and both nisin and lactacin are 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 1,000 arbitrary units (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 lactacin 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 lactacin 3147, two potent class I bacteriocins.



**FIG 2** Colony mass spectrometry analysis of *L. lactis* CSK2775, *L. lactis* CSK3281 (nisin transconjugant, Nis<sup>+</sup>), *L. lactis* CSK3594 (lactacin transconjugant, Ltn<sup>+</sup>), and *L. lactis* CSK3533 (nisin and lactacin double producer, Nis<sup>+</sup>, Ltn<sup>+</sup>). Masses corresponding to the bacteriocins are indicated. Inset photos show inhibition zones produced by each strain against the indicator strain *L. lactis* HP.



**FIG 3** Acidification profiles of *L. lactis* CSK2775 (□), *L. lactis* CSK3594 (lactacin) (■), *L. lactis* CSK3281 (nisin) (○), and *L. lactis* CSK3533 (nisin and lactacin) (●) grown in 10% reconstituted skim milk.

**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 nonfermenting colonies could be observed when plated on lactose indicator agar (LIA). This mixed culture was subsequently attributed to the loss of a large plasmid (>50 kb) present in CSK3533 (which was 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 the acidification profiles of each transconjugant and the recipient strain revealed that the bacteriocin-free recipient, CSK2775, was the most efficient acidifier (Fig. 3). Although the lactacin 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.

**Spectrum of inhibition and bacteriophage resistance.** The activities 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 bacteria, pathogenic bacteria, LAB, and nonstarter LAB (NSLAB) (Table 1). The single lactacin 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 the lactacin or nisin single producer with regard to *Clostridium tyrobutyricum* inhibition, producing a 6-mm zone while the lactacin producer, CSK3594, and the nisin producer, CSK3281, produced zones of 4 mm and 3 mm, respectively. Interestingly, the recipient strain, CSK2775, also produced a 1-mm zone against *C. 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 harbors 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 lactacin may be due to a lower cell density in the seeded plate as a consequence of a lower growth rate, although this has not been confirmed.

Bacteriophage sensitivity assays confirmed that CSK2775, the nonbacteriocinogenic recipient, was sensitive to all bacteriophages analyzed (Table 2). The nisin producer, CSK3281 was resistant to 50% of the bacteriophages analyzed, while the lactacin single producer and the double producer were each resistant to 80% of bacteriophages analyzed (Table 2).

**Laboratory-scale cheese production.** To analyze the *in situ* inhibitory activity of the bacteriocin producers (single, double, and in combination with the plantaricin

**TABLE 1** Antimicrobial spectrum of the *L. lactis* isogenic family of nisin and lactacin transconjugants

Indicator strain	Growth medium	Zone size (mm) for strain:				Source
		CSK2275	CSK3594 (Ltn)	CSK3281 (Nis)	CSK3533 (Ltn, Nis)	
<i>Bacillus cereus</i> DPC6085/6086	BHI <sup>b</sup>	No zone	No zone	1	1	TFRC <sup>e</sup>
<i>Bacillus subtilis</i> DPC6511	BHI <sup>b</sup>	No zone	No zone	No zone	No zone	TFRC
<i>Enterococcus faecalis</i> DPC5055/LMG 7973	BHI <sup>b</sup>	No zone	No zone	3	3	TFRC
<i>Enterococcus faecium</i> DPC5056 <sup>a</sup>	BHI <sup>b</sup>	No zone	No zone	6	6	TFRC
<i>Escherichia coli</i> P1432-DPC6054	BHI <sup>b</sup>	No zone	No zone	No zone	No zone	TFRC
<i>Clostridium sporogenes</i> DPC6341 <sup>a</sup>	RCM <sup>b</sup>	No zone	1.5	1.5	1	TFRC
<i>Clostridium tyrobutyricum</i> DPC6342 <sup>a</sup>	RCM <sup>b</sup>	1	4	3	6	TFRC
<i>Lactobacillus casei</i> DPC6125	MRS <sup>d</sup>	No zone	No zone	7	7	TFRC
<i>Lactobacillus acidophilus</i> DPC5378	MRS <sup>b</sup>	No zone	1	3	3	TFRC
<i>Lactobacillus delbrueckii</i> subsp. <i>delbrueckii</i> DPC5385	MRS <sup>b</sup>	No zone	No zone	6	6	TFRC
<i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i> DPC5387	MRS <sup>d</sup>	No zone	1.5	6	6	TFRC
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> DPC5383	MRS <sup>d</sup>	No zone	2	9.5	9.5	TFRC
<i>Lactobacillus helveticus</i> DPC4571	MRS <sup>b</sup>	No zone	1.5	8	8	TFRC
<i>L. lactis</i> subsp. <i>lactis</i> biovar <i>diacetylactis</i> CSK1411	LM17 <sup>c</sup>	No zone	1.5	4	4	CSK, The Netherlands
<i>L. lactis</i> subsp. <i>cremoris</i> HP DPC5718	LM17 <sup>d</sup>	No zone	2.5	4.5	4.5	TFRC
<i>L. lactis</i> subsp. <i>lactis</i> DPC4268/303	LM17 <sup>d</sup>	No zone	No zone	0.5	0.25	TFRC
<i>L. lactis</i> subsp. <i>lactis</i> CSK2775	LM17 <sup>d</sup>	No zone	2.5 mm	4.5 mm	4.5 mm	CSK, The Netherlands
<i>L. lactis</i> subsp. <i>lactis</i> CSK3594	LM17 <sup>d</sup>	No zone	No zone	4.5 mm	4.5 mm	CSK, The Netherlands
<i>L. lactis</i> subsp. <i>lactis</i> CSK3281	LM17 <sup>d</sup>	No zone	2.5 mm	No zone	4 mm	CSK, The Netherlands
<i>L. lactis</i> subsp. <i>lactis</i> CSK3533	LM17 <sup>d</sup>	No zone	No zone	No zone	No zone	CSK, The Netherlands
<i>Leuconostoc lactis</i> DPC3838	MRS <sup>d</sup>	No zone	No zone	1.5	1.5	TFRC
<i>L. innocua</i> DPC6578	GM17 <sup>b</sup>	No zone	No zone	No zone	No zone	TFRC

<sup>a</sup>Cultures were grown anaerobically for up to 48 h.

<sup>b</sup>Cultures were grown at 37°C for up to 48 h.

<sup>c</sup>Cultures were grown at 35°C for up to 48 h.

<sup>d</sup>Cultures were grown at 30°C for up to 48 h.

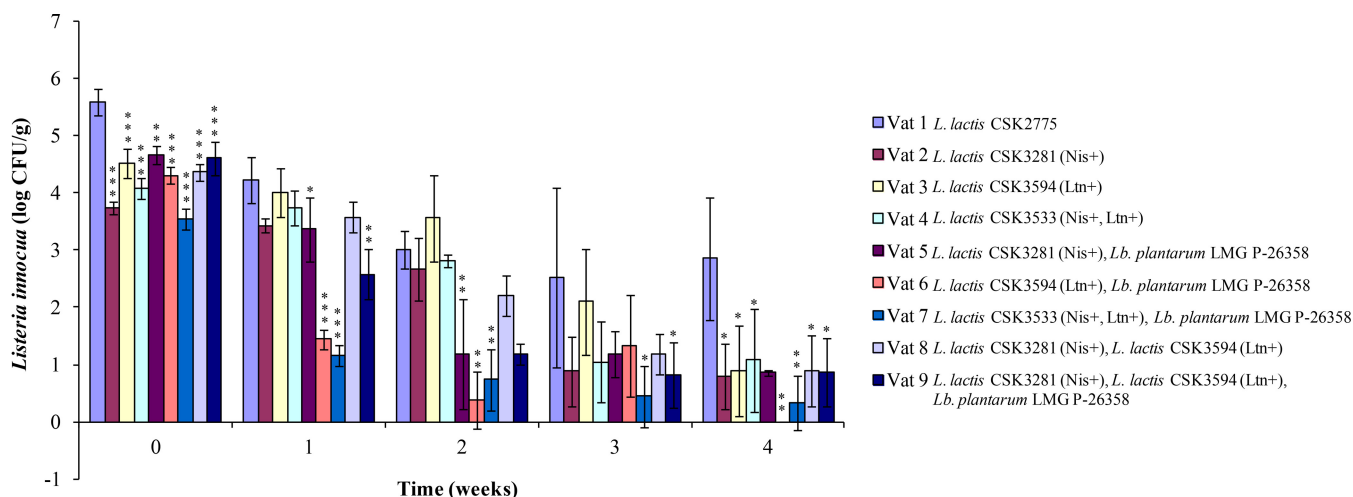
<sup>e</sup>TFRC, Teagasc Food Research Centre, Moorepark, Fermoy, County Cork, Ireland.

producer *Lb. plantarum* LMG P-26358), laboratory-scale cheeses were manufactured with the fast acidifier *L. lactis* DPC4268 and the bacteriocin producers served as protective cultures. The cheeses were spiked with 10<sup>4</sup> CFU/ml of *L. innocua*. Each cheese was ripened for 4 weeks at 7°C; *Listeria* was enumerated weekly during the ripening period. Figure 4 shows *Listeria* viable cell counts over the 4-week period where bacteriocin-containing vats were compared with vat 1 (no bacteriocin) at each week. At week 0, *Listeria* numbers were significantly different between vat 1 (5.6 log CFU/g) and all other vats, with the lowest *Listeria* numbers recorded for vat 7 (CSK3533 and *Lb. plantarum*) at 3.5 log CFU/g ( $P < 0.001$ ), followed by vat 2 (CSK3281) at 3.8 log CFU/g ( $P < 0.001$ ). *Listeria* numbers in the remaining vats were reduced by 0.9 to 1.5 logs compared to those in vat 1. By week 1, *Listeria* numbers in vat 7 continued to decrease significantly compared to those in vat 1, with a 3-log reduction recorded ( $P < 0.001$ ).

**TABLE 2** *L. lactis* transconjugants surveyed for bacteriophage sensitivity

Bacteriophage	Sensitivity <sup>a</sup> of <i>L. lactis</i> strain:			
	CSK2775	CSK3281 (Nis)	CSK3594 (Ltn)	CSK3533 (Nis, Ltn)
5410F	+	+	–	–
5163F	+	–	–	–
5210 F	+	+	+	+
5167F	+	–	–	–
5385F (Bacteriophage cocktail)	+	–	–	–
5386F (Bacteriophage cocktail)	+	+	–	–

<sup>a</sup>+, bacteriophage sensitivity observed by a clearing of the bacterial population; –, bacteriophage insensitivity observed as growth (turbidity) of the bacterial population.



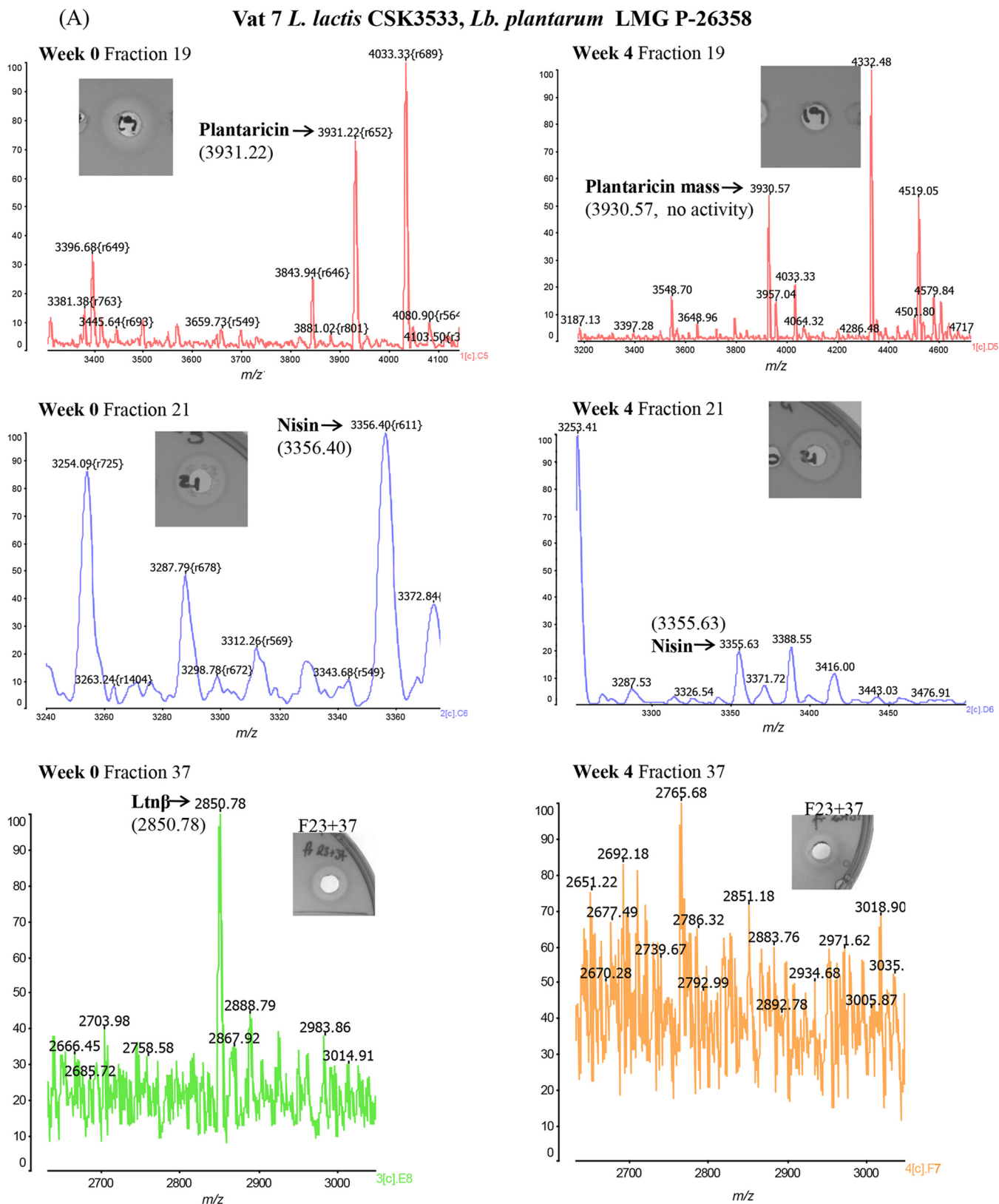
**FIG 4** Counts of viable *L. innocua* cells in laboratory-scale cheeses. Bacteriocin-containing vats were compared to vat 1 (no bacteriocin) at each week. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

*Listeria* numbers in vat 6 (CSK3594 and *Lb. plantarum*) were also significantly different from those in vat 1, with a 2.7-log reduction ( $P < 0.001$ ). Significant reductions were also observed for vat 9 (CSK3281, CSK3594, and *Lb. plantarum*) (1.6-log reduction;  $P < 0.01$ ) and vat 5 (CSK3281 and *Lb. plantarum*) (0.8-log reduction;  $P < 0.05$ ). At week 2, the lowest *Listeria* numbers were recorded for vat 6 (0.4 log CFU/g) and vat 7 (0.7 log CFU/g), representing 2.6- and 2.3-log reductions compared to numbers in vat 1 (3 log CFU/g) ( $P < 0.01$ ). Vat 5 (CSK3281 and *Lb. plantarum*) was also significantly different from vat 1 (1.8-log reduction;  $P < 0.01$ ). By week 3, vat 7 exhibited the lowest *Listeria* numbers (0.4 log CFU/g), followed by vat 9 (0.8 log CFU/g), which were both significantly different from vat 1 (2.5 log CFU/g) ( $P < 0.05$ ). By week 4, *Listeria* numbers for vat 1 (2.9 log CFU/g) were significantly different from those for most other vats, with no *Listeria* detected in vat 6 ( $P < 0.01$ ) and numbers reduced to 0.3 log CFU/g for vat 7 ( $P < 0.01$ ). *Listeria* numbers in the remaining vats (vats 2, 3, 4, 8, and 9) ranged from 0.8 to 1 log CFU/g, representing significant differences compared to vat 1 ( $P < 0.05$ ). While *Listeria* numbers in vat 5 were not deemed significantly different from those in vat 1, they approached a significant reduction ( $P = 0.07$ ).

In terms of the lacticin and nisin transconjugants (without *Lb. plantarum*), no significant differences were observed between the double producer (vat 4) and either of the single producers (vats 2 and 3) with regard to *Listeria* numbers at any week. We then compared vat 8, which consists of the two single producers (CSK3281 and CSK3594) (4.4 log CFU/g at week 0) with vats 2, 3, and 4. While vat 8 was found to be significantly different from vat 2 (3.7 log CFU/g) at week 0 ( $P < 0.05$ ) whereby the nisin producer generated greater *Listeria* reductions than the combination of nisin and lacticin single producers, no significant differences were observed for weeks 1 to 4. Likewise, no significant differences were observed between vat 8 and vat 3 or 4 over the ripening period.

**Bacteriocin detection in vats 4, 5, 6, and 7.** The correct masses for nisin (fraction 21), Ltn $\beta$  (fraction 37), and plantaricin (fraction 19) were detected by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) in vat 7 (double producer and plantaricin) at week 0 (Fig. 5A). Antimicrobial assays also revealed that these fractions contained activity (lacticin activity was restored by combining fractions 23 [Ltn $\alpha$ ] and 37).

At week 4, a mass corresponding to plantaricin was detected in fraction 19, but there was no antimicrobial activity. Nisin was detected in fraction 21 at week 4, and activity was also confirmed. The correct mass for Ltn $\beta$  could not be detected at week 4. Despite this, combining fractions 23 and 37 did yield a zone of inhibition against the indicator strain, suggesting that the bacteriocin is present.

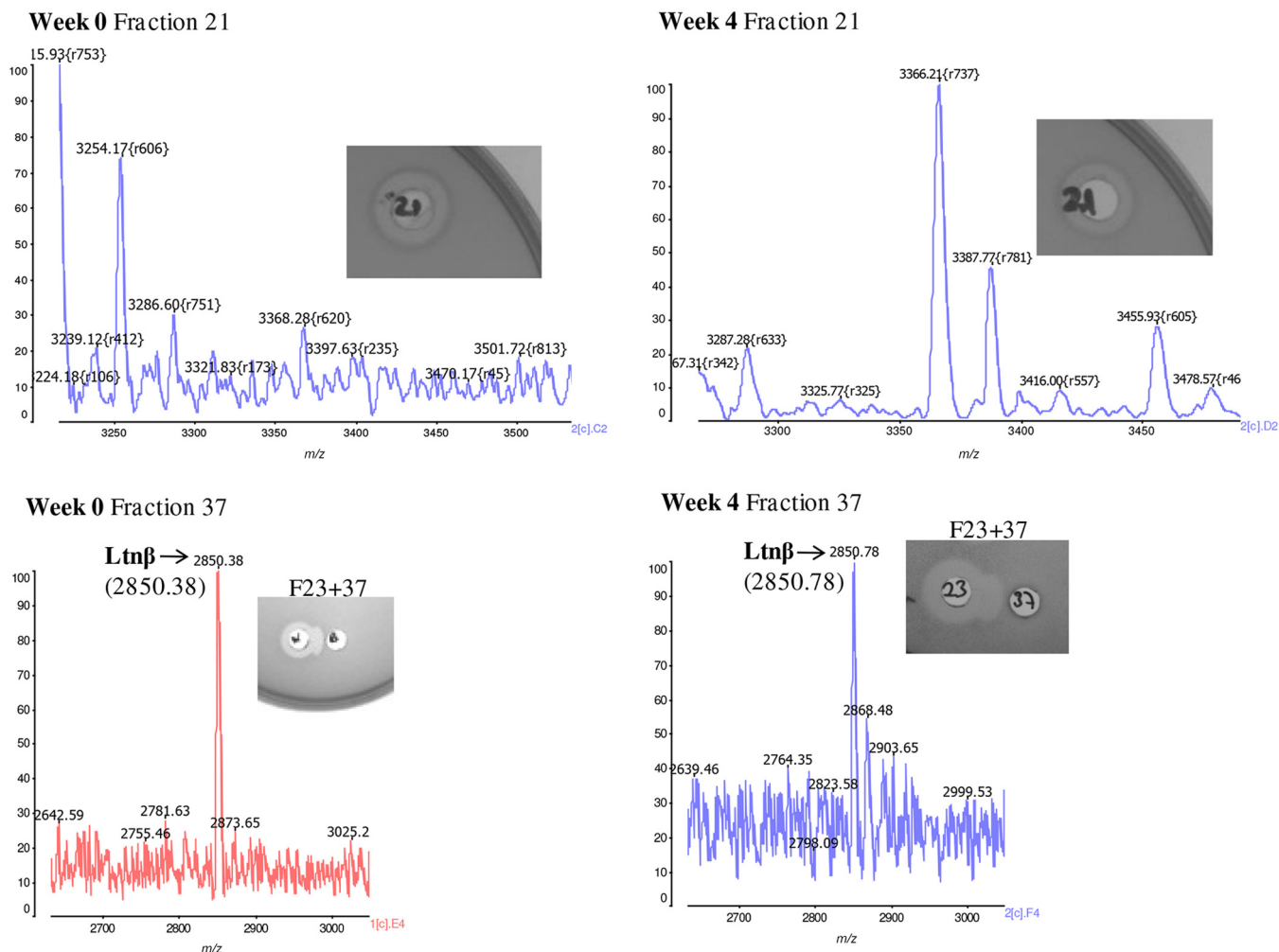


**FIG 5** MALDI-TOF MS analysis of vat 7 (lactacin, nisin, and plantaricin) (A), vat 4 (lactacin and nisin) (B), vat 5 (nisin and plantaricin) (C), and vat 6 (lactacin and plantaricin) (D). Masses corresponding to the bacteriocins are indicated. Inset photos show inhibition zones produced by correct-mass-containing fractions against the indicator strain *L. lactis* HP (nisin and lactacin) or *L. innocua* (plantaricin). F, fraction.



(B)

**Vat 4 *L. lactis* CSK3533**



**FIG 5** (Continued)

The correct mass for nisin was not detected in vat 4 (double producer) at week 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 that 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 that the peptide eluted from the high-performance liquid chromatography (HPLC) column. 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 than at week 4. In the case of lacticin, combining fractions 24 (*Ltnα*) and 37 confirmed antimicrobial activity.

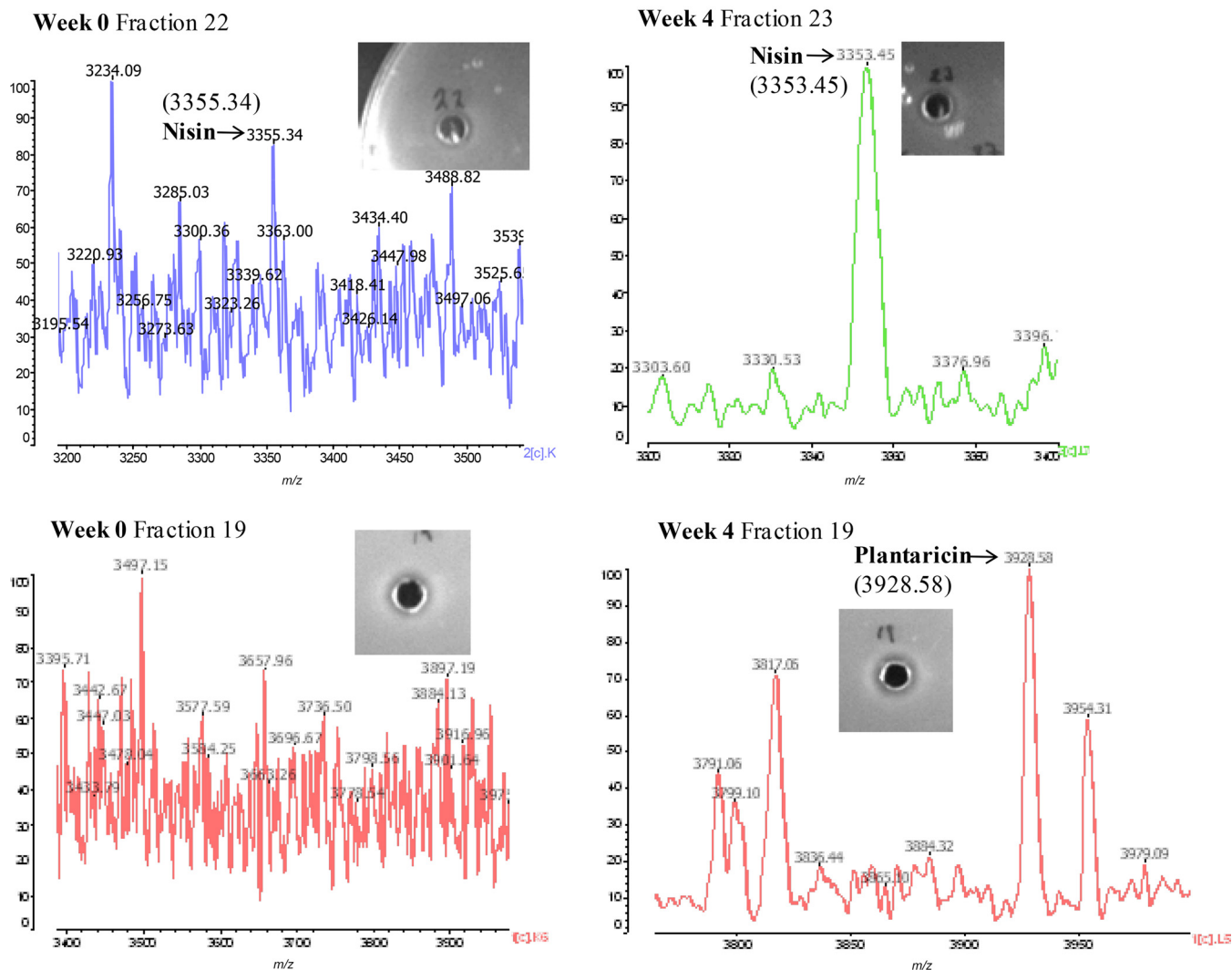
(C) **Vat 5 *L. lactis* CSK3281, *Lb. plantarum* LMG P-26358**

FIG 5 (Continued)

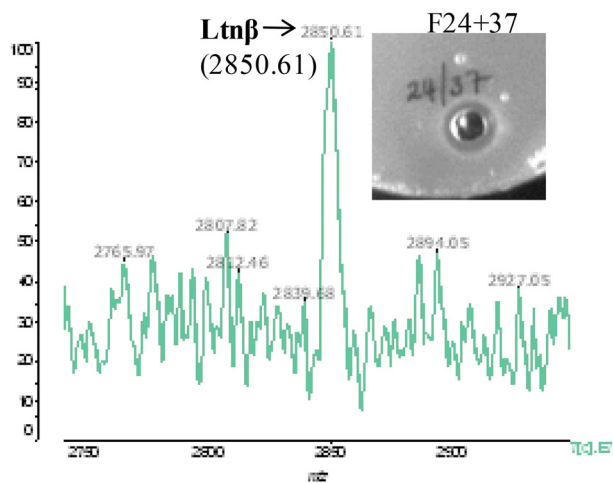
**Development of bacteriocin tolerance.** The frequency of tolerance/resistance development in *L. innocua* was assessed using 1,000 AU/ml of each bacteriocin. The frequency of resistance against 1,000 AU/ml of nisin was calculated to be  $6.56 \times 10^{-4}$ . Resistance development could not be observed when *Listeria* was exposed to 1,000 AU/ml lactacin or 1,000 AU/ml nisin and lactacin. On the other hand, the frequencies of resistance development against 320 AU/ml nisin or 320 AU/ml lactacin (representing arbitrary concentrations in cheese) were much lower, at  $4.9 \times 10^{-1}$  and  $3.02 \times 10^{-1}$ , respectively. Simultaneous exposure to lactacin and nisin at 320 AU/ml decreased the frequency of resistance to  $3.18 \times 10^{-2}$ . However, bacteriocin-resistant colonies remained sensitive to 1,000 AU/ml, indicating that *Listeria* cells were tolerant rather than completely resistant.

## DISCUSSION

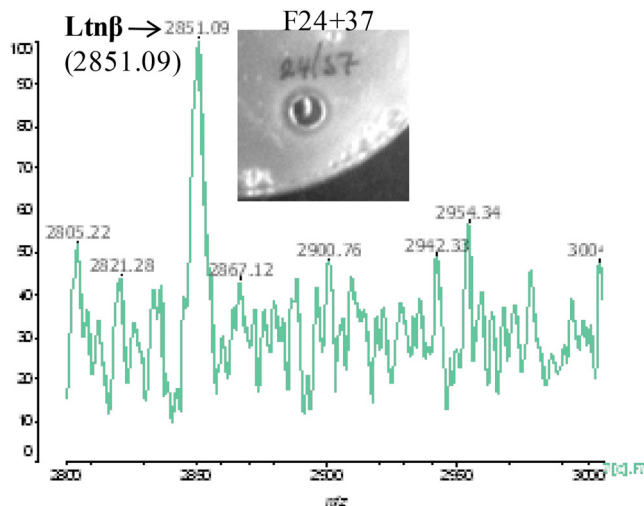
In this study, an isogenic family of nisin and lactacin transconjugants was developed with a view to better understand the impact of multiple bacteriocin production and genetic load on starter culture functionality. Genotypic and phenotypic analyses, including PCR, well diffusion assays, and CMS, confirmed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

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

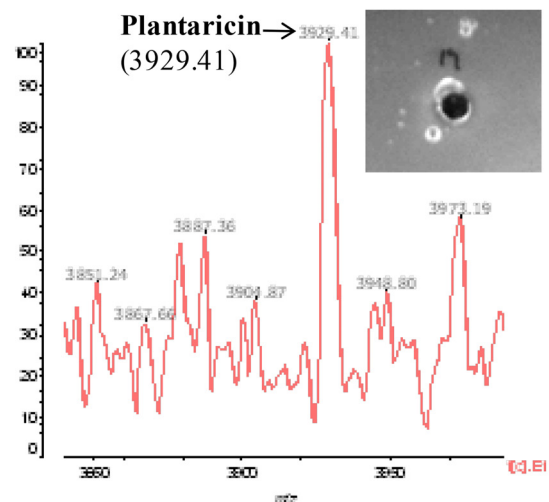
**Week 0 Fraction 37**



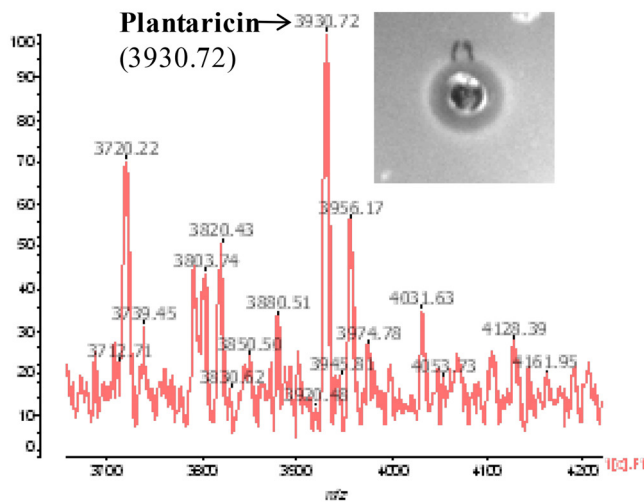
**Week 4 Fraction 37**



**Week 0 Fraction 19**



**Week 4 Fraction 19**



**FIG 5 (Continued)**

In agreement with previous findings (29) 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 lactacin transconjugant, as did the presence of the nisin transposon in the nisin transconjugant. However, bacteriocin stacking resulted in the slowest acidification rates, but this 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 (30). 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 (>50-kb) 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 (29, 31). This is supported by the fact that the addition of lactose to M17 broth maintains the lactose utilization phenotype in the double producer.

Antimicrobial activity assays confirmed that coproduction of nisin and lactacin by CSK3533 was as effective as its single-producing counterparts against most of the indicator strains tested. This indicates that bacteriocin production is not affected by the lower growth rate observed in the double producer. Furthermore, with the exception of *C. tyrobutyricum*, the coproduction of the two potent bacteriocins did not result in an increase in antimicrobial activity. Studies suggest that bacteriocin production and subsequent inhibition may be influenced by the growth medium (32, 33). The multi-bacteriocinogenic strain *L. lactis* INIA 415 was capable of producing the class I bacteriocin lactacin 481 in M17 broth only and produced nisin in milk only (32). Therefore, it is possible that growth of CSK3533 under different conditions could result in higher levels of nisin or lactacin 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 derivatives of pMRC01, which is known to harbor an abortive infection mechanism (34).

Laboratory-scale cheese inoculated with *Listeria* was used to assess the efficacy of single- and double-bacteriocin producers alone and in combination with the plantaricin producer *Lb. plantarum* LMG P-26358 *in situ*. The latter strain was previously shown to have a narrow spectrum of inhibition, inhibiting *Listeria* and enterococcal strains but not clostridia, *Escherichia coli*, *Bacillus* species, *Salmonella*, or members of the LAB (18). The strain proved to be an effective adjunct for controlling *Listeria* growth in a cheese model (18). In the present study, CSK3594, CSK3281, and CSK3533 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 compared to those for 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 the 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 lactacin transconjugant and *vice versa*, whereas the double producer is immune to both bacteriocins. The combination of the lactacin producer with *Lb. plantarum* LMG P-26358 also significantly reduced *Listeria* numbers by week 1, and by week 4, *Listeria* could not be detected in this vat. Overall, the double producer combined with the plantaricin producer, followed by the lactacin producer combined with plantaricin, exhibited the most significant reductions in *Listeria* numbers over the ripening period.

In general, a similar inhibitory trend was observed among the single class I producers, the double producer, or the combined single producers, which were significantly different from vat 1 at week 0 and week 4. While the double producer did not alter *Listeria* numbers significantly 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 lactacin, suggesting that bacteriocin stacking could be an effective method to prevent pathogen growth in food applications. However, combining bacteriocins from different classes or subclasses is considered most effective for reducing the emergence of resistance (35), which explains the increased antimicrobial efficacy for vats containing the class I and class II bacteriocins in this study.

Interestingly, cheese prepared with nonbacteriocinogenic CSK2775 also resulted in reduced *Listeria* numbers over the 4-week period, although to a lesser extent than the bacteriocin-containing cheeses. Therefore, it is probable that bacterial competition coupled with unfavorable 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 (36, 37).

MALDI-TOF MS of cheeses from vats 4, 5, 6, and 7 indicated that nisin and lactacin were present in the appropriate cheeses, implying that bacteriocin integrity was not compromised in the cheese environment. The presence of plantaricin could not be confirmed in vat 7 cheese (double producer and plantaricin) at week 4, but it was present in vats 5 and 6 at both times, as expected. MALDI-TOF MS is not quantitative and is also subject to preferential ionization in that some peptides ionize better than others. The peptide content in a cheese increases during ripening due to the breakdown of casein, so a number of bacteriocin purification steps were performed to increase the chances of detecting bacteriocin masses. Cheeses were passed through  $C_{18}$  solid-phase extraction, columns and peptides were further separated using reverse-phase HPLC (RP-HPLC). Each HPLC fraction potentially contains numerous peptides, making it difficult to detect the bacteriocin masses which are present at low concentrations. Usually the bacteriocin mass and a concomitant zone of inhibition are taken as proof of the presence of bacteriocin, but in the case of a cheese fraction, the presence of a zone of inhibition alone may be taken as indicative of bacteriocin presence.

Natural isolates capable of producing multiple bacteriocins have been reported in the literature (38–42). Most recently, *L. lactis* LMG2081 was shown to produce two different classes of bacteriocins, a novel lantibiotic and the class IIb bacteriocin lactococcin G (43). However, the ability to generate a multibacteriocin producer from an already-established culture through food-grade enabling technologies has several benefits. First, 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 harbor 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 (44, 45). Therefore, transconjugants can be used in food applications in a manner similar to that for the recipient strain (46). 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-lactacin transconjugants were unsuccessful, which was often attributed to the incompatibility of bacteriocin modification machinery or bacteriocin sensitivity (13, 34). Traditionally, the discovery of technologically valuable industrial strains has focused on large-scale screening strategies from a variety of sources (47). However, the successful transfer of lactacin and nisin to commercial starter cultures as reported in this study may provide additional avenues for the development of multihurdle protective cultures using food-grade methods.

## MATERIALS AND METHODS

**Bacterial strains and media.** The bacterial strains used in this study are listed in Tables 1 and 3. *L. lactis* strains were routinely propagated at 30°C in M17 medium (Difco Laboratories, Detroit, MI, USA) supplemented with 0.5% (wt/vol) lactose (LM17) or glucose (GM17). *Lb. plantarum* was grown in MRS medium (48) (Difco Laboratories) at 30°C. *L. innocua* was routinely propagated in GM17 broth at 37°C containing 500  $\mu$ g/ml streptomycin (Sigma-Aldrich, Ireland). Other media used in this study include brain heart infusion (BHI) broth (Oxoid Ltd., Basingstoke, Hampshire, England) and reinforced clostridial medium (RCM) (Merck, Darmstadt, Germany). All strains were stored in 50% glycerol at –20°C.

**Strain construction and analytical tests. (i) Strain construction.** The conjugation method of Coakley et al. (34) was used with slight modifications to generate lactacin 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 ( $16,000 \times g$  for 1 min) and rinsed twice with GM17 broth. After the final rinse, each strain was resuspended in 50  $\mu$ l of GM17 broth. The concentrated recipient and donor ( $20\times$ ) were then mixed with each other at ratios of 1:1, 2:1, and 20:1. Each mixture was spotted onto the center of a GM17 agar plate and incubated for 18 h at 30°C.

**TABLE 3** Bacterial strains used in this study

Bacterial strain	Relevant detail(s)	Relevant phenotype <sup>a</sup>	Source or reference
<i>L. lactis</i> HP	Bacteriocin-sensitive indicator strain	Ltn <sup>-</sup> Nis <sup>-</sup>	TFRC <sup>b</sup>
<i>L. lactis</i> MG1363 (pMRC01)	Donor strain harboring pMRC01, lactacin producer	Lac <sup>-</sup> Ltn <sup>+</sup> Nis <sup>-</sup>	TFRC
<i>L. lactis</i> CSK2583	Donor strain harboring Tn5276, nisin producer	Lac <sup>-</sup> Nis <sup>+</sup> Ltn <sup>-</sup>	CSK, The Netherlands
<i>L. lactis</i> CSK2775	Recipient strain	Lac <sup>+</sup> Nis <sup>-</sup> Ltn <sup>-</sup>	CSK, The Netherlands
<i>L. lactis</i> CSK3281	CSK2775 transconjugant, nisin producer	Lac <sup>+</sup> Nis <sup>+</sup>	This study
<i>L. lactis</i> CSK3594	CSK2775 transconjugant harboring pMRC01, lactacin producer	Lac <sup>+</sup> Ltn <sup>+</sup>	This study
<i>L. lactis</i> CSK3533	CSK3281 transconjugant harboring pMRC01, nisin-lactacin double producer	Lac <sup>+</sup> Nis <sup>+</sup> Ltn <sup>+</sup>	This study
<i>Lb. plantarum</i> LMG P-26358	Plantaricin 423 producer	Pln <sup>+</sup>	18
<i>L. lactis</i> DPC4268	Starter culture for cheese manufacture	Lac <sup>+</sup>	TFRC

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

<sup>b</sup>TFRC, Teagasc Food Research Centre, Moorepark, Fermoy, County Cork, Ireland.

The following day, spots were harvested in 1 ml of maximum recovery diluent (MRD) (Oxoid) and serially diluted before plating on LIA containing lactacin (400 AU/ml) as described previously (34). Following 48 h of incubation at 30°C, the lactacin-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. (49) with modifications. Inocula of the donor (1.5%) and recipient (2%) were grown for 4 h in GM17 broth at 30°C. The donor and recipient were then mixed at ratios of 1:10 and 1:100 in the presence of 400 µg/ml α-chymotrypsin (Sigma-Aldrich). The cells were collected onto membrane filters (0.45-µm pore size; Merck, Millipore, Darmstadt, Germany), after which the filters were placed on GM17 agar plates (cell side down). Following 18 h of incubation at 30°C, cells were harvested from the filter and added to 10% reconstituted skim milk (RSM) containing 400 AU/ml nisin (Sigma-Aldrich) and incubated at 30°C for 24 to 48 h. Clotted samples were serially diluted and plated on LIA, and following 48 h of incubation at 30°C, yellow colonies were selected for further analysis.

**(ii) Bacteriocin production and immunity.** Bacteriocin production and immunity was assessed by performing the agar well diffusion assay as described by Ryan et al. (50). Indicator organisms are listed in Table 1. Bacteriocin sensitivity was scored according to the diameter of the zone of inhibition surrounding the well which contained cell-free supernatant from the bacteriocin producer. The concentration of bacteriocin produced by the double producer was measured by agar well diffusion assay using a serial 2-fold dilution of the filtered culture supernatant, and bacteriocin activity was calculated as the inverse of the last dilution that gave a definite zone of clearance after overnight incubation, where AU were expressed per ml.

**(iii) CMS.** CMS was performed according to the method described by Field et al. (51).

**(iv) PCR scan.** Genomic DNA was extracted from 1.5 ml of 18-h cultures according to the method of Hoffman and Winston (52) slightly modified as described previously (53). Primer pairs used to scan strains for the presence of pMRC01 as well as the genes associated with nisin production are listed in Table 4. PCR was performed in a Hybaid PCR express unit (Hybaid Ltd., Middlesex, UK) using MyTaq Red Mix polymerase (Bioline Ltd., London, UK) according to the manufacturers' specifications combined with an annealing temperature of 55°C.

**(v) PFGE.** PFGE was performed as described by Mills et al. (53) using the restriction enzyme SmaI (New England BioLabs, Hertfordshire, UK). DNA fragments were run on a CHEF-DR III pulsed-field system (Bio-Rad Laboratories, Hercules, CA, USA) at 6 V/cm for 22 h with a 1- to 30-s linear ramp pulse time. Molecular size markers (N0340S and N0350S) were purchased from New England BioLabs.

**TABLE 4** Primer pairs used in this study

Primer	Sequence	Target gene(s)	Size (bp)
27-F	5'-GGGGAACAATCTTACCTA	<i>orf27</i>	326
27-R	5'-ATTATTTTTATTGCATTCTACTA		
49-F	5'-CCAATACCCGCCAAAATAAAGT	<i>orf49</i>	347
49-R	5'-CTAAGCGCAGAGGAAATACAACC		
51-F	5'-TTCTCAAATCATCAAATCAAGT	<i>orf51</i>	293
51-R	5'-GTACGAACAGGAGCGAAAAA		
52-F	5'-CCTAAGTTGTCTATTCTGTCCA	<i>orf52</i>	210
52-R	5'-ATTAGGTGAGTGCTCTGATTTTC		
nisA-F	5'-CAAAAGATTTTAACTTGGATTG	<i>nisA</i>	163
nisA-R	5'-ACGTGAATACTACAATGACAAG		
nisFG-F	5'-GGTTTAATTTCTGCAGATACTG	<i>nisFEG</i>	1,573
nisFG-R	5'-GTAATTATCCAGATCATTGCTG		

(vi) **Bacteriophage assays.** Bacteriophages were propagated according to a method outlined previously (54). Sensitivity to bacteriophage infection was performed by the double agar layer plaque assay as described previously (34).

(vii) **Characterization 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 (55).

**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% (vol/vol) into 10% (wt/vol) 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% (vol/vol) yeast extract and 0.2 g/liter  $MnSO_4 \cdot 4H_2O$  as previously reported (18).

One-liter vats of whole milk heated to 31°C were inoculated with the 18-h RSM cultures as follows: vat 1, 0.75% (vol/vol) *L. lactis* DPC4268 and 0.75% (vol/vol) *L. lactis* CSK2775 (no bacteriocin); vat 2, 0.75% (vol/vol) *L. lactis* DPC4268 and 0.75% (vol/vol) *L. lactis* CSK3281 (nisin producer); vat 3, 0.75% (vol/vol) *L. lactis* DPC4268 and 0.75% (vol/vol) *L. lactis* CSK3594 (lactacin producer); vat 4, 0.75% (vol/vol) *L. lactis* DPC4268 and 0.75% (vol/vol) *L. lactis* CSK3533 (nisin-lactacin double producer); vat 5, 0.75% (vol/vol) *L. lactis* DPC4268, 0.5% (vol/vol) *L. lactis* CSK3281 (nisin producer), and 0.5% (vol/vol) *Lb. plantarum* LMG P-26358 (plantaricin producer); vat 6, 0.75% (vol/vol) *L. lactis* DPC4268, 0.5% (vol/vol) *L. lactis* CSK3594 (lactacin producer), and 0.5% (vol/vol) *Lb. plantarum* LMG P-26358 (plantaricin producer); vat 7, 0.75% (vol/vol) *L. lactis* DPC4268, 0.5% (vol/vol) *L. lactis* CSK3533 (nisin-lactacin double producer), and 0.5% (vol/vol) *Lb. plantarum* LMG P-26358 (plantaricin producer); vat 8, 0.75% (vol/vol) *L. lactis* DPC4268, 0.5% (vol/vol) CSK3594 (lactacin producer), and 0.5% (vol/vol) CSK3281 (nisin producer); and vat 9, 0.75% (vol/vol) *L. lactis* DPC4268, 0.5% (vol/vol) CSK3594 (lactacin producer), 0.5% (vol/vol) CSK3281 (nisin producer), and 0.5% (vol/vol) *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 minutes after inoculation, 150 international milk clotting units/ml Kalase rennet (CSK Food Enrichment, The Netherlands) was added according to the 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 molds for 20 min before pressing overnight. After 24 h, the cheeses were submerged in a brine bath (23% [wt/vol] NaCl, 0.22% [vol/vol] phosphoric acid, 0.1% [wt/vol] NaOH, 0.6% [wt/vol]  $CaCl_2$ ) at 10 to 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 homogenizing 1 g of cheese in 2% sterile trisodium citrate and plating serial dilutions on selective medium (GM17 agar with 500  $\mu$ g/ml of streptomycin). The cheese trial was performed in triplicate, and sampling for each trial was performed in duplicate.

Nisin (3,352  $\pm$  3 Da), lactacin (Ltn $\beta$ ) (2,847  $\pm$  4 Da), and plantaricin (3,928  $\pm$  3 Da) present within cheese samples from vats 4, 5, 6, and 7 were verified by MALDI-TOF MS as described previously (18). In the case of lactacin, the presence of the correct mass for Ltn $\beta$  was indicative of lactacin, 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 (lactacin and nisin against *L. lactis* HP and plantaricin against *L. innocua*), where mass and concomitant activity were indicative of bacteriocin presence. Fractions expected to contain the lactacin peptides (fractions 23 and 24, Ltn $\alpha$ ; fraction 37, Ltn $\beta$ ) were combined or wells were positioned near each other to assess lactacin 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 1,000 AU/ml or 320 AU/ml of the appropriate bacteriocin or bacteriocin combination; the latter concentration represents the arbitrary *in situ* concentration of the bacteriocins in the cheeses. Plates were incubated aerobically at 37°C for up to 48 h, at which time the frequency of bacteriocin resistance/tolerance was calculated as described previously (56). All experiments were performed in triplicate.

**Statistical analysis.** *Listeria* counts in laboratory-scale cheeses were statistically analyzed using one-way analysis of variance (ANOVA). *Post hoc* multiple comparisons were done by Tukey's test, and differences were considered to be statistically significant at a *P* value of <0.05. Statistical tests were performed using XLSTAT statistical software.

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## REFERENCES

- Mills S, O'Sullivan O, Hill C, Fitzgerald GF, Ross RP. 2010. The changing face of dairy starter culture research: from genomics to economics. *Int J Dairy Technol* 63:149–170. <https://doi.org/10.1111/j.1471-0307.2010.00563.x>.
- O'Sullivan L, Morgan SM, Ross RP. 2007. Bacteriocins: changes in cheese flora and flavour, p 326–348. In Weimer BC (ed), *Improving the flavour of cheese*. Woodhead Publishing, Cambridge, England.
- O'Connor PM, Ross RP, Hill C, Cotter PD. 2015. Antimicrobial antagonists against food pathogens: a bacteriocin perspective. *Curr Opin Food Sci* 2:51–57. <https://doi.org/10.1016/j.cofs.2015.01.004>.
- Cotter PD, Hill C, Ross RP. 2005. Bacteriocins: developing innate immunity for food. *Nat Rev Microbiol* 3:777–788. <https://doi.org/10.1038/nrmicro1273>.
- Alvarez-Sieiro P, Montalbán-López M, Mu D, Kuipers OP. 2016. Bacterio-

- cins of lactic acid bacteria: extending the family. *Appl Microbiol Biotechnol* 100:2939–2951. <https://doi.org/10.1007/s00253-016-7343-9>.
6. Martínez RC, Staliano CD, Vieira AD, Villarreal ML, Todorov SD, Saad SM, Franco BD. 2015. Bacteriocin production and inhibition of *Listeria monocytogenes* by *Lactobacillus sakei* subsp. *sakei* 2a in a potentially synbiotic cheese spread. *Food Microbiol* 48:143–152. <https://doi.org/10.1016/j.fm.2014.12.010>.
  7. Diaz-Ruiz G, Omar NB, Abriouel H, Canamero MM, Galvez A. 2012. Inhibition of *Listeria monocytogenes* and *Escherichia coli* by bacteriocin-producing *Lactobacillus plantarum* EC52 in a meat sausage model system. *Afr J Microbiol Res* 6:1103–1108. <https://doi.org/10.5897/AJMR10.175>.
  8. Garde S, Avila M, Arias R, Gaya P, Nunez M. 2011. Outgrowth inhibition of *Clostridium beijerinckii* spores by a bacteriocin-producing lactic culture in ovine milk cheese. *Int J Food Microbiol* 150:59–65. <https://doi.org/10.1016/j.jfoodmicro.2011.07.018>.
  9. Gómez-Sala B, Herranz C, Díaz-Freitas B, Hernández PE, Sala A, Cintas LM. 2016. Strategies to increase the hygienic and economic value of fresh fish: biopreservation using lactic acid bacteria of marine origin. *Int J Food Microbiol* 223:41–49. <https://doi.org/10.1016/j.jfoodmicro.2016.02.005>.
  10. Mills S, Stanton C, Hill C, Ross RP. 2011. New developments and applications of bacteriocins and peptides in foods. *Annu Rev Food Sci Technol* 2:299–329. <https://doi.org/10.1146/annurev-food-022510-133721>.
  11. Horn N, Martínez MI, Martínez JM, Hernández PE, Gasson MJ, Rodríguez JM, Dodd HM. 1999. Enhanced production of pediocin PA-1 and coproduction of nisin and pediocin PA-1 by *Lactococcus lactis*. *Appl Environ Microbiol* 65:4443–4450.
  12. Reviriego C, Fernández L, Rodríguez JM. 2007. A food-grade system for production of pediocin PA-1 in nisin-producing and non-nisin-producing *Lactococcus lactis* strains: application to inhibit *Listeria* growth in a cheese model system. *J Food Prot* 70:2512–2517. <https://doi.org/10.4315/0362-028X-70.11.2512>.
  13. O'Sullivan L, Ryan MP, Ross RP, Hill C. 2003. Generation of food-grade lactococcal starters which produce the lantibiotics lactacin 3147 and lactacin 481. *Appl Environ Microbiol* 69:3681–3685. <https://doi.org/10.1128/AEM.69.6.3681-3685.2003>.
  14. Breukink E, Wiedemann I, van Kraaij C, Kuipers OP, Sahl HG, de Kruijff B. 1999. Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. *Science* 286:2361–2364. <https://doi.org/10.1126/science.286.5448.2361>.
  15. Lubelski J, Rink R, Khusainov R, Moll GN, Kuipers OP. 2008. Biosynthesis, immunity, regulation, mode of action and engineering of the model lantibiotic nisin. *Cell Mol Life Sci* 65:455–476. <https://doi.org/10.1007/s00018-007-7171-2>.
  16. Wiedemann I, Bottiger T, Bonelli RR, Wiese A, Hagge SO, Gutschmann T, Seydel U, Deegan L, Hill C, Ross P, Sahl HG. 2006. The mode of action of the lantibiotic lactacin 3147—a complex mechanism involving specific interaction of two peptides and the cell wall precursor lipid II. *Mol Microbiol* 61:285–296. <https://doi.org/10.1111/j.1365-2958.2006.05223.x>.
  17. Suda S, Cotter PD, Hill C, Ross RP. 2012. Lactacin 3147—biosynthesis, molecular analysis, immunity, bioengineering and applications. *Curr Protein Pept Sci* 13:193–204. <https://doi.org/10.2174/138920312800785021>.
  18. Mills S, Serrano LM, Griffin C, O'Connor PM, Schaad G, Bruining C, Hill C, Ross RP, Meijer WC. 2011. Inhibitory activity of *Lactobacillus plantarum* LMG P-26358 against *Listeria innocua* when used as an adjunct starter in the manufacture of cheese. *Microb Cell Fact* 10(Suppl 1):S7. <https://doi.org/10.1186/1475-2859-10-S1-57>.
  19. Cui Y, Zhang C, Wang Y, Shi J, Zhang L, Ding Z, Qu X, Cui H. 2012. Class IIa bacteriocins: diversity and new developments. *Int J Mol Sci* 13:16668–16707. <https://doi.org/10.3390/ijms131216668>.
  20. Van Tassel ML, Ibarra-Sánchez LA, Takhar SR, Amaya-Llano SL, Miller MJ. 2015. Use of a miniature laboratory fresh cheese model for investigating antimicrobial activities. *J Dairy Sci* 98:8515–8524. <https://doi.org/10.3168/jds.2015-9967>.
  21. Schelegueda LI, Delcarlo SB, Gliemmo MF, Campos CA. 2016. Effect of antimicrobial mixtures and modified atmosphere packaging on the quality of Argentine hake (*Merluccius hubbsi*) burgers. *LWT Food Sci Technol* 68:258–264. <https://doi.org/10.1016/j.lwt.2015.12.012>.
  22. Olle Resa CP, Gerschenson LN, Jagus RJ. 2014. Natamycin and nisin supported on starch edible films for controlling mixed culture growth on model systems and Port Salut cheese. *Food Control* 44:146–151. <https://doi.org/10.1016/j.foodcont.2014.03.054>.
  23. Fernández MV, Jagus RJ, Mugliaroli SL. 2014. Effect of combined natural antimicrobials on spoilage microorganisms and *Listeria innocua* in a whey cheese “Ricotta.” *Food Bioprocess Technol* 7:2528–2537. <https://doi.org/10.1007/s11947-013-1243-0>.
  24. Mingming G, Jin TZ, Wang L, Scullen J, Sommers CH. 2014. Antimicrobial films and coatings for inactivation of *Listeria innocua* on ready-to-eat deli turkey meat. *Food Control* 40:64–70. <https://doi.org/10.1016/j.foodcont.2013.11.018>.
  25. Morgan SM, Ross RP, Beresford T, Hill C. 2000. Combination of hydrostatic pressure and lactacin 3147 causes increased killing of *Staphylococcus* and *Listeria*. *J Appl Microbiol* 88:414–420. <https://doi.org/10.1046/j.1365-2672.2000.00975.x>.
  26. Scannell AG, Ross RP, Hill C, Arendt K. 2000. An effective lactacin bio-preservative in fresh pork sausage. *J Food Prot* 63:370–375. <https://doi.org/10.4315/0362-028X-63.3.370>.
  27. Soriano A, Ulmer HM, Scannell AGM, Ross RP, Hill C, Garcia-Ruiz A, Arendt EK. 2004. Control of food spoiling bacteria in cooked meat products with nisin, lactacin 3147, and a lactacin 3147-producing starter culture. *Eur Food Res Technol* 219:6–13. <https://doi.org/10.1007/s00217-004-0910-9>.
  28. Scannell AG, Hill C, Ross RP, Marx S, Hartmeier W, Elke Arendt K. 2000. Development of bioactive food packaging materials using immobilised bacteriocins lactacin 3147 and nisaplin. *Int J Food Microbiol* 60:241–249. [https://doi.org/10.1016/S0168-1605\(00\)00314-7](https://doi.org/10.1016/S0168-1605(00)00314-7).
  29. Fallico V, McAuliffe O, Fitzgerald GF, Hill C, Ross RP. 2009. The presence of pMRC01 promotes greater cell permeability and autolysis in lactococcal starter cultures. *Int J Food Microbiol* 133:217–224. <https://doi.org/10.1016/j.jfoodmicro.2009.04.029>.
  30. Smith JS, Hillier AJ, Lees GJ. 1975. The nature of the stimulation of the growth of *Streptococcus lactis* by yeast extract. *J Dairy Res* 42:123–138. <https://doi.org/10.1017/S0022029900015156>.
  31. Friehs K. 2004. Plasmid copy number and plasmid stability. *Adv Biochem Eng Biotechnol* 86:47–82.
  32. Bravo D, Rodríguez E, Medina M. 2009. Nisin and lactacin 481 coproduction by *Lactococcus lactis* strains isolated from raw ewes' milk. *J Dairy Sci* 92:4805–4811. <https://doi.org/10.3168/jds.2009-2237>.
  33. Avonts L, E Van Uytven, De Vuyst L. 2004. Cell growth and bacteriocin production of probiotic *Lactobacillus* strains in different media. *Int Dairy J* 14:947–955. <https://doi.org/10.1016/j.idairyj.2004.04.003>.
  34. Coakley M, Fitzgerald G, Ross RP. 1997. Application and evaluation of the bacteriophage resistance- and bacteriocin-encoding plasmid pMRC01 for the improvement of dairy starter cultures. *Appl Environ Microbiol* 63:1434–1440.
  35. Bastos Mdo C, Coelho ML, Santos OC. 2015. Resistance to bacteriocins produced by Gram-positive bacteria. *Microbiology* 161:683–700. <https://doi.org/10.1099/mic.0.082289-0>.
  36. Donnelly CW. 2004. Growth and survival of microbial pathogens in cheese, p 541–560. *In* Fox PF, McSweeney PLH, Cogan TM, Guinee TP (ed), *Cheese chemistry, physics and microbiology*, 3rd edition, vol 1. Elsevier Academic Press, London, England.
  37. Johnson EA, Nelson JH, Johnson M. 1990. Microbiological safety of cheese made from heat-treated milk, part II. *Microbiology J Food Prot* 53:519–540. <https://doi.org/10.4315/0362-028X-53.6.519>.
  38. O'Shea EF, O'Connor PM, Raftis EJ, O'Toole PW, Stanton C, Cotter PD, Ross RP, Hill C. 2011. Production of multiple bacteriocins from a single locus by gastrointestinal strains of *Lactobacillus salivarius*. *J Bacteriol* 193:6973–6982. <https://doi.org/10.1128/JB.06221-11>.
  39. Quadri LE, Sailer M, Roy KL, Vederas JC, Stiles ME. 1994. Chemical and genetic characterization of bacteriocins produced by *Carnobacterium piscicola* LV17B. *J Biol Chem* 269:12204–12211.
  40. Rodríguez E, González B, Gaya P, Nuñez M, Medina M. 2000. Diversity of bacteriocins produced by lactic acid bacteria isolated from raw milk. *Int Dairy J* 10:7–15. [https://doi.org/10.1016/S0958-6946\(00\)00017-0](https://doi.org/10.1016/S0958-6946(00)00017-0).
  41. Kojic M, Strahinic I, Fira D, Jovic B, Topisirovic L. 2006. Plasmid content and bacteriocin production by five strains of *Lactococcus lactis* isolated from semi-hard homemade cheese. *Can J Microbiol* 52:1110–1120. <https://doi.org/10.1139/w06-072>.
  42. Himeno K, Fujita K, Zendo T, Wilaipun P, Ishibashi N, Masuda Y, Yoneyama F, Leelawatcharamas V, Nakayama J, Sonomoto K. 2012. Identification of enterocin NKR-5-3C, a novel Class IIa bacteriocin produced by a multiple bacteriocin producer, *Enterococcus faecium* NKR-5-3. *Biosci Biotechnol Biochem* 76:1245–1247. <https://doi.org/10.1271/bbb.120089>.
  43. Mirkovic N, Polovic N, Vukotic G, Jovic B, Mijlkovic M, Radulovic Z, Diep



- DB, Kojic M. 2016. *Lactococcus lactis* LMG2081 produces two bacteriocins, a nonlantibiotic and a novel lantibiotic. *Appl Environ Microbiol* 82:2555–2562. <https://doi.org/10.1128/AEM.03988-15>.
44. Derx PMF, Janzen T, Sørensen KI, Christensen JE, Stuer-Lauridsen B, Johansen E. 2014. The art of strain improvement of industrial lactic acid bacteria without the use of recombinant DNA technology. *Microb Cell Fact* 13(Suppl 1):S5. <https://doi.org/10.1186/1475-2859-13-S1-S5>.
45. Pedersen MB, Iversen SL, Sørensen KI, Johansen E. 2005. The long and winding road from the research laboratory to industrial applications of lactic acid bacteria. *FEMS Microbiol Rev* 29:611–624. <https://doi.org/10.1016/j.fmrre.2005.04.001>.
46. Hill C, Ross RP. 1998. Starter cultures for the dairy industry, p 174–192. In Roller S, Harlander S (ed), *Genetic modification in the food industry: a strategy for food quality improvement*. Springer Science, Dordrecht, The Netherlands.
47. Hansen EB. 2002. Commercial bacterial starter cultures for fermented foods of the future. *Int J Food Microbiol* 78:119–131. [https://doi.org/10.1016/S0168-1605\(02\)00238-6](https://doi.org/10.1016/S0168-1605(02)00238-6).
48. De Man JC, Rogosa M, Sharpe ME. 1960. A medium for the cultivation of lactobacilli. *J Appl Microbiol* 23:130–135.
49. Gireesh T, Davidson BE, Hillier AJ. 1992. Conjugal transfer in *Lactococcus lactis* of a 68-kilobase-pair chromosomal fragment containing the structural gene for the peptide bacteriocin nisin. *Appl Environ Microbiol* 58:1670–1676.
50. Ryan MP, Rea MC, Hill C, Ross RP. 1996. An application in Cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147. *Appl Environ Microbiol* 62:612–619.
51. Field D, Begley M, O'Connor PM, Daly KM, Hugenholtz F, Cotter PD, Hill C, Ross RP. 2012. Bioengineered nisin A derivatives with enhanced activity against both Gram positive and Gram negative pathogens. *PLoS One* 7:e46884. <https://doi.org/10.1371/journal.pone.0046884>.
52. Hoffman CS, Winston F. 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene* 57:267–272. [https://doi.org/10.1016/0378-1119\(87\)90131-4](https://doi.org/10.1016/0378-1119(87)90131-4).
53. Mills S, Griffin C, Coffey A, Meijer WC, Hafkamp B, Ross RP. 2010. CRISPR analysis of bacteriophage insensitve mutants (BIMs) of *Streptococcus thermophilus*—implications for starter design. *J Appl Microbiol* 108: 945–955. <https://doi.org/10.1111/j.1365-2672.2009.04486.x>.
54. Mills S, Coffey A, McAuliffe OE, Meijer WC, Hafkamp B, Ross RP. 2007. Efficient method for generation of bacteriophage insensitive mutants of *Streptococcus thermophilus* yoghurt and mozzarella strains. *J Microbiol Methods* 70:159–164. <https://doi.org/10.1016/j.mimet.2007.04.006>.
55. Harrington A, Hill C. 1991. Construction of a bacteriophage-resistant derivative of *Lactococcus lactis* subsp. *lactis* 425A by using the conjugal plasmid pNP40. *Appl Environ Microbiol* 57:3405–3409.
56. Gravesen A, Jydegaard Axelsen AM, Mendes da Silva J, Hansen TB, Knochel S. 2002. Frequency of bacteriocin resistance development and associated fitness costs in *Listeria monocytogenes*. *Appl Environ Microbiol* 68:756–764. <https://doi.org/10.1128/AEM.68.2.756-764.2002>.