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Control of Cheese Microflora using Bacteriocins

R.P. Ross and C. Hill

Lacticin 3147 - a natural antimicrobial agent, can be used to control microflora development during cheese-making to enhance flavour and improve product quality and consistency.

Control of Cheese Microflora using Bacteriocins

Armis No. 4542

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Summary and Conclusions

Bacteriocins are proteins, produced by some bacteria which are capable of inhibiting other bacteria. The overall aim of this project was the development and exploitation of bacteriocins such as Lacticin 3147 (produced by a food-grade microorganism), as biological tools to control the microflora of foods.

Lacticin 3147-producing strains were evaluated for their ability to improve the microbial quality of a variety of dairy products and in particular, Cheddar cheese. The manipulation of cheese flora using bacteriocins should offer manufacturers greater control in the consistency and quality of the final product, in addition to improving its safety.

In concert with these studies, Lacticin 3147 was studied in detail at the molecular level resulting in its biochemical and genetic analysis. These studies have demonstrated the complexity and uniqueness of this potent antimicrobial.

Main conclusions and achievements

* *Lacticin 3147 was shown to inhibit the dangerous food pathogen* Listeria monocytogenes *Scott A in Cottage cheese.*

The study demonstrated the effectiveness of Lacticin 3147 as an inhibitor of L. monocytogenes *in a food system where post-manufacture contamination by this organism could present a risk to the consumer.*

* *Commercial cheese starter strains were successfully manipulated to produce Lacticin 3147 without significantly affecting their cheese-making properties. This was confirmed by commercial scale trials.*

Simultaneously, Lacticin 3147-tolerant adjunct cultures were developed to be used in conjunction with the Lacticin 3147-producing starter strains, for enhancement of cheese flavour.

This new technology facilitates greater control over the growth of cheese microflora, which in turn should enhance flavour, quality and consistency.

** Reduced fat Cheddar cheese manufactured with a Lacticin 3147 producer can be ripened at an elevated temperature which can be difficult to achieve with traditional starters due to spoilage by non-starter populations.*

** Biochemical analysis has identified unusual residues such as lanthionine, dehydrated amino acids and D-alanine residues in Lacticin 3147. This is the first two-component, D-alanine containing lantibiotic reported to date, demonstrating the uniqueness of this potent antimicrobial.*

** The genes involved in Lacticin 3147 production and immunity have been cloned, sequenced and through mutational analysis, have been functionally characterised. Such analyses have demonstrated that:*

- The two component peptides of Lacticin 3147 require separate modification enzymes for activity.

- The gene ltnI *has been identified as conferring immunity to Lacticin 3147.*

- Lacticin 3147 uses a regulatory mechanism not previously identified in lantibiotic systems.

- A role for Lacticin 3147 as a food-grade selectable marker has been demonstrated.

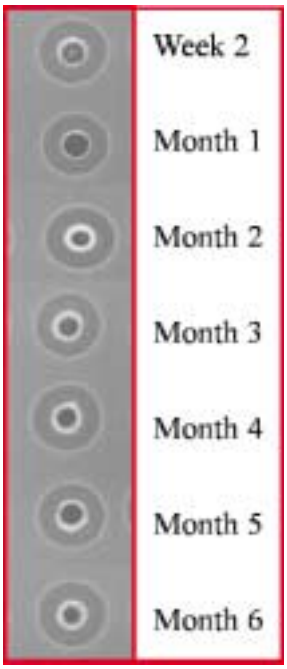

Fig. 1: Lacticin 3147 activity in cheese during 6 months of ripening.

Research and Results

Members of the lactic acid bacteria play an essential role in a wide variety of food fermentations with *Lactococcus* subsp. being the primary starter cultures exploited for Cheddar cheese manufacture. In addition, a population of adventitious microflora, otherwise known as non-starter lactic acid bacteria (NSLAB) can proliferate during ripening and often form the dominant flora in the cheese.

The precise role of NSLAB strains in flavour development remains unclear, however whether positive or negative, they certainly contribute to the unpredictability associated with Cheddar cheese quality. Importantly, NSLABs have also been associated with defects in cheese such as the formation of calcium lactate crystals and slit formation.

Given increasing consumer demand, the challenge now facing the cheesemaker is the development of a safe, well-flavoured, consistent Cheddar. One approach to improving cheese flavour has been the deliberate addition of selected, adjunct lactobacilli during manufacture which would impart beneficial qualities. Although some studies reported cheese with improved flavour, other adjunct cultures were responsible for flavour defects. In addition, comparisons with control vats were difficult to interpret due to contamination with the adjunct strain or with adventitious NSLABs, which eventually reach levels similar to the test vat.

Hence, in order to study the relevance of NSLABs, as well as examining the role of adjunct cultures in Cheddar flavour, there is a requirement for an effective and simple method of controlling adventitious strains during manufacture and ripening.

Consequently, the known ability of Lacticin 3147-producing lactococcal starters to inhibit NSLABs during cheese ripening has been exploited and evaluated in these studies.

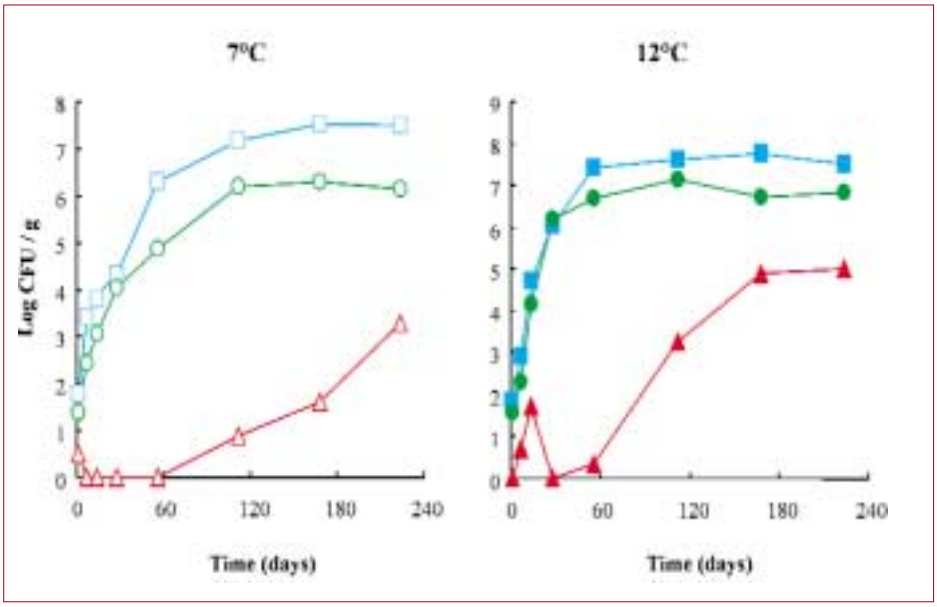

Fig. 2: Growth of non-starter lactic acid bacteria in reduced fat Cheddar cheese - a) open symbols: ripened at 7ºC and b) filled symbols: ripened at 12ºC. Blue and green lines represent counts for control cheeses, manufactured in the absence of Lacticin 3147 producng strains. Red lines represent counts of non-starter lactic acid bacteria in cheese manufactured with a Lacticin 3147 producer.

Use of Lacticin-producing starters in cheese-making

Cheddar

The bacteriocin Lacticin 3147 inhibits a broad-range of Gram positive bacteria including food pathogenic and spoilage organisms such as *Listeria*, *Clostridia* and *Staphylococci* and formed the focus of this research project. The genes encoding Lacticin 3147 are located on a conjugative plasmid, pMRC01 and during the course of this project the genetic determinants for Lacticin 3147 were transferred to a range of commercial cheese starters with the aim of generating a bank of improved bacteriocin-producing starter cultures.

Importantly these strains are not GMOs as they were generated by natural matings between bacteria.

These Lacticin 3147 producing strains were evaluated for their ability to influence the developing non-starter bacterial population in cheese.

In all pilot and commercial scale trials performed, results indicate that these strains can be used to successfully manufacture Cheddar as they reduce the pH within the desired time period. Furthermore, the level of Lacticin 3147 in the cheese remained constant throughout ripening and correlated with significant reductions in the levels of adventitious microflora.

Reduced Fat Cheddar

A Lacticin 3147-producing transconjugant strain was also evaluated in reduced fat Cheddar which was ripened at the elevated temperature of 12ºC with the aim of controlling NSLAB populations.

Pilot-scale trials demonstrated that even though the rate of NSLAB growth was significantly increased at this temperature, sufficient Lacticin 3147 was produced to markedly reduce NSLAB proliferation in experimental vats, thereby providing a means of controlling the developing microflora at increased ripening temperatures. The significance of these results is that cheese may be ripened at higher temperatures if Lacticin 3147 producing starters are used in manufacture. This may yield improvements in flavour/quality since the vast majority of NSLABs are inhibited by Lacticin 3147.

Mould- and Smear-Ripened Cheese

In addition to their contribution to cheese quality, Lacticin 3147-producing strains were also evaluated as surface protection cultures for the inhibition of *Listeria* in mould- and smear-ripened cheese. In recent years there has been a number of high profile outbreaks of foodborne illness due to ingestion of food contaminated with the pathogenic microorganism *Listeria*

monocytogenes. For this reason, a Lacticin 3147-producing strain was investigated as a protective culture on the surface of mould-ripened and smear-ripened cheese. The Lacticin 3147-producing strain was sprayed onto the surface of the cheese which was subsequently spiked with *Listeria monocytogenes*.

During ripening, microbiological analysis revealed significant reductions in levels of listerial contamination in both cheese types. The studies demonstrate the potential of Lacticin 3147 producing strains for the protection of cheese from contamination by Listeria*.*

Cottage Cheese

The efficacy of using a Lacticin 3147-producing starter to protect Cottage cheese against contamination with the harmful human pathogen *L. monocytogenes* Scott A was also investigated. A Lacticin 3147-producing strain was used as a starter in the manufacture of Cottage cheese which was spiked with approximately 10^4 cfu/g *L. monocytogenes* Scott A.

Fig. 3: Mould-ripened cheese.

Lacticin 3147 activity was detected throughout the 6-day storage period and was associated with 1,000-fold reductions in Listeria *numbers in cheese stored at 4ºC. The kill rate was even more rapid in cheeses stored at higher temperatures. These results demonstrate the effectiveness of Lacticin 3147 as an inhibitor of* L. monocytogenes *in a food system where post-manufacture contamination by this organism could be problematic.*

Development of Cheese Adjunct Strains Tolerant to Lacticin 3147

In order to further manipulate cheese microflora, a *Lactobacillus* strain with increased tolerance to Lacticin 3147 was generated by subculturing in increasing concentrations of bacteriocin. This strain, *Lactobacillus paracasei* DPC5337 was assessed as a cheese-making adjunct with a Lacticin 3147-producing starters and its performance was compared to the sensitive parent, *Lb. paracasei* DPC5336.

Pilot-scale trials were performed and in each case, the level of Lacticin 3147 activity in the cheese correlated with a reduction in NSLAB proliferation

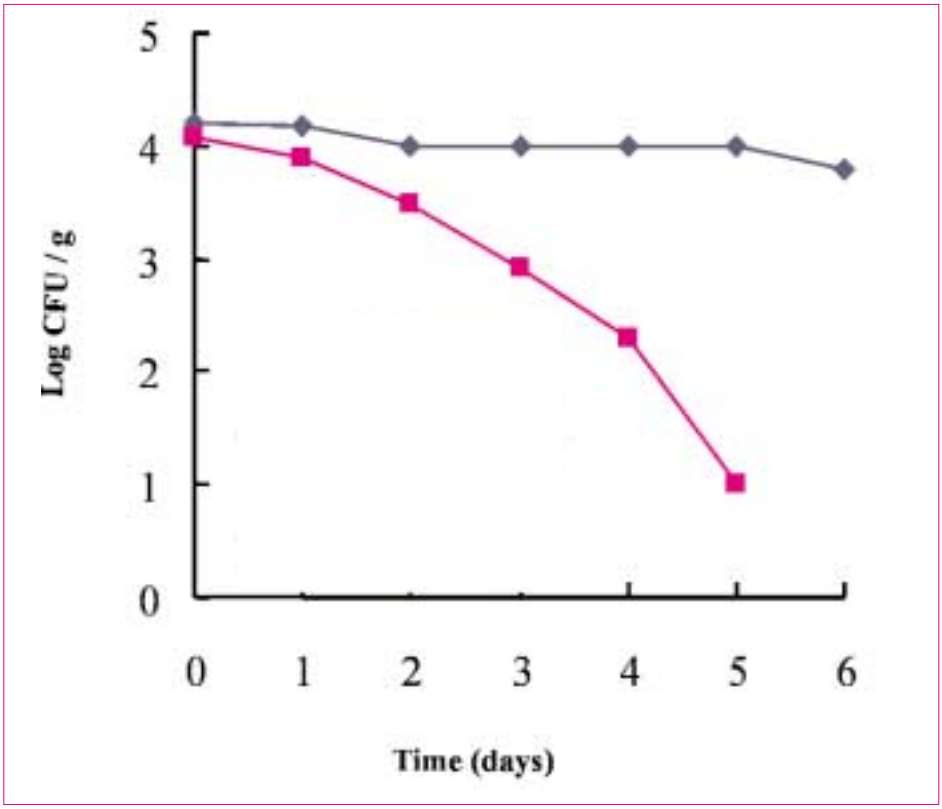

Fig. 4: The survival of Listeria monocytogenes *in Cottage cheese, manufactured in the presence (■) and absence (◆) of a Lacticin 3147-producing culture.*

throughout ripening. In contrast, the tolerant adjunct strain survived the concentration of Lacticin 3147 in the cheese, reaching counts of 10^7 CFU/g during ripening, compared to the sensitive strain which was present at 10^4 to 10^5 CFU/g. RAPD-PCR was employed to demonstrate that the tolerant adjunct strain comprised the dominant microflora in the experimental cheeses.

Hence, the use of Lacticin 3147-producing cultures in combination with tolerant adjuncts may be exploited to yield a more predictable cheese microflora which concomitantly improves product consistency. Importantly, this example demonstrates the versatility of Lacticin 3147 in providing a useful tool to the cheesemaker, for the microbial control of cheese during manufacture and ripening.

Expression of Lacticin 3147 in *Enterococcus faecalis*

Many hospital-acquired infections such as endocarditis have been linked to *Enterococcus faecalis*, several strains of which produce a substance with combined haemolytic/bactericidal activity, termed cytolysin. Cytolysin is composed of two peptides, both of which contain lanthionines. These peptides are encoded by large, pheromone-responsive, conjugative plasmids such as pAD1, indicating a potential for transfer of cytolytic capabilities to other bacteria. Given its associated toxic nature, concern has been raised for future protein engineering of lantibiotics since such alterations could conceivably cause previously non-toxic bacteriocins to acquire an activity against mammalian cells.

Alternatively, it has been suggested that cytolysin may form the basis of a potential therapeutic target in the control of such infections.

Although cytolysin was the first two-peptide bacteriocin to be identified among the lantibiotics, the more recent identification of Lacticin 3147 and *Staphylococcin* C55 suggests that these complex, two-component systems may be more widespread. Given the clinical significance of enterococcal strains expressing cytolysin, it is of interest to compare the biological activity of this bacteriocin to other two-component lantibiotic systems.

The use of Lacticin 3147 as a biological tool for ensuring a predictable microflora in Cheddar cheese.

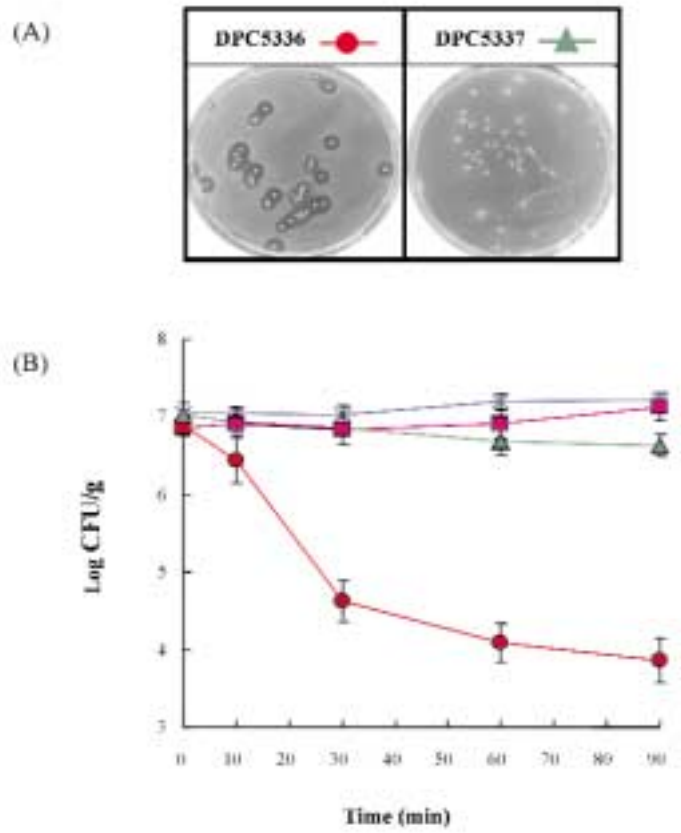

Fig. 5: (A) The agar plates contain isolated colonies of the Lacticin 3147-producing strain, Lactococcus lactis *DPC3147 which are overlaid with a lawn of* Lactobacillus paracasei *DPC5336 and DPC5337, respectively. The absence of zones of inhibition in the* Lb. paracasei *DPC5337 lawn indciates that this strain is tolerant to the levels of Lacticin 3147 produced by* L. lactis *DPC3147 colonies while strain* Lb. paracasei *DPC5336 remains sensitive. (B) The graph represents the bactericidal activity of Lacticin 3147 on* Lb.paracasei *DPC5336 (●) and DPC5337 (▲). Bacteriocin was added at a final concentration of 1,000 AU/ml to washed log-phase cells. Controls to which no bacteriocin was added are represented by* Lb. paracasei *DPC5336 (■) and DPC5337 (×).*

In particular, since Lacticin 3147 and Lacticin 3147-producing strains show considerable promise in food and biomedical applications it is important to determine whether there are any undesirable features associated with this lantibiotic.

Although Lacticin 3147 is produced by a strain commonly used as a starter in food fermentations and has been consumed safely for many years, it is imperative to establish that the genes encoding this lantibiotic do not confer toxic activity. Hence, a comparative study between the biological activities of Lacticin 3147 and cytolysin was carried out.

A plasmid containing the entire Lacticin 3147-encoding region was constructed and designated pOM02. Expression of cytolysin (encoded by pAD1) and Lacticin 3147 (encoded by pOM02) in an enterococcal background facilitated the comparison of their respective activities against eukaryotic and bacterial cells.

A study was performed to compare the biological activity of Lacticin 3147 with cytolysin. The Lacticin 3147 encoding determinants were heterologously expressed in *Enterococcus faecalis* FA2-2, a plasmid free strain, to generate *Enterococcus faecalis* pOM02, thereby facilitating a direct comparison with *Enterococcus faecalis* FA2-2.pAD1, a cytolysin producer.

Both heterologously expressed Lacticin 3147 and cytolysin exhibited a broad spectrum of activity against bacterial targets. Furthermore, enterococci expressing active Lacticin 3147 did not exhibit haemolytic activity against equine blood cells.

The results indicated that the Lacticin 3147 biosynthetic machinery can be heterologously expressed in an enterococcal background resulting in the production of the bacteriocin with no detectable haemolytic activity.

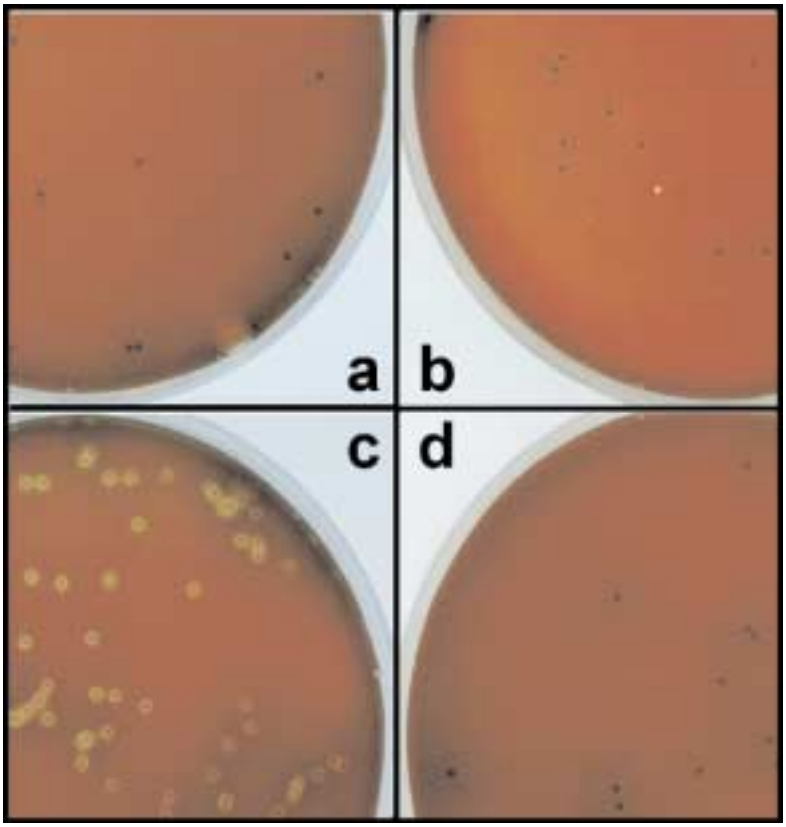

Fig. 6: Haemolytic assays demonstrate that heterologous expression of Lacticin 3147 in Enterococcus faecalis *FA2-2 does not result in lysis of equine bloods cells (d). The parent Lacticin 3147-producer strain* Lactococcus lactis *DPC3147 (a) and the plasmid-free strain,* Enterococcus faecalis *FA2-2 (b) were plated as controls and do not exhibit haemolysis. However, isolated colonies of* Enterococcus faecalis *FA2-2 pAD1, (cytolysin-producing) gave rise to distinct zones of clearing as indciated in plate c.*

Biochemical and Genetic Analysis of Lacticin 3147

- Lacticin 3147 is a novel, two component, D-alanine containing lantibiotic that undergoes extensive post-translational modification which may account for its potent antimicrobial activity against a wide range of Gram-positive bacteria.

- Production and immunity of Lacticin 3147 is encoded by the 60.2 plasmid pMRC01. The gene ltnI*, encodes a 116 amino acid protein and confirmation of the role of* ltnI *in immunity was obtained when it was observed that disruption of* ltnI *resulted in a complete loss of immunity.*

- A role for Lacticin 3147 as a food-grade selectable marker has been demonstrated. Results indicate that high expression levels of the immunity gene, ltnI*, confers a level of immunity which can be used to select for transformants in the absence of an antibiotic selection.*

- Each peptide of the two component lantibiotic Lacticin 3147 requires a separate modification enzyme for activity. Creation of a number of double mutants confirmed that LtnM1 is required to produce mature LtnA1, while LtnM2 is required to produce mature LtnA2.

- Lacticin 3147 uses a regulatory mechanism not previously identified in lantibiotic systems for the regulation of immunity.

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