Use of Bacteriocins to Improve Cheese Quality and Safety

Armis No. 4207

Project Team:

Paul Ross and Colin Hill (Leaders)
Máire Ryan
Alan Cunniffe
Olivia McAuliffe
Deirdre Murray
Triona O'Keefe
Mary Rea

The Dairy Products Research Centre
Moorepark, Fermoy, Co. Cork

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Summary and Conclusions

Bacteriocins are inhibitory peptides produced by a number of Lactic Acid Bacteria capable of killing other bacteria. These natural inhibitors have widespread applications in the preservation of foods, since they can kill a number of pathogenic and spoilage bacteria. A novel broad spectrum bacteriocin designated lacticin 3147*was discovered as part of this project. The bacterial strain which produces lacticin 3147 (*Lactococcus lactis*subsp.*lactis*DPC3147) is food-grade and similar to strains used for commercial cheese making. Lacticin 3147 is effective in the inhibition of all Gram-positive bacteria tested including the food pathogens* Listeria monocytogenes *and* Staphylococcus aureus *and food spoilage bacteria such as* Clostridia*and* Bacillus *species. In a collaborative effort between the Microbiology Department, UCC and the Dairy Products Research Centre, Moorepark a detailed genetic and biochemical characterisation of this bacteriocin has been performed, which demonstrates that it is a unique bacteriocin composed of two individual peptides. The genetic determinants encoding lacticin 3147 have been transferred to a range of lactococcal starter cultures, conferring on these strains the ability to control the microflora of fermented products, through the production of the bacteriocin. For example lacticin 3147 producing starters have the ability to reduce or eliminate non-starter lactic acid bacteria during cheese ripening, or to eliminate the pathogen* Listeria monocytogenes *from certain susceptible varieties of cheese. (The elimination of* L. monocytogenes*is of extreme importance given that the fatality rate for individuals infected with this organism is 30-40%).*

In a different study, a narrow spectrum bacteriocin producer which secretes three bacteriocins (lactococcin A, B and M) was used in the manufacture of Cheddar cheese. The combined effect of the three bacteriocins induced lysis of the companion starter cells, resulting in release of intracellular enzymes. Increased starter cell lysis resulted in improved

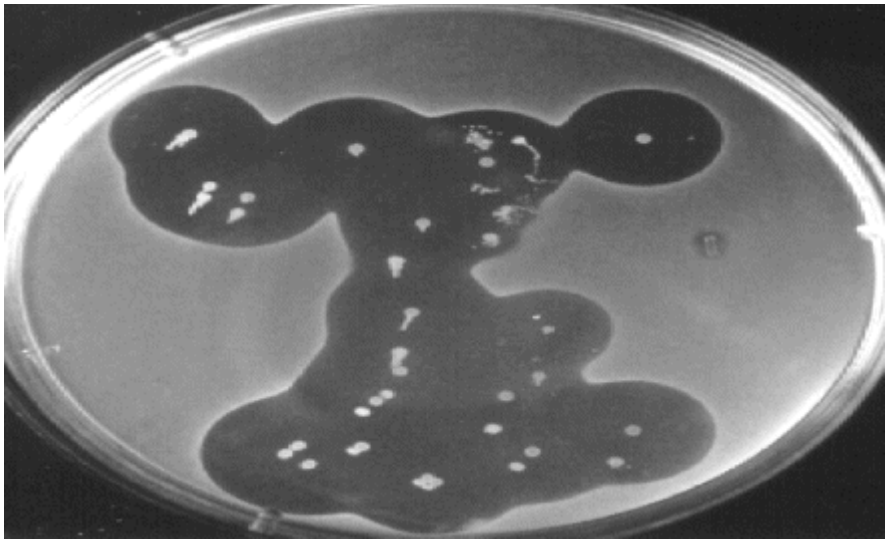
flavour development and accelerated cheese ripening, thereby potentially offering a substantial saving to the manufacturers of ripened cheeses.

Hence the objectives of this project were to generate, characterise and exploit a range of novel bacteriocin producing starter cultures to improve both the safety and the quality of fermented dairy foods.

The main conclusions were as follows:

- Lacticin 3147 is a broad spectrum bacteriocin which inhibits a wide range of Gram-positive bacteria including lactobacilli, clostridia and Listeria.
- The bacteriocin has been purified by chromatographic procedures and has been shown to be composed of two peptides, both of which are required for biological activity.
- The mechanism of action of lacticin 3147 has been elucidated.
- The entire plasmid encoding lacticin 3147 has been sequenced and the bacteriocin in distinct from any previously characterised lactococcal bacteriocin.
- The Food Grade introduction of the bacteriocin genes into cheese starters was carried out.
- Lacticin 3147 producing starters have been used to control the pathogen Listeria monocytogenes on the surface of mould ripened cheese.
- Lacticin 3147 producing starters have been used to control the non-starter lactic acid bacteria complement in Cheddar cheese during the ripening process.
- A novel starter system using a bacteriocin (lactococcin)- producing adjunct has been designed which gives increased cell lysis during Cheddar cheese manufacture while ensuring that efficient acid production is not compromised.

In summary these studies have found that naturally occurring antimicrobials such as bacteriocins have a wide range of applications in the food industry for improving both the quality and safety of fermented dairy products.

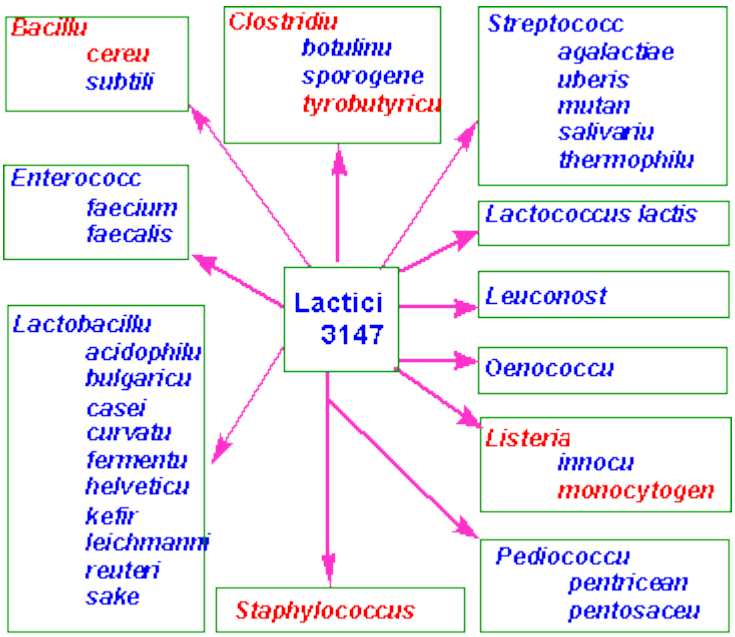


Lactococcus lactis subsp. *lactis* DPC3147 colonies on an agar plate showing zones of inhibition due to the presence of the bacteriocin lacticin 3147.

Research and Results

Spectrum of Lacticin 3147 Activity

Lacticin 3147 has a broad spectrum of activity and inhibits all Gram-positive bacteria tested to date including potential food-borne pathogens such as staphylococci, clostridia, and *Listeria*. In addition, lacticin 3147 is effective at killing all mastitic staphylococcal and streptococcal isolates tested including *Staphylococcus aureus,Streptococcus dysgalactiae, Strep. agalactiae* and *Strep. uberis*. However, lacticin 3147 is not effective at killing Gram-negative bacteria.

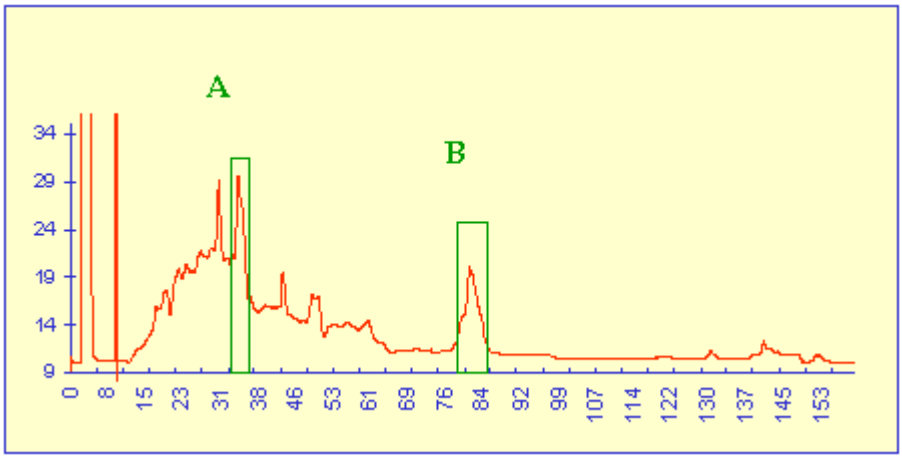


These results demonstrate that lacticin 3147 has a broad spectrum of inhibition capable of inhibiting all Gram-positive bacteria tested including food-borne pathogens (in red).

Physical properties

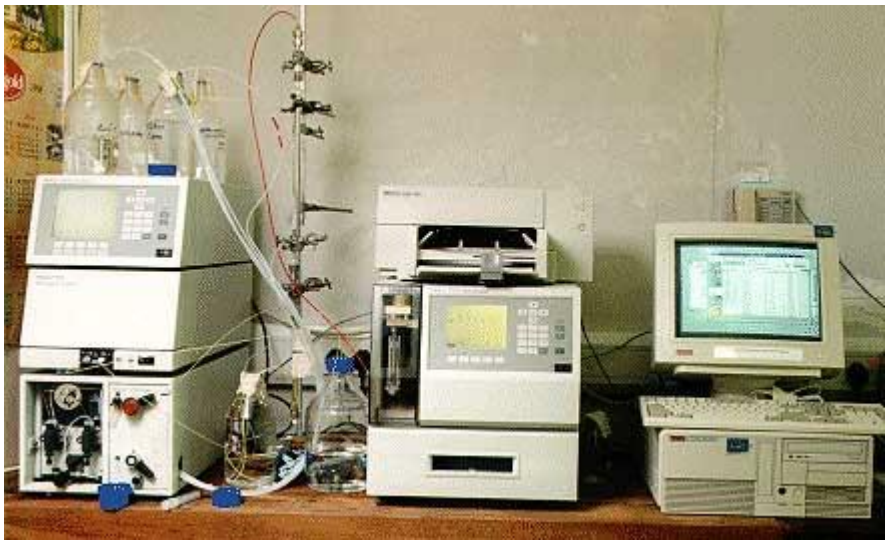
Like most other proteins lacticin 3147 is sensitive to proteolytic enzymes including digestive enzymes. Treatment of preparations of lacticin 3147 with trypsin, a-chymotrypsin, proteinase K and pronase E, inactivated this bacteriocin. Lacticin 3147 is also extremely active over a wide pH range, including physiological pH and thus, unlike nisin should prove to have a number of applications in foods with pH close to neutrality. Lacticin 3147 is also extremely heat resistant, particularly at low pHs and can withstand autoclaving temperatures.

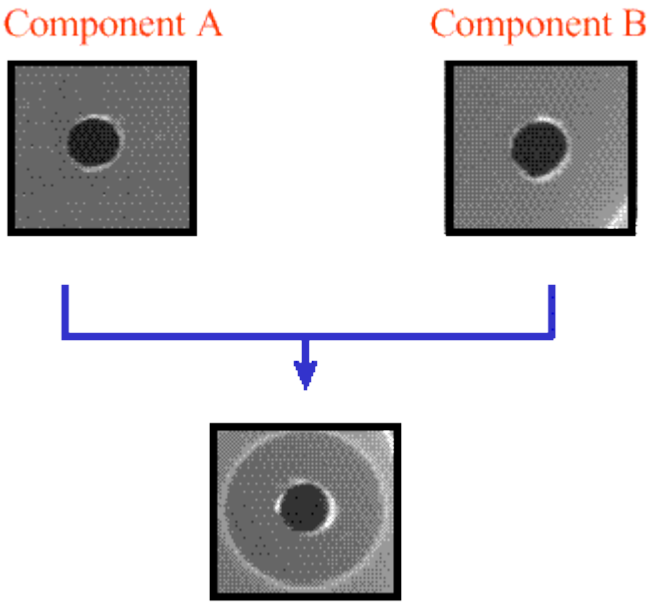
The bacteriocin has been purified by chromatographic procedures and has been shown to be composed of two peptides, both of which are required for biological activity.



Component A and B required for activity shown here on a chromatogram.

(SPLC apparatus used to purify LACTICIN 3147)



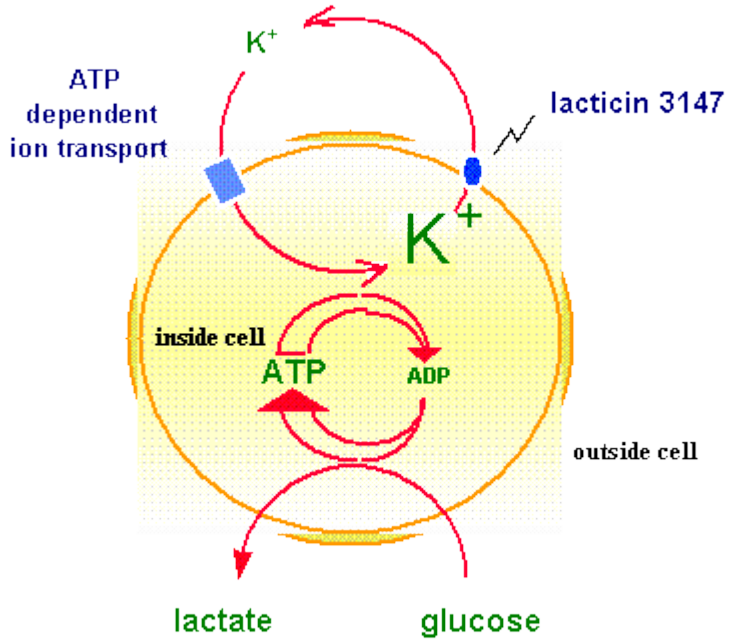


Inhibitory activity demonstrated when compenent A and B are combined.

Results indicate that lacticin 3147 is active over a wide pH range, including physiological pH, and that its activity is dependent on the presence of two peptides.

Mechanism of action

Lacticin 3147 elicits a bactericidal effect on sensitive cells by first interacting with the cell membrane. This causes the membrane to become porous for K+ and inorganic phosphate which leak out of the cells. In an attempt to re-accumulate these ions, ATP-dependent uptake systems lead to hydrolysis of internal ATP. As ATP is required for the maintenance of essential cellular functions, such as the pH gradient at the cell membrane, cellular functions are disrupted and the cell eventually loses energy and dies.

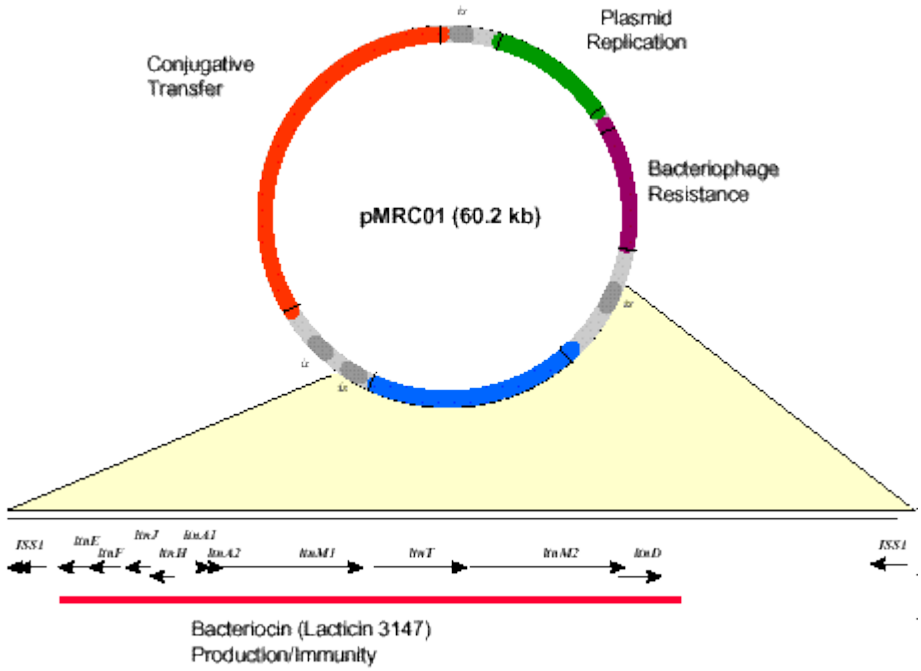


A schematic diagram showing the mechanism of action of lacticin 3147.

Lacticin 3147 induces cell death by causing the membranes of sensitive cells to become porous to K+ and inorganic phosphate.

Genetics of lacticin 3147 production and immunity

Lacticin 3147 is encoded in *Lactococcus lactis* ssp. *lactis* 3147 on a piece of DNA which lies outside its chromosome, called a plasmid. The plasmid which encodes lacticin 3147 production and immunity has been designated pMRC01. This plasmid can readily be mated between different lactococcal strains, in a food grade manner. The entire nucleotide sequence of this 60,232 bp pMRC01 plasmid was determined, representing the largest lactococcal plasmid sequenced to date. Detailed analysis of the genetic loci involved in the production of lacticin 3147 are provided in the paper by Dougherty et al., 1998. A synopsis of these data is provided here.



Map of pMRCO1, which can be divided into a number of basic components delineated by insertion sequences (IS): a region encoding conjugative functions; a region encoding phage resistance and plasmid replication and maintenance; and a region encoding lacticin 3147 production and immunity functions. The lacticin 3147-encoding region is flanked by two iso-ISS1 elements and contains 10 open reading frames (ORFs), which are believed to be associated with bacteriocin functions.

Knowledge of the DNA sequence permits genes to be identified, which in turn allows the sequence of amino acids in the proteins produced to be determined. In many cases, the function of these genes and the products they encode can be inferred by comparing the DNA or deduced protein sequences with known sequences present in databases. Comparative sequence analysis coupled with experimental data has permitted the region of plasmid pMRC01 required for bacteriocin activity to be identified and up to 10 genes are believed to be directly associated with lacticin 3147 activity. These genes are arranged in two clusters with *ltnA1, ltnA2, ltnM1, ltnT, ltnM2* and *ltnD* transcribed in the same direction and *ltnH, ltnJ, ltnF* and *ltnE* transcribed in the opposite direction.

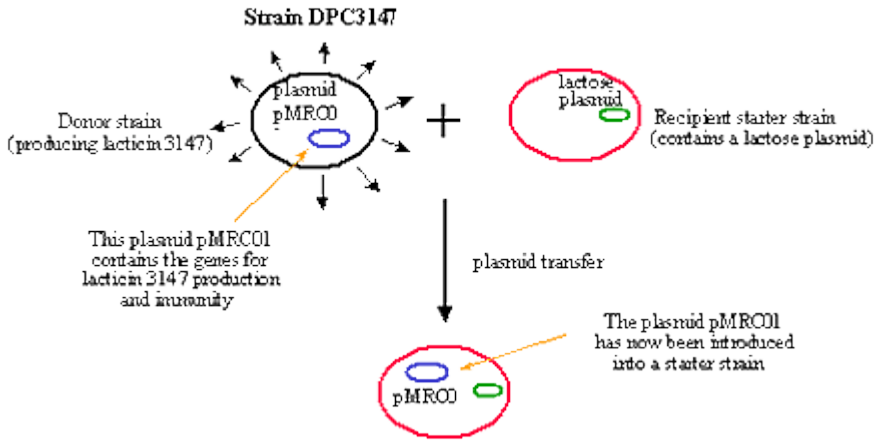
The first genes in the larger cluster, *ltnA1* and *ltnA2*, encode two relatively short peptides which are most likely the precursor of the active lacticin 3147 bacteriocin. These peptides are probably required to undergo a number of modifications before they are activated. The *ltnM1* and *ltnM2* gene products (modification proteins) are likely to perform this role. Lacticin 3147 is unusual in that to date it is the only bacteriocin known to have two modification proteins, a feature presumably linked to the two different peptide components of the active bacteriocin. After the LtnA1 and LtnA2 peptides have been modified the cell needs to export them to the exterior so that the bacteriocin can mediate its effect against other organisms. Transport is probably mediated by an ABC (ATP Binding Cassette) transporter which is encoded by *ltnT*. If the concentration of lacticin 3147 in the immediate vicinity of the producer cell gets too high, it may kill the producer cell. To prevent this, the producer cell has to have a certain level of immunity or resistance to its own bacteriocin. Immunity is probably provided (at least in part) by the products of *ltnE* and *ltnF*. The gene product of another gene, *ltnH*, has characteristics of a regulatory protein which binds to DNA indicating that the cell has a control mechanism to alter expression of the genes associated with bacteriocin activity. The function of the two genes, *ltnD* and *ltnJ* is unknown, however, their genetic location suggests that they are associated with lacticin 3147 functions.

Lacticin 3147 is encoded by the conjugative plasmid designated pMRC01. The lacticin 3147 region codes for at least 10 proteins, which are believed to be associated with bacteriocin functions.

Introduction of the bacteriocin genes into cheese starters

The food grade introduction of the bacteriocin genes into cheese starters was carried out, by a bacterial mating process known as conjugation. This is made possible by the fortuitous association of the bacteriocin genes with a plasmid which can be transferred between strains. Conjugation experiments revealed that the presence of lacticin 3147 in media is sufficient to select for the presence or absence of the associated immunity gene(s). Genetic determinants which encode lacticin 3147 immunity thus provide an excellent tool as a food grade marker for the development of improved starters. This research has resulted in the generation of a range of novel starters which now both produce and are immune to lacticin 3147.

When grown in milk these strains now secrete the bacteriocin into their environment, resulting in fermented products such as cheese, which contain the inhibitor, lacticin 3147. Since lacticin 3147 is produced naturally by the starter culture in the product, it is not considered a food additive and therefore does not require labelling. One of the lacticin 3147 producing starters has been evaluated in the production of Cheddar cheese, low fat cheese, Cottage cheese and mould ripened cheese. In each case the lacticin producing starter was used to either improve cheese quality or improve cheese safety.



A schematic drawing showing the development of a food grade marker system based on lacticin 3147.

1. An example of a lacticin 3147 producing starter used to improving cheese quality.

In cheeses such as Cheddar, it is desirable that the manufacturer has control over flavour development. Since non-starter lactic acid bacteria have been implicated in off flavour defects, control of their growth is desirable. Trials carried out with a bacteriocin producing starter demonstrated that after six months of ripening non-starter counts were at a low enough level that they would not interfere with flavour development. The use of bacteriocin producers to control the growth of advantageous bacteria gives the manufacturer control of flavour development in cheese, and prevents economic losses which may occur if off flavours develop. A new project aimed at further exploiting lacticin 3147 for Cheddar cheese uses is currently underway.

In Cheddar cheese manufactured with a lacticin 3147 producing starter substantial amounts of bacteriocin (1,280 AU/ml) was detected in the cheese. The presence of the bacteriocin correlated with a reduction in non-starter lactic acid bacteria numbers.

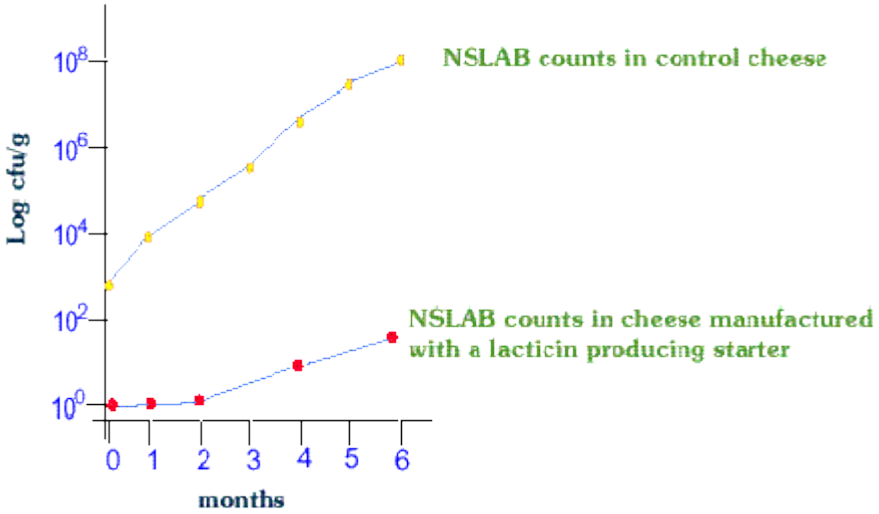


Figure showing the inhibition of non-starter lactic acid bacteria (NSLAB) growth in Cheddar cheese manufactured in the presence and absence of a lacticin 3147 producing starter.

Bacteriocins may offer cheeesemakers more control over which bacteria proliferate in cheese.

2. An example of a lacticin 3147 producing starter used to improving cheese safety.

Certain mould ripened cheeses may be at risk of contamination with pathogenic bacteria such as *Listeria*. During ripening the pH at the surface of these cheeses can excede pH 7, which provides a suitable environment for the proliferation of many undesirable bacteria such as *Listeria*. Trials have been carried out whereby a lacticin 3147 producer applied to the surface of a mould ripened cheese resulted in effective killing of *Listeria*. Here again an opportunity exists for manufacturers to benefit from the use of a natural antimicrobial to control the micro-flora present in cheese.

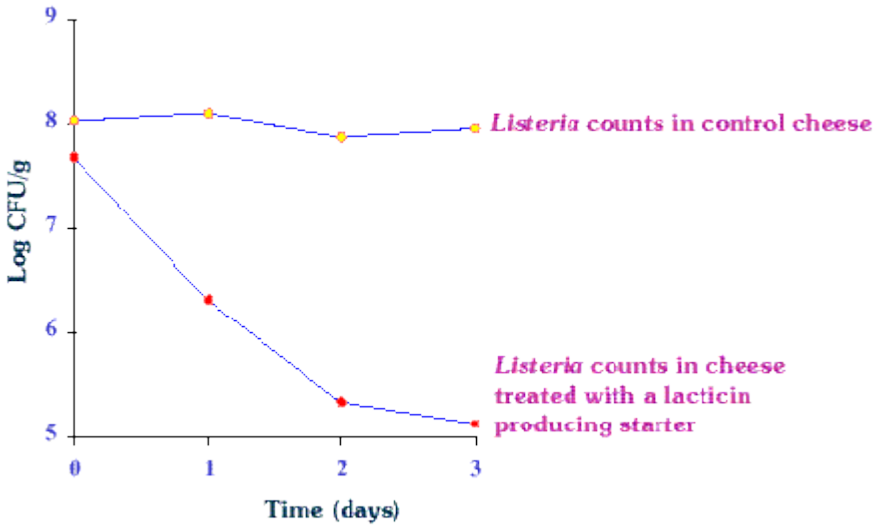
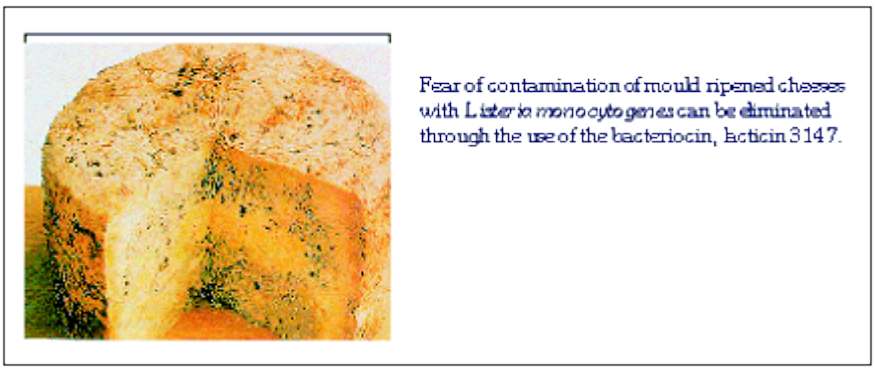


Figure showing the inhibition of *Listeria* on mould ripened cheese manufactured in the presence and absence of a lacticin 3147 producing starter.



Fear of contamination of mould ripened cheeses with *Listeria monocytogenes* can be eliminated through the use of the bacteriocin, lacticin 3147.

*Lacticin 3147 immunity has been used as a tool in the development of a food grade marker system for the development of improved starters. These lacticin 3147 producing starters, when used in Cheddar cheese manufacture, resulted in a reduction in non-starter lactic acid bacteria numbers. Lacticin 3147 producing starters were also effective at reducing the numbers of*Listeria*on the surface of mould ripened cheese.*

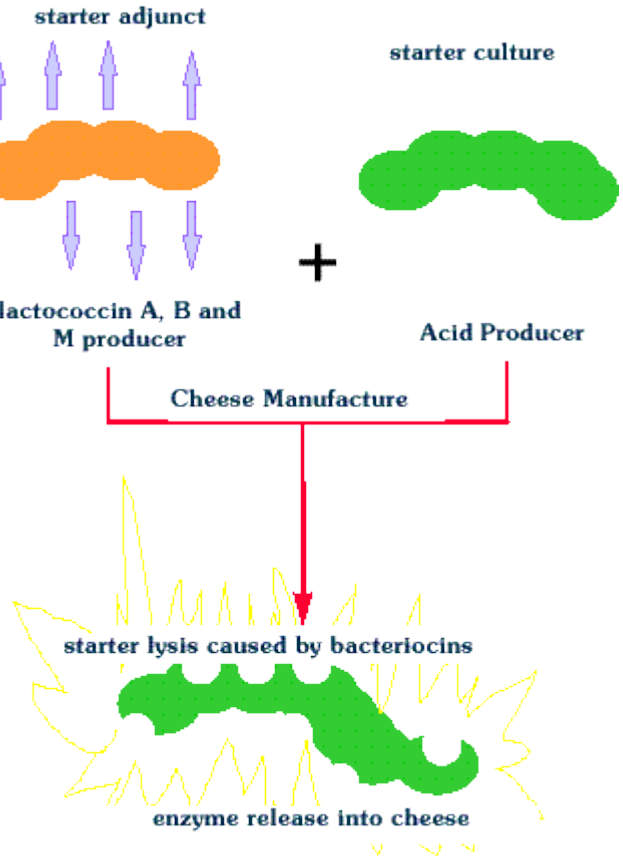
Bacteriocins in accelerated cheese ripening

The usefulness of bacteriocins in accelerated cheese ripening was also assessed as part of this project. A novel starter system using a bacteriocin producing adjunct (producing lactococcin A, B and M) has been designed which gives increased cell lysis during Cheddar cheese manufacture while ensuring that efficient acid production is not compromised.

Cheddar cheese manufactured with the lactococcin A, B and M producer demonstrated higher concentrations of intracellular enzymes, indicating that increased starter cell lysis had occurred during manufacture and ripening. These cheeses also exhibited higher levels of free amino acids, indicating that a greater degree of secondary proteolysis had occurred. In addition, Cheddar manufactured with the bacteriocin producing adjunct were awarded higher grading scores than control cheeses.

This novel starter system using a bacteriocin (lactococcin)- producing adjunct has been designed to give increased starter cell lysis during Cheddar cheese manufacture. Cheeses manufactured with the bacteriocin producing adjunct were favoured during commercial cheese grading.

A diagram showing how the lactococcin A, B and M producer, when used as an adjunct to the acid producing starter culture, induces lysis of the starter cells.



Increased starter cell lysis in cheeses manufactured with the bacteriocin producing adjunct resulted in these cheeses being favoured during commericial cheese grading.

List of Publications

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For Further Information, please contact: Dr. R. Paul Ross / Dr. Colin Hill