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Assessment and Control of Foodborne Pathogens in Ireland

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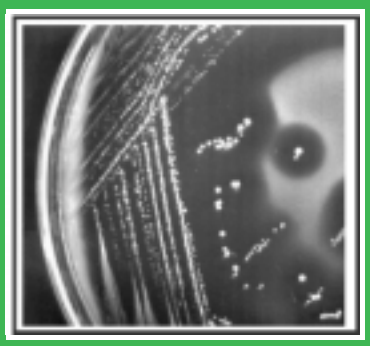
and D. van Sinderen

Commercially available unpasteurised milk cheeses, smear-ripened and surface-ripened cheeses, i.e. those most likely to harbour pathogens, were found to be free of E. coli *O157:H7,* Listeria monocytogenes *and* Salmonella *spp.*
Lacticin 3147, a natural microbial inhibitor, was successfully incorporated into a food grade spray-dried powder and its effectiveness in inhibiting or killing spoilage and pathogenic bacteria in a wide range of foods was demonstrated.
Lacticin 3147 was also shown to inhibit some clinically important human pathogens, including some antibiotic resistant strains.



Assessment and Control of Foodborne Pathogens in Ireland

ARMIS No. 4541

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Summary and Conclusions

Consumers are increasingly demanding food that is free from pathogens, but with less preservatives and additives. As a response to these conflicting demands, current trends in the food industry include minimal processing, and the investigation of alternative inhibitors for use in foods.

Additionally, the manufacture of an increasing range of novel foods, and the inclusion of non-dairy ingredients into dairy products, and vice versa, poses additional dangers with respect to safety. Furthermore, the dramatic increase in incidence of food-borne illness internationally, as a result of contamination with food-borne pathogens such as Listeria monocytogenes*, is a cause of considerable consumer concern.*

Bacteriocins are inhibitory peptides produced by a number of Lactic Acid Bacteria which are 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.

The broad spectrum bacteriocin Lacticin 3147 (discovered in a previous project and patented - see DPRC No. 3) is produced by Lactococcus lactis *subsp.* lactis *DPC3147, a food-grade strain, similar to strains used for commercial cheese manufacture. 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.*

As part of this project the bacteriocin Lacticin 3147 was assessed as a food preservative for improving food safety via inhibition of pathogenic organisms.

Thus the project plan followed a "twin-track" approach to assessing and controlling the food safety aspects of Irish food.

The first of these was designed to investigate the current safety status of Irish dairy products.

The second approach involved an attempt to exploit natural antimicrobial substances, including Lacticin 3147, to protect foods from pathogenic bacteria.

Main Conclusions and Achievements

Assessment of Foodborne Pathogens:

** A representative sample of commercially available smear-ripened, surface-ripened and unpasteurised milk cheeses, were found to be free of* E. coli *O157:H7,* Listeria monocytogenes *and* Salmonella *spp. The dairy products investigated are those considered most likely to harbour these dangerous pathogens.*

** It was demonstrated that both* Listeria monocytogenes *and* E. coli *O157:H7, if present, could grow rapidly during cheese-making, and subsequently survive in significant numbers during ripening which could pose a serious threat to consumers.*

Because of its pathogenicity and low infective dose, the presence, even in low numbers, of E. coli *O157:H7 in particular, in raw milk destined for cheese manufacture, is a particular concern.*

These studies also highlighted the necessity for representative sampling of both the rind and core of the cheese to ensure accurate pathogen detection.

** Highly sensitive and specific methods (PCR) were adapted and successfully used to trace the origin of* Listeria monocytogenes *in dairy processing plants, and to detect* E. coli *O157:H7 and* Listeria monocytogenes *in dairy products.*

In cheese, E. coli *O157:H7 can now be detected at an extremely low level of 1/g (compared to 10^3 cfu/g by the current method) facilitating improved detection of the pathogen as well as the study of microbial survival kinetics at very low cell numbers.*

A rapid screening procedure was also developed for detection of inhibitors to pathogens.

** Acidity (low pH) and salt are used traditionally for food preservation. However studies of* E. coli *O157:H7 show that exposure to (relatively high) pH 5.5 for one hour can enhance subsequent survival of the organism at pH 3.0 - normally considered lethal for the pathogen.*

Similarly it was shown that in some circumstances sodium chloride may in fact promote both the recovery and growth of stressed bacterial cells.

Cells grown in milk were more stress tolerant than those grown in laboratory media.

Control of Foodborne Pathogens:

** Following optimisation of Lacticin 3147 production in reconstituted demineralised whey powder, a pilot scale food grade spray-dried Lacticin 3147 powder preparation was manufactured. Bacteriocin activity remained constant throughout manufacture, indicating that no loss in activity occurred during processing.*

Lactococcus lactis *DPC3147 (the Lacticin 3147 producing strain) was immobilised in double-layered calcium alginate beads providing an effective, stable and long term method of producing the bacteriocin.*

** Lacticin 3147 was successfully immobilised on packaging film where it reduced counts of* Listeria *and* Staphylococcus aureus *on the surface of ham and cheese.*

** The effectiveness of Lacticin 3147 as an inhibitor of both spoilage and pathogenic bacteria was demonstrated in a wide variety of food systems including infant food formula, powdered soup, cottage cheese, natural yogurt and fresh pork sausage.*

** Lacticin 3147 in combination with high hydrostatic pressure resulted in greater pathogen inhibition than either treatment alone.*

** Lacticin 3147 was found to inhibit a range of clinically important human (gram positive) pathogens including some antibiotic resistant strains.*

Research and Results

Section 1:

ASSESSMENT OF FOODBORNE PATHOGENS

Screening of Dairy Products for the Presence of Pathogenic Microorganisms

In order to reassure consumers of the safety of Irish dairy products and to pre-empt problems it is important to determine if food pathogens could be detected in dairy products. Some of the higher risk dairy products include raw (unpasteurised) milk cheese, smear-ripened cheese and surface-ripened cheese. On two independent occasions 30 cheeses from these categories were obtained. The samples were tested for the presence of *E. coli* O157, *Listeria monocytogenes* and *Salmonella* spp. using the EiaFoss rapid detector, which is an immunologically based method that was shown to be capable of detecting 1 cell/50 g of cheese.

None of the three food pathogens were detected in any of the cheeses.

Pathogen Survival in Cheese

Although the process of cheese ripening represents an environment unsuitable for survival of *E. coli* O157:H7 and *Listeria monocytogenes*, the process of manufacture can encourage growth of these pathogens.

We developed a laboratory scale process for the manufacture and ripening of a semi-soft smear-ripened cheese made from raw milk to study the growth and death kinetics of *E. coli* O157:H7 and *Listeria monocytogenes* during the different stages of processing. The cheesemilk was inoculated with 33 ± 4 cfu/ml *E. coli* O157:H7 or 5 x 10^3 cfu/ml *Listeria monocytogenes*. In both cases pathogen growth during manufacture was sufficient to allow considerable survival during ripening.

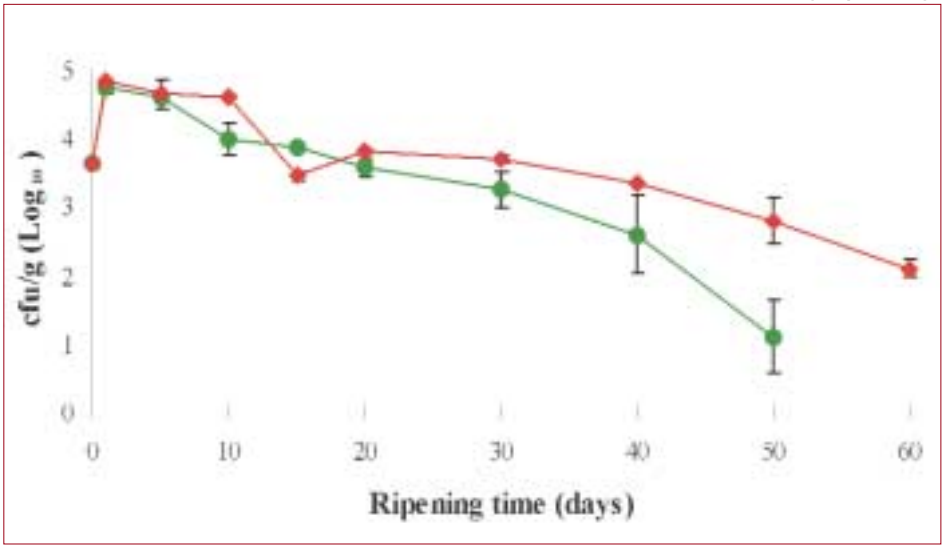

Fig. 1: The death kinetics of Listeria monocytogenes *CI 2 in the rind (exterior) (●), and core (interior) (♦) of the cheese during ripening. Values are the average of duplicate vats. The initial increase in cell numbers represents growth during manufacture.*

Death of Listeria monocytogenes *occurred in the core (interior) of the cheese, but even after 60 days a considerable number survived (Fig. 1). Death occurred at a faster rate on the rind (exterior), possibly due to competitive inhibition by the surface smear flora.*

The results emphasise the importance of representative product sampling, including rind and core, for detection and estimation of pathogen numbers in cheese.

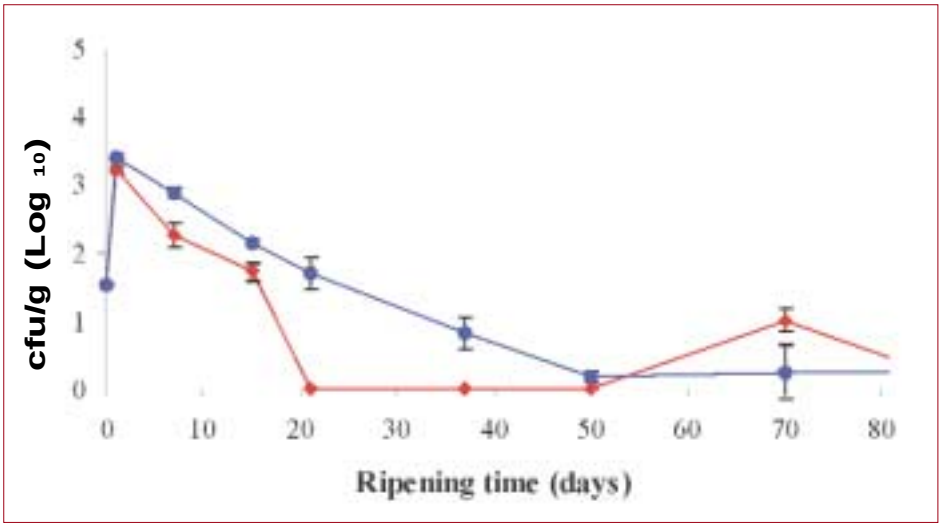

Fig. 2: The death kinetices of E. coli *O157:H7 in the rind (●), and core (♦) of the cheese during ripening. Values are the average of quadruplicate vats. The initial increase represents growth during manufacture.*

Growth of *E. coli* O157:H7 occurred during manufacture of the cheese. During ripening the rate of death was greater in the core of the cheese than on the rind *(Fig. 2)*. In both the rind and core of the cheese the organism could be isolated after 70 days.

Because of the low infective dose of this pathogen low numbers can pose a threat to the consumer.

These results again indicate the necessity for proper sampling of the rind and core of the cheese. They also indicate that the manufacturing procedure encouraged substantial growth of E. coli *O157:H7 to levels that permitted survival during ripening and extended storage.*

The presence of low numbers (33 cfu/ml) of E. coli *O157:H7 in milk, destined for raw milk cheese manufacture, could constitute a threat to the consumer.*

Stress Responses in Pathogenic Microorganisms

Many bacteria can produce responses to a particular stress that enable them to survive that stress. In addition, they can induce adaptive tolerance responses (ATR), where exposure to a non-lethal stress can induce responses that allow survival of a lethal stress. Some ATRs are specific in that they only confer resistance to the stress used to induce them. Others are global responses that confer cross resistance to other stress factors.

During this project we examined the induction of ATR by *E. coli* O157:H7.

We have shown that the induction of these responses can alter the death kinetics of the organism (Fig. 3).

Induction of the ATR at pH 5.5 for 1 h can enhance survival at pH 3.0.

This increased tolerance to pH 3.0 was shown to be due to a change in proton flux across the cell membrane. Using two dimensional electrophoresis, it was possible to view the proteins of the cell membrane. Differences in the membrane proteins of adapted and non-adapted cells were observed.

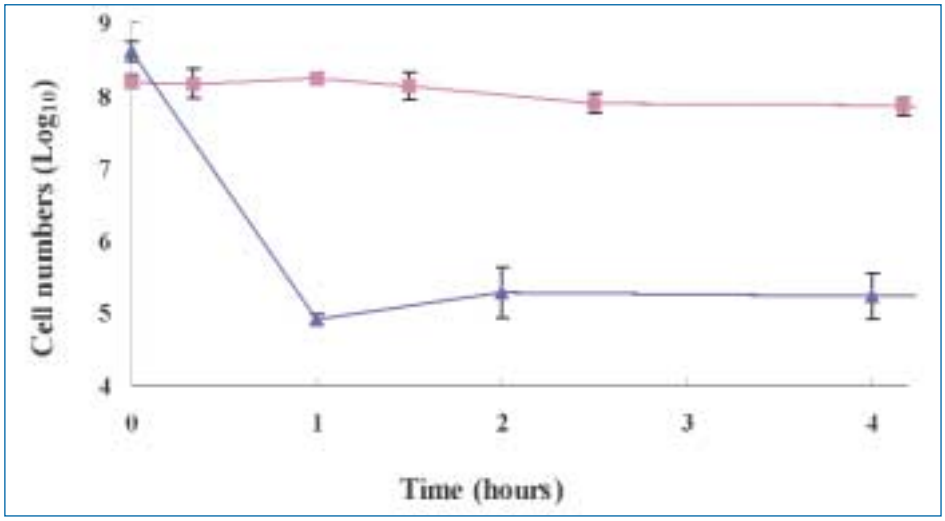

Fig. 3: The survival of E. coli *O157:H7 (strain P1432) at pH 3.0, before (▲), and after (■) adaptation at pH 5.0 for 1 h prior to challenge. Prior to acid challenge cultures were grown to mid-exponential phase (pH 7.0) at 30ºC.*

Expression of the protein (highlighted by arrow in *Fig. 4* was also considerably increased with induction by lactate and acetate, but not by induction with heat. The level of this protein was quantified by using a computer to measure the density of the protein spot in terms of the pixel intensity. Cross resistance to other acids, but not to heat, was conferred with acid induction. The *rpo*S related proteins are associated with the stationary phase of bacterial growth and confer global stress resistance. Increased expression of the protein was not seen in stationary phase cells, suggesting that *rpo*S was not involved. It was concluded that increased expression of this protein is a specific response to acid and not a global response.

Transposon mutagenesis is a method of interrupting gene expression by randomly inserting an extra piece of DNA into a gene. Since the insertion is random, a large number of isolates must be screened to find a strain with the required gene interruption. Using this method a mutant of *E. coli* that has an ATR gene interrupted and is, therefore, unable to induce an

ATR to HCl was isolated. This mutant will be used to identify the interrupted gene and to study the role of ATR in different environments.

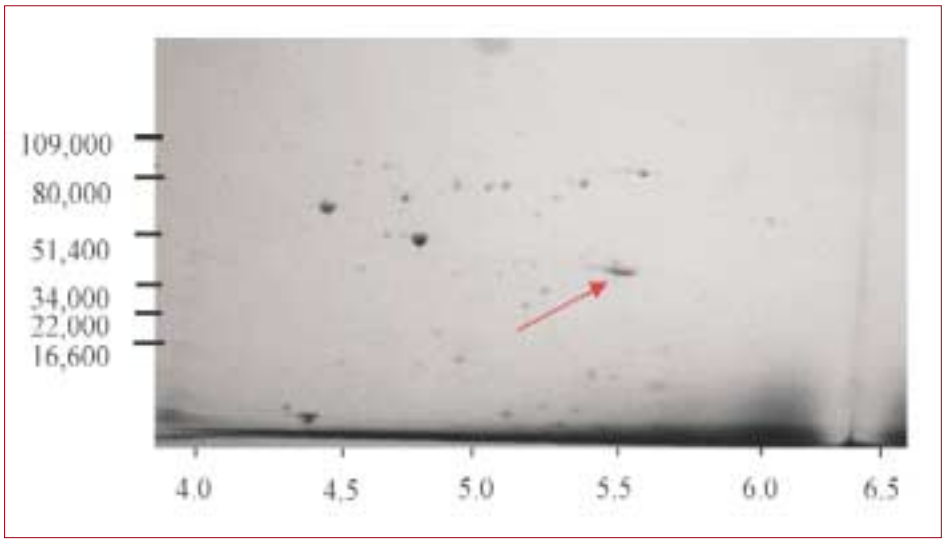

Fig. 4: The membrane proteins of E. coli *O157:H7 (acid adapted) as separated by two dimensional gel electrophoresis. One particular protein (shown by an arrow) showed almost a 10-fold increase in expression when cells were adapted.*

Sodium chloride was shown to increase the recovery and growth of E. coli *O157:H7 cells that were stressed with lactate (Table 1). It is generally accepted that sodium chloride is an additional stress factor when added to food. However, these results indicate that this may not always be the case, as the sodium chloride may promote recovery and growth of stressed cells.*

Table 1: The effect of different sodium chloride and lactate concentrations on the growth rate of acid stressed cells of strain P1432 in broth at pH 5.0.

Lactate	Salt concentration %			
%	0	4	5	6
0	0.79 ± 0.03	1.21 ± 0.11	1.32 ± 0.05	1.32 ± 0.09
0.5	0.65 ± 0.02	1.20 ± 0.13	1.06 ± 0.09	1.08 ± 0.03
1.0	0.61 ± 0.01	1.59 ± 0.39	1.12 ± 0.06	1.28 ± 0.04
1.5	NG[1]	1.22 ± 0.06	0.94 ± 0.04	1.22 ± 0.29

NG[1] - no growth

Section 2:

CONTROL OF FOODBORNE PATHOGENS

Screening for Bacteriocin-Producing Strains

A number of natural sources including raw milk, Kefir grains, dairy products and the brewing environment were screened for producers of inhibitory compounds. A procedure was developed for the rapid screening of dairy produce for the presence of inhibitory substances. The procedure enables the screening of a large number of samples in a short length of time. A number of potential inhibitors to both *Listeria* and *E. coli* were identified *(Fig. 5)*, in addition to Lactic Acid Bacteria capable of inhibiting *B. cereus*. The inhibition of *Listeria* by Kefir fermentates could be attributed to bacteriocin activity in a number of cases. With regard to

the inhibition of *E. coli*, inhibition appears to be due perhaps to the combination of acid and oxidative killing.

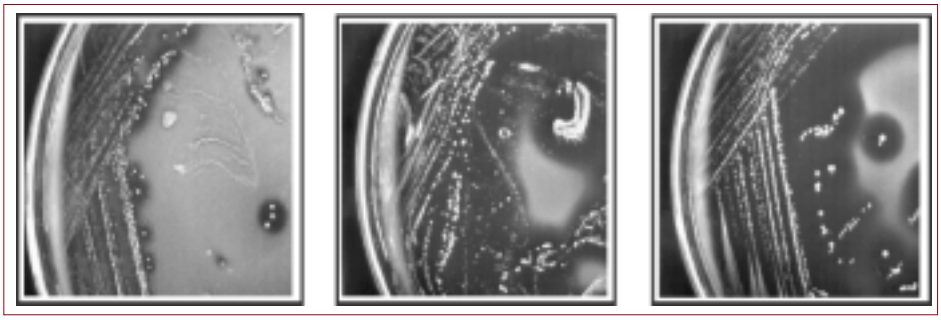

Fig. 5: The inhibitory effects of a number of potential bacteriocin producing strains isolated from a Kefir fermentate. Zones of clearing indicate inhibitory activity.

As part of the above study, five hundred raw milk samples were screened for bacteriocin producing bacteria. Of these, 362 strains exhibited antimicrobial activity. *Two bacteriocins, designated Lacticin 104 and Lacticin 115, were subsequently identified which inhibited a wide range of Gram positive bacteria including* Listeria monocytogenes.

Characterisation of Novel Inhibitory Activities

The two novel bacteriocins isolated during the screening of raw milk samples are produced by *L. lactis* cultures.

Evidence suggested that Lacticin 104 had a medium spectrum of inhibition, capable of inhibiting Listeria *and* Staphylococcus *strains. Inhibitory activity was active over a wide pH and temperature range.*

Inhibitory activity was purified by ammonium sulphate precipitation, application to XAD beads, cation exchange chromatography and reverse phase high pressure liquid chromatography. This revealed that this strain

produces a bacteriocin identical to a well-characterised lactococcal bacteriocin, namely Lacticin 481. The genetic determinants for inhibitory activity are located on a 60 kb conjugative plasmid. The strain demonstrates an unusual phenomenon in that it induces a leaky phenotype (increased release of intracellular contents) in lactococci, a property under investigation for enhancing starter cell lysis in cheese making.

Scale-up of Lacticin 3147 Production to Pilot Scale

A major portion of this project involved the production of the broad-spectrum bacteriocin Lacticin 3147 for food applications. For this reason Lacticin 3147 production was optimised, by detailed modelling of culture performance during pH controlled fermentation studies.

Increased Lacticin 3147 activity could be obtained by maintaining the pH of the growth medium constant at pH 6.5. Levels of bacteriocin activity reached 10,240 AU/ml in 10% reconstituted demineralised whey powder when the pH of the growth medium was held constant at pH 6.5 compared to 640 AU/ml when no pH control was imposed *(Fig. 6)*. Following the optimisation of Lacticin 3147 production in reconstituted demineralised whey powder, suitable spray drying conditions were identified that had no adverse effect on Lacticin 3147 activity.

A pilot scale spray-dried Lacticin 3147 powder preparation was manufactured and bacteriocin activity remained constant throughout manufacture, indicating that no loss in bacteriocin activity occurred during processing.

In parallel with the fermentor studies outlined above, the application of immobilised cell technology for bacteriocin production was also assessed. The suitability of a number of natural polymers as immobilisation supports for *L. lactis* DPC3147 (the Lacticin 3147 producer) revealed that alginate was the most suitable.

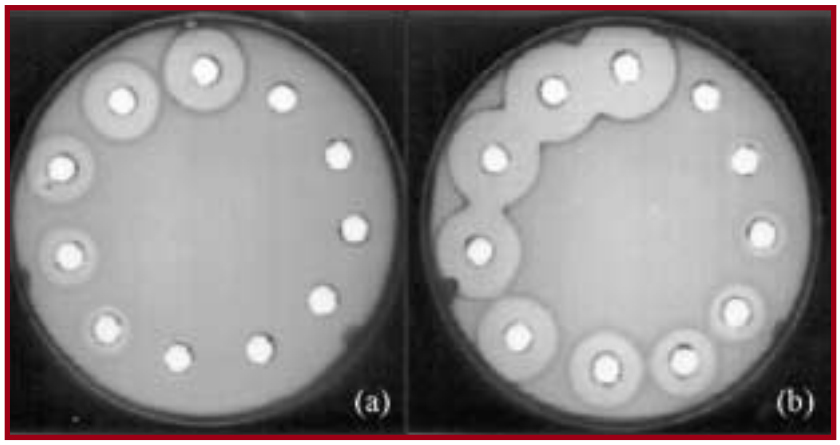

Fig. 6: Lacticin 3147 fermentations in 10% demineralised whey with: (a) no pH control and (b) pH controlled at 6.5. More (and larger) zones of inhibition represent greater activity.

L.lactis DPC3147 was immobilised on double layered calcium alginate beads and bacteriocin production was detected after 3 h and had stabilised after 24 h. Bacteriocin production from the immobilised cells remained constant for the duration of the experiment (180 h), compared to production by free cells which had declined after 80 h.

Results of this study suggest that the immobilisation of L. lactis *DPC3147 may offer a more stable and long-term means of producing Lacticin 3147.*

Effectiveness of a powdered Lacticin 3147 product for the control of Gram positive spoilage and pathogenic microorganisms.

The powdered Lacticin 3147 was assessed for the inhibition of food spoilage and pathogenic microorganisms in a number of food systems including infant food formula, powdered soup, cottage cheese and natural yogurt.

Powdered Lacticin 3147 was very effective in killing pathogens and spoilage organisms in a number of different food systems investigated.

Starting cell numbers in each case were approximately 10^5 cell forming units per gram or millilitre. The main findings are summarised hereunder.

- In the infant milk formulation the use of the Lacticin 3147 powder resulted in greater than a 99% kill of *L. monocytogenes* Scott A within 3 h at 30ºC.

- The effect of Lacticin 3147 powder on the inhibition of *L. monocytogenes* Scott A in natural yogurt demonstrated that greater than 98.3% of the culture was killed within 5 min at 30ºC and within 60 min no viable cells remained.

- In the case of Cottage cheese 40% of the *L. monocytogenes* Scott A population was killed within 5 min at 30ºC and within 160 min only 14% of the population remained viable *(Fig. 7)*.

- The effect of a range of concentrations of Lacticin 3147 in powdered soup against *B. cereus* at 30ºC, demonstrated that following 24 h incubation greater than a 99.9% kill was observed.

- A similar study was carried out to determine the effect of various concentrations of Lacticin 3147 powder on the survival of *L. monocytogenes* Scott A in powdered soup. Interestingly the effect on *Listeria* was not as marked as that observed for the inhibition of *B. cereus*.

- A study was also performed to determine the effect of Lacticin 3147 powder on *Bacillus* spores. A spore preparation exposed to Lacticin 3147 demonstrated that while the Lacticin 3147 powder does limit the germination of spores, no killing effect was observed.

These results suggest that this bio-active Lacticin 3147 food ingredient may find applications in many different foods, including those with pH close to neutrality.

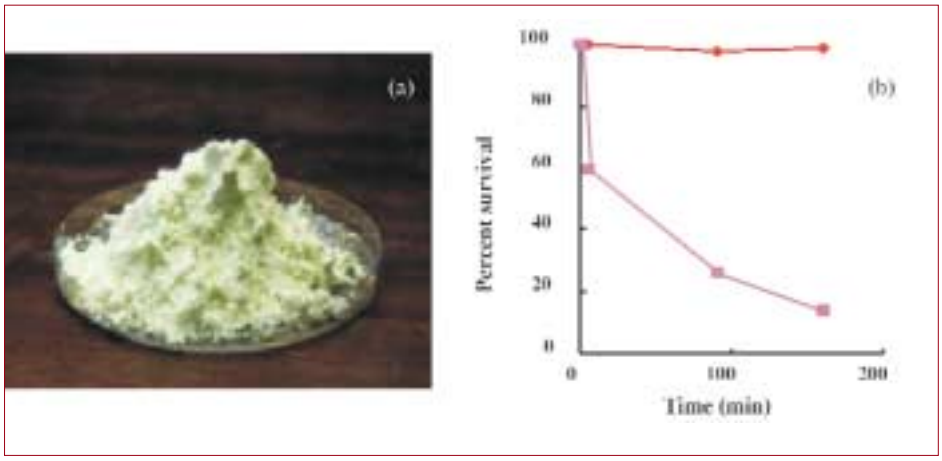

Fig. 7: (a) the Lacticin 3147 powder product, and (b) inhibition of Listeria monocytogenes *Scott A in Cottage cheese using the Lacticin 3147 powder.*

Lacticin 3147 as a Preservative in Fermented Sausage

Lacticin 3147 has been investigated as a preservative in fermented sausage. The bacteriocin producing transconjugant strain *L. lactis* DPC4275 and the non-producing parent strain DPC4268 are sensitive to high levels (100 ppm) of sodium nitrite. As levels of nitrite were increased, starter growth and rates of acidification of the fermented sausage were reduced. An inverse relationship between bacteriocin activity and nitrite concentration existed. The inclusion of a culture pre-enrichment step prior to inoculation into the meat mix enhanced strain performance resulting in higher numbers of recoverable starter cells. In addition, both starter growth and rates of acidification were enhanced with the addition of manganese or magnesium, particularly when both manganese and magnesium were combined.

Hence, Lacticin 3147 cultures may be suitable for fermented sausage manufacture.

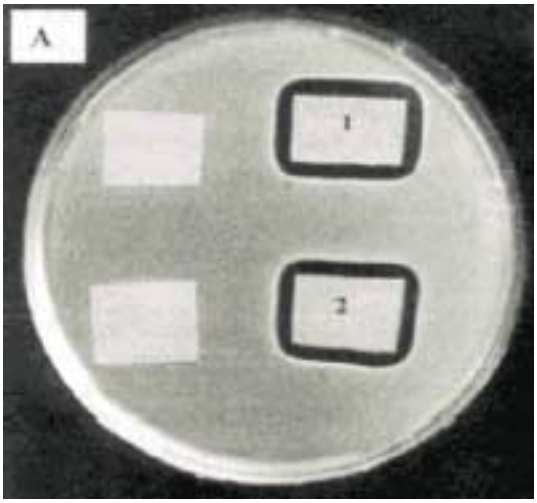

Fig. 8: The inhibitory effect of lacticin (1) and nisin (2) immobilised on a cellulose based packaging material. Zones of clearing (dark rectangular areas) demonstrate inhibitory activity. Controls on the left.

Immobilised Lacticin 3147 on packaging film.

Both nisin (a commercial bacteriocin preparation) and Lacticin 3147 produced effective stable bioactive packaging material.

When applied to the surface of ham and cheese slices artificially inoculated with L. innocua *and* S. aureus *and stored at refrigeration temperatures under modified atmosphere packaging (MAP), bioactive inserts reduced the recoverable population in both cases. In addition, there was a positive inhibition observed in total aerobic plate counts and lactic acid bacteria for both the ham and cheese product over the 24-day period. Cheese stored under vacuum packaging, in bioactive vacuum pouches also demonstrated reductions in non-starter lactic acid bacteria,* L. innocua *and* S. aureus *during the 84-day test period compared to the control (Fig. 8).*

Lacticin 3147 in combination with High Hydrostatic Pressure

The use of hydrostatic pressure and Lacticin 3147 treatments were evaluated in milk and whey with a view to combining both treatments for improving the quality of minimally processed dairy foods. The system was evaluated using two foodborne pathogens: *Staphylococcus aureus* ATCC6538 and *Listeria innocua* DPC1770. Trials against *Staph. aureus* ATCC6538 were performed using concentrated Lacticin 3147 prepared from culture supernatant.

Results demonstrated greater than an additive effect when both treatments were used in combination *(Fig. 9)*, for example, the combination of 250 MPa (2.2 log reduction) and Lacticin 3147 (1 log reduction) resulted in more than 6 logs of kill of *L. innocua*.

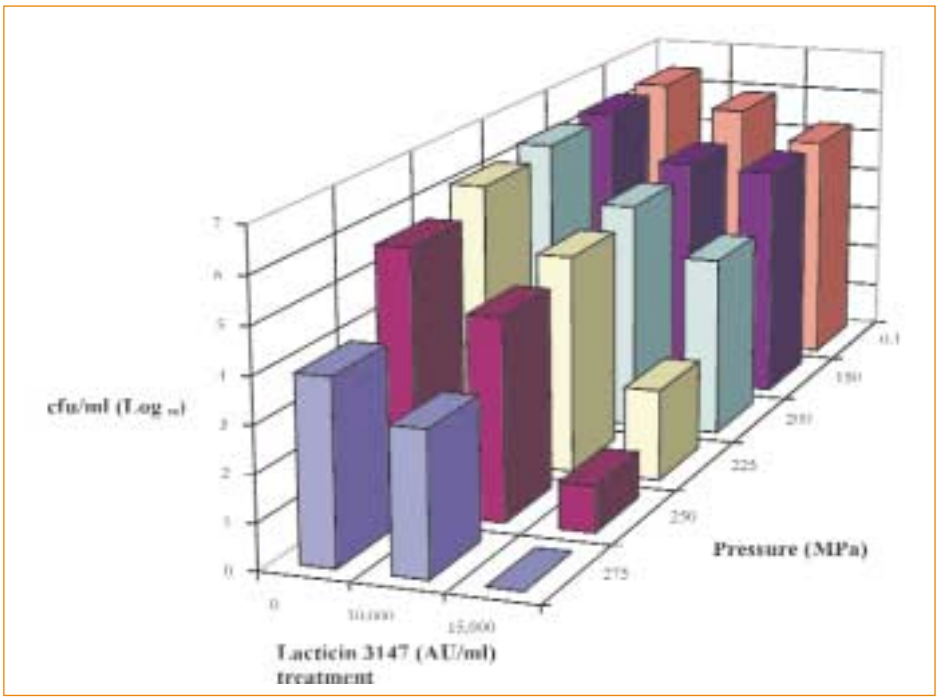

Fig. 9: The effect of high hydrostatic pressure and Lacticin 3147 (alone and in combination) on the viability of L. innocua *DPC1770.*

Similar results were obtained when a foodgrade powdered form of Lacticin 3147 (developed from a spray-dried fermentation of reconstituted demineralised whey powder) was evaluated for the inactivation of *L. innocua* DPC1770. Furthermore, it was observed that treatment of Lacticin 3147 preparations with pressures greater than 400 MPa yielded an increase in bacteriocin activity (equivalent to a doubling of activity).

These results indicate that a combination of high pressure and Lacticin 3147 may have potential for improving the quality of minimally processed foods at lower hydrostatic pressure levels

Suitability of Lacticin 3147 for treatment of clinically important Gram positive human pathogens.

The application of Lacticin 3147 for biomedical uses was investigated.

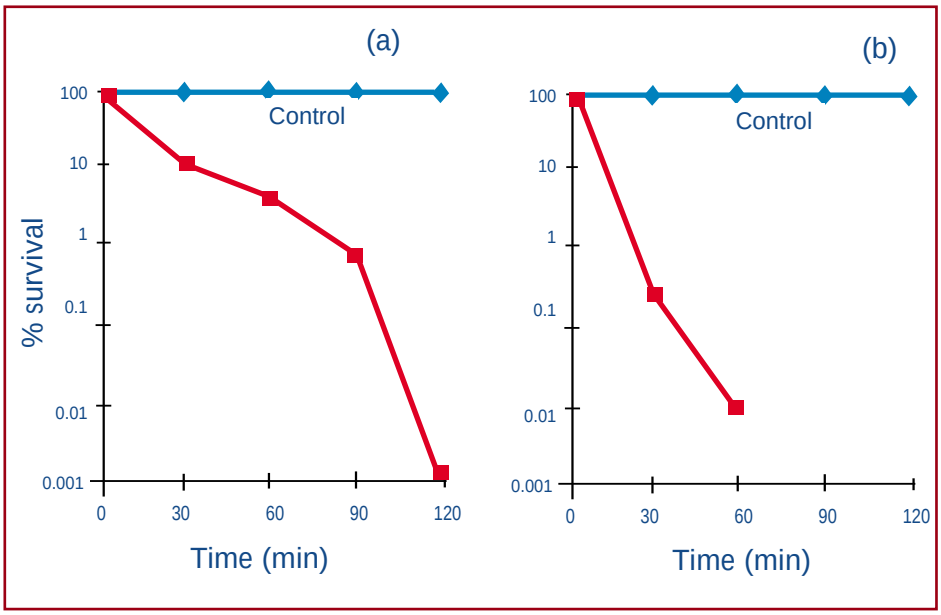

Fig. 10: The bactericidal effect of Lacticin 3147 (20,000 AU/ml) on the viability of (a) methycillin resistant Staphylococcus aureus *and (b)* Streptococcus mutans.

Lacticin 3147 was found to inhibit a range of clinically important Gram positive pathogens (Fig. 10) including methicillan resistant Staphylococcus aureus *(MRSA), vancomycin resistant* Enterococci *(VRE), penicillin resistant* Pneumococcus *(PRP),* Propionibacterium *and* Streptococcus mutans*.*

Further studies and trials are required to assess its potential as an alternative therapeutic compound in serum and topical creams.

Patents

An anti-microbial effective against Gram positive pathogens: IE Patent Application No. 980499; International Patent Application No. PCT/IE99/00057.

A spray-dried bacteriocin powder with anti-microbial activity: IE Patent Application No. 980500; International Patent Application No. PCT/IE99/00058.

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