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Shining Light on Food Microbiology; Applications of Luciferase-tagged Microorganisms in the Food Industry

Ruth Morrissey

Shining Light on Food Microbiology: Applications of Luciferase-tagged Microorganisms in the Food Industry

Ollscoil na hÉireann, Corcaigh

THE NATIONAL UNIVERSITY OF IRELAND, CORK

A Thesis presented to the National University of Ireland for the

Degree of Doctor of Philosophy by

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Declaration

I hereby declare that the research presented in this thesis is my own work and effort and that it has not been submitted for any other degree, either at University College Cork or elsewhere. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

This work was completed under the guidance of Prof. Colin Hill and Dr. Máire Begley at the Alimentary Pharmabiotic Centre, Biosciences Institute (Microbiology Department), University College Cork.

Signature:……

Date:……

Thesis Abstract

Bioluminescence is the production of light by living organisms as a result of a number of enzyme catalysed reactions caused by enzymes termed luciferases. The *lux* genes responsible for the emission of light can be cloned from one bioluminescent microorganism into one that is not bioluminescent. The light emitted can be monitored and quantified and will provide information on the metabolic activity, quantity and location of cells in a particular environment, in real-time.

The primary aim of this thesis was to investigate and identify several food industry related applications of *lux*-tagged microorganisms. Throughout this thesis, a variety of *lux*-tagged Gram negative and Gram positive bacteria were examined, under standard laboratory conditions and in complex food systems. Initial experiments investigated the effect of introducing the *lux* plasmid into *Cronobacter sakazakii* and found the *lux* genes had no negative effect on the growth characteristics of the strain. The potential of using the *lux*-tagged *C. sakazakii* strain to monitor growth of this bacterium in clear and opaque liquids was examined and results demonstrated that bacterial bioluminescence could be detected when the *lux*-tagged strain was grown in Luria-Bertani broth, skim milk, and infant milk formula. We also showed that bioluminescence of the strain could be detected when the *lux*-tagged strain was cultured in the presence of competing bacteria and that the effect of antimicrobial agents on growth of the *lux*-tagged *C. sakazakii* could be monitored by measuring bioluminescence.

Following this, we investigated the use of a bioluminescence based early warning system for starter culture disruption by antimicrobial agents. To achieve this, a new conjugative *lux*-tagged plasmid (pRH001*lux*), capable of mobilisation between strains, was constructed. We subsequently transformed this plasmid into several

bacteria and developed a bank of bioluminescent cheese starter culture strains. When the effect of introducing this new *lux* plasmid into starter cultures it was was examined, it was determined that the *lux* plasmid had no negative effect on the growth characteristics of the strain. We used this bank to establish assays capable of rapidly monitoring antimicrobial agents, such as antibiotics, in laboratory media and milk.

The final section of this thesis examined the activity of bioengineered Nisin derivatives, Nisin V and S29A, against foodborne pathogens in laboratory media and selected foods. For the first time, we report the enhanced antimicrobial activity of Nisin V against Gram negative bacteria in agarose assays and laboratory media. We have also highlighted the enhanced bioactivity of S29A and V against *Listeria monocytogenes* in a commercially produced frankfurter maintained under sub-optimal conditions and demonstrated that Nisin V outperforms both the wild-type nisin and S29A, with respect to controlling growth of *Listeria* over a 24 hour period.

When Nisin V and S29A was examined against Gram negative bacteria in powdered infant formula (PIF), apple juice and liquid whole egg no inhibitory effect was observed.

In conclusion, this thesis demonstrates that there are many food related applications of *lux*-tagged microorganisms in the food industry. In particular, the *lux* system can be used to monitor growth of tagged micro-organisms in clear and opaque liquids, as an early warning system for starter culture disruption by antibiotic residues and as a real-time method for tracking the antimicrobial effect of Nisin variants in selected model food systems.

Chapter I

Literature review

Shining Light on Food Microbiology; Applications of *Lux*-tagged Microorganisms in the Food Industry

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1. Abstract

Bioluminescence is the production of light by living organisms. The luciferase genes responsible for the generation of light (*lux* genes) can be cloned from a bioluminescent microorganism into one that is not naturally bioluminescent. Light output can be monitored in real time and will provide information on the metabolic state, location and quantity of cells in a given environment. Herein we review the applications of *lux*-tagged microorganisms in the food industry including their uses in the development of novel food products and processes, for problem solving and for determining parameters important for the control of food quality and safety.

2. Introduction

Bioluminescence is the emission of light by living organisms as a consequence of an enzymatic reaction catalysed by enzymes termed luciferases. Over 80% of luminescent organisms have been isolated from marine habitats and include bacteria, fungi, crustaceans, jellyfish, worms and beetles (Widder, 2010). The biological functions of light emission include communication, illumination, camouflage, attraction and repulsion (Meighen, 1993). The *lux* genes responsible for bioluminescence have been identified and can be transferred and expressed in several microorganisms including foodborne pathogens and food spoilage bacteria. Herein we briefly summarise approaches used to create bioluminescent bacteria and discuss the applications of bioluminescent technology to food microbiology.

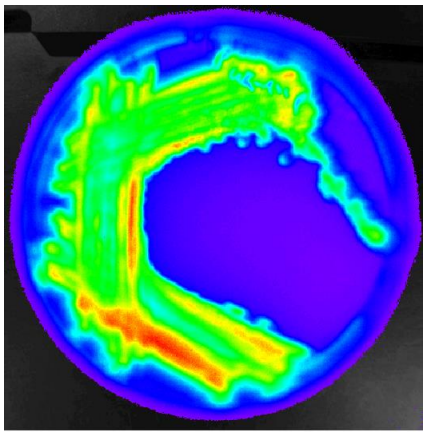
3. *Lux* system

While the majority of luciferases have been identified in marine organisms the most frequently used bioluminescent reporter system, the *lux* system, is derived from the Gram negative bacterium *Photorhabdus luminescens* that is found in terrestrial environments. In the presence of oxygen the luciferase enzyme catalyses the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long chain aliphatic aldehyde to yield flavin mononucleotide (FMN) and a long chain fatty acid (Fig. 1). As a consequence of this reaction, light is emitted at a wavelength of 490nm (Baker *et al.*, 1992, Dostalek and Branyik, 2005). The luciferase enzyme is encoded by the *luxAB* genes and the fatty acid reductase complex required for regeneration of the aldehyde is coded for by the *luxCDE* genes. The *lux* genes can be transferred from *P. luminescens* and expressed in other bacteria and the resulting *lux*-tagged bacteria emit light. A large number of bacteria of importance in food have been successfully *lux*-tagged (Table 1). Light output from Gram positive bacteria is usually lower than that from Gram negatives and it has been suggested that this is the result of poor expression of *lux* genes (Jacobs *et al.*, 1995, Griffiths, 2000). This problem can be overcome by introducing translational signals optimized for Gram positive bacteria in front of *luxA, luxC* and *luxE* (Qazi *et al.*, 2001).

(A)

$$FMN*H_2 + O_2 + R\text{-}CHO \xrightarrow{\text{Luciferase}} FMN + R\text{-}COOH + H_2O\ (+\ LIGHT\ 490nm)$$

(B)



(C)

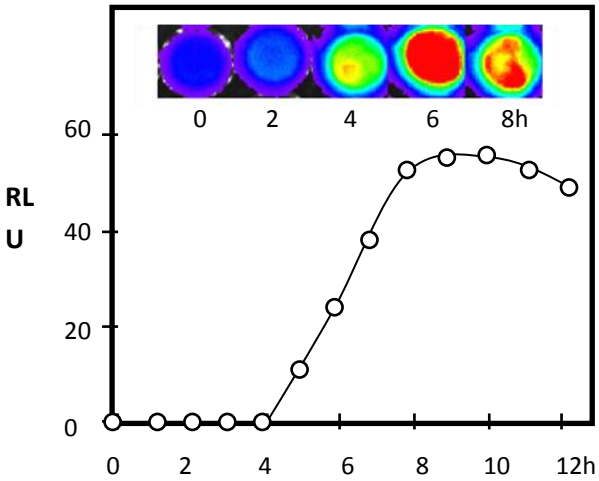


Fig 1. (A) Schematic of the *lux* reaction. **(B)** *Lux*-tagged *C. sakazakii* on Luria Bertani (LB) agar viewed with a Xenogen IVIS 100 Imager. **(C)** Growth of *lux*-tagged *C. sakazakii* in LB broth. Relative light units (RLU) were measured with a Luminoskan luminometer. Inset picture on Fig. 1 (C) represent growth of the tagged strain over 8 h. Image was viewed with a Xenogen IVIS 100 Imager.

Table 1. Summary of plasmids and transposons that have been used to *lux*-tag microorganisms of relevance to the food industry.

Bacterium	Plasmid/Transposon	Relevant information	References
Listeria monocytogenes	pPl2*lux*	Contains *luxABCDE* from *P. luminescens* and p_{help} promoter for the constitutive expression of light. Integrates into $tRNA^{ARG}$ locus on chromosome.	(Riedel *et al.*, 2007b)
Staphlyococcas aureus	pMK4 *luxABCDE* (Also known as p-XEN-1)	Contains the *luxABCDE* derived from *P. luminescens*. Functional in Gram positive and negative bacteria. Random host DNA fragments were used as promoters.	(Francis *et al.*, 2000, Moulton *et al.*, 2009)
Streptococcus thermophilus	P*Lux*72 & pTC*Lux*72	*Lux*AB genes derived from *V. harveyi*. Exogenous aldehyde (decanal) must be added.	(Jacobs *et al.*, 1995)
Bacillus cereus	*B. cereus* F4810/ 72(pMDX[P_1/*LuxABCDE*])	Contains the *LuxABCDE* from *P. luminescens* under the control of the *ces* promoter.	(Dommel *et al.*, 2010)
Bacillus subtilus	pSB357-*sspB:: LuxAB*	Contains *LuxAB* genes from *V. harveyi* under the control of the *B. subtilus sspB* promoter. The promoter encodes small acid soluble proteins which only induce bioluminescence upon germination of the *Bacillus* spores. Exogenous aldehyde must be added to the reaction.	(Hill *et al.*, 1994)
Bacillus anthracis	pSB357 containing *lux* genes	Contains the *LuxABCDE* from *P. luminescens* and is under the control of the spore small acid-soluble protein promoter *sspB*. Bioluminescence is induced upon germination of the *Bacillus* spores.	(Sanz *et al.*, 2008)
Escherichia coli, Cronobacter sakazakii, Citrobacter rodentium, Salmonella enterica, Yersinia enterocolitica, Pseudomonas aeruginosa, Shigella flexneri	p16S*lux*	Contains *LuxABCDE* genes from *P. luminescens*. Contains p_{help} promoter for the constitutive expression of light. Plasmid integrates into 16S region of Gram negative bacterial chromosome.	(Riedel *et al.*, 2007a)
Salmonella enterica	pBEN276	*LuxCDABE* genes from *P. luminescens* are under control of constitutive *E. coli frr* promoter	(Howe *et al.*, 2010)

		which encodes the genes responsible for the ribosome recycling factor. Also contains *tnsABCD* genes required for transposition. The plasmid inserts at the *attTn7* site in the genome.	
Escherichia coli	*Lux* pCGLS1 and pCGLS1R	*LuxABCDE* genes come from *X. luminescens*. Under the control of the *lac* promoter.	(Frackman *et al.*, 1990)
Salmonella enterica	pAK1-Lux1	Contains *luxCDABE* genes from *P. luminescens* controlled by the *lacZ* promoter. Strains carrying pAK1-*Lux*1 constitutively expressed the *lux* operon.	(Karsi *et al.*, 2008)
Salmonella enterica	pSB230 *LuxAB*	Contains the *Lux* genes *LuxAB* and contains *trp* pol (promoter operator leader). The *lux* genes are derived from *V. harveyi* Exogenous substrate dodecanal must be added.	(Hill *et al.*, 1991)
Lactococcus lactis	pNZ8048 containing *lux* genes	Contains the modified *lux* genes *ABCDE* from *P. luminescens* under the control of nisin inducible promoter *nisA* in pNZ8048. Induces luminescence upon detection of nisin.	(Immonen and Karp, 2007)
Bifidobacterium breve	pLuxMC1	Contains the *LuxABCDE* genes derived from *P. luminescens*. Integrates into the chromosome and allows for the constitutive expression of light with the aid of either the P_{rep} or the P_{help} promoter.	(Cronin *et al.*, 2008)
Lactococcus lactis	PNZ5519	Contains *LuxAB* derived from *V. harveyi*. FMN or riboflavin is added as the luminous reaction is dependent on intracellular $FMNH_2$ concentration. Exogenous substrate is added in the form of nonanal.	(Bachmann *et al.*, 2007)
Mycobacterium tuberculosis	pMV306hsp +*LuxAB*	Contains the genes *LuxABCDE* derived from *P. luminescens* under the control of the inducible promoter *Phsp60*. Exogenous aldehyde in the form of decanal must be added.	(Andreu *et al.*, 2010)
Pseudomonas syringae	pJF*Lux*1	Contains *LuxCDABE* from *P. luminescens* and under the control of a constitutive *TT4*	(Fan *et al.*, 2008)

		promoter.	
Salmonella enteritdis	pDB30 (GCDE/GCDE)	*LuxAB* genes from *V. harveyi* are carried by the Tn-5 containing plasmid pDB30. The bioluminescence marker was expressed in the presence of n-decanal.	(Chen and Griffiths, 1996a)
Yersinia enterocolitica	pUT-mini-Tn*5* *luxCDABE*-Km^r	*Lux* mutants of *Y. enterocolitica* were generated by transposon mutagenesis using a promoterless, complete *lux* operon (*luxCDABE*) derived from *P. luminescens.* Tn*5 lux* transposon integrates randomly into the *Yersinia* chromosome. The *lux* genes are under the control of a promoter active in foodstuff at low temperatures.	(Maoz *et al.*, 2002)
Listeria monocytogenes	pAUL- A *Tn4001 LuxABCDE*	The *Lux* genes (*ABCDE*) were derived from *P.luminescens* and were modified, by introduction of Gram-positive ribosome binding site, to be functional in Gram positive and Gram negative.	(Francis *et al.*, 2000)
Clavibacter michiganensis	Tn*1409*::*Lux*	Contains promoterless *LuxCDABE* operon and Tn*1409* transposon. Inserts into chromosome. *Lux* genes were derived from *P. luminescens*. No exogenous substrate added. The strain was constitutively bioluminescent.	(Xu *et al.*, 2010)
Escherichia coli	Mini-MuLux	Combines the mini-mu transposon with *luxCDABE* genes from *V. fischeri*. Mini-mu*lux* transposon lacks the *lux* operon promoter so expression of the *lux* genes was regulated by promoters located outside of the Mu right end. The *lux*-transposon was capable of inserting at random or in either orientation into chromosomal or plasmids DNA.	(Engebrecht *et al.*, 1985)

Early efforts at *lux*-tagging microorganisms involved cloning the *luxAB* genes which encode the catalytic luciferase enzyme and the substrate for the reaction was added exogenously. It is now more common and more efficient to clone the entire *lux* gene cluster, *luxA-E*, so that in addition to producing the luciferase enzyme the recombinant strain also produces the substrate. The *lux* genes can be placed under the control of a variety of promoters depending on the particular application. For constant expression, the *lux* genes can be placed under the control of a constitutive promoter which can be optimized for the host strain. For example Riedel and co-workers altered the constitutive *Lactococcus* consensus promoter P_{CP25} by introducing the 5' untranslated region of the *Listeria monocytogenes hlyA* gene. This new promoter P_{help} (help; highly expressed *Listeria* promoter) gives a strong signal in *Listeria* and other genera (Riedel *et al*., 2007a; Riedel *et al.*, 2007b). The *lux* system can also be used as a reporter of gene expression. The promoter of any gene of interest can be fused to the *lux* genes on a plasmid vector. When environmental conditions induce expression of the promoter the *lux* genes are expressed and light is emitted. For example a *hly* promoter fusion study confirmed that this *L. monocytogenes* promoter was induced by heat shock (Park *et al.*, 1992) and similarly a *ces* promoter fusion study was used to examine expression of the *Bacillus cereus* cereulide toxin gene in various foods (Dommel *et al.*, 2010).

In certain cases, the stability of plasmids used to *lux*-tag strains may be an issue and antibiotic addition may be required to maintain the plasmid. This problem may be addressed by using plasmids which integrate into specific sites on the bacterial chromosome. Examples include plasmid p16S*lux* which integrates into the 16S rRNA gene of Gram negative bacteria, including *Escherichia coli, Cronobacter sakazakii*, *Citrobacter rodentium*, *Salmonella* Typhimurium and *Pseudomonas*

aeruginosa (Riedel *et al.*, 2007a) or plasmid pPL2*lux* which integrates into the $tRNA^{Arg}$ locus in the *L. monocytogenes* chromosome (Bron *et al.*, 2006). Single copy integration systems may be preferred over multi-copy plasmid systems when expression levels of promoters are assayed. Howe *et al.* (2010) generated a plasmid where *luxA-E* genes under the control of the constitutive *E. coli frr* promoter are flanked by Tn7 transposon arms. The plasmid also contains *tnsABCD* genes required for transposition and the plasmid inserts at the *attTn7* site in the genome. This system was used to successfully and stably confer bioluminescence to eleven *Salmonella enterica* strains (Howe *et al.*, 2010).

3.1 Transposons

Bioluminescent strains can also be generated by transposon mutagenesis where *lux* genes are transferred on transposons which usually integrate randomly into the bacterial chromosome. The *lux* genes can be placed under the control of a constitutive promoter e.g. EM7 promoter for Tn*5* (Burns-Guydish *et al.*, 2005). Alternatively, promoterless *lux* genes can be used and the *lux* genes are then expressed via a promoter it acquires upon transposition. In this case the bank of transposants must first be screened for mutants with strong light emission (e.g. Maoz *et al.*, 2002). It has been noted that creation of *lux*-tagged strains using transposons may not be suitable for all studies as integration of a transposon may potentially influence bacterial pathogenesis. For example in a study by Hardy and coworkers (2004) the *lux*-tagged *L. monocytogenes* transposon mutant used was significantly attenuated when compared to the parent strain; the LD50 was 4-fold higher than that of the parent strain (Hardy *et al.*, 2004).

3.2 Benefits and limits of *lux*-based assays

One of the major advantages of luminescent reporters over other classical reporters such as green fluorescent protein (GFP) is the shorter half-life of luciferases, which allows for repeated measurement in quick succession (Waidmann *et al.*, 2011). In addition, fluorescent reporters have to be excited prior to measurements, which can result in a high background of autofluorescence (Shaner *et al.*, 2005). This problem was not encountered with luciferase reporters and background luminescence is low. Bioluminescent imaging is a faster and less labour intensive method of tracking bacterial growth when compared to conventional methods such as performing viable plate counts. While the latter requires preparation of media, overnight incubation of plates and enumeration the following day, bioluminescence readings can be measured in real-time and the output is instantaneous. The use of automated microtitre plate readers allows large numbers of samples to be assayed at a time and experiments can easily be performed over long time frames without the need for researcher input, thereby increasing laboratory efficiency. Bioluminescence methods are non-destructive as measurements can be made *in situ* in food products with minimal or no matrix disruption. Information can be obtained with regard to the precise location of bacteria in food and can determine hotspots of contamination. Other methods used to assess food samples require complete disruption of the food matrix before bacterial enumeration, resulting in a loss of spatial information. Bioluminescence-based methods may be of use in monitoring growth of bacteria in opaque liquids such as milk which are not conducive to optical density measurements. Growth in opaque liquids is generally examined by performing plate counts or indirect methods such as measuring pH. Monitoring growth by measuring

bioluminescence is now being considered as a more attractive alternative (Morrissey *et al.*, 2011).

The production of reduced flavin mononucleotide is limited by a functional electron transport chain, meaning only metabolically active cells can emit light (Alloush *et al.*, 2006, Howe *et al.*, 2010). However, this may also be considered beneficial as the monitoring of light output over time may tell if a microorganism is growing (increase in light emission), dead (decrease in light emission) or injured (light output remains constant) (Griffiths, 2000). A shortcoming of using the luciferase system is that oxygen is required for light emission meaning that light output cannot be measured under anaerobic conditions. Another potential drawback of *lux*-tagging is that the strain of interest must be engineered to contain the *lux* genes and this is not always possible for all strains. It is also possible that the expression of the luciferase and accessory proteins and the process of light emission may place a burden on bacterial metabolism and may impair growth of some strains. It should also be noted that certain compounds may quench or enhance the luciferase reaction (e.g. strong disinfectants and detergents) so it is imperative that appropriate controls are included in experiments.

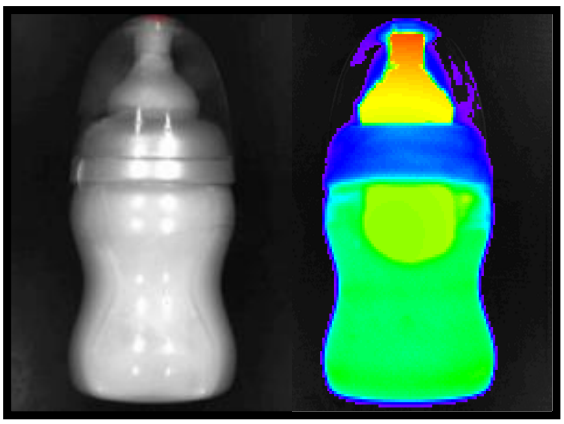
4. Applications of *lux*-tagged microorganisms in the food industry

Bioluminescence can be employed to track bacteria in real-time in a fast, reliable, non-destructive manner. The following sections describe studies which highlight the potential applications of *lux*-tagged microorganisms in the food industry and their advantages over conventional approaches.

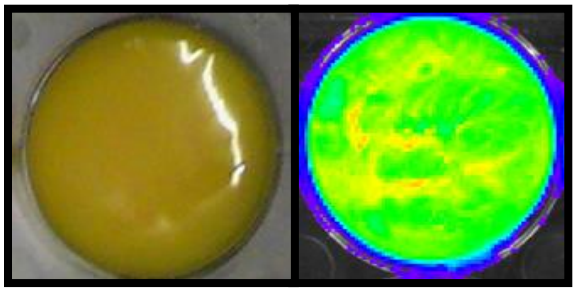
4.1 Reporter of the microbial ecology of foods

Bioluminescence-based systems can be employed to study the microbial ecology of foods and offer a rapid and simple method of establishing whether food products can support the growth of a particular microorganism. Experiments can be performed *in situ* in the food product and bioluminescence can be measured in opaque matrices and in the presence of background microflora (See Fig. 2 for examples). Bioluminescent reporter organisms can be used to monitor survival of bacteria during simulated manufacturing processes and show potential as tools for developing quantitative risk assessment models, where the data obtained could be applied to determine appropriate preservation regimes and storage conditions. Bioluminescence-based systems can also provide information on the spatial distribution of bacteria in food and identify hot-spots for contamination. Specific studies from the literature have been selected to highlight the aforementioned applications of bioluminescence-based assays.

(A)



(B)



(C)

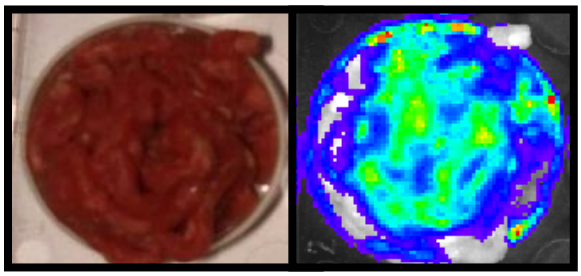


Fig 2. Bioluminescent images of **(A)** *lux*-tagged *C. sakazakii* in infant formula, **(B)** *lux*-tagged *S. enterica* serovar Typhimurium in egg yolk emulsion and **(C)** *lux*-tagged *L. monocytogenes* on minced meat. All images were taken with an Xenogen IVIS 100 Imager.

Bioluminescent *E. coli* and *Listeria* have been used to determine post-processing survival of these bacteria in yoghurt and cheese, *lux*-tagged *Campylobacter jejuni* were used to assess egg shell colonization and penetration in eggs and the survival of bioluminescent *E. coli* in fermented sausage has also been examined (Chen and Griffiths, 1996a, Hudson *et al.*, 1997, Fratamico *et al.*, 1997, Tomicka *et al.*, 1997, Ramsaran *et al.*, 1998, Allen and Griffiths, 2001). Riedel *et al*. (2007b) investigated growth of a *lux*-tagged *L. monocytogenes* strain in hot dogs and Camembert cheese, two food products commonly associated with *Listeria* contamination, by measuring bioluminescence and performing viable plate counts. Results showed that *Listeria* grew well in hot dog homogenate and that during exponential growth bioluminescence readings closely correlated with plate counts. High standard errors were recorded for plate counts performed on cheese homogenates and no significant growth of *Listeria* was observed (Riedel *et al.*, 2007b). In comparison, luminescence readings were more reproducible and they indicated a two-fold increase in bacterial numbers (Riedel *et al.*, 2007b). Low standard errors were recorded for luminescent results and therefore these results were deemed to be more accurate than manual plate counts recorded. Chen & Griffith (1996a) used *lux*-tagged *Salmonella* enteritidis to determine whether the strain could penetrate the membrane of an egg and grow in the egg under varying storage conditions. Results revealed that the strain could not penetrate the egg membrane but could grow in the space between the shell and the membrane. It was also shown that the *lux*-tagged strain remained metabolically active in the egg for up to four weeks at 4°C (Chen and Griffiths, 1996a). A study by Maoz *et al*. (2002) used a bioluminescent mutant of *Yersinia enterocolitica* to monitor growth of the strain on Camembert cheese. Bioluminescent readings determined that although the entire surface of the cheese samples were

initially contaminated with *Y. enterocolitica*, over time certain areas on the cheese became more highly contaminated than others (Maoz *et al.*, 2002). This implies that the local micro-heterogeneity of food plays an important role in pathogen distribution on the surface of the food and that this can be tracked using bioluminescence. A similar study used bioluminescent readings to show that light emitting *L. monocytogenes* only grew in Camembert cheese on sites close to the surface of the cheese. This confirmed that the altered pH in this region, due to growth of *Penicillium camembertii*, promoted growth of the pathogen (Ramsaran *et al.*, 1998). Howe *et al.* (2010) developed a reporter system for *lux*-tagging *Salmonella* strains and evaluated the performance of the strains on chicken skin sections at two temperatures in an aqueous environment. The authors suggest that their model may be employed to evaluate the *in vitro* efficacy of pathogen mitigation strategies such as antimicrobial compounds, and examine processing parameters that may be used in the poultry processing industry to control *Salmonella* (Howe *et al.*, 2010).

Chapter II of this thesis demonstrates the benefits of *lux*-technology in monitoring growth of bacteria in opaque liquids. This study showed that growth of the pathogen *C. sakazakii* could easily be measured in real-time in reconstituted infant formulae by monitoring bioluminescence even when low initial inocula were used. It was also noted that light could be detected in the presence of competing bacteria (Morrissey *et al.*, 2011). This is important as infant formula is not produced in a sterile environment and contains a resident microflora. Overall, results of this study suggest that *lux*-technology could potentially be employed to examine the influence of various ingredients and inhibitors on the growth of *C. sakazakii* and assist in the development of novel infant formula.

Lux-tagged strains can be employed to understand the effects of storage time on the microbial food safety of products. This is important as when products approach the end of their shelf life the concentration of preservatives may be rapidly decreasing due to their instability or interaction with the food matrix. Bioluminescence-based biosensors have been used to determine the levels of bacteriocins such as Nisin in food samples (Immonen and Karp, 2007). The assay was very sensitive; having the ability to detect 0.1 pg/ml in pure solution and 3 pg/ml in opaque solution (Immonen and Karp, 2007). This technique therefore allows for extensive dilutions of food samples prior to nisin quantification; this subsequently minimizes the impact of possible interfering factors in food matrices such as milk (Immonen and Karp, 2007). Bioluminescence also has applications in the study of pathogen colonization dynamics in plants. *Clavibacter michiganensis* subsp. *michiganensis*is is a Gram positive bacterium that causes wilting and bacterial canker in tomatoes, resulting in economic loss for growers. The method of infection in tomatoes is known; however, the mechanism of seed-to-seedling transmission is not well understood. Xu *et al.* (2010) constructed a *lux*-tagged *C. michiganensis* strain that allowed bioluminescent tracking of the colonization dynamic in tomato plants. When healthy tomato seedlings were inoculated with the bioluminescent strain by soaking, and careful dissection, it was revealed that the *lux*-tagged strain aggregated at highest levels on the hypocotyles and cotyledons (found in plant seedlings), during the early stages of germination (Xu *et al.*, 2010). It was also shown that the colonization dynamics of *C. michiganensis* infection of on seedlings promoted future infection when real-time imaging showed that BL-cmm17 aggregated on the hypocotyls and cotyledons (Xu *et al.*, 2010). With this information, attempts can be made to prevent or control *C.*

michiganensis aggregation, during the germination stage, which in turn could contribute to the improved shelf-life of tomato plants.

4.2 Studying injury and survival of pathogenic and spoilage microorganisms

Bioluminescence-based assays can be used to understand the factors affecting the growth and survival of pathogens in foods or food-related environmental conditions (e.g. low temperature), and may aid in determining appropriate storage conditions or the development of novel bioprocessing technologies.

Ellison and co-workers (1991) used bioluminescent imaging (BLI) as a real-time monitor of cell injury and recovery of *S.* Typhimurium following freezing at -20°C. Frozen cells were thawed and subsequently compared to the non-frozen cells. Bioluminescence was measured and manual plate counts were performed. Though both methods yielded the same results, the bioluminescent measurements were produced in real-time and showed that immediately after thawing there was a five-fold decrease in the number of viable cells recovered (Ellison *et al.*, 1991). Chen and Griffiths used bioluminescence as a real-time reporter of the effects of heat treatments (50°C, 55°C and 65°C) and pH (from 1 to 7) on the survival, growth and recovery of *Salmonella* cells (Chen and Griffiths, 1996a, Chen and Griffiths, 1996b). The *lux*-tagged strains were also used to monitor growth and survival of the pathogen in food samples including homogenized chicken meat, whole liquid egg and fluid milk. Duffy & co-workers (1995) used bioluminescence to model the thermal inactivation of *Salmonella* Typhimurium. This revealed that, while severely heat-treated cells could not be detected by viable plate counts, bioluminescence recovery curves could be extrapolated to study lower survival levels (Duffy *et al.*, 1995). The authors conclude that for their thermal inactivation experiments the bioluminescence

and plate count data showed complete equivalence and suggest that bioluminescence could be used for future experiments without parallel plate counts. It was also noted that production of bioluminescence was unaffected by the presence of competing microorganisms which included *E. coli*, *Citrobacter freundii* and *Pseudomonas fluorescens*.

Duncan and colleagues (1994) used bioluminescence to detect the activity of starved and viable but nonculturable bacteria (*Vibrio harveyi*, *E. coli* and *P. fluorescens*). Measurement of luminescence was found to be significantly more sensitive, rapid and convenient in quantifying metabolic activity following nutrient amendment (addition of yeast extract and nalidixic acid) than measurement of changes in cell length (Duncan *et al.*, 1994). This technique could potentially be applied to the food industry to assess nutrient limitations in a number of food systems. Liu and co-workers (2008) used a *lux*-tagged *L. monocytogenes* strain to demonstrate that heterologous production of the bacteriocin enterocin A by a *Lactococcus* starter strain could decrease numbers of the pathogen in cottage cheese (Liu *et al.*, 2008). *Lux* technology has also been used to demonstrate the enhanced activity of a variant of the bacteriocin Nisin against *L. monocytogenes* in frankfurters (Field *et al.*, 2010c).

Bacterial spores are generally not considered a hazard in foods as they are metabolically dormant. However, spores can germinate under favourable conditions and the subsequent growth, proliferation and/or toxin production can result in food spoilage or food poisoning. Food manufacturers commonly use heat treatments to reduce spores in foods; however, these treatments may negatively affect the taste and organoleptic quality. Furthermore, the effects of preservation treatments used to target spores are not fully understood; for example, it is not always known if

treatments kill or injure spores or affect spore germination and vegetative outgrowth (Ciarciaglini *et al.*, 2000). Bioluminescence-based experiments may be of use in addressing this lack of information. Ciarciaglini *et al*. (2000) used a strain of *Bacillus subtilis* in which the *lux* genes were placed under the control of the promoter for *sspB* (encoding the spore coat associated small acid soluble protein). This system resulted in light emission only from germinating spores and was used to monitor the effects of preservative treatments on germination. It was demonstrated that the combination of mild acidity, lactic acid and a pasteurization step resulted in enhanced inhibition of spore germination compared with the effects of the individual treatments alone (Ciarciaglini *et al.*, 2000). The authors highlight the advantages of the bioluminescence-based system over traditional methods of monitoring germination including rapidity, automated data capture and the ability of performing high throughput assays. Experiments can be performed in microtiter plates meaning that only small quantities of spores are required but more importantly it easily allows the study of potentially hundreds of treatment conditions. Germination-induced bioluminescence reporter systems can be employed to optimize existing preservation treatments and study the effects of new treatments or combination of treatments in order to develop milder preservation systems.

4.3 Reporters of virulence gene expression and pathogenesis

Lux-based gene expression experiments may provide information on how food ingredients, food compositions and environmental storage conditions affect expression of virulence genes. For example, *lux*-based approaches can be used to examine the expression of bacterial toxins in foods. This could identify potential sources of risk to the consumer as it could be used to assign foods and food

ingredients into different risk categories according to their ability to support toxin production. This application of *lux*-technology has been elegantly demonstrated in *B. cereus* by Dommel *et al*. (2010). The promoter of the cereulide toxin gene *ces* was fused to *lux* genes and the activity of the promoter in different foods was monitored by measuring bioluminescence. High promoter activity was observed in cooked rice while only intermediate promoter activity was observed in Camembert cheese (Dommel *et al.*, 2010). This finding is interesting as reports of emetic food poisoning implicate starchy foods rather than proteinaceous foods. A selection of retail food products (crème fraiche, pastry snack, Quark dessert, Camembert cheese, sauce Bernaise and cooked pasta) were inoculated with the bioluminescent *B. cereus* reporter strain and examination of bioluminescence allowed division of foods into categories by their potential to support toxin synthesis (low risk, risk and high risk). The amount of cereulide toxin extracted from spiked foods correlated well with the bioluminescence data. The authors conclude that as methods required for cereulide quantification are time consuming, laborious and error prone (especially from fat-rich foods), the bioluminescence-based approach developed may be an attractive alternative (Dommel *et al.*, 2010). Sheehan and co-workers (1992) fused the promoter of the *S. aureus eta* gene (encoding epidermolytic toxin A) to *lux* genes and examined the effects of environmental conditions on promoter expression by measuring bioluminescence (Sheehan *et al.*, 1992). A study by Cotter *et al.* (2008) investigating Listeriolysin S (LLS), a peptide haemolysin associated with *L. monocyogenes* strains responsible for the majority of listeriosis outbreaks, used a *lux* reporter system to demonstrate that *lls* is induced by oxidative stress (Cotter *et al.*, 2008).

Lux-tagged pathogens can be employed in *in vitro* and *in vivo* models of infection. For example, bioluminescent high-throughput assays can be employed to examine the interaction of bacteria with mammalian intestinal cells *in vitro* (Brovko *et al.*, 2011). Shimizu and co-workers (2011) constructed a bioluminescent reporter system to examine expression of Shiga toxin genes *stx1* and *stx2* in enterohemorrhagic *E. coli* 0157. This revealed that expression of *stx1* and *stx2* was enhanced upon contact with intestinal epithelial cells and within macrophages (Shimizu *et al.*, 2011). Bioluminescence can also be used to examine pathogen infection in animal models such as mice and permits monitoring of the spatiotemporal distribution in various organs as demonstrated by Bron *et al*. (2006) and Hardy *et al*. (2004) for *L. monocytogenes*. Live animals can be imaged several times over the course of an experiment allowing a reduction in the number of animals required for a given experiment (See Fig. 3). Experiments performed with *lux*-tagged *L. monocytogenes* strains have revealed that the ability to grow in the gall bladder may be an important facet of listerial pathogenicity. Hardy *et al*. (2004) demonstrated that the bacterium could grow extracellularly in the lumen of murine gall bladders. Further bioluminescence-based investigations by this group demonstrated that *Listeria* could be released from the gall bladder into the intestine *via* bile providing a means of re-infecting the same animal or allowing excretion in feces (Hardy *et al.*, 2006). Dowd and co-workers (2011) demonstrated growth of *lux*-tagged *L. monocytogenes* in *ex vivo* porcine gall bladders (Dowd *et al.*, 2011).

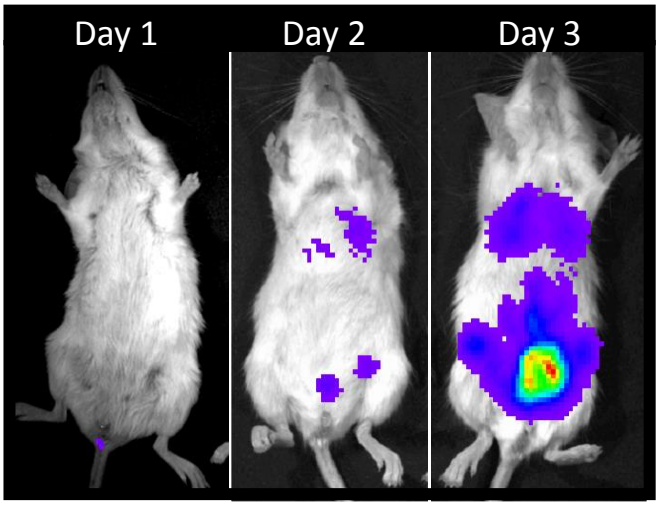


Fig 3. Visualisation of a *L. monocytogenes*-infected mouse over a three day period. The mouse was orally infected with *lux*-tagged *L. monocytogenes* and images were taken daily with a Xenogen IVIS 100 Imager. (Hill, unpublished image).

4.4 Detection of pathogens

The detection of food pathogens is critical for food safety. Traditional detection and identification methods are primarily phenotype-based and measure biochemical and antigenic properties. These approaches are usually laborious, time consuming and costly so alternative approaches are desirable. Bioluminescence-based methods for detection of bacteria have been investigated. This involves cloning the *lux* genes into a host specific bacteriophage and upon infection the *lux* genes are transferred to the host bacterium, transcribed and produce light (Griffiths, 2000). Luminescence cannot be expressed and emitted until the *lux* genes have been transferred to the bacterial host, as the bacteriophage do not contain the intracellular biochemistry to transcribe the genes themselves (Griffiths, 2000). This approach has successfully been applied

to several foodborne pathogens including *E. coli*, *L. monocytogenes*, *S. aureus*, *Mycobacterium tuberculosis* and *S.* Typhimurium (Ulitzur and Kuhn, 1987, Chen and Griffiths, 1996b, Loessner *et al.*, 1997).

Ulitzur & Kuhn (1987) added *lux*-modified L4 bacteriophage to *E. coli* W3110 and observed that the amount of light produced was directly related to the initial inoculum of the *E. coli* present in the medium and that as few as 10 cells could be detected within 100 minutes (Ulitzur and Kuhn, 1987). The *lux* L4 phage was also capable of detecting *E. coli* W3110 in sterile milk and could detect 10 *E. coli* cells/ml of milk within 30 minutes (Ulitzer and Kuhn 1987). Turpin & co-workers (1993) developed a *lux*-tagged phage to detect *Salmonella*. The method was found to be selective, it could detect *Salmonella* cells present in mixed cultures and in environmental samples, and there was an excellent correlation between luminescence and standard plate counts. It was also noted that no false positives or negatives were obtained (Turpin *et al.*, 1993). Chen & Griffiths (1996b) used *lux*-tagged phages to detect *Salmonella* in eggs. Eggs contaminated with 10^3 CFU of *Salmonella* became luminescent after 16 h of incubation, however, the limit of detection could be decreased to a little as 63 *Salmonella* cells when the incubation period was extended to 24 hours. Loessner & co-workers (1996) constructed a *lux*-tagged bacteriophage for detection of *L. monocytogenes* and established that following infection and a 2 h incubation period between 10^2 and 10^3 *L. monocytogenes* cells per ml of media could be detected. The authors subsequently demonstrated that with a short enrichment step (20 h) the phage was capable of detecting very low (less than 1 cell per gram of contaminated food) initial bacterial numbers in foods artificially contaminated with *L. monocytogenes* (Loessner *et al.*, 1996). These foods included ricotta cheese, chocolate pudding and cabbage, and foods with large background flora such as

minced meat and soft cheese (Loessner *et al.*, 1997). When this *lux*-tagged phage method was used for detection of *L. monocytogenes* in natural food and environmental samples from within the dairy plant, it was found to be as sensitive as standard plating procedures. It was noted, however, that the time required for detection of *Listeria* with the luciferase phage assay was 24 h compared to the four days needed for the plating method.

These examples illustrate that bioluminescence-based pathogen detection methods are sensitive, reliable and capable of detecting pathogens in the presence of background bacteria. The sensitivity of assays can be improved further by including an enrichment step. The construction of "locked" *lux*-tagged phages has been reported. These phages carry *lux* genes but because they lack an essential gene they cannot grow until it infects a host that synthesizes the missing protein. These "locked" phages may prevent accidental release of the genetically modified phage into the environment (Kuhn *et al.*, 2002).

4.5 Biofilm formation and biocide efficiency

Biofilms are sessile communities of cells which attach to surfaces and are known to enhance resistance of cells to environmental stresses (Kumar and Anand, 1998, Marshall, 1992) and provide protection against routine disinfectants and sanitizers (Kim *et al.*, 2006, Kim *et al.*, 2007a). Biofilms have been shown to occur in food preparation areas, on utensils, and conveyer belts, as well as on a number of other surfaces in food preparation facilities (Kumar and Anand, 1998). They are of major concern to the food industry as they cannot be removed by normal cleaning regimes and increase the risk of food-borne illness through cross contamination with raw ingredients. Biofilm formation and attachment has been demonstrated in a variety of

bacteria including *E. coli*, *L. monocytogenes*, *C. sakazakii* and *Pseudomonas putida* (Siragusa *et al.*, 1999, Hibma *et al.*, 1996, Hurrell *et al.*, 2009, Chumkhunthod *et al.*, 1998).

Bioluminescence provides a fast and easy way of monitoring biofilms and enables one to determine how various environmental factors influence their formation and growth. Hibma *et al*. (1996) employed a *lux*-tagged *L. monocytogenes* strain to study the attachment of biofilms to stainless steel surfaces and intravenous rubber tubing. Results showed that the luminescent signals recorded were similar to that of the manual plate counts and in some instances, the bioluminescent bacteria proved more efficient, as it gave results in real time whereas manual counts required longer periods of time for colony formation (Hibma *et al.*, 1996). Siragusa *et al*. (1999) used a *lux*-tagged *E. coli* strain to examine the ability of the organism to adhere to beef carcass tissue. Analysis of the bioluminescent output was higher on the lean fascia covered tissue than on adipose fascia covered tissue. This suggests that the physical topography and structure of a surface influences the bacterial association of biofilms to that surface (Siragusa *et al.*, 1999). It was noted that luminescence strongly correlated with bacterial counts.

Bioluminescence can be used to rapidly test the efficacy of sanitizers and disinfectants. Fabricant & co-workers (1995) investigated the effects of five biocides on four strains of bioluminescent *E. coli*. Results demonstrated that bioluminescent *E. coli* responds rapidly to varying concentrations of biocides (Fabricant *et al.*, 1995). Robinson & co-workers (2011) investigated the use of a *lux*-tagged *E. coli* as a whole-cell bioreporter of the very early stage of kill of fast-acting biocides under clean conditions and in the presence of organic soil. Their findings showed that even among biocides with similar mechanisms of action or with common active agents

there are differences in their activity during the very early phase of kill. The authors note that these differences would not be measurable using traditional recovery count methods due to the time required to transfer samples to neutralising diluents (Robinson *et al.*, 2011). This study highlights how bioluminescent-based experiments can provide a greater understanding of the mechanism and kinetics of microbial kill by biocides.

Bioluminescent strains have been employed to rapidly monitor the efficacy of santizers against biofilms. For example Chumkhunthod *et al.* (1998) used luminescent *P. putida* to examine the effect that two sanitizers employed in dairy plants for cleaning in place (a non-foaming acid sanitizer and a liquid hypochlorite sanitizer) had on this organism. Results demonstrated that neither sanitizer could completely eliminate biofilm-associated *P. putida*. The authors concluded that microbial bioluminescence was better for assessing the effectiveness of sanitizers against microbial biofilms as compared to manual plate counts as bioluminescence produced results in real-time (Chumkhunthod *et al.*, 1998).

Bioluminescence-based approaches may be of use in evaluating the efficacy of biocides, but it is important to note that some stronger disinfectants and detergents may either enhance or quench the luminescent reaction. For example ethanol at 1% v/v inhibited the luminescent reaction by 15% while ethanol at 4% v/v enhanced the luminescent reaction (Calvert *et al.*, 2000).

Bioluminescence can be used to examine the expression of specific genes associated with biofilm formation (Rice *et al.*, 1995, Wallace *et al.*, 1994). For example, Wallace *et al.* (1994) investigated the role of the exopolysaccharide alginate in adherence and biofilm formation by *P. aeruginosa* by creating a *algG-lux* reporter

fusion plasmid and were easily able to demonstrate the effect of environmental stimuli and carbon sources on *algG* expression (Wallace *et al.*, 1994).

4.6 Probiotics

In recent years there has been growing interest in the addition of probiotic bacteria to functional food products for the maintenance of general gastrointestinal health and the prevention of intestinal infections (FAO/WHO, 2001, Ross *et al.*, 2005). In order for probiotic bacteria to elicit their positive effects on the host they must be successfully delivered to and survive in the gastrointestinal tract. Bioluminescence-based methods are currently used in research to elucidate the behaviour of probiotic strains both *in vitro* and *in vivo*. Non-invasive, *lux*-based reporters could be used to monitor probiotic bacteria capable of reaching the gut in high numbers and subsequently interacting in a positive way with the gastrointestinal tract. Using this knowledge, robust probiotic strains could be identified and used to develop novel functional foods. This technology could help the food industry to identify the most effective mode of probiotic delivery to the gastrointestinal tract (Jiang *et al.*, 2006). Advances in genetic tools for manipulation of probiotic strains means that it should be possible to transform them with *lux* genes. Indeed, Jiang *et al.* (2006) transformed lactic acid bacteria with *lux* genes and Loessner and colleagues (2009) *lux*-tagged *E. coli* Nissle (Loessner *et al.*, 2009). Cronin and colleagues (2008) developed a *lux*-based reporter system for tracking of *Bifidobacterium breve* in mice and the authors were able to track the colonisation potential and persistence of this probiotic species in real-time (Cronin *et al.*, 2008).

4.7 Monitoring starter culture activity

Starter culture strains used in the production of fermented foods can be inhibited by phage or antibiotics. In order to limit any potential negative effects of phage or antibiotics, starter strain rotation strategies (SSR), which involve rotating the number and type of starter strains used in the batch fermenter, are employed. However, SSR requires detailed information on each starter used and results in inconsistent flavours and textures. Currently the most popular method of assessing starter culture activity is direct measurement of lactic acid levels using the Heap-Lawrence starter culture activity test (Durmaz and Klaenhammer, 1995, Sing and Klaenhammer, 1993). This test requires several hours to yield a result and can only be carried out once the starters are added to the fermentation tanks.

Lux genes have been introduced into strains of lactic acid bacteria including *Lactococcus lactis*, *Lactobacillus casei* and *Lactobacillus plantarum* and bioluminescent-based assays have been successfully used to detect antibiotics and bacteriophage in whole milk. Ahmad & Stewart used a bioluminescent strain of *Lactococcus lactis* subsp. *diacetylactis* and were able to detect bacteriophage active against that organism at concentrations as low as 10^5/ ml in 100 minutes (Ahmad and Stewert, 1988, Ahmad and Stewart, 1991, Stewart, 1990). Using a luminescent derivative of *Lactobacillus casei*, low levels of antibiotics could be detected in milk; for example 0.3 µg/ml of penicillin G could be detected in milk in less than 30 minutes (Dostalek and Branyik, 2005, Ulitzur, 1986). These experiments highlight the potential of using *lux*-tagged starter strains to develop rapid and robust methods to assess the presence of bacteriophage or antibiotics in milk. Non-*lux*-tagged counterparts can subsequently be used in the cheese making process.

5. Conclusion and future directions

It is evident that bioluminescence-based assays have numerous potential food industry-related applications and offer many advantages over traditional methods, which are usually microbiologically based. Bioluminescence-based assays may be of particular benefit to the field of food safety. Ensuring a safe food supply is a major challenge to the food industry especially since there is increased consumer demand for minimally processed, ready-to-eat foods. Bioluminescence-based assays can be used to examine the growth and survival of pathogens in various foods and under a variety of storage conditions. The availability of the entire genome sequences of food-borne pathogens will facilitate *lux*-based promoter probe assays, which may aid in elucidating the mechanisms employed by pathogens to adapt to the conditions encountered in food processing surroundings. It is becoming increasingly obvious that the environment to which a food-borne pathogen is exposed prior to ingestion can affect the outcome of infection. *Lux*-tagged strains can be used to easily examine the effects of various food compositions or storage conditions on the ability of pathogens to cause disease in animal models. Taken together, the results of these assays may aid in the design of optimal processing regimes or the development of novel preventative and control strategies which in turn may ultimately lead to a safer food supply. Bioluminescence-based assays may also have many potential uses in the area of beneficial food microbiology including the development of functional foods containing probiotics or monitoring starter culture activity. *Lux*-technology may also be of benefit to the improvement of hygiene in food processing environments by providing important insights into bacterial biofilm formation and biocide resistance.

In conclusion, *lux*-based technologies offer a sensitive, real-time, spatial and temporal methodology for monitoring the presence, quantity and behaviour of

bacteria *in situ* in complex food systems, something which has been a goal of food microbiologists since the first recognition of the importance of bacteria in food systems.

Aims of this thesis

The objectives of this thesis were to examine the potential applications of luciferase-tagged microorganisms to the food industry. The first aim was to monitor a *lux*-tagged *Cronobacter sakazakii* in reconstituted infant milk formula, in real-time. The second aim was to investigate a bioluminescent-based early warning system for starter culture disruption by bacteriophages and antibiotic residues. The third of this thesis was to examine the use of a bioluminescent-based assay to test the activity of bioengineered Nisin derivatives M21V and S29A against foodborne pathogens in laboratory media and selected foods.

Chapter II

Real-Time Monitoring of Luciferase-Tagged *Cronobacter sakazakii* in Reconstituted Infant Milk Formula

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Abstract

The aim of this study was to examine the potential of using a *lux*-tagged *Cronobacter sakazakii* strain to monitor growth of the bacterium in various liquids. *C. sakazakii* was transformed with plasmid p16S*lux* and integration of the plasmid at the desired site on the chromosome was confirmed by PCR. The growth of the *lux*-tagged strain was similar to that of the non–*lux*-tagged strain and the integrated plasmid was stable when cells were cultured in the absence of antibiotic. Growth of the *lux*-tagged strain was monitored in real-time in Luria-Bertani broth, skim milk and infant milk formula by using both the Luminoskan luminometer and the Xenogen IVIS imager. Bioluminescence could be detected when the *lux*-tagged strain was co-cultured with other bacteria. The effect of monocaprylin and nisin on the growth of *C. sakazakii* in milk was monitored by measuring bioluminescence. In conclusion, growth of a *lux*-tagged *C. sakazakii* can be monitored in real time in both clear and opaque liquids by measuring bioluminescence. *Lux*-tagged *C. sakazakii* strains could be potentially employed in high-throughput assays to monitor the effects of various infant milk formula compositions on growth of the bacterium.

Introduction

Enterobacter sakazakii was described as a new species in 1980 (Farmer *et al.*, 1980, Kucerova *et al.*, 2010). Members of this species were shown to be relatively heterogenous, and extensive geno- and phenotypic evaluations led to the definition of six new species that were moved to a new genus, named *Cronobacter* (Iversen *et al*., 2007). Although *Cronobacter* infections in adults have been reported, infants and children are considered to be most at risk. Infections can result in necrotizing enterocolitis, sepsis and meningitis and mortality rates can be as high as 40 to 80% (Back *et al.*, 2009, Breeuwer *et al.*, 2003, Cawthorn *et al.*, 2008, Iversen and Forsythe, 2003, Liu *et al.*, 2006, Willis and Robinson, 1988). While *Cronobacter sakazakii* has been isolated from a wide variety of environments and foods (e.g., fresh water, plants, spices and vegetables), Powered infant formula (PIF) is thought to be the primary transmission vehicle (Al-Holy *et al.*, 2010, Bowen and Braden, 2006, Drudy *et al.*, 2006, Kandhai *et al.*, 2004, Kandhai *et al.*, 2006, Molloy *et al.*, 2009, Mullane *et al.*, 2008a, Mullane *et al.*, 2008b, Nazarowec-White and Farber, 1997). PIF is manufactured in a non-sterile environment and so, given the ubiquitous nature of *C. sakazakii*, contamination with this pathogen is difficult to prevent. Microbiological analyses of commercially available PIF's using the method devised by the U.S. Food and Drug Administration have isolated the pathogen from 2.5 to 14% of samples tested (Iversen *et al.*, 2004, Muytjens *et al.*, 1988). As it is now evident that this isolation method has a number of limitations (Lampel and Chen, 2009), these figures may not reflect the true occurrence of *C. sakazakii* in PIF.

Research is currently focusing on updating detection methods by using a combination of microbiological and molecular methods (Lampel and Chen, 2009). Given the serious consequences of *C. sakazakii* infections and the economic costs of

product recalls, PIF manufacturers are devoting considerable efforts to preventing product contamination by the bacterium. Research is also focusing on identifying anti-*Cronobacter* agents, such as antimicrobial peptides, monoglycerides, etc., that can inhibit or kill the pathogen if present in the product (Hayes *et al.*, 2009, Nair *et al.*, 2008). Furthermore, as PIF manufacturers are continuously developing food ingredients destined for consumption by infants, to ameliorate milk allergies and influence the composition of gut microbiota, it will be vital to examine the effect of varying PIF compositions on the growth and survival of *C. sakazakii*. As reconstituted PIF is opaque and does not lend itself to optical density measurements, growth of the bacterium is generally examined by performing direct plate counts (Nair *et al.*, 2008) or, indirectly, by measuring pH changes (Chang *et al.*, 2010) or alterations in electrical impedance. These methods can be time-consuming; thus, a faster method of monitoring *C. sakazakii* growth would be beneficial.

A method for *lux*-tagging Gram-negative bacterial strains by using p16S*lux* has recently been developed in our laboratory (Riedel *et al.*, 2007a). This plasmid contains the *lux* operon derived from *Photorhabdus luminescens*, under the control of the constitutive promoter P_{help} (the subscript ‘‘help’’ is an acronym for ‘‘highly expressed *Listeria* promoter’’) (Riedel *et al.*, 2007b) and facilitates homologous recombination–based integration into the 16S rRNA gene of Gram-negative bacteria. The aim of the present study was to examine the growth characteristics of a *lux*-tagged *C. sakazakii* strain and explore its potential use in monitoring real-time growth of the bacterium in liquids including reconstituted infant formula (RIF).

Materials and Methods

Bacterial strains, media, and chemicals

C. sakazakii NCTC 8155 was transformed with plasmid p16S*lux* as previously described (Riedel *et al.*, 2007a). *Escherichia coli, Salmonella* Typhimurium, *Listeria monocytogenes, Bacillus licheniformis*, and *Bacillus cereus* strains were obtained from the University College Cork culture collection. The bacilli were isolated from commercial PIF (Begley, Unpublished data). *C. sakazakii, E. coli, Salmonella* Typhimurium, and *Bacillus* strains were routinely grown in Luria-Bertani (LB) medium (Merck, Darmstadt, Germany). *L. monocytogenes* was grown in brain heart infusion broth (Oxoid, Basingstoke, UK) at 37° C with shaking. When required, the antibiotic erythromycin (Sigma-Aldrich, MO, USA) was added at a concentration of 500 mg/ml. Stock solutions of nisin (10 mg/ml; 2.5% nisin from *Lactococcus lactis* N5764 (Sigma-Aldrich, MO, USA) were prepared in sterile water. Stock solutions (1 M) of monocaprylin (1-octanoyl-rac-glycerol; Sigma-Aldrich, MO, USA) were prepared in ethanol.

Plasmid stability

The *lux*-tagged *C. sakazakii* strain was grown overnight (approximately 16 h) in triplicate in LB broth containing 500 mg of erythromycin per ml. One millilitre of each culture was centrifuged at 9000 x g for 8 min, washed, and resuspended in an equal volume of ¼ -strength Ringer's solution (Merck, Darmstadt, Germany). Two hundred microliters was transferred into 10 ml of fresh LB broth without antibiotic. Tubes were incubated with shaking at 37 °C for approximately 8 h, after which time 200 µl of culture was transferred into fresh broth and incubated at 37 °C with shaking overnight. This passaging was repeated for a further 3 days. At every

passage, 100 µl aliquots were serially diluted in ¼ -strength Ringer's solution and spread plated onto LB agar. Plates were incubated at 37 °C. Fifty random colonies from each passage were scored for antibiotic resistance by patching colonies onto LB agar and LB agar plus 500 mg/ml of erythromycin.

Molecular biology experiments

A PCR was performed using primers (Eurofins MWG, Ebersberg, Germany) 16S_fwd_new (5' ACACTGGAACTGAGACACGGTCCAGACTCC- 3') and 16S_int_rev 5' TTGTAAAACGACGGCCAGTGAGCGCGCG- 3') to confirm the integration of p16S*lux*. Reactions were carried out in 50-µl volumes consisting of primers, Taq polymerase (Biotaq, Bioline, Luckenwalde, Germany), and deoxynucleotide triphosphates (dATP, dTTP, dGTP, and dATP) (Roche Diagnostics GmbH, Mannheim, Germany), which were used according to manufacturers' recommendations. Colonies were lysed prior to PCR by suspending a colony in 5 µl of water and microwaving at 800 W for 5 min. Amplification of the target region was achieved with 30 cycles of 95 °C for 2 min, 53.5 °C for 1 min, and 72 °C for 1 min. The PCR was carried out in a T3000 Thermocycler (Biometra, Gottingen, Germany). Four microlitres (µl) of the PCR mixture was run on a 1% agarose gel (Invitrogen, Carlsbad, CA) at 90 V and 2 A for 60 min with a Power pack 200 (Bio-Rad, Hercules, CA). The gel was imaged under UV light in an Innovate gel doc system (Alpha Innotech, San Leandro, CA).

Monitoring of growth in LB broth by performing viable plate counts and measuring bioluminescence

One millilitre of overnight cultures was centrifuged at 9000 x g for 8 min, washed, and resuspended in an equal volume of ¼ -strength Ringer's solution. A 2% inoculum was added to fresh LB broth. Viable plate counts were performed at intervals by diluting 100 µl aliquots in ¼ -strength Ringer's solution and plating onto LB agar. Two hundred microliters of the freshly inoculated LB broth was added to a 96-well white plate (Nunc, Thermo Fisher Scientific, Roskilde, Denmark), and bioluminescence measurements were recorded every h in relative light units (RLU) by a Luminoskan luminometer automatic plate reader (Thermo Fisher Scientific, Roskilde, Denmark). Two hundred microliters was also added to 96-well clear plates (Starsted Ltd., Numbrecht, Germany), and bioluminescence was measured in photons per second per square centimeter with the IVIS Xenogen Imaging 100 system (Xenogen, Alameda, CA) with a binning of 16 and an exposure time of 1 min.

Monitoring bioluminescence in the presence of other bacteria

C. sakazakii, *B. licheniformis*, *B. cereus* (two strains), *L. monocytogenes*, *Salmonella* Typhimurium, and *E. coli* overnight cultures (approximately 16 h) were washed in Ringer's solution as previously described. Approximately 4 CFU/ml of *C. sakazakii* per ml was transferred into a fresh tube of LB broth, and an equal level of *B. licheniformis* was added. The tube was vortexed and 200 µl was transferred to a well of a 96-well white plate; bioluminescence was measured every h with a Luminoskan luminometer. This was repeated for the other bacteria. In each case equal levels of *C. sakazakii* and the competing bacterium were used.

Monitoring bioluminescence in milk

Six popular brands of PIF and two brands of skim milk powder were purchased from a local supermarket. RIF was prepared by adding 10 g of powder to 100 ml of boiled water cooled to 70 °C, in bottles that were sterilized in a Smartec electronic steam sterilizer (Mothercare, Watford, UK). Reconstituted skim milk (RSM) was prepared by adding 10 g of skim milk powder to 100 ml of deionized water and autoclaving at 110 °C for 10 min. Ten-millilitre aliquots of room temperature RSM and RIF were transferred into universal containers (Sterilin, Caerphilly, UK) and inoculated with 200 µl of washed *C. sakazakii* overnight cultures (1 x 10^9 CFU). Aliquots of 200 µl were added to 96-well plates and bioluminescence was measured every h by both a Luminoskan luminometer and a Xenogen IVIS imager as previously described. For experiments involving nisin and monocaprylin, the required levels of freshly prepared stocks were added to RSM prior to addition of bacteria. As monocaprylin is prepared in ethanol, equivalent levels of ethanol alone (i.e., minus monocaprylin) were added to RSM to serve as an appropriate control. Aerobic plate counts were performed for infant formula by performing serial dilutions in maximum recovery diluent (Oxoid, Basingstoke, UK) and plating onto standard plate count agar (Oxoid, Basingstoke, UK). Plates were incubated at 37 °C for 48 h.

Reproducibility and statistical analysis

All experiments were performed in triplicate from three separate cultures (i.e., three biological repeats) and repeated twice on two other separate days. The data presented are for results obtained on one particular day, but similar results were obtained for repeat experiments. Analysis of the scientific data was carried out using the basic

statistical software Excel. Statistical significance was determined by Student's t test ($P < 0.05$).

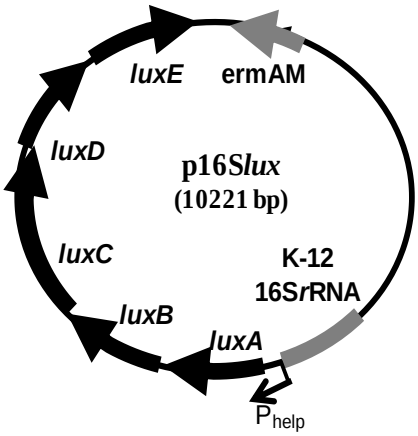
Results

Analysis of a *lux*-tagged *C. sakazakii* strain

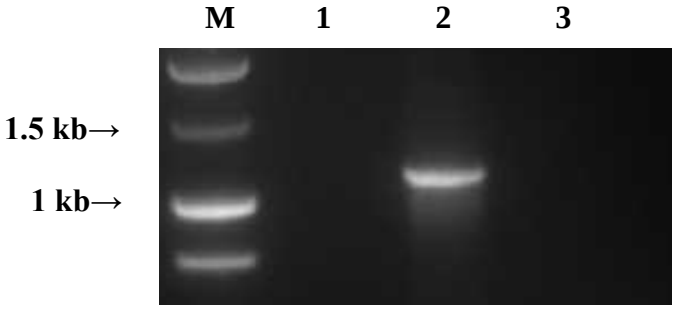
C. sakazakii NCTC 8155 was transformed with plasmid p16S*lux* by electroporation as previously described (Oh and Kang, 2005). This plasmid contains the *lux* operon derived from *P. luminescens* under the control of the constitutive promoter P_{help} (Riedel *et al.*, 2007b) and facilitates homologous recombination–based integration into the 16S rRNA gene of Gram-negative bacteria (Fig. 1A). Using primers based on the integration site, PCR amplification of a 1,163-bp product in the *lux*-tagged *C. sakazakii* strain confirmed that p16S*lux* had integrated at the desired location. As expected, a PCR product was not amplified from the non–*lux*-tagged strain (Fig. 1B). Examination of strains streaked onto LB agar revealed that, as expected, the *lux*-tagged strain emitted light whereas the non-tagged strain did not (Fig. 1C). In order to investigate whether the presence of p16S*lux* or the generation of light interfered with bacterial growth under standard laboratory conditions, growth of the strains in LB broth was followed by performing viable plate counts at intervals. It was observed that the two strains had similar growth rates (Fig. 2A). It was also noted that colony shape, size, and morphology were identical for both strains on all media examined (LB, tryptic soya agar [TSA] and Druggan Forsythe Iversen (DFI) selective agar) and that both strains produced yellow-pigmented colonies on TSA incubated at 25 °C (data not shown). Because the entire plasmid is present in the strain, albeit integrated into the chromosome, it is possible that it will excise and be

lost in the absence of selective pressure. Bearing that in mind, the stability of the integrated p16S*lux* plasmid was examined by continuously passaging the strain in LB broth in the absence of erythromycin. It was determined that the integrated plasmid was 100% stable over two transfers and 98% stable over four transfers but was slightly unstable over longer time periods (Fig. 2B). Therefore, the p16S*lux*-tagged *C. sakazakii* can be used in short-term assays, lasting no longer than 2 days, in the absence of selective pressure.

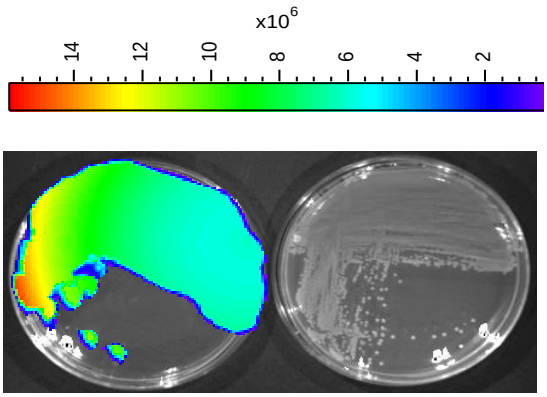
(A)



(B)



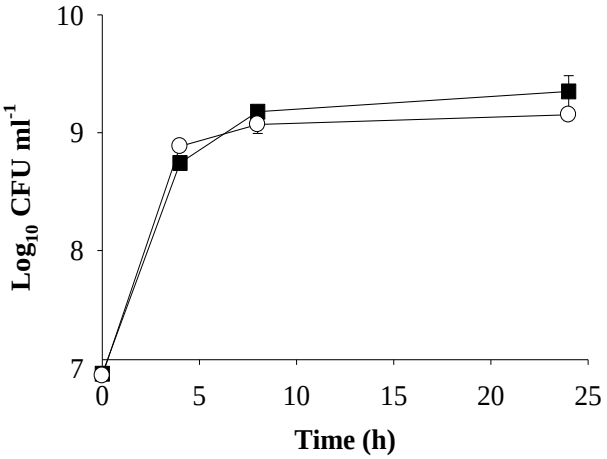
(C)



Lux tagged Non *lux* tagged

Fig. 1. **(A)** Map of p16S*lux* showing P_{help} promoter, *E. coli* DH10B 16S gene and *lux* operon (*luxABCDE*). **(B)** Gel picture of PCRs performed to check the integration of p16S*lux* into the chromosome. M, molecular marker (1 kb; Promega); lane 1, nontagged *C. sakazakii* used as template DNA; lane 2, *lux*-tagged *C. sakazakii* used as template DNA; lane 3, negative control (water). **(C)** *C. sakazakii lux*-tagged and non-tagged strains streaked onto LB and viewed with a Xenogen IVIS 100 imager. The colour bar represents the bioluminescent signal measured in photons per second per square centimetre.

(A)



(B)

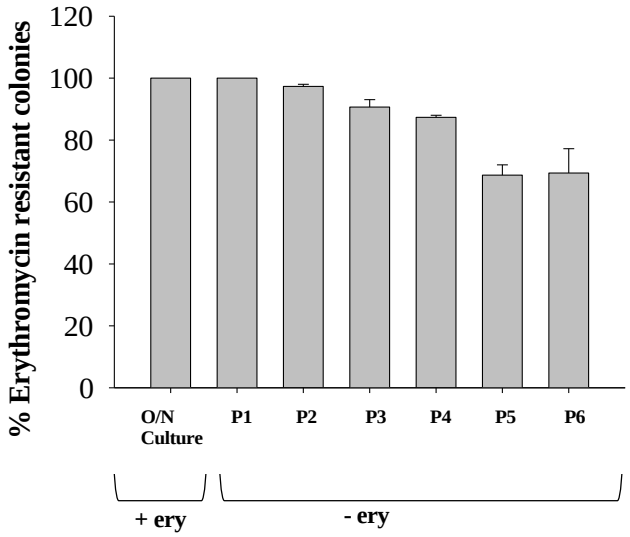
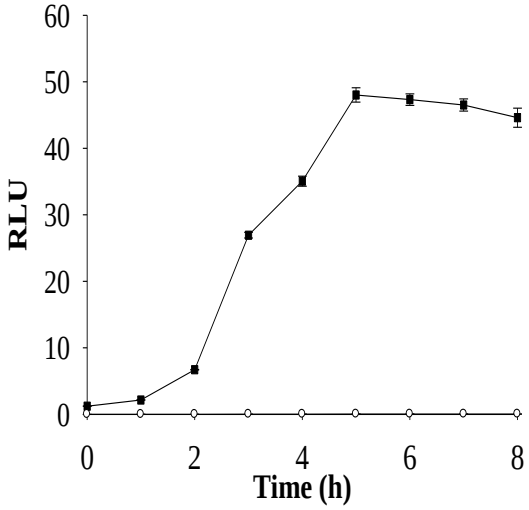


Fig 2. (A) Growth of *lux*-tagged (■) and non-tagged (o) *C. sakazakii* strains in LB broth at 37 °C. Data are presented as average Log CFU per milliliter +/- standard deviations for three biological repeats. **(B)** Stability of p16S*lux*. Shown are percentages of erythromycin-resistant colonies following passaging of the *lux*-tagged *C. sakazakii* every 8 hours in the absence of antibiotic selection. P, passage number; O/N, overnight; ery, erythromycin.

Monitoring of bioluminescence in LB broth

Growth of the *lux*-tagged strain was monitored in LB broth by measuring bioluminescence with both a luminometer and a Xenogen IVIS imager (Fig. 3A and 3B, respectively; OD data presented in appendix). The amount of bioluminescence increased over time as cell numbers increased. Bioluminescence was not detected for the non–*lux*-tagged strain or LB broth alone, confirming that there was no background interference. Results were reproducible; biological repeats within one experiment and repeat experiments performed on different days all gave similar results. It was noted that RLU readings can be correlated with viable plate counts (R^2 ~ 0.84; correlation scatter shown in Fig. 1 appendix).

(A)



(B)

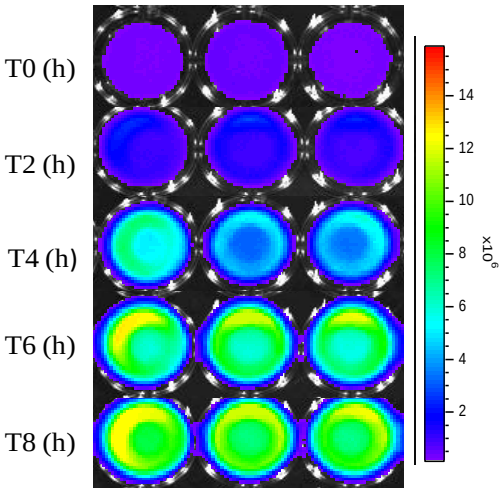


Fig 3. (A) Growth of *lux*-tagged (■) and non-tagged (o) *C. sakazakii* strains in LB broth at 37 °C. Bioluminescence was measured by a Luminoskan luminometer. Error bars represent the standard deviations of triplicate experiments. (B) Growth of *lux*-tagged *C. sakazakii* in LB broth at 37 °C. Bioluminescence was measured with a Xenogen IVIS 100 imager.

Monitoring of bioluminescence in the presence of other bacteria

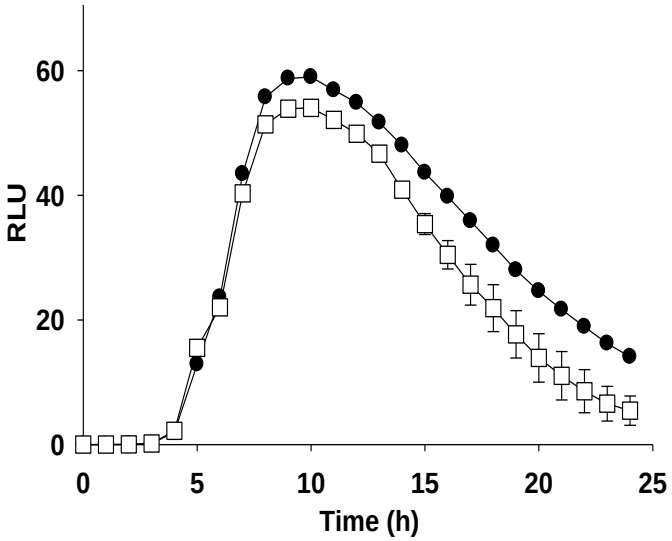
In order to examine whether growth of the *lux*-tagged strain could be monitored in the presence of other bacteria by measuring bioluminescence, the *lux*-tagged strain was co-cultured with other foodborne pathogens from our laboratory culture collection and also *Bacillus* strains previously isolated from PIF. Figure 4A shows the results obtained for the experiments performed with *B. licheniformis*. It was observed that bioluminescence readings in the presence of *B. licheniformis* were similar to those obtained when *C. sakazakii* alone was grown in LB. Similar results were obtained when *C. sakazakii* was co-cultured with equal concentrations of *B. cereus, L. monocytogenes, E. coli*, and *Salmonella* Typhimurium (Fig. 4 appendix). These experiments show that the ability of the *lux*-tagged *C. sakazakii* strain to emit light is unaffected and growth can be monitored in the presence of competing bacteria by measuring bioluminescence.

Monitoring bioluminescence in milk

Growth of the *lux*-tagged strain was examined in two different brands of RSM and six different brands of commercially available PIF. Similar to the results obtained for LB broth, it was observed that bioluminescence readings increased over time as cell numbers increased (luminometer data are presented in Fig. 4B; Fig. 1 appendix). As PIF is not produced in a sterile environment, it usually contains an inherent microbial flora, which we also expected to increase in numbers over the time course of our assay. Indeed, aerobic plate counts performed with PIF after the 24-h incubation revealed average counts of 10^8 CFU/ml, again demonstrating that the *lux*-tagged *C. sakazakii* can be detected in the presence of competing microflora. Bioluminescence

results obtained for RSM and PIF experiments were reproducible; biological repeats within one experiment and repeat experiments performed on different days all gave similar results (Fig. 4 appendix).

(A)



(B)

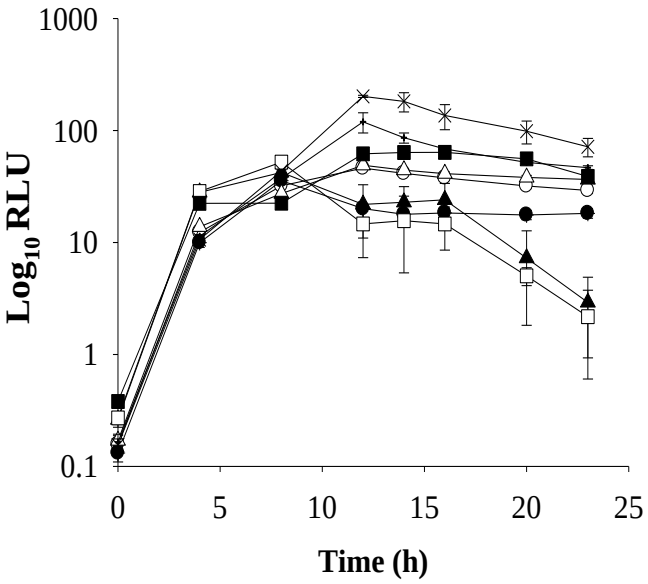
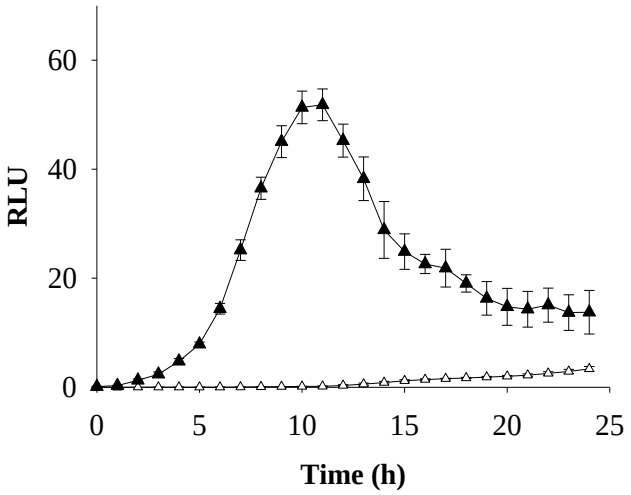


Fig 4. **(A)** Growth of *lux*-tagged *C. sakazakii* alone in LB (●) or in the presence of *B. licheniformis* (□). Error bars represent the standard deviations of triplicate experiments. **(B)** Growth of *lux*-tagged *C. sakazakii* in six different brands of RIF (●, o, Δ, ■, +, X) and two different brands of reconstituted skim milk (▲, □). Bioluminescence was measured with a Luminoskan luminometer. Error bars represent the standard deviations of triplicate experiments.

Examination of the effects of monocaprylin and nisin on the growth of *C. sakazakii* in milk

Monocaprylin and nisin have previously been investigated for their potential to inhibit *C. sakazakii*. In these studies, bacterial numbers were monitored by performing viable plate counts (Lee and Jin, 2008, Nair *et al.*, 2004). We decided to carry out similar experiments to investigate whether growth of *C. sakazakii* in the presence of these compounds could be monitored by measuring bioluminescence rather than performing plate counts. Nair *et al*. demonstrated that monocaprylin rapidly inactivates *C. sakazakii*; the bacterium was reduced by 5 log units after a 60-min exposure to 50 mM monocaprylin (Nair *et al.*, 2004). The results of our bioluminescence-based experiments agree with these findings (Fig. 5A). Monocaprylin stocks were prepared in ethanol as this fatty acid does not dissolve in water. Ethanol alone (2.5%, vol/vol) was added to RSM to serve as an appropriate control. It was observed that ethanol affected growth of *C. sakazakii* (as determined by plate counts), hence explaining why RLU readings are lower than those normally obtained for *C. sakazakii* in RSM alone (Fig. 5B). In agreement with a previous study (Lee and Jin, 2008), addition of various concentrations of nisin (100 to 500 mg of nisin powder/ml) did not have any effect on the growth of *C. sakazakii*. Results obtained for 100 mg/ml nisin are shown in Figure 5B.

(A)



(B)

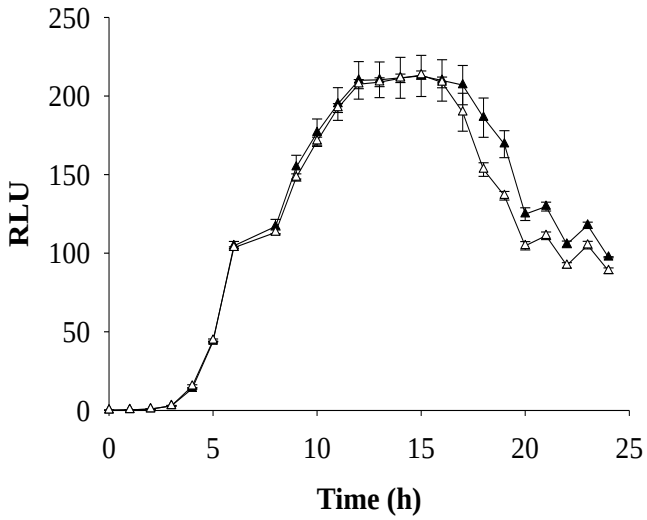


Fig 5. **(A)** Growth of *lux*-tagged *C. sakazakii* in RSM containing 2.5% (vol/vol) ethanol (▲) and in RSM supplemented with 25 mM monocaprylin (Δ). **(B)** Growth of *lux*-tagged *C. sakazakii* in RSM (▲) and in RSM supplemented with 100 mg/ml nisin (Δ). Data are presented as average relative light units +/- standard deviations for three biological repeats.

Sensitivity of bioluminescence measurements

C. sakazakii is usually only present in low numbers in natural environments such as contaminated PIF (Muytjens *et al.*, 1988); therefore, it is likely that future experiments aimed at examining the growth potential of the bacterium in various surroundings will use low starting cell numbers. Hence, we decided to investigate the sensitivity of measuring bioluminescence as an indicator of *C. sakazakii* growth. Media (LB, RSM, and RIF) were inoculated with various initial inocula and bioluminescence was measured over 25 h periods. It was observed that even with the lowest initial inoculum (100 CFU/ml); growth of the bacterium could be easily detected within the time course of the assay (Fig. 6 shows data obtained for RSM; Fig. 2 appendix (PIF data)).

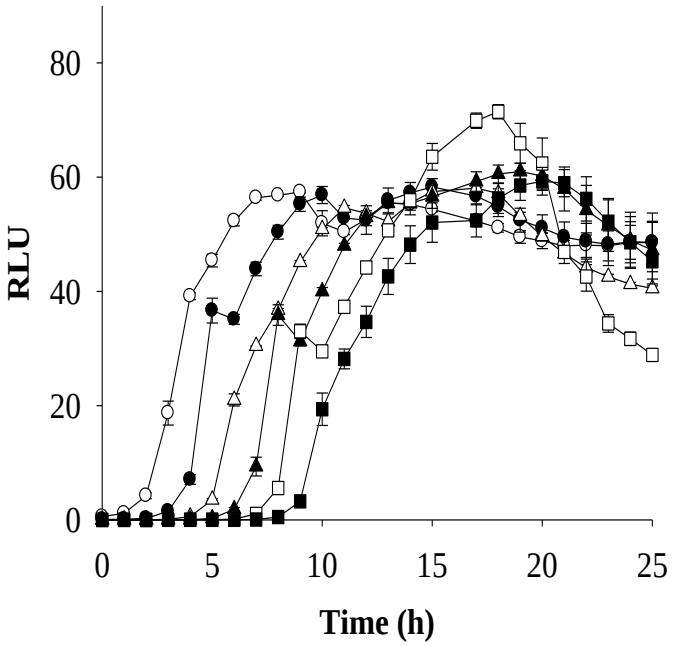


Fig 6. Growth of *lux*-tagged *C. sakazakii* in RSM. Varying initial inocula were used: 2 Log CFU/ml (■), 3 Log CFU/ml (□), 4 Log CFU/ml (▲), 5 Log CFU/ml (Δ), 6 Log CFU/ml (●), and 7 Log CFU/ml (o). Bioluminescence was measured with a Luminoskan luminometer. Data are presented as average relative light units +/- standard deviations for three biological repeats.

Discussion

C. sakazakii infections can cause a number of severe and life-threatening conditions including meningitis, bacteremia, and necrotizing enterocolitis in neonates and infants. PIF has been implicated as the primary vehicle of transmission of the bacterium (Bowen and Braden, 2006, Codex Alimentarius Commission, 2008). PIF manufacturers are therefore interested in identifying ingredients that may inhibit the growth of *C. sakazakii*. Casein-derived antimicrobial peptides, bacteriophages, monoglycerides, etc., have been investigated in this regard (Hayes *et al.*, 2009, Kim *et al.*, 2007b, Nair *et al.*, 2008). Furthermore, as manufacturers are continuously aiming to improve PIF formulations by the addition of constituents that will enhance infant nutrition and health, they will be interested in examining the ability of *C. sakazakii* to grow and survive in the resulting products. Real-time methods for monitoring growth of the bacterium in RIF would greatly assist these endeavours. As milk is opaque, growth of *C. sakazakii* in milk cannot be assessed by direct measurement of optical density. At present, growth of the bacterium in milk is primarily monitored by performing viable plate counts and plating onto chromogenic differential agar such as Druggan Forsythe Iversen agar (Back *et al.*, 2009, Breeuwer *et al.*, 2003, Gurtler and Beuchat, 2007, Lampel and Chen, 2009, Nair *et al.*, 2008). Indirect methods include monitoring pH changes by the addition of pH indicators such as bromocresol blue and measurement of alterations in electrical conductance; for example, the Don Whitley RABIT (Rapid Automated Bacterial Impedance Technique) technology (Forsythe, 2009, Iversen *et al.*, 2008). Oh and Kang have also described a method for the fluorogenic detection of *C. sakazakii* by the addition of 4 -methylumbelliferyl-α-D-glucoside to broth, which is hydrolysed as the bacterium produces α-glucosidase (Oh and Kang, 2005). The aim of the present study was to

examine the potential of using *lux*-tagged *C. sakazakii* as a faster method of following growth of the bacterium in liquids, including standard laboratory media, skim milk and RIF. While attempts by other researchers to *lux*-tag *C. sakazakii* failed (Forsythe, 2009), we succeeded by using the integrative plasmid p16S*lux*. Initial experiments focused on examining the effect of introducing the plasmid on the general growth characteristics of *C. sakazakii*. It was found that the plasmid did not negatively impact growth and the *lux*-tagged strain behaved in a manner similar to that of the non-tagged counterpart under standard laboratory conditions. Continuous passaging of the strain in the absence of antibiotic confirmed that the plasmid was sufficiently stable for monitoring growth over relatively short time frames. Subsequent experiments showed that growth of the *lux*-tagged strain could be monitored in real time in LB broth, milk and RIF by using both a luminometer and a Xenogen IVIS imager. Tracking of a *lux* strain by measuring bioluminescence proved to be very reproducible and bioluminescence was shown to be unaffected by the presence of other competing bacteria including *Bacillus* spp., which are the major competitors in infant formula. Bioluminescent tracking of *C. sakazakii* in milk is a faster and less labour-intensive method of monitoring growth than other, conventional methods, such as performing viable plate counts. The latter can require preparation of large amounts of media and a limited number of samples can be analysed at a specific time as samples must be serially diluted and plated. Furthermore, plates require overnight incubation, after which colonies must be counted. Bioluminescence- based experiments offer many advantages. Readouts from the luminometer are instantaneous, so growth can be monitored in real time. The use of automatic microplate readers allows larger numbers of samples to be analysed, experiments can easily be performed over longer time frames (e.g.,

overnight) and as the system is automated, researchers are free to conduct other work simultaneously. Bioluminescence-based tracking could be of benefit to high-throughput assays. A potential drawback is that the *C. sakazakii* strains of interest must be engineered to contain the *lux* plasmid and while we did not encounter problems with the *C. sakazakii* strain that we used, not all *C. sakazakii* strains may be as readily genetically manipulated. Future efforts should also investigate the potential of *lux*-tagging the other six *Cronobacter* species (*C. malonaticus, C. turicensis, C. muytjensii, C. condimentii, C. universalis* and *C. dublinensis*). While it has been reported that certain factors (such as alcohol) can affect luciferase and hence the generation of light by causing either enhancement or quenching effects, these factors will not likely be encountered when performing experiments mimicking ‘‘real-life’’ PIF conditions. In summary, our study demonstrates that bioluminescent imaging facilitates real-time tracking of *C. sakazakii* in a culture-independent manner, in opaque food matrices such as infant formula, which are not conducive to optical density measurements. This method could be employed to examine the influence of various ingredients and inhibitors on the growth of *C. sakazakii* in infant milk formula.

Conclusion

In conclusion we have examined the potential of using a *lux*-tagged *C. sakazakii* strain to monitor growth of the bacterium in various liquids. The tagged strain was shown to integrate at the desired site on the chromosome and growth of the *lux*-tagged strain was similar to that of the non–*lux*-tagged strain. The integrated plasmid was stable when cells were cultured in the absence of antibiotic. Growth of the *lux*-tagged strain was monitored in real-time in Luria-Bertani broth, skim milk and infant milk formula by monitoring bioluminescence. Bioluminescence could be detected when the *lux*-tagged strain was co-cultured with other bacteria. The effect of antimicrobial agents on the growth of *C. sakazakii* in milk was monitored by measuring bioluminescence. Growth of the *lux*-tagged *C. sakazakii* could be monitored in real time in both clear and opaque liquids by measuring bioluminescence.

Chapter III

Investigation of a Bioluminescence Based 'Early-Warning' System for Starter Disruption by Antibiotic residues and Bacteriophages

A manuscript based on this chapter is in preparation

Abstract

The presence of antimicrobial agents in bulk milk can inhibit or slow *Lactococcus* proliferation during cheese fermentation having a negative impact on cheese quality or result in complete failure of cheese batches. A rapid method of tracking starter culture activity would therefore be useful to the cheese industry. As starter culture strains are not always easily manipulated by means of electroporation, the aim of this study was to construct a bioluminescent panel of *Lactococcus* strains using a conjugative *lux*-vector. These tagged strains would subsequently be used to monitor the action of antimicrobial agents in opaque environments such as milk.

The newly constructed plasmid pRH001*lux* consisting of an *Escherichia coli-Lactococcus* shuttle vector ligated to *luxABCDE* genes isolated from p16S*lux* was a suitable vector for expressing the *lux* genes and in the presence of pMRC01 facilitated conjugative transfer of the *lux* plasmid between *Lactococcus* strains. As current methods of phage/antibiotic detection are slow, the newly developed plasmid could help identify those strains suitable for starter strain rotation strategies and provide techniques for identifying antibiotic residues in whey. In conclusion, we have constructed a stable conjugative *lux* vector which can be monitored in clear and opaque environments by measuring bioluminescence. The *lux*-tagged starter strains are capable of monitoring the effects of various antibiotics on the growth of a variety of tagged starter culture strains.

Introduction

Lactococcus lactis cultures are extensively used in the dairy industry where they are employed as starter culture strains for the production of a variety of cheeses. During cheese production these Lactic Acid Bacteria (LAB) utilise lactose and citrate and excrete lactic acid, which in turn coagulates the milk; an essential step for successful cheese production. However, the bulk milk can become contaminated with antimicrobial agents through the presence of phage or antibiotics and this may lead to subsequent starter culture failure, resulting in huge economic and time loss for cheese manufactures (Ahmad and Stewart, 1991, Guinee and O'Brien, 2010). At the moment a range of antibiotics are used as veterinary treatments for bovine infections such as mastitis. If sufficient levels of antibiotic are present in milk obtained from antibiotic treated animals, the starter culture inoculum can be slow to ferment and in severe cases may fail to develop altogether (Ahmad and Stewart, 1991). Other antimicrobial agents which negatively influence cheese production include *L. lactis* bacteriophage. *L. lactis* phages have been assigned to 10 species (Marvig *et al.*, 2011, Deveau *et al.*, 2006). The most common *Lactococcus* phages that are problematic in the dairy industry include; 936, c2 types and P335 types, with c2 and 936 being isolated most frequently (Mahony *et al.*, 2008, Moineau *et al.*, 1996). Thermal inactivation is most commonly used for phage inactivation but many c2 phages survive pasteurisation temperatures as well as traditional cheese milk temperatures if present in high enough numbers (10^4 PFU/ml) (Marvig *et al.*, 2011, Atamer *et al.*, 2010, Garneau and Moineau, 2011).

A number of phage defence mechanisms have evolved to counter-act the action of phage with much success. Three of these mechanisms are prevention of phage adsorption, restriction and modification, and abortive infection (Murray, 2000, Sing

and Klaenhammer, 1993, Tükel *et al.*, 2006, Chopin *et al.*, 2005, Madera *et al.*, 2003, Pingoud *et al.*, 2005, Garneau and Moineau, 2011). These mechanisms are often encoded on plasmids and in some cases multiple genes can be present on the same plasmid providing elevated levels of phage resistance (Sing and Klaenhammer, 1993). In addition, advances in our understanding of novel phage resistance mechanisms have led to the use of natural mutations or genetically modified defence strategies such as Bacteriophage Insensitive Mutants (BIM's), phage-encoded resistance (Per), antisense RNA and superinfection exclusion (Sie) (Mahony *et al.*, 2008, McGrath *et al.*, 1999). However phage can overcome these resistance mechanisms.

Due to the ubiquitous nature of bacteriophage and the on-going use of antibiotics for the treatment of bovine infections, complete elimination of antimicrobial substances from the cheese production environment is not possible. Methods designed to protect and monitor starter culture strains used in the dairy industry have been developed and include starter strain rotation (SSR) strategies (protects against phage attack) and measurement of lactic acid levels (indicating presence of phage and/or antibiotics) using the Heap-Lawrence Starter Activity Test (SAT) (Sing and Klaenhammer, 1993, Heap and Lawrence, 1976). However, SSR can lead to cheese products of variable properties while SAT's requires continuous passaging of test milk samples and can require up to 24 h or more to yield a result. For these reasons a rapid assay for screening starter culture strains against antimicrobial agents would be very useful to the dairy industry.

One such solution to this problem could involve bioluminescence and use of the bacterial *lux* system. Bioluminescence is the production of light by living organisms as a result of a number of enzyme catalysed reactions. These enzymes are known as

luciferases and in the presence of oxygen the enzyme catalyses the oxidation of reduced flavin mononucleotide and a long chain aliphatic aldehyde to yield flavin mononucleotide (FMN) and a long chain fatty acid and light. The luciferase enzyme is encoded by the *luxAB* genes and the fatty acid reductase complex responsible for regeneration of the aldehyde is coded for by the *luxCDE* genes. Previously, *lux*-tagging microorganisms involved cloning the *luxAB* genes, encoding the catalytic luciferase enzymes, from a naturally luminescent host to the target microorganism with the substrate for the reaction being provided exogenously (Baker *et al.*, 1992, Ahmad and Stewart, 1991). However, now an entire *lux* cassette can be transferred from its original host and expressed in other bacteria with the resulting *lux*-tagged bacteria emitting light independently of exogenously provided substrates (Morrissey *et al.*, 2013). Previously, Ahmad & Stewart (1991) constructed a promoter-*lux* plasmid which was transferred into *Lactobacillus casei*, *Lactococcus lactis* and *Lactococcus lactis* subsp. *diacetylactis* by electroporation (Ahmad and Stewart, 1991). When examined all transformants expressed the bioluminescent phenotype in the presence of exogenous aldehyde. These could be used to successfully monitor antimicrobial substances in milk active against starter culture strains for example; antibiotics (Ahmad and Stewart, 1991). Stewart & Ahmad also showed that a bioluminescent strain of *L. lactis* subsp. *diacetylactis* was able to detect bacteriophage active against that organism at concentrations as low as 10^5 PFU/ml in 100 mins (Ahmad and Stewart, 1991, Stewart, 1990, Ahmad and Stewert, 1988). However, this method of monitoring bacteriophage was not a highly automated method with one ml samples being withdrawn and tested every hour in order to obtain bioluminescent measurements. Secondly, the assay involves cloning the *luxAB* genes from *Vibrio fischeri* which encode the catalytic luciferase enzyme and the

subsequent addition of exogenous aldehyde for light production. Nowadays, it is considered more efficient and less labour intensive to clone the entire *lux* operon, *luxABCDE*, so as well as producing the luciferase enzyme the recombinant strain also produces the substrate necessary for the reaction (Morrissey *et al.*, 2013). Finally, the plasmid was only shown to be transferrable by electroporation which is not always possible with industrially important starter strains.

Other applications of bioluminescent bacteria have been explored through the years; however, studies involving bioluminescent *Lactococcus* reporters have been few and far between with a few exceptions (Jiang *et al.*, 2006, Immonen and Karp, 2007, Geoffroy *et al.*, 2000, Eaton *et al.*, 1993).

With this in mind, we set out to construct a new *Lactococcus lux* plasmid capable of mobilisation between strains by conjugation. Using this new plasmid we will construct, insofar as we can determine, the first bank of highly bioluminescent cheese starter culture strains and subsequently develop an assay capable of rapidly monitoring antimicrobial substance active against starter cultures strains in milk. As not all *Lactococcus* plasmids are readily genetically manipulated and electroporation as a means of plasmid transfer is not always possible, a plasmid capable of DNA transfer by conjugation and electroporation must first be generated.

Materials and methods

Bacterial strains, media and chemicals

A summary of strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) broth (Merck, Darmstadt, Germany) was used routinely for growing *Escherichia coli* cultures and *Lactococcus* cultures were grown in GM17 broth (M17 broth supplemented with 0.5% glucose (Oxoid, Basingstoke, UK). Microbiological agar (Merck, Darmstadt, Germany) was added to LB and GM17 broth when solid agar was required. *E. coli* cultures were grown at 37°C, while *Lactococcus* cultures were grown at 30°C. When required, the antibiotic erythromycin (Sigma-Aldrich, MO, USA) was added at a concentration of 500 µg/ml for *E. coli* and 5 µg/ml for *Lactococcus* strains. Chloramphenicol (Cm) (Sigma-Aldrich, MO, USA) was used at a concentration of 10 µg/ml for *E. coli*, 5 µg/ml for *Lactococcus* for electroporation experiments and 15 µg/ml for conjugation experiments.

Table 1. List of *lactococcal* strains/plasmids used in study, including source. UCC = University College Cork

Strains/Plasmids	Strain/Plasmid details	Reference
MG1363	*L. lactis subsp. cremoris*	UCC collection
MG1614	*L. lactis subsp. lactis Strep*^{r}	UCC collection
Il1403	*L. lactis subsp. lactis*	UCC collection
158	*L. lactis subsp. cremoris*	UCC collection
WM1	*L. lactis subsp. lactis*	UCC collection
275	*L. lactis subsp. lactis*	UCC collection
DPC 5101	Industrial chedder cheese starters	Hickey *et al.*, 2001
DPC 4272	Industrial chedder cheese starters	Hickey *et al.*, 2001
DPC 743	Industrial chedder cheese starters	Hickey *et al.*, 2001
DPC 745	Industrial chedder cheese starters	Hickey *et al.*, 2001
DPC 4935	Industrial chedder cheese starters	Hickey *et al.*, 2001
DPC 5250	Industrial chedder cheese starters	Hickey *et al.*, 2001
pCI372	*E.coli-Lactococcus* shuttle vector, Cm^r	UCC collection
p16S*lux*	Integrates into 16S locus chromosome, Ery^r	Riedel *et al.*, 2007a
pRH001	pCI372 containing *oriT* region, Cm^r	Hickey *et al.*, 2001
pRH001*lux*	pRH001 + *luxABCDE* operon +P_{help} promoter, Cm^r,	This study
MG1363 + pMRC01	*L. lactis* subsp. *cremoris* containing pMRC01	Hickey *et al.*, 2001
158*lux*	158:: pRH001*lux*, Cm^r	This study
WM1*lux*	WM1:: pRH001*lux*, Cm^r	This study
275*lux*	275::pRH001*lux*, Cm^r	This study
5101*lux*	5101:: pRH001*lux*, Cm^r	This study
4272*lux*	4272:: pRH001*lux*, Cm^r + Rif ^{r}	This study
743*lux*	743:: pRH001*lux*, Cm^r + Rif ^{r}	This study
745*lux*	745:: pRH001*lux*, Cm^r + Rif ^{r}	This study
4935*lux*	4935:: pRH001*lux*, Cm^r + Rif ^{r}	This study
5250*lux*	5250:: pRH001*lux*, Cm^r + Rif ^{r}	This study

[a]Cm^r, Chloroamphenicol resistant. Ery^r, Erythromycin resistant. Strep^r, Streptomycin resistant.

Transformation of p16S*lux* into *Lactococcus*

To determine whether the *lux* genes could be expressed in *Lactococcus,* plasmid p16S*lux* (Riedel *et al.*, 2007a) was transformed into two commonly used *Lactococcus* strains (*L. lactis subsp. cremoris* MG1363 and *L. lactis subsp. lactis* IL1403). p16S*lux* plasmid was extracted from its *E. coli* host strain using the Invitrogen Quick plasmid miniprep kit (Invitrogen, Löhne, Germany) and transformed by electroporation as described by Holo and Nes, but with minor modifications (Holo and Nes, 1989). *Lactococcus* cultures were grown in GM17 broth to an OD600 nm of 0.5–0.8, and then diluted 100-fold in SGM17 (GM17 supplemented with 0.5 M sucrose and 0.5 M glucose). Incubation at 30°C was continued until an OD600 nm of 0.4–0.6 was reached. Cells were harvested at 4 °C by centrifugation at 2500 x g for 10 mins. After two washings with ice-cold washing buffer (0.5 M sucrose containing 10% glycerol) the cells were suspended in 4 mls of washing buffer. For electroporation assays 50 µl of these competent cells were transformed with ~ 2 µg of purified plasmid in a 2-mm cuvette (Bio-Rad, Mississauga, ON, Canada). The pulse was delivered by a Gene Pulser Xcell (Bio-Rad, Mississauga, ON, Canada), set at 25 µF, 2 kV, and 200 ohms. The cells were resuspended in 945 µl of GM17MC (GM17 containing 20 mM $MgCl_2$ and 2 mM $CaCl_2$) and the cell suspension was incubated at 30 °C for 2 h. Transformants were selected by plating on GM17 agar containing erythromycin (5 ug/ml). Plates were incubated for 24 h at 30 °C. Bioluminescence was measured (photons per second per square centimetre) using the IVIS Xenogen Imaging 100 system (Xenogen, Alameda, CA) with a binning of 16 and an exposure time of 1 min.

Construction of new recombinant plasmid – pRH001*lux*

To construct the bioluminescent *Lactococcus* strain, the *luxABCDE* genes plus P_{help} promoter were amplified from plasmid p16S*lux* by PCR using 2X extensor long range PCR master mix (Thermo Scientific, Hempstead, UK) according to manufacturer's instructions and cloned into the multiple cloning site of pRH001 (PCI372 containing 312-bp *oriT*). Amplification was carried out using *luxABCDE* primers for (5'- CACCGCTACACCTGGAAT-3') and rev (5'-GAACTAGTGGATCCCCCG-3') (Eurofins MWG, Ebersberg, Germany). Amplification of the target region was achieved with 30 cycles of 94°C for 2 min, 10 cycles of 94°C for 10 sec, 57 °C for 30 sec, 68° C for 4 min, 20 cycles of 94°C for 10 sec, 57 °C for 30 sec, 68° C for 4 min and 1 cycle of 68°C for 7 min. The PCR was carried out in a T3000 Thermocycler (Biometra, Gottingen, Germany).

Following amplification the PCR product was purified with Purelink PCR Purification kit (Invitrogen, Männheim, Germany) according to manufacturer protocols and subsequently digested for 4 h at 37°C with *Pst* I restriction enzyme (Roche, UK). The plasmid pRH001 was digested with the same restriction enzyme under the same conditions and again purified using the PCR Purification kit. The ligation was performed in 1x buffer with T4 ligase, and incubated in ice-water for 16 h overnight (Roche, UK).

L. lactis chedder cheese starter culture strains (WM1, 158, 275 and 5101) were transformed with plasmid pRH001*lux* following electroporation protocols detailed above. Transformants were selected by plating on GM17 agar supplemented with chloramphenicol (5 µg/ml). Bioluminescence was measured with the IVIS Xenogen Imaging 100 system. PCR was performed to confirm the presence of the new *lux* plasmid based on PCI372 multiply cloning site primers PCI372 For (5'-

ACACTGGAACTGAGACACGGTCCAGACTCC-3') PCI372 Rev (5' TTGTAAAACGACGGCCAGTGAGCGCGCG-3') (Eurofins MWG,). *L. lactis* MG1363, L. Lactis MG1363 + pMRC01 and *L. lactis* IL1403 were also transformed with plasmid pRH001*lux.* All starter culture strains used in this study were isolated from whey samples obtained from the dairy industry and provided by University College Cork or the Agriculture and Food Development Authority, Teagasc, Fermoy, Cork (Table 1).

Generation of spontaneous *L. lactis* rifampicin resistant recipients

Ten millilitres of overnight culture starter strains cultures listed in table 1 were centrifuged at 2500 x g for 10 min. The supernatant was removed and the pellet was resuspended in 200 µl sterile ¼ strength Ringer's solution (Merck, Darmstadt, Germany). One hundred microlitres of this suspension was plated onto GM17 agar containing 30 µg/ml rifampicin. Plates were incubated at 30 °C overnight. The following day a colony was picked and re-streaked onto GM17 agar containing 100 µg/ml rifampicin (Smalla *et al.*, 2000).

Mobilization of pRH001*lux* into industrial starter strains by conjugation

Filter matings were performed in triplicate using *L. lactis* MG1363 containing pMRC01 plus pRH001*lux* as the donor strain. The recipients used were rifampicin resistant variants of chedder cheese starter culture strains isolated from the dairy industry (Table 1). Ten millilitres of overnight culture of the recipient and 10 ml of overnight cultureof the donor were centrifuged at 2500 x g for 10 min, washed in Ringers' solution, and resuspended in 10 ml GM17 broth (Smalla *et al.*, 2000). Ten millilitres of washed culture of the recipient strain were passed through a 0.45 µm

nitrocellulose membrane filter (Millipore, Billerica, MA, USA) followed by 500 µg/ml of proteinase-K and subsequently held for 5 min at room temperature. Ten millilitres of the donor strain was passed through the same nitrocellulose membrane. Filters were placed facedown onto GM17 agar and incubated overnight at 30°C. The following day, filters plus the underlying agar were placed in sterile tubes containing 10 ml ¼ strength Ringers' solution and gently agitated. One hundred microlitres of the ringers mix was spread plated onto GM17 agar (100 µg/ml rifampicin and 15 µg/ml chloramphenicol) to identify recipient cells containing the *lux* plasmid. Positive transconjugants were confirmed by bioluminescence using the IVIS imager. To ensure only the *lux* plasmid (and not the lacticin 3147-encoding pMRC01 plasmid) was being mobilised into the recipient, bacteriocin agar well diffusion assays were carried out on the transconjugants. These assays were performed by seeding GM17 agar with an appropriate indicator strain (*L. lactis* HP). Once the agar had set, wells were made in the agar (4.6 mm in diameter) and 50 µl of washed overnight cultures of the lux-tagged starter cultures (WM1, 158, 275, 5101, 4935, 4272, 743, 745, 5250) was added to the wells. Fifty microliters of washed MG1363-pMRC01 overnight culture was used as a positive control. Plates were incubated at 30 °C overnight. The following day plates were observed for zones of clearing around wells indicating Lacticin 3147 production.

Biological characteristics of the new *lux* plasmid- Plasmid stability

The *lux*-tagged starter culture strains generated by transformation and conjugation were grown overnight (approximately 16 h) in triplicate in GM17 broth containing chloramphenicol (5 µg/ml). Three millilitres of culture was centrifuged, washed and resuspended in an equal volume of ¼ strength Ringer's solution. Two hundred

microliters was transferred into 10 ml of fresh GM17 broth without antibiotic. Tubes were incubated (statically) at 30 °C for approximately 8 h, after which time 200 µl of culture was transferred into fresh broth and incubated at 30°C overnight. This passaging was repeated for an additional 2 days. At every passage, 100 µl aliquots were serially diluted in ¼ strength Ringer's solution and spread plated onto GM17 agar. Plates were incubated at 30°C. Fifty random colonies from each passage were scored for antibiotic resistance by patching on GM17 agar and GM17 plus chloramphenicol (5 µg/ml). Plates were incubated at 30°C overnight. These plasmid stability assays were repeated for each *lux*-tagged starter culture strain.

Monitoring growth of the *lux*-tagged starter strains in GM17 broth and whole milk

Growth patterns of the newly tagged transformant and transconjugant starter culture strains and the wild-type starter culture strains were monitored by measuring relative light units (RLU) and by viable plate counts (Log CFU/ml). Three millilitres of luminescent overnight culture was centrifuged (2500 x g**,** for 10 min), washed and resuspended in ¼ strength Ringers' solution. A 2% inoculum of each strain was added to fresh GM17 broth. Two hundred microlitres of this freshly inoculated GM17 broth was added to a 96-well white plate (Nunc, Thermo Fisher Scientific, Roskilde, Denmark) and bioluminescent and optical density measurements were recorded hourly at 30 °C, in relative light units (RLU) using a Luminoskan luminometer (Thermo Fischer Scientific, Roskilde, Denmark) and OD595nm (Tecan, Männedorf, Germany). Viable plate counts were performed at hourly intervals by diluting 100 µl aliquots in Ringers' solution and spread plating onto GM17 agar. This experiment was repeated in pasturised whole milk. Acid production was

determined by measuring pH values following 24 h incubation at 30 °C. pH was measured using a pH meter (Mettler Toledo, Gießen, Germany). Finally, growth curves of *lux*-tagged starter strains transformed by electroporation and conjugation were also completed to investigate whether various methods of plasmid transfer interfere with bacterial growth or stability under standard laboratory conditions.

Susceptibility of *L. Lactis* starter strains to bacteriophage

The susceptibility of each of the *L. Lactis* starter strains to a collection of bacteriophage was determined by the bacteriophage spot test (Parasion *et al.*, 2012, Gutiérrez *et al.*, 2010). Bacteriophage used in this study were propagated on their associated host strains as follows. The host strain was grown to an approximate optical density (OD595) of 0.15 in 10 ml GM17 broth. Calcium chloride was then added at a final concentration of 1 mM and the relevant phage was added. Following incubation at 30 °C, lysis of the broth occurred. The lysates were filtered through a 0.45 µm pore filter to remove any residual bacterial contamination and stored at 4°C until required.

To perform bacteriophage well assays, 10 ml of each starter culture strain was grown overnight in GM17 broth. Three hundred microliters of washed overnight culture was mixed with 4 ml of molten soft GM17 agar (0.7 %) and overlaid on the surface of solidified GM17 agar (1.5 %). Starter strain sensitivity to bacteriophage was established by spotting 30 µl of each phage onto the surface of the agar seeded with the starter culture strain. Lysis (indicated by a zone of clearing) occurs only at the spot where starter strains are sensitive to a particular phage.

Once starter strains susceptible to phage atack was determined plaque, plaque assays were carried out to estimate phage titres necessary for starter strain inhibition. To do

this 350 µl of overnight culture, 100 µl of 1 M $CaCl_2$ and 100 µl of phage dilutions (ranging between 10^{-1} and 10^{-5}) were added to 3 ml of M17 overlay (0.7% agar). The contents were mixed, poured onto M17 agar, and incubated at 30 °C for 18 h. The following day plates were examined for plaques and the number of plaques and the phage dilution were recorded. Efficiency of plaquing (EOP) of a phage on a particular host was determined by dividing the phage titer on the test strain by the titer on the homologous phage-sensitive host (Terzaghi and Sandine, 1975, Coakley *et al.*, 1997).

Each of the bacteriophages used in this study (POO8, SK1, JJ50 340, 397, 531, 949 WRP3, Tuc2009 c2 and Q54) were specifically selected as they were previously isolated from the dairy industry.

Challenging *L. Lactis* 5250*lux* with bacteriophage

Once starter strains sensitive to phage were identified and phage susceptibility ranges determined, assays challenging one of the *lux*-tagged starter strains (L. Lactis 5250-*lux*) with phage were carried out. As all starter strains used in cheese fermentations are initially propagated in reconstituted skim milk (RSM), *lux*-tagged starter strain *L. lactis* 5250*lux* was cultured in commercially prepared RSM (10%) and incubated for 16 h at 30°C. Following overnight incubation, RSM cultures was centrifuged and resuspended in Ringer's solution and approximately 1 x 10^9 CFU/ml of *Lactococcus* culture was added to fresh whole milk. Freshly inoculated milk was subsequently spiked with of 1 x 10^9 Plaque forming units (PFU) of c2 phage reaching a multiplicity of infection of 1.0. Assays were carried out in triplicate and the necessary controls to ensure adequacy of milk to support growth of the microorganism and sterility of broth were performed. Two hundred microliter

samples were transferred to a 96-well plate and bioluminescent measurements were recorded hourly in a luminoskan luminometer. The assays were carried out in triplicate and appropriate controls were included (Tuc2009 phage alone was used as the negative control) (Santos *et al.*, 2010, Tükel *et al.*, 2006). Assays were also carried out an MOI of 10.

Challenging *L. Lactis* 5250*lux* with antibiotic

Overnight cultures of *L. Lactis* 5250*lux* were centrifuged, and washed and resuspended in ¼ strength Ringer's solution. A 2% inocula of this was added to fresh pasturised whole milk and the antibiotic kanamycin was added at a concentration of 5 µg/ml. Two hundred microlitres of this was added to a 96-well white plate and bioluminescent measurements were recorded hourly in relative light units (RLU) using a Luminoskan luminometer. Similar experiments were performed using the antibiotics ampicillin and streptomycin (5 ug/ml). This assay were based on similar experiments preformed by ahmad and stewert, 1991.

Interpretation of data and statistical analysis

All experiments were performed in triplicate from three biological repeats and were repeated at least twice. The results shown represent the mean +/- standard deviations (error bars) for one particular experiment. Means and standard deviations were calculated using a basic Excel programme. Statistical significance was determined via Sigma plot t-test. In all cases, a P value less than 0.05 were considered to be statistically significant.

Results

Construction of pRH001*lux* plasmid

Initial experiments focused on determining whether the *lux* genes in the p16S*lux* plasmid could be expressed in *L. lactis*. Following electroporation, *L. lactis* MG1363 and *L. lactis* IL1403 were successfully transformed with the p16S*lux* plasmid. Examination of re-streaked transformants revealed that *lux*-tagged strains emitted high levels of bioluminescence whereas, the non-tagged controls did not. The *lux*-tagged strains could be maintained under antibiotic pressure when grown at 30 °C. Given that the *lux* genes under P_{help} promoter (highly expressed *Listeria* promoter) control could be expressed in *L. lactis*, we constructed a new *lux* plasmid (pMRC01*lux*) which could be maintained under antibiotic pressure and could potentially be transferred between *L. Lactis* strains by conjugation and electroporation.

This new conjugative vector (pRH001*lux*) consists of pRH001 and the *luxABCDE* genes plus P_{help} promoter (Fig. 1 A, 1C), was sucessfully transformed into *L. lactis* MG1363, IL1403 and four *L. Lactis* starter culture strains (158, WM1, 275, 5101) by electroporation (Fig. 1B). To confirm the presence of the *luxABCDE* genes, a PCR was performed using primers based on the PCI372 multiple cloning site and analysed by 1% agarose gel electrophoresis (data not shown). Repeated attempts to transform pRH001*lux* into other starter culture strains (5250, 743, 745, 4935 and 4272) failed. Therefore only four starter cultures out of a possible nine were sucessfully transformed. We initally speculated that this was perhaps due to the large size of the plasmid. Attempts to transform a smaller plasmid (pCI372) into several starter strains (*L. Lactis* 743 and 745) were successful; however, starter strains *L. Lactis* 4935 and 4272 could not even be transformed with pCI372. Attempts to transform other

plasmids (pNZ44 and pRH001) into competetent 4935 and 4272 starter strains were also unsuccessful. When electro-competent 4935 and 4272 cells alone were serially diluted and plated on GM17 agar, high cell numbers were recorded (~ 10^9 CFU/ml). Despite these high cell numbers, electroporation was not always a suitable method of plasmid transfer to *Lactococcus* starter strains.

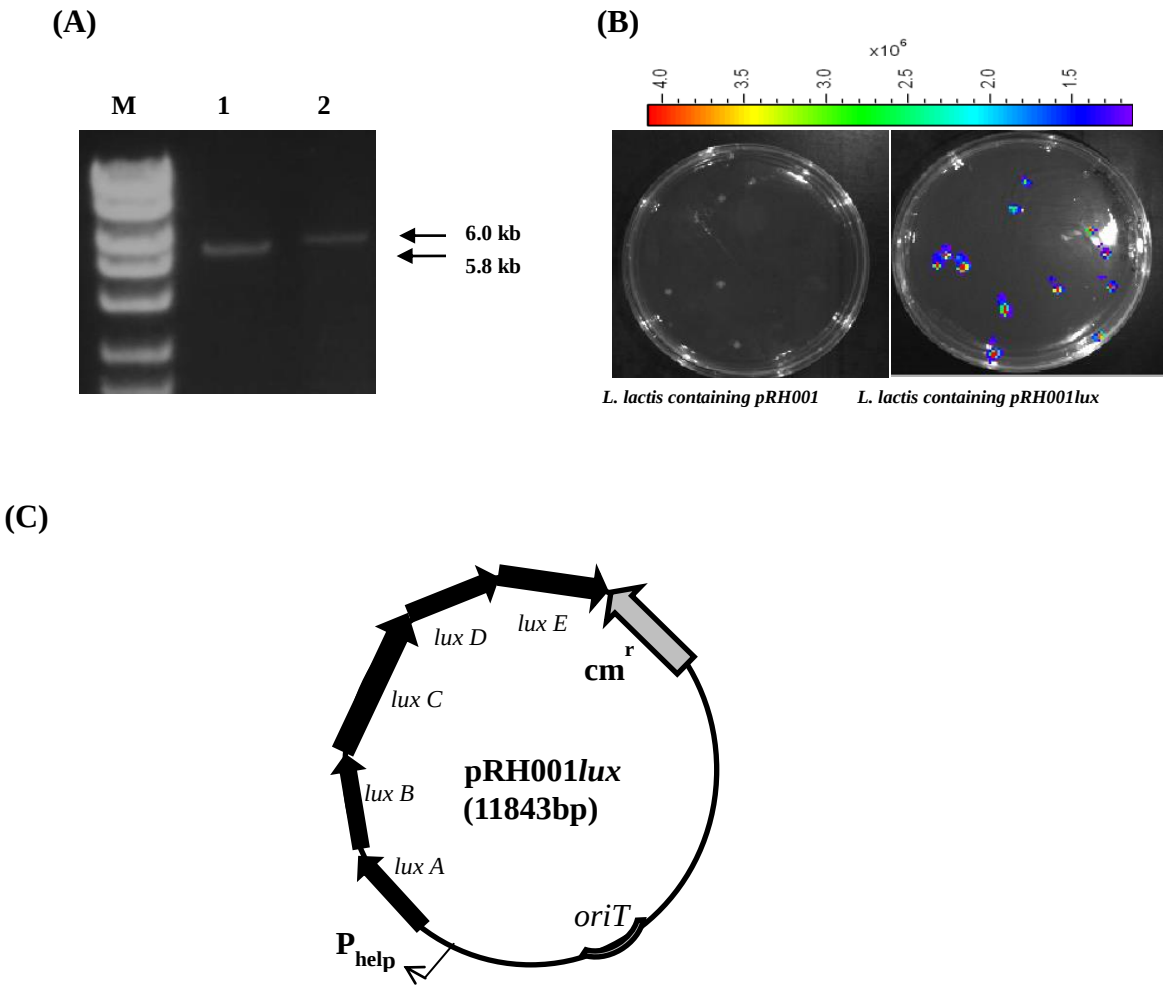


Fig 1. (A) Analysis of the recombinant plasmid pRH001*lux* by 0.7% agarose gel electrophoresis, run under 100 V for 60 min in 1X TAE buffer. The *lux* region was PCR amplified as described in the material and methods. M, molecular marker (1kb Bioline), lane 1, amplified *luxABCDE* + P_{help} promoter region (isolated from p16S*lux*) *Pst1* digested, lane 2, pRH001 (pCI372 plus *oriT), Pst* 1 digested. **(B)** *L. lactis* MG1363 containing pRH001 (empty plasmid) and pRH001*lux*, viewed with a Xenogen IVIS 100 imager. The colour bar represents the bioluminescent signal measured in photons per second per square centimeter. **(C)** Map of pRH001*lux* showing *luxABCDE* genes, P_{help} promoter and origin of transfer region (*oriT*).

Mobilization of pRH001*lux* into industrial starter strains by conjugation

In order to determine whether the new *lux*-plasmid could be transferred by conjugation as well as electroporation, attempts were made to mobilize pRH001*lux* into nine industrial *L. Lactis* starter strains (158, WM1, 275, 5101, 5250, 743, 745, 4935 and 4272) by conjugation. All filter matings were successfully carried out but in all cases transfer efficiencies were low (~ 3.75 x 10^3 transconjugates/µg); however, transconjugates were found to exhibit high levels of luminescence. To ensure pMRC01 (which contains the genes that encode lacticin 3147) was not co-mobilized during the matings all nine starter strains were examined for the presence of pMRC01 using well diffusion assays testing for Lacticin 3147 production. All transconjugants tested negative for Lacticin 3147 production when tested against the sensitive indicator strain *L. lactis* HP with the exception of *L. lactis* MG1363 + pMRC01 which was used as positive control.

Growth of transformants/transconjugants

These experiments focused on examining the effect that the *luxABCDE* genes had on their *Lactococcus* host. A major concern of genetic manipulation of any strain is that the strain will be negatively affected in terms of general growth characteristics. However, introduction of the *lux* genes into starter strains either by transformation or conjugation had no negative effect on the general growth characteristics of the strains and the *lux*-tagged strains grew in a manner similar to that of the non-*lux*-tagged counterpart (Fig. 2). In fact, no statistical difference was observed between growth of the *lux*-tagged strains and wild-type strains when examined using plate counts ($P < 0.05$; Fig. 1 appendix).

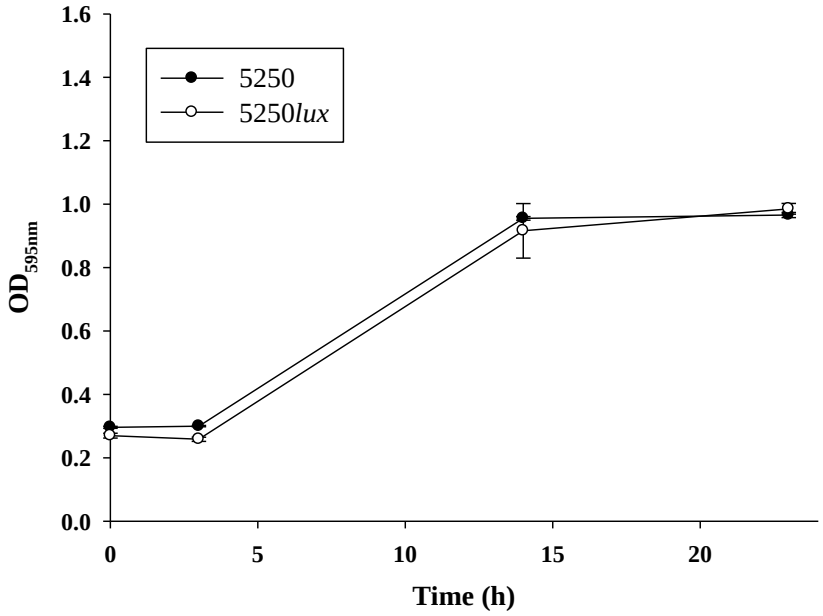


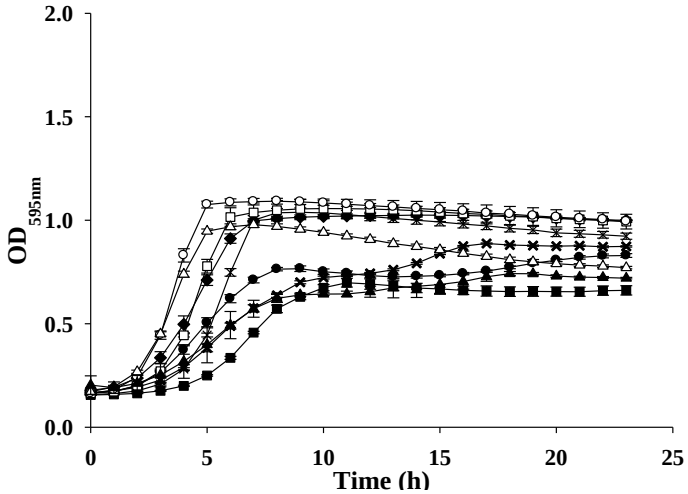
Fig 2. Growth curve of non-*lux* tagged *L. Lactis* 5250 (●) and the *lux*-tagged 5250 transconjugants (o), in GM17 broth at 30 °C. Data are presented as mean OD_{595nm} +/- standard deviations for three biological repeats.

Growth of the *lux*-tagged strains could easily be monitored in GM17 broth when measuring optical density (OD) (Fig. 3A) or light emissions (RLU). Bioluminescent measurements increased over time as cell numbers increased (data not shown) and, as expected, bioluminescence was not detected for the non–*lux*-tagged strain or in uninoculated broth, confirming that there was no background interference. Results were reproducible, produced in real-time and RLU readings correlated with viable plate counts (R^2 for *L. lactis* 5250*lux* ~ 0.8885; correlation scatter plot shown in Fig.1 appendix). Growth of the *lux*-tagged strain was also examined in whole milk (Fig. 3B). Similar to the results obtained for GM17 broth, it was observed that bioluminescence readings increased over time as cell numbers increased (data not

shown). When acidity of the *lux*-tagged strain was determined by measuring pH values after milk cultures were incubated at 30° C; it was determined that light could be emitted between pH's of 4.1 and 6.6, confirming that luminescence can be emitted in mildly acidic environments.

When strains transferred by conjugation were compared to those transformed by electroporation (by measuring growth patterns and plasmid stability) no significant differences were noted ($P < 0.05$) (Fig. 2 and Fig. 15 appendix). Starter culture strains created by electroporation and conjugation had similar growth and stability patterns and no significant difference were noted ($P < 0.05$; Fig. 4 – 18 appendix).

(A)



(B)

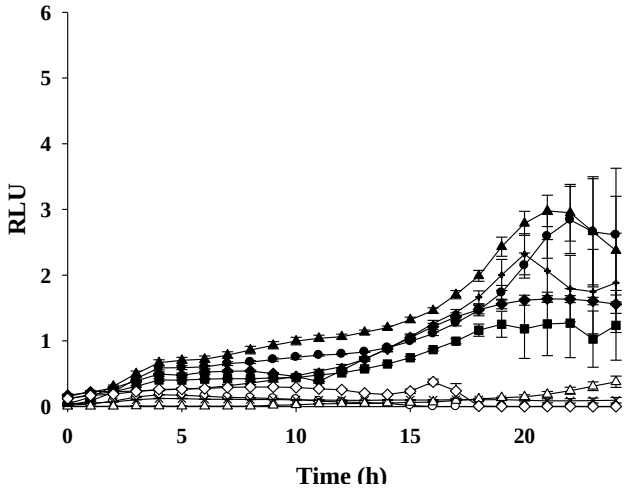


Fig 3. (A) Growth of each *lux*-tagged starter culture strain (transconjugants), 158 (●), 4272 (o), WM1 (■), 4935 (□), 5250 (+), 5101 (x), 745 (▲), 743 (Δ), 275 (♦), in GM17 broth at 30 °C. Data are presented as mean OD_{595nm} +/- standard deviations for three biological repeats. **(B)** Growth of *lux*-tagged starter culture strains (transconjugants), 158 (●), 4272 (o), WM1 (■), 4935 (□), 5250 (+), 5101 (x), 745 (▲), 743 (Δ), 275 (♦), in whole milk at 30 °C. Data are presented as mean RLU's (Relative Light Units) +/- standard deviations for three biological repeats. Bioluminescence was measured by a Luminoskan luminometer.

Stability of plasmid

When *lux*-tagged strains were examined for stability by continuous passaging in the absence of antibiotic, strains were found to be stable. Results presented in figure 4 represent assays carried out with *lux*-tagged *L. lactis* 5250*lux*. In this case the plasmid was found to be 96% stable over the first transfer, 92% stable over the next passage but approximately 70% stable after passage 5 (Fig. 4). Stability assays were repeated for each *lux*-tagged strain and in each instance similar results were observed (Fig. 4 - 13 appendix). This confirms that the plasmid was sufficiently stable for monitoring growth over relatively short time frames of approximately 24 h.

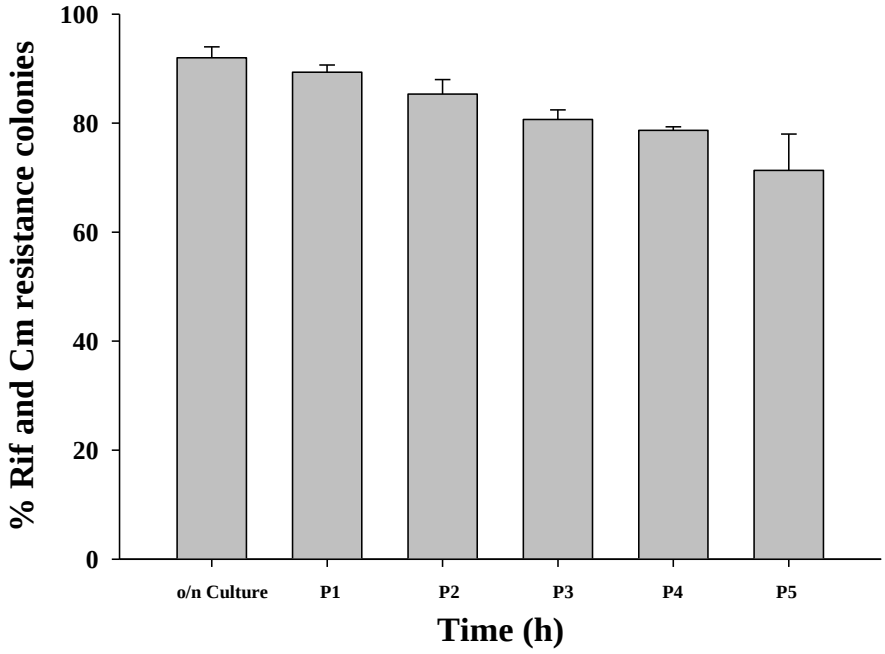


Fig 4. Stability of pRH001*lux* in *L. lactis* 5250*lux* represented as a percentage of chloramphenicol resistant colonies following passaging every 8 h in the absence of antibiotic. Data presented are the means of triple replicates +/- standard error. Stability assays were repeated for each *lux*-tagged starter strain (data not shown).

Susceptibility of starter strains to bacteriophage

Bacteriophage spot assays revealed that four *lux*-tagged starter strains (5101*lux*, 743*lux*, 5250*lux* and WM1*lux*) were sensitive to phage c2 when starter culture overlays were spotted with a number of industrial relevant bacteriophage (Fig. 5A). Eight other phage tested had no inhibitory effect on the remaining five starter strains. Plaque assays confirmed this and determined the phage concentration necessary for starter strain inhibition (Fig. 20 appendix). However, when the bioluminescent phage- sensitive starter 5250*lux* was challenged with c2 phage, a significant increase

(P> 0.05) in luminescence was observed when compared to the *lux*-tagged strain grown alone. This was unexpected and suggested that growth of the *lux*-tagged starter was increased in the presence of phage c2. However, plate counts confirmed c2 phage was in fact inhibiting bacterial numbers by approximately 1 Log after 2 h. In milk samples inoculated with *L. lactis* 5250*lux* together with the control bacteriophage Tuc2009, a significant increase in bioluminescence was again noted over a 24 h period (Fig. 5B). As expected, when plate counts were performed, Tuc2009 samples showed no reduction in cell numbers and grew similarly to the *lux*-tagged strain grown in the absence of phage. Similar results were observed when assays were performed in GM17 broth. The strains used to propagate phage were grown in the absence of phage and filtered, and the filtrate was examined for luminescence. No luminescence was observed.

(A)

5250lux ***5250lux + Tuc2009*** ***5250lux + c2***

Fig. 5 (A) Spot assay demonstrating the effect of c2 phage and Tuc2009 phage (negative control) on growth of *lux*-tagged starter culture strain *L. lactis* 5250*lux* as determined using a conventional technique (Solid agar surface and soft agar). *L. lactis* 5250*lux* (*left*), *L. lactis* 5250*lux*+ Tuc2009 phage *(centre*) and *L. lactis* 5250*lux* plus c2 phage (*right*).

(B)

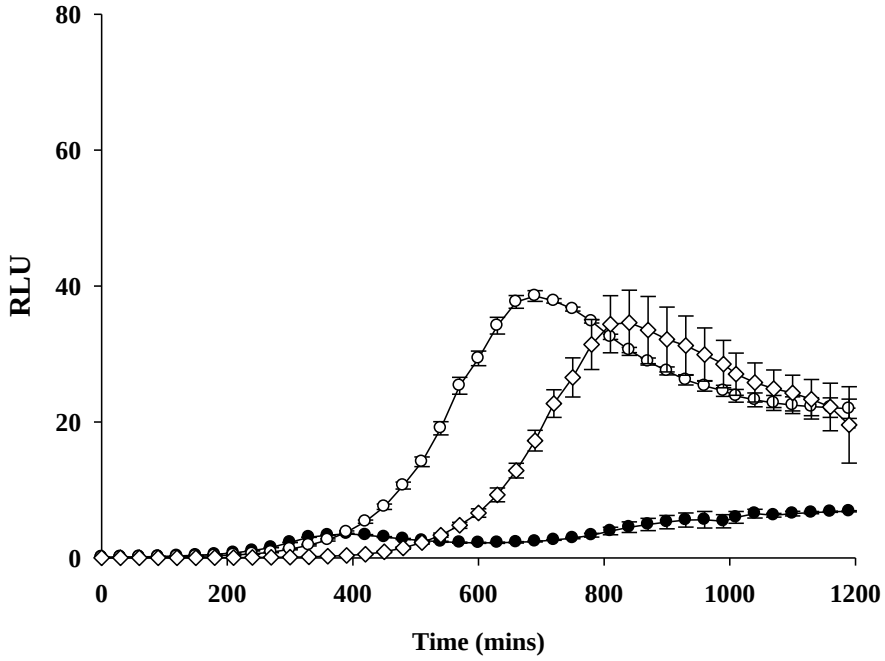


Fig. 5 (B) Mean effect of no phage (●), c2 bacteriophage (o) or Tuc2009 bacteriophage (◊) on the level of luminescence produced by the *lux*-tagged starter culture strain *L. lactis* 5250*lux* in whole milk, as determined by the luminoskan luminometer. RLU = Relative Light Units.

Susceptibility of starter strains to antibiotics

Antibiotic susceptibility assays revealed that addition of kanamycin had a bacteriostatic effect on *L. lactis* 5250*lux* over the time course of the assay (Fig. 6A). This was particularly evident when Kanamycin was added at concentrations of 5 µg/ml. Similar results were also observed when the antibiotic ampicillin (added at the same concentration as kanamycin) was tested against 5250*lux* (Fig. 19 appendix). The antibiotic streptomycin was also examined against the *lux*-tagged starter and was

found to have little inhibitory effect on growth of the strain (Fig. 6B). Viable plate counts supported this finding (data not shown). The influence of antibiotics on the growth of *lux*-tagged starter strains in milk can easily be tracked by measuring bioluminescence.

(A)

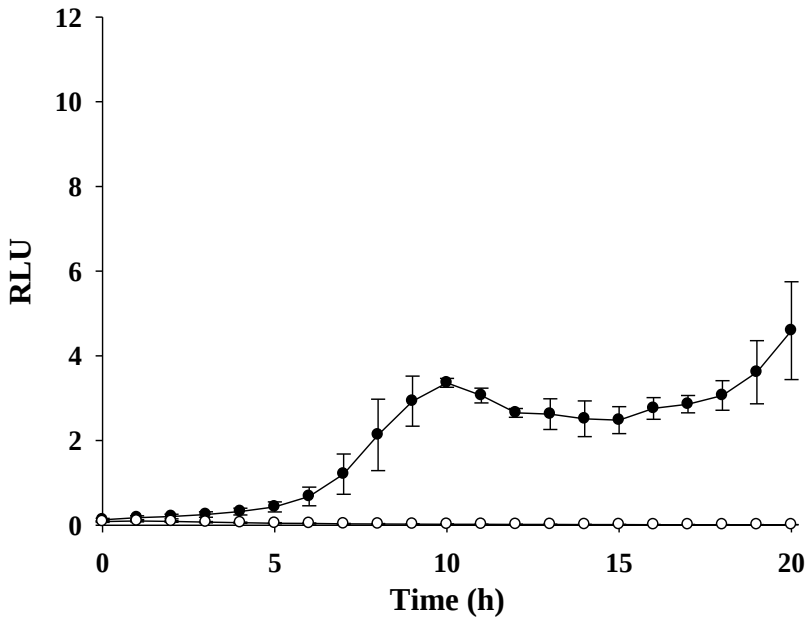


Fig. 6 (A) Bioluminescence as a measure of the antimicrobial activity of Kanamycin in whole milk as determined by the luminoskan luminometer over 20 h. (●) represents *L. lactis* 5250*lux*, (o) represents *L. lactis* 5250*lux* plus 5 µg/ml of Kanamycin. RLU = Relative Light Units.

(B)

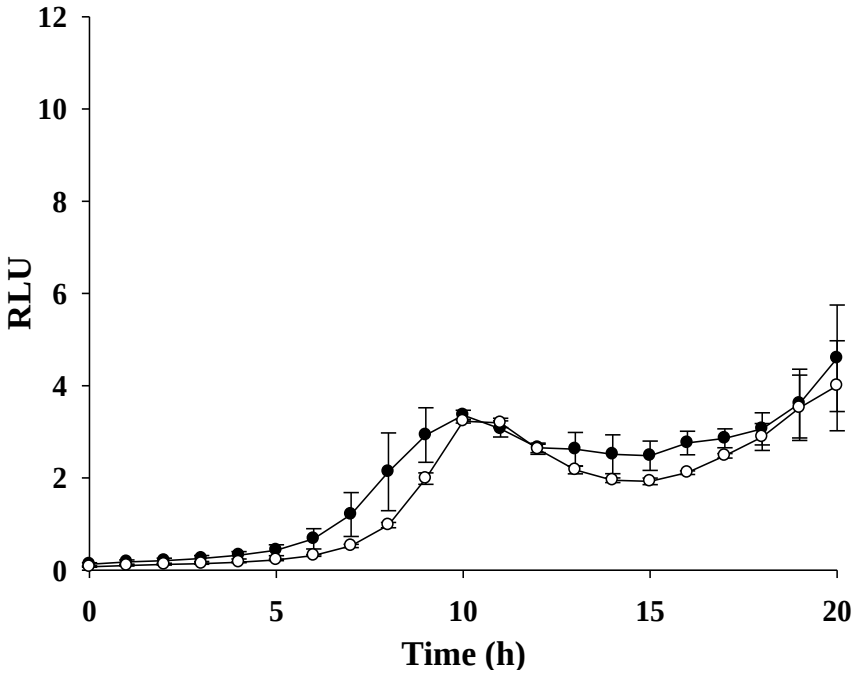


Fig. 6 (B) Bioluminescence as a measure of the antimicrobial activity of Streptomycin in whole milk as determined by the luminoskan luminometer over 20 h. (●) represents *L. lactis* 5250*lux*, (o) represents *L. lactis* 5250*lux* plus 5 µg/ml of Streptomycin. RLU = Relative Light Units.

Discussion

Fermentation of milk is an essential step in the production of cheese and despite extensive control measures, the presence of antimicrobial agents i.e. antibiotics and bacteriophage in the dairy industry is still a significant problem (McGrath *et al.*, 1999, Quiberoni *et al.*, 2006, Marvig *et al.*, 2011). If present in bulk milk, antimicrobial substances can either destroy the *Lactococcus* culture or, in complex starters, destroy the balance of inoculated strains with significant effects on product flavour and texture (Stewart, 1990). At present, Starter Activity Tests (SAT's) are used to evaluate antimicrobial activity in milk; however, this requires several hours of incubation before an assessment can be made (Stewart, 1990). Methods to prevent starter strain contamination and subsequent economic loss have been established; for example; Starter Strain Rotation (SSR), improved hygiene and thermal treatment of milk. However, improved hygiene practices are not always successful and SSR strategies can lead to dairy products of inferior or variable quality (e.g poor/variable aroma, proteolysis etc) (Ledeboer *et al.*, 2002). Techniques for improvement of phage resistance of starter cultures for example transfer of phage resistant plasmids to starter strains, as well as phage detection mechanisms have been developed (Coakley *et al.*, 1997, Wilson, 1935). However, a rapid assay for screening starter strains against antimicrobial agents would be very useful to the dairy industry.

With this in mind, we set out to construct a new *lux* plasmid (pRH001*lux*) capable of continuously expressing the *lux* genes in a Gram positive host. This plasmid was transformed into a variety of cheese starter culture strains to produce a bank of bioluminescent stable starter strains. These strains could potentially be used within the cheese industry to monitor bulk milk samples for the presence of antimicrobial

agents, such as antibiotics. However it is important to note that due to their genetically modified status the *lux*-tagged starter strains developed in this study are only suitable for use as a research or monitoring tool for the dairy industry. The *lux*-tagged strains are unsuitable for direct use in cheese production where the non-*lux*-tagged counterparts would continue to be used.

Initial experiments focused on determining whether the *lux* genes in p16S*lux* plasmid could be expressed in *L. lactis* strains. The p16S*lux* plasmid contains *luxABCDE* genes derived from *Photorhabdus luminescens* and the *E. coli* DH10B 16S rRNA genes. However, this plasmid does not contain the necessary elements for plasmid transfer by conjugation and so it was not suitable for application in this study. However, p16S*lux* could be sucessfully transformed into *L. lactis* MG1363 and *L. lactis* IL1403 by electroporation, indicating the *lux* genes can be expressed in *L lactis*. Previous attempts by Riedel *et al*. (2006a) to transform this plasmid into *L. lactis* NZ9000 were also successful; however attempts to integrate the plasmid into the bacterial chromosome failed. Replacement of the 16S sequence (from p16S*lux*) with a 16S sequence from a Gram positive bacterium did not promote integration of the plasmid (p16SLla*lux*). Riedel postulated that Gram positive bacteria are more sensitive to the disruption of the 16S rRNA gene than Gram negative bacteria (Riedel *et al.*, 2007a). Based on these finding, no attempts were made to construct a chromosomal integration vector based on the 16S rRNA gene during this study.

Next we set out to develop a bank of bioluminescent starter culture strains capable of being monitored in real-time in whole milk. In order to develop pRH001*lux*, the native *Lactococcus* plasmid pMRC01 was exploited due to its self-transmissible nature. Plasmid pMRC01 is a 63-kb conjugative *Lactococcus* plasmid which encodes bacteriophage resistance genes as well as genes encoding production of and

immunity to the broad spectrum bacteriocin Lacticin 3147. This plasmid was previously identified as containing putative genes for conjugal transfer of the plasmid and an origin of transfer region (*oriT*) (Ryan *et al.*, 1996). In its current form, pMRC01 was deemed inappropriate for this study due to the absence of *lux* genes and the presence of bacteriophage resistance genes. However, by combining the *oriT* from pMRC01 with pCI372 (a high copy number *E. coli-Lactococcus* shuttle vector) and ligating it with the *lux* cassette the resulting plasmid can be successfully mobilised into starter culture strains (Hickey *et al.*, 2001).

In terms of molecular construction of the new conjugative-*lux* plasmid, the P_{help} promoter plus the *luxABCDE* cassette, derived from *P. luminescens,* was cloned from p16S*lux* into a 6-kb broad range host vector known as pRH001. This plasmid consists of 5.7-kb plasmid pCI372 and the 0.3-kb *oriT* derived from pMRC01. As well as being the original host of the *oriT*, pMRC01 also contain a 19.6-kb region which includes putative genes for conjugal transfer of the plasmid and genes necessary for bacteriocin production (Hickey *et al.*, 2001, Coakley *et al.*, 1997). In the presence of the mobilization genes (*oriT*) and pMRC01, the new *lux* plasmid could be transferred by filter matings and expressed in Gram positive bacteria. Up to nine starter strains were transformed in this manner with all transconjugates exhibiting strongly bioluminescent phenotypes, confirming that the *lux* genes can be expressed in *Lactococcus*.

To prevent co-mobilising pMRC01, the bacteriocin selective pressure during the filter mating was relieved by the addition of proteinase K to the filter (Hickey *et al.*, 2001). Co-mobilizing pMRC01 would result in bacteriophage resistant transconjugates which would not be suitable for this study but would be useful if constructing a bank of *lux*-tagged bacteriophage resistant starter culture strains.

In the event that transformation by conjugation was not possible (perhaps due to incompatibility with a resident plasmid or difficulties replicating efficiently in the recipient), strategies to transform the plasmid by electroporation were also established (Coakley *et al.*, 1997). Electroporation is commonly used as a means of plasmid transfer (Dupont *et al.*, 2004, Coakley *et al.*, 1997); however, in many countries it is not regarded as a food grade approach and was not always possible during the course of this work. In some instances, the transformation efficiency into *Lactococcus* was very low or the plasmid simply would not transform. We speculate that this was at least in part due to the relatively large plasmid size.

We also investigated whether luminescence was affected by pH when the *lux*-tagged starter strains were left to ferment in milk overnight. Previously Jiang *et al*. (2006) had determined that media components influenced the reaction of the luminescent cultures to pH. The mechanisms by which media components affected the *lux*-tagged *L. lactis* response to pH are unknown. However, the bioluminescent *L. lactis* could emit a strong luminescent signal within a large pH range (approximately 2.5–9.5) thereby increasing the usefulness of *lux*-tagged *L. lactis* as reporters in different foods and under different environmental conditions; for example, in milk or during cheese production (Jiang *et al.*, 2006). In this study, acidity was determined by measuring pH values after milk cultures were incubated at 30° C. After 24 hours pH values were measured and it was determined that light could be emitted between pH's of 4.1 and 6.6, confirming that luminescence can be emitted in mildly acidic environments.

To our surprise, challenging the *lux*-tagged starter strains with bacteriophage yielded an increase in luminescence despite a reduction in cell number being recorded when plate counts were performed. This occurred even when the strain was not susceptible

to the added phage (e.g. Tuc2009). This response does not occur when the tagged strains are exposed to another bactericidal agent such as an antibiotic. The increased bioluminescence must result from either (i) an increase in cell numbers, (ii) an increase in enzyme levels or (iii) and increase in enzyme activity. We were able to rule out an increase in cell numbers and speculate that this increase in luminescence is most probably as a result of increased activity, perhaps due to an increase in ATP levels in the presence of bacteriophage. It may well be that bacteria respond to phage attack by increasing metabolic activity to respond to the threat posed by the bacteriophage. However, it is unusual that the same response was observed in the presence of a phage which does not inhibit the luminescent strain. Despite extensive research, we have found no other explanation in the literature to support this finding. Further research will be necessary to confirm and/or explain this finding. While our initial hypothesis was that bacteriophage disruption would be detected by a decrease in luminescence, an increase would be equally useful for detection purposes however, we will have to establish if this is a general phenomenon. In the case of antibiotic residue detection, the strategy worked as originally envisaged, in that the presence of antibiotics prevents the development of a bioluminescent signal. The current study demonstrates a rapid method of monitoring the action of antibiotic residues on growth of bioluminescent cheese starter culture strains, in real time. This assay highlights the speed and ease at which biomonitoring assays can be performed when compared to plate assays.

Using bioluminescent-based technology to monitor starter culture strains has many advantages. It is a rapid method of monitoring growth of the *lux* tagged strains when compared to other conventional methods such as plate counts. The latter requires serial dilutions of samples and plating on previously prepared agar plates which, in

the case of large sample loads, can be slow and labour intensive. Bioluminescent output can be measured in real-time and the result is instantaneous. Bioluminescent reporters give an accurate representation of starter strain growth given that there is no significant difference in growth rates between *lux*-tagged and wild-type strains (P-value < 0.05). These reporters allow results to be tracked in real-time, at intervals determined by the researcher and over long or short time frames. In this study and in chapter II, it was shown that bioluminescence techniques may be used to monitor growth of bacteria in opaque environments such as whole milk (Morrissey *et al.*, 2011). This is very useful as opaque liquids are not conducive to optical density measurements and require other indirect methods of measurement such as measuring pH or cell counts.

Conclusion

In brief, we have developed a method of transformation of the *lux* genes into *Lactococcus* strains to produce starters with a stable luminescent phenotype. These strains can be constructed using conjugation or electroporation and the physiological characteristics (growth, stability and acid resistance) of these strains were not significantly affected. The newly developed plasmid pRH001*lux* was successfully transformed into several industrially important starter culture strains by electroporation and conjugation and all transformants exhibited the luminescent phenotype. The plasmid pRH001*lux* was suitable for expressing *luxABCDE* genes in starter culture strains and tagged strains capable of being used as biomonitoring reporters for the dairy industry. Though it was not possible to monitor the presence of bacteriophage in milk, these reporters were used sucessfully to monitoring antibiotic residues in milk. These reports cannot be used for product manufacture as there are GMO's (genetically modified organisms); however they could be beneficial to the dairy industry as an important research tool prior to the production of cheese.

Chapter IV

Use of Bioluminescence-Based assays to test the Activity of Bioengineered Nisin Derivatives M21V and S29A against Foodborne Pathogens in Laboratory Media and Selected Foods

A manuscript based on this chapter is in preparation

Abstract

Nisin A is the best known bacteriocin and has been the subject of much research in particular with regard to its protective role in foods. It is also the only commerically available bacteriocin to date. Nisin is a 34- amino acid lantibiotic peptide, characterised by the presence of post translationally modified amino acids including lanthionine and/or methyllanthionine groups. Several Nisin variants with enhanced activity against Gram positive and negative bacteria have been identified. With this in mind, we set out to examine the effect of Nisin and two recently bioengineered Nisin variants (V and S29A) on a number of luciferase-tagged foodborne pathogens both *in vitro* and in food models at room temperature. We used bioluminescent reporter strains and modified agarose-based assays to quantitatively assess how each of these peptides performed against a range of foodborne pathogens, under sub-optimal conditions. We also used a bioluminescent-based assay to test how well each of the peptides preformed in a model food system when combined with a an antimicrobial agent (EDTA). For the first time, we report the enhanced bioactivity of Nisin V against Gram negative bacteria (*Escherichia coli* and *Cronobacter sakazakii*) in agarose assays and laboratory media (*E. coli)*. However, further analysis of Nisin V against Gram negative bacteria in powdered infant formula (PIF), apple juice and liquid whole egg revealed no such inhibitory effect. The enhanced bioactivity of Nisin S29A and V against *Listeria monocytogenes* in a commercially produced frankfurter maintained under sub-optimal conditions is also reported. In this instance, we demonstrated that Nisin V outperforms both the wild-type Nisin and S29A with respect to controlling growth of *Listeria* over a 24 hour period. Finally, we report that combinations of variants (M21V & S29A) with EDTA displayed

enhanced bioactivity against *C. sakazakii* in PIF, when compared to the wild-type nisin.

Introduction

Nisin A is easily the most extensively characterised lantibiotic and was the first approved as an antimicrobial agent in food by the Joint FAO/WHO expert committee on food additives in the late sixties (Delves-Broughton, 2005, Molloy *et al.*, 2012). Since then, it has been used as a food preservative in over 50 countries worldwide and in europe has been given the food additive number E234 (Piper *et al.*, 2009, Delves-Broughton, 2005). It functions by a distinctive dual mode of action involving binding of Nisin to lipid II, an essential precursor of the bacterial cell wall, followed by insertion into the membrane of the target cell to form a pore (Piper *et al.*, 2009, Cotter *et al.*, 2005, Wiedemann *et al.*, 2006, Breukink *et al.*, 1999, Field *et al.*, 2008). In recent years and facilitated by the gene-encoded nature of lantibiotics, bioengineered Nisin A derivatives with enhanced bioactivity against Gram positive bacteria have been generated (Field *et al.*, 2010a). Early attempts focussed on the generation of derivatives through targeted mutagenesis approaches in a bid to improve the solubility, stability and/or antimicrobial properties of Nisin A (Liu and Hansen, 1992, Kuipers *et al.*, 1992, Rink *et al.*, 2007, Rollema *et al.*, 1995). Despite the large numbers of derivatives generated by these studies, relatively few Nisin variants were found with enhanced activity against pathogenic micro-organisms (Yuan *et al.*, 2004, Rink *et al.*, 2007, Kuipers *et al.*, 1996). In recent years the combination of site-directed and non-targeted mutagenesis strategies, as well as specific targeting of a short 3 amino acid stretch in the peptide chain known as the hinge region, have resulted in the discovery of new derivatives with enhanced

activity against a range of pathogenic micro-organisms (Yuan *et al.*, 2004, Field *et al.*, 2008). Initial success was demonstrated through the generation of two hinge mutants, N20K and M21K, that only exhibited enhanced antimicrobial activity against Gram negative bacteria, including *Shigella*, *Pseudomonas* and *Salmonella* spp. (Yuan *et al.*, 2004). However, four years later the goal of generating Nisin derivatives with enhanced activity against Gram positive pathogens was achieved via a non-targeted approach (Field *et al.*, 2008). The screening of a large bank of randomly mutated Nisin mutants led to the discovery of one variant which encoded a K22T change (located within the hinge-region) that yielded a Nisin variant with enhanced activity against several Gram positive pathogens. A more in depth randomization of the hinge residues (Asn20-Met21-Lys22) led to the identification of further derivatives of note including N20P, M21V and K22S (Field *et al.*, 2008). Of these, Nisin M21V, subsequently renamed Nisin V, was noteworthy by virtue of its enhanced antimicrobial activity against a wide range of Gram positive targets, including medically significant pathogens (heterogeneous vancomycin-intermediate *S. aureus*, methicillin-resistant *S. aureus* and vancomycin resistant enterococci), as well as *Clostridium difficile*, *Streptococcus agalactiae* (a human and bovine pathogen) and *Listeria monocytogenes* (Field *et al.*, 2008, Field *et al.*, 2010b).

The first instance of a Nisin derivative showing simultaneous enhanced activity against both Gram positive and Gram negative bacteria was reported in 2012 (Field *et al.*, 2012). Using a targeted mutagenesis approach, derivatives of Nisin modified Serine 29, namely S29G, S29A, S29D and S29E, were found to possess superior antimicrobial activity against a range of Gram positive drug resistant clinical, veterinary and food pathogens. In addition, a number of the Nisin S29 derivatives displayed superior antimicrobial activity to Nisin A when assessed against a range of

Gram negative food-associated pathogens, including *Escherichia coli*, *Salmonella enterica* serovar Typhimurium and *Cronobacter sakazakii*.

Increasing consumer demand for minimally processed foods and ingredients ensures that Nisin A remains the most commercially important bacteriocin. To date, much of the work demonstrating the antimicrobial activity of Nisin A has been carried out in the form of food trials (Chollet *et al.*, 2008, Davies *et al.*, 1999, Murray, 1997). However, the activity of Nisin A variants in different food matrices has not been comprehensively examined, with the exception of two recent studies. Rouse *et al*. (2012) demonstrated the enhanced activity of the Nisin hinge variants SVA and NAK compared to Nisin A in controlling the growth of *L. monocytogenes* in chocolate milk stored under varying conditions. Field *et al.* (2010b) also examined the impact of the Nisin variant V compared to wild type Nisin A on *L. monocytogenes* in frankfurter meat stored at 37°C for 1 h.

With this in mind, we set out to compare the bioactivity of Nisin A and the most promising lead variants V and S29A against Gram positive and Gram negative bacteria, in laboratory media and food matrices stored under sub-optimal conditions (representing temperature abuse situations). Once the potency of each peptide was established in agarose, we used *lux*-tagged foodborne pathogens to determine whether the enhanced activity of the Nisin derivatives could be replicated in laboratory media and in food matrices stored under sub-optimal conditions.

Materials and methods

Bacterial strains and growth conditions

A summary of the Nisin producing strains and *lux*-tagged bacterial strains used in this study are listed in Table 1. Luria-Bertani (LB) broth (Merck, Darmstadt, Germany) was used to grow *E. coli,* S. *enterica* and *C. sakazakii* cultures. Microbiological agar (Merck, Darmstadt, Germany) was added to LB broth when solid agar was required. *Lactococcus lactis* strains were grown in M17 broth (Oxoid, Basingstokes, UK) supplemented with 0.5% glucose, GM17 (Sigma-Aldrich, MO, USA) or GM17 agar (Oxoid, Basingstoke, UK) at 30°C. *Listeria* strains were grown in brain heart infusion (BHI) broth or BHI agar (Oxoid, Basingstoke, UK) at 37°C. *E. coli* and *C. sakazakii* cultures were grown at 37°C, while S. *enterica* cultures were grown at 37°C. When required, antibiotics were used where indicated at the following concentrations: chloramphenicol (Sigma-Aldrich, MO, USA) 7.5 mg/ml^{-1} for *Listeria* and 10 mg/ml^{-1} for *L. lactis* and erythromycin (Sigma-Aldrich, MO, USA) at 500 mg/ml^{-1} for *E. coli*, *C. sakazakii,* and *S.* Typhimurium. Stock soltions of 1 M EDTA (Sigma-Aldrich, MO, USA) was prepared in 100% ethanol.

Table 1. List of Nisin-producing strains, *lux*-tagged bacterial strains used in this study, including relevant characteristics and references.

Strains/*Lux*-tagged bacteria	Relevant characteristics or source of strains	Reference
Lactococcus lactis Nisin A (UCC 5010)	Wild-type Nisin-producing strain	(Field *et al.,* 2008)
Lactococcus lactis Nisin V (UCC 5012)	Wild-type Nisin-producing strain + alteration at position 21 on peptide chain	(Field *et al*., 2010b)
Lactococcus lactis NZ9800 PCI372-NisA S29A	Wild-type Nisin-producing strain + alteration at position 29 on peptide chain	(Field et al., 2012)
Listeria monocytogenes:: F2365 ::pPL2*lux* -P_{help}	Isolated from cheese and transformed with pPL2*lux*-P_{help} plasmid	UCC culture collection
Cronobacter sakazakii NCTC 8155::p16S*lux* -P_{help}	Isolated from dried milk powder and transformed with p16*Slux*-P_{help} plasmid	UCC culture collection (Chapter II)
Escherichia coli 0157:H7 TUV 93-0:p16S*lux*- P_{help}	Derived from strain EDL933 and transformed with p16*Slux*-P_{help} plasmid	This study
Salmonella Typhimurium UK-1::p16S*lux* -P_{help}	Chicken-passaged isolate of a highly virulent *S*. Typhimurium strain, originally isolated from infected horse. Transformed with p16*Slux*- P_{help} plasmid	UCC culture collection

Nisin purification

L. lactis Nisin A (UCC 5010) or the mutant nisin strains of interest, *L. lactis* NZ9800 PCI372-nisA S29A (S29A) and *L. lactis* Nisin V (UCC 5012) (V) were sub-cultured twice in GM17 broth at 1% at 30°C before use. Two liters of modified Tryptic yeast broth was inoculated with the culture at 0.5% and incubated at 30°C overnight. The culture was centrifuged at 3,287 X g for 20 min at 4 ˚C. The cell pellet was re-suspended in 300 ml of 70% isopropanol (IPA) containing 0.1% trifluoroacetic acid (TFA) and stirred at room temperature for approximately 3 h. Cell debris was removed by centrifugation at 3,287 g for 20 min and the supernatant was retained. The isopropanol was evaporated from the supernatant using a rotary evaporator (Buchi) and the pH was adjusted to 4.5 before applying to a 10 g (60 ml) Varian C-18 Bond Elut Column (Varian, Harbor City, CA) pre-equilibrated with methanol and water. The column was washed with 100 ml of 30% ethanol and the inhibitory activity was eluted in 100 ml of 70% IPA 0.1% TFA. Aliquots (10 ml) were concentrated to 2 ml through the removal of IPA by rotary evaporation. Aliquots (1.5 ml) were applied to a Phenomenex (Phenomenex, Cheshire, UK) C12 reverse phase (RP)-HPLC column (Jupiter 4u proteo 90 Å, 250 x 10.0 mm, 4 µm) previously equilibrated with 25% IPA, 0.1% TFA. The column was subsequently developed in a gradient of 30% IPA containing 0.1% TFA to 60% IPA containing 0.1% TFA from 10 to 45 min at a flow rate of 1.2 ml min^{-1}.

Mass spectrometry

For colony mass spectrometry analysis, bacteria was collected with sterile plastic loops and mixed with 50 ml of 70% isopropanol adjusted to pH 2 with HCl. The suspension was vortexed, the cells were spun down in a benchtop centrifuge at 13,148 x g for 2 min and the supernatant was removed for analysis. Mass spectrometry in all cases was performed with an Axima CFR plus matrix-assissted laser desorption/ionisation Time-of-flight (MALDI TOF) mass spectrometer (Shimadzu Biotech, Manchester, UK). A 0.5 ml aliquot of matrix solution [alpha-cyano-4-hydroxy cinnamic acid (CHCA), 10 mg ml^{-1} in 50% acetonitrile-0.1% (v/v) TFA] was placed onto the target and left for 1–2 min before being removed. The residual solution was then air dried and the sample solution (re-suspended lyophilized powder or colony mass spectrometry supernatant) was positioned onto the pre-coated sample spot. Matrix solution (0.5 ml) was added to the sample and allowed to air dry. The sample was subsequently analyzed in positive-ion reflectron mode.

Well diffusion assays for antimicrobial activity

Agarose-based deferred antagonism assays were carried out as follows: Underlay agar (0.03%) was prepared with 10 mM sodium phosphate buffer (SPB) at pH 7.4 and agarose (1% w/v) was added, autoclaved and cooled to 50 °C. Bacteria grown to mid-logarithmic phase (OD595nm ~ 0.5nm) were harvested by centrifugation and washed with 10 mM SPB at pH 7.4. Bacterial cells were then added to 15 ml cooled underlay medium to obtain a concentration of 2×10^7 colony-forming units (CFU)/ml. The inoculated medium was rapidly transferred into sterile Petri dishes,

allowed to solidify and dried. Wells (4.6mm in diameter) were then made in the seeded plates. Freeze dried purified Nisin A and mutants was weighed and resuspended in BHI/LB broth to a final concentration of 60 µM for Gram negative bacteria and 30 µM for Gram positive bacteria. Ten microliters of these resuspended Nisin peptides were then added to the wells and the plates were incubated at 37 °C (Gram positive strains) or room temperature (Gram negative strains) for 3 hours. The plates were then overlaid with 15 ml of autoclaved double strength BHI/LB agarose (1% w/v agarose) overlay medium pre-cooled to 50 °C. The plates were then incubated overnight (~ 16 h) at the relevant temperature.

Minimum inhibitory concentration assays

Minimum inhibitory concentration (MIC) determinations for Gram positive organisms were carried out in triplicate in microtitre plates (Sarstedt, Numbrecht, Germany). Ninety-six well microtitre plates were pre-treated with bovine serum albumin (BSA) (Sigma-Aldrich, MO) prior to addition of the peptides. Briefly, to each well of the microtitre plate 200 µl of phosphate buffered saline (PBS) (Gibco, Paisley, UK) containing 1% (w/v) bovine serum albumin (PBS/BSA) was added and incubated at 37 °C for 30 min. The wells were washed with 200 µl PBS and allowed to dry. Target strains (Table 1) were grown overnight using the appropriate conditions and broth medium, sub-cultured into fresh broth and allowed to grow to an OD595 of ~ 0.5 and diluted to a final concentration of 10^5 CFU/ml. Wild type Nisin and Nisin mutant peptides were adjusted to a 7.5 µM (*L. monocytogenes*) starting concentration and 2-fold serial dilutions of each peptide were added to the wells containing the target strain. After incubation for 16 h at 37°C the MIC was

determined as the lowest peptide concentration causing inhibition of visible growth. For Gram negative bacteria (*E. coli*, *C. sakazakii* and *S.* Typhimurium), strains were grown to mid-logarithmic phase and then harvested by centrifugation, washed with 10 mM SPB at pH 7.4, and diluted to 2×10^5 CFU/ml in SPB. Nisin and Nisin derivative purified peptides were resuspended in sterile HPLC water and 50 µl aliquots were added to wells containing 2×10^5 CFU of bacteria in a volume of 50 µl. Plates were incubated at room temperature for 2 hours with agitation. Double strength LB broth (100 µl) was added and plates were incubated at room temperature overnight (~ 16 h). The MIC was taken as the lowest concentration of peptide at which growth was inhibited. All assays were carried out in triplicate.

Food models

Infant milk formula Trial with *C. sakazakii*

Commerically available powdered infant formula (PIF) was prepared according to manufacturers instructions, brought to room temperature and the pH of the milk was determined. An overnight culture of *C. sakazakii* NCTC 8155::*lux* was diluted and inoculated into reconstituted PIF at a final concentration of 1×10^5 CFU/ml. To this, Nisin A or the Nisin variants were added at a concentration of 160.1 mg/L (48 µM). Reconstituted PIF inoculated with the pathogen alone and PIF containing neither pathogen nor peptide served as contols for the experiment. One hundred microliters of test and control samples were transferred to a 96-well white plate (Nunc, Thermo Fisher Scientific, Roskilde, Denmark) and bioluminescence measurements were recorded every hour for 24 h at sub-optimal temperature in relative light units (RLU) by a Luminoskan luminometer automatic plate reader (Thermo Fisher Scientific,

Roskilde, Denmark). *C. sakazakii* counts levels were determined through dilution and plating of samples on LB and DFI agar. The experiments was carried out in triplicate.

Apple Juice trial with *E. coli 0157:H7*

Commerically available apple juice was brought to room temperature and the pH of the juice determined. An overnight culture of *E. coli:0157:*H7::*lux* was diluted and inoculated into the juice at a final concentration of 1 x 10^5 CFU/ml. To this, Nisin A or the Nisin variants were added at a concentration of 160.1 mg/L (48 µM). Apple juice inoculated with the pathogen alone and juice containing neither pathogen nor peptide served as controls for the experiment. One hundred microliters of test and control samples were transferred to a 96-well white plate and bioluminescence measurements were recorded every hour for 24 h at ambient temperature in relative light units (RLU) by a Luminoskan luminometer automatic plate reader. *E. coli* 0157:H7 counts were determined through dilution and plating of samples on LB agar. The experiment was carried out in triplicate.

Egg trial with *S*. Typhimurium

Large Grade A eggs were purchased from a local supermarket and egg shells were washed with 70% ethanol and allowed to air dry. The sanitised eggs were aseptically broken, transferred into a sterile stomacher bag (Seward, UK) and homogenised for 2 mins in a stomacher (Alvarez *et al.*, 2007). The liquid egg (yolk plus white) was stored at 4 °C until required. The pH of the liquid egg was determined. An overnight

culture of *S.* Typhimurium::*lux* was diluted and inoculated into liquid egg at a final concentration of 1 x 10^5 CFU/ml. To this, Nisin A or the Nisin variants were added at a concentration of 160.1 mg/ml (48 µM). Egg inocultaed with the pathogen alone and egg containing neither pathogen or peptide, served as controls for the experiment. One hundred microliters of both test and control samples were transferred to a 96-well white plate and bioluminescence measurements were recorded every hour for 24 h at ambient temperatures in relative light units (RLU) by a Luminoskan luminometer automatic plate reader. *S.* Typhimurium counts were determined through dilution and plating of samples on LB agar. The experiment was carried out in triplicate.

Frankfurter meat trial *with Listeria monocytogenes*

Thirty-five grams of frankfurter meat (78% pork meat and 12% pork fat) was weighed and placed into a sterile blender and homogenized on full speed for 30 s with 35 ml of PBS. One ml of the meat homogenate was placed into sterile 1.5 ml eppendorf tubes. The samples were then inoculated with approximately 1x10^5 CFU/ml *L. monocytogenes* F2365*lux*. To this, Nisin A or the Nisin variants were added at a concentration of 7.5 mg/L (2.24µM). The effect of Nisin against *Listeria* was examined by two techniques: 200 µl of treated homogenate was transferred to 96-well plates and kill of *Listeria* monitored by bioluminescence using the Luminoskan Luminometer. Similarly, 200 µl of treated homogenate was transferred to a sterile eppendorf tube, incubated at ambient temperature and *Listeria* counts were monitored by serial dilution and plate count technique using BHI and listeria

selective (LSA) agar (Oxoid, Basingstoke, UK). Samples were withdrawn and plated after 1, 3, 5 and 24 h. The test was conducted in triplicate (Field *et al.*, 2010b).

Infant milk formula Trial with *C. sakazakii* and EDTA

Commerically available powdered infant formula (PIF) was prepared according to manufacturers instructions, brought to room temperature and the pH of the milk was determined. An overnight culture of *C. sakazakii* NCTC 8155::*lux* was diluted and inoculated into reconstituted PIF at a final concentration of 1 x 10^5 CFU/ml. To this, Nisin A or the Nisin variants were added at a concentration of 160.1 mg/L (48 µM). EDTA at a concentration of 20 Mm was also added. Reconstituted PIF inoculated with the pathogen alone, PIF containing neither pathogen nor peptide and PIF containing EDTA served as controls for the experiment. One hundred microliters of test and control samples were transferred to a 96-well white plate (Nunc, Thermo Fisher Scientific, Roskilde, Denmark) and bioluminescence measurements were recorded every hour for 24 h at sub-optimal temperature in relative light units (RLU) by a Luminoskan luminometer automatic plate reader (Thermo Fisher Scientific, Roskilde, Denmark). *C. sakazakii* counts levels were determined through dilution and plating of samples on LB and DFI agar. The experiments was carried out in triplicate.

Note: Growth curves examining *lux*-tagged *Listeria monocytogenes, Cronobacter sakazakii, Escherichia coli* and *Staphylococcus aureus* at various pH's and salt concentrations were carried out. Result of this work can be found in the appendix.

Interpretation of data and statistical analysis.

All experiments were performed in triplicate from three separate cultures (i.e. three biological repeats) and repeated at least twice. The results shown represent the means +/- standard deviations (error bars) for one particular experiment. Mean and standard deviations were calculated using a basic Excel programme. Statistical significance was determined via Sigma plot t-test. In all cases, a P-value less than or equal to 0.05 was considered to be statistically significant.

Results

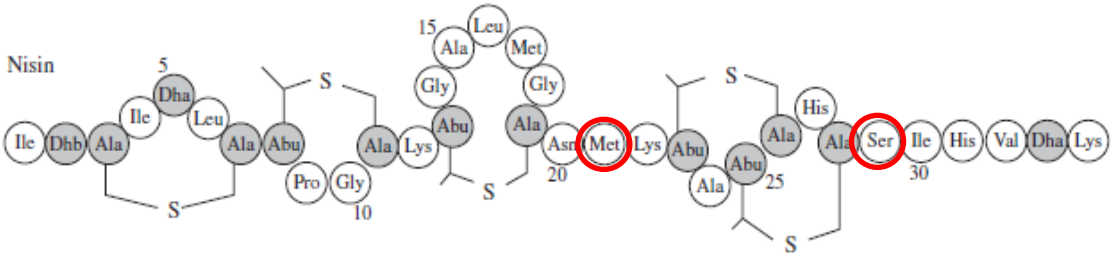
Nisin purification and Mass spectrometry

Previously Field *et al*. (2010a) generated Nisin variants (V and S29A) with enhanced activity through alterations of single amino acid residues (Fig. 1A). Following purification and freeze drying of these Nisin variants, colony mass spectrometry (CMS) analysis was performed. Peptide masses of 3,321 Da, 3,336 Da and 3,353 Da were obtained for Nisin peptides V, S29A and Nisin A, respectively, confirming the correct mass and purity of each peptide (Fig. 1B).

Bioassays for antimicrobial activity and minimum inhibitory concentration (MIC)

The antimicrobial activity of each peptide was initially determined by means of a modified agarose diffusion assay using equimolar concentrations of the purified peptides as previously described (Field *et al.*, 2012). The peptides were assessed against a range of targets which included *L. monocytogenes*, *C. sakazakii*, *E. coli* and *S.* Typhimurium (Table 2). Results revealed that the Nisin variant V displayed enhanced activity when compared to wild-type Nisin A, when assessed against *L. monocytogenes* (16.13mm and 13.16mm, respectively), *E. coli* (13.70 and 12.32mm, respectively) and *C. sakazakii* (13.99mm and 12.25mm, respectively). Notably, the V variant was not enhanced against *S.* Typhimurium. In contrast, the Nisin S29A derivative exhibited enhanced bioactivity against all the pathogens tested when compared to the wild-type peptide.

(A)



(B)

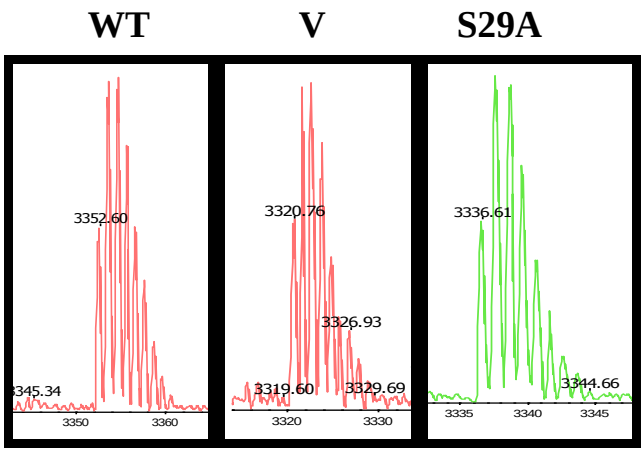


Fig. 1 (A) The structure of Nisin A (wild-type). Red circles indicate where amino acids were altered resulting in the generation of Nisin variants with enhanced activity. **(B)** Colony Mass Spectrometry analysis of Nisin A (3352.6 Da), V (3320.76 Da) and Nisin S29A (3336.6 Da) producing strains utilized in this study.

Strains/Plasmids	Nisin A	V	S29A
	mm^2	mm^2	mm^2
L. monocytogenes F2365::pPL2*lux*	13.16±0.17	**16.13**±0.19	**14.73**±0.16
		(p=0.0003)	(p=0.002)
C. sakazakii NCTC 8155::p16S*lux*	12.25±0.24	**13.99**±0.36	**13.39**±.078
		(p=0.017)	(p=0.015)
E. coli 0157:H7 TUV 93-0::p16S*lux*	12.32±0.18	**13.70**±0.14	**13.51**±0.20
		(p=0.004)	(p=0.012)
S. Typhimurium UK-1::p16S*lux*	9.70±0.17	9.64±0.28	**10.28±0.12**
		(p=0.86)	(p=0.05)

Table 2. Activity of purified peptides of Nisin A, V and S29A against *C. sakazakii*, *E. coli* and *S.* Typhimurium (60 µM) and *L. monocytogenes* (30 µM) as determined by agarose gel diffusion assay. Results are expressed as total area of inhibitory zone expressed in mm^2. p -values represent statistical difference between Nisin variants and wild type Nisin A. Values in bold reached statistical significance compared to the Nisin A control (Student's t-test: $p < 0.05$).

To further confirm the specific activity of the peptides, and to ensure that these results were not simply a result of improved solubility in solid agar, broth-based MIC determination assays were also carried out using purified peptides against the same Gram positive and Gram negative targets (Table 3). The lower the MIC recorded, the higher the activity of the peptide. The results closely matched the results obtained using the agarose-based diffusion assays. S29A exhibited at least a two-fold increase in specific activity against all of the pathogen targets tested. In the case of Nisin V, enhanced specific activity compared to Nisin A was observed for *L. monocytogenes* (1.87µM and 3.75µM, respectively) and notably *E. coli* (3.75µM and 7.50µM, respectively). In contrast to the agar-diffusion assays however, the specific activity of Nisin V was comparable to Nisin A when examined against *C. sakazakii* and *S.* Typhimurium. These results demonstrate for the first time the enhanced activity of Nisin V against a Gram negative strain (*E. coli*) in a broth-based MIC assay.

Strains/Plasmids	**Nisin A**	**V**	**S29A**
	mg/L (µM)	**mg/L (µM)**	**mg/L (µM)**
L. monocytogenes F2365::pPL2*lux*	25.14(7.5)	**12.57(3.75)**	**6.28(1.87)**
C. sakazakii NCTC 8155::p16S*lux*	12.57(3.75)	12.57(3.75)	**6.28(1.87)**
E. coli 0157:H7 TUV 93-0 p16S*lux*	25.14(7.5)	**12.57(3.75)**	**12.57(3.75)**
S. Typhimurium UK-1:: p16Slux	25.14(7.5)	25.14(7.5)	**12.57(3.75)**

Table 3. MIC of purified Nisin A, V and S29A against representative Gram negative strains & one Gram positive strain. Results are expressed (in mg/L and µM) as the mean of triplicate assays. Values in bold reached statistical significance compared to the Nisin control (Student's t-test: $p < 0.05$).

Food Trials

Having established the potency of Nisin V and S29A against Gram positive and negative bacteria in both agar and broth, we set out to determine if the observed enhanced antimicrobial activity could translate to different food matrices stored at ambient temperature to simulate a temperature abuse situation. In food trials using reconstituted PIF (pH ~ 6.68) spiked with *C. sakazakii*, we observed that addition of wild-type Nisin A and the Nisin variants (V and S29A) had no inhibitory effect on bacterial growth (measured in RLU) over the time course of the assay (Fig. 2). In fact, samples containing Nisin A shared a similar growth pattern to samples containing either Nisin A or S29A. Viable plate counts taken at regular intervals support these findings (data not shown).

When apple juice samples containing *E. coli* were spiked with wild-type Nisin A, V and S29A it was noted that no inhibitory effect on bacterial growth was observed for any of the samples tested (Fig. 3). Samples containing Nisin A peptide showed similar results to samples containing either Nisin A or S29A. Due to the low pH associated with apple juice (pH of apple juice ~ 3.16), it was not possible to monitor bacterial growth using bioluminescence. This was confirmed when higher inocula were also added to apple juice and luminescence was monitored. Instead viable plate counts were performed.

In food trials using liquid whole egg (pH ~ 6.9) spiked with *S.* Typhimurium, we observed that addition of wild-type Nisin A and Nisin variants (V and S29A) had no inhibitory effect on bacterial growth (measured in RLU) over the time course of the assay (Fig. 4). Again samples containing Nisin A shared a similar growth pattern to samples containing either Nisin A or S29A. Viable plate counts taken at regular intervals support these findings (data not shown).

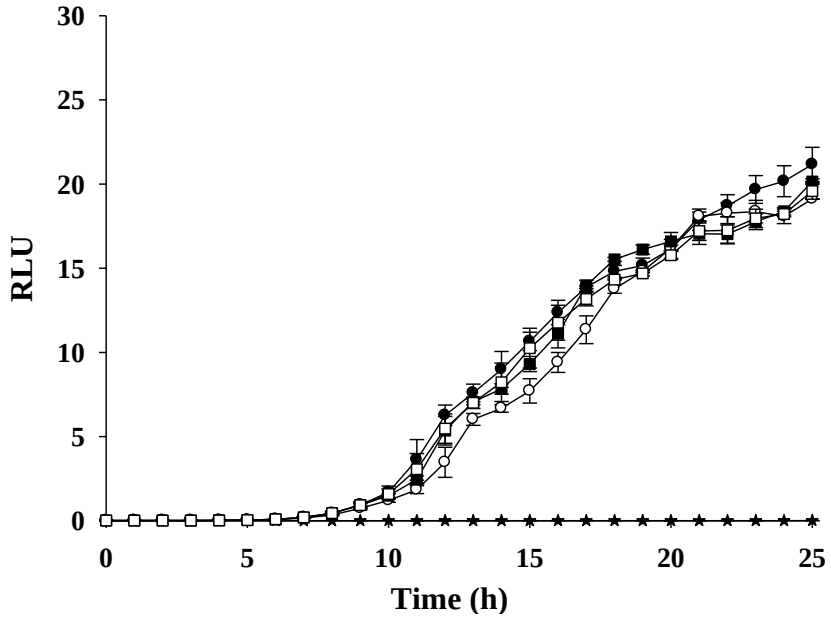


Fig. 2 Growth of *lux*-tagged *C. sakazakii* (1 x 10^5 CFU/ml) in PIF alone (●) and in plus 48 µM wild-type Nisin (o), and in PIF plus 48 µM V variant (■), plus 48 µM S29A (□) at room temperature as measured for 24 h on the luminoskan luminometer. (*) represents control containing PIF alone. Error bars represent the standard deviations of triplicate experiments (Students T-test: $p < 0.05$).

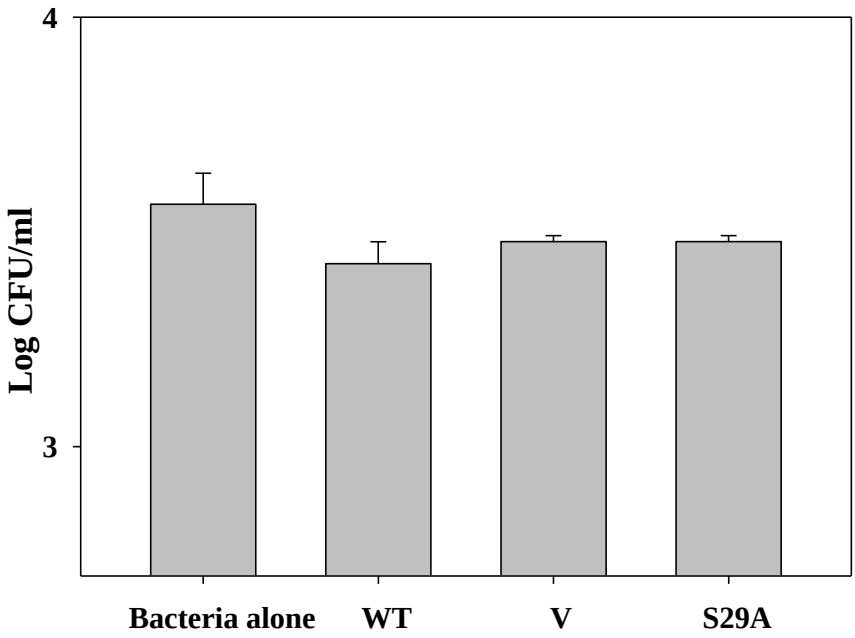


Fig. 3 Growth of *lux*-tagged *E. coli* (1 x 10^5 CFU/ml) in apple juice, and in apple juice plus 48 µM wild-type Nisin, and in apple juice plus 48 µM V variant, or in apple juice plus 48 µM S29A, at ambient temperature and measured after 2 h. Error bars represent the standard deviations of triplicate experiments (students t-test $p<0.05$).

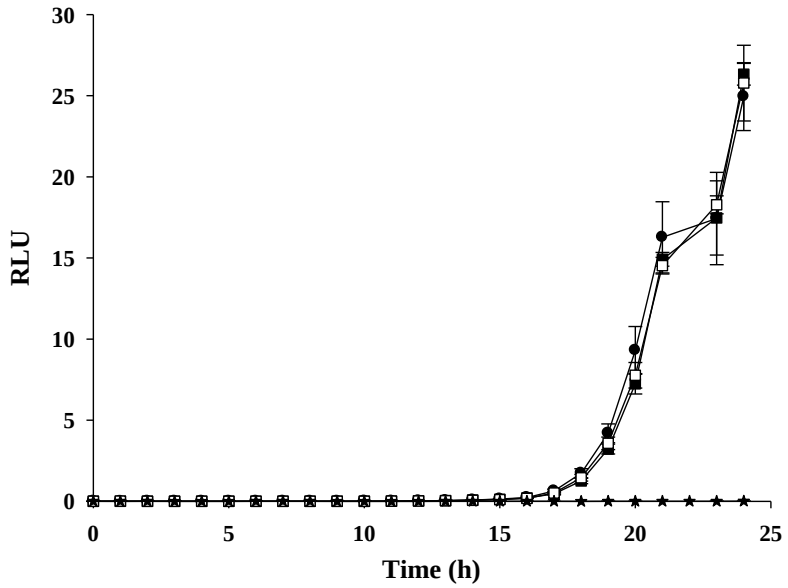


Fig. 4 Growth of *lux*-tagged *S.* Typhimurium (1 x 10^5 CFU/ml) in liquid egg alone (●), and in liquid egg plus 48 µM wild-type Nisin (o), and in liquid egg plus 48 µM V variant (■), and in liquid egg plus 48 µM S29A (□), at room temperature and measured for 24 h on the luminoskan luminometer. (*) represents control of egg alone. Error bars represent the standard deviations of triplicate experiments (student's t-test $p < 0.05$).

Having established the potency of Nisin V and S29A against *L. monocytogenes* in broth and agar, we sought to determine whether the enhanced bioactivity could be observed in a meat model at a sub-optimal temperature. When variants were assessed in frankfurters meat spiked with 2 x 10^5 CFU/ml of *L. monocytogenes* and Nisin V peptide at sub-optimal temperature, a significant reduction ($P < 0.05$) in *Listeria* counts was observed in samples taken after 1, 3, 5 and 24 h (Fig. 5). A significant difference was also observed in samples containing S29A after 1 and 3 h ($p < 0.05$) when compared to wild-type Nisin A samples. Plate count analysis of samples after 5 and 24 h revealed that samples containing S29A demonstrated recovery of *Listeria* and a 2 Log increase in bacterial numbers. Due to the low initial inoculum of *Listeria* used and the reduced levels of luminescence emitted from Gram positive bacteria it was not possible to monitor bacterial growth using bioluminescence. However, the assay demonstrates that Nisin V provided significantly enhanced protection against *L. monocytogenes* relative to the control peptide and S29A under the temperature abuse circumstances outlined above. Previous investigations carried out with Nisin V and A involved monitoring growth of *Listeria* in a model food system for just 1 h and at 37°C. In addition, for the first time, S29A has been shown to demonstrate enhanced activity relative to the control peptide in frankfurter meat against *L. monocytogenes* for approximately 5 h. Notably, *Listeria* numbers regrew in both the Nisin A and S29A samples to levels equivalent to the control containing no peptide after 24 h. In contrast, *Listeria* levels in the sample containing Nisin V remained unchanged at all time points.

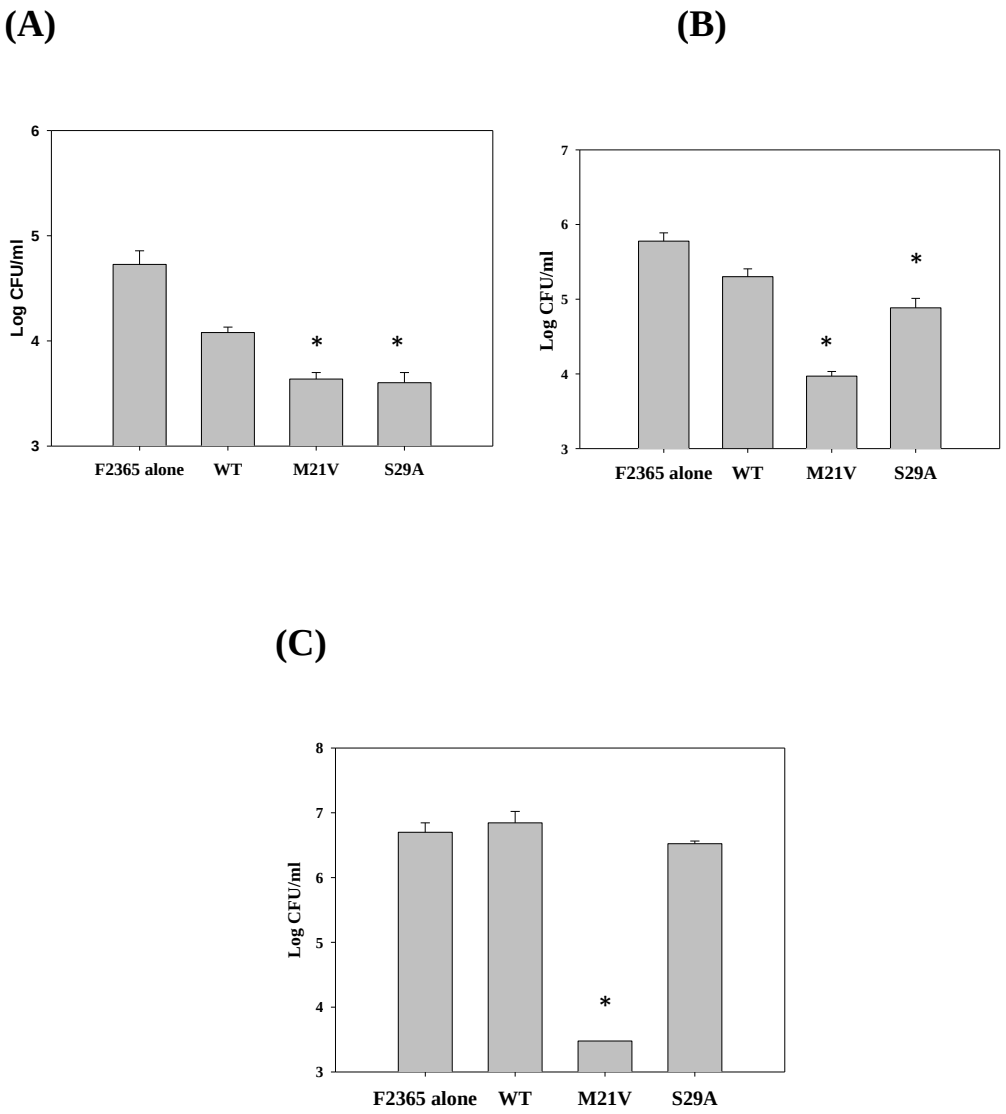


Fig. 5 Growth of *lux*-tagged (1 x 10^5 CFU/ml) *L. monocytogenes f2365-lux* in frankfurter mix plus 7.5 µg/ml wild-type Nisin, in frankfurter mix plus 7.5 µg/ml Nisin V, in frankfurter mix plus 7.5 µg/ml S29A, at room temperature and measured after 1 h **(A),** 3 h **(B)** and 24 h **(C).** Error bars represent the standard deviations of triplicate experiments. (*) represents Nisin variants statistically enhanced against the wild-type control ($p < 0.05$).

Having established that Nisin V and S29A alone had no enhanced antimicrobial effect on *C. sakazakii* growth in food models, we sought to determine whether an antimicrobial effect would be observed in a food model containing Nisin variants in combination with EDTA. When variants were assessed in reconstituted PIF spiked with *C. sakazakii*, Nisin V peptide and EDTA at a sub-optimal temperature, a significant reduction ($P < 0.05$) in *Cronobacter* counts was observed in samples taken after 8 h (Fig. 6). A significant difference was also observed in samples containing S29A and EDTA after 8 h ($p < 0.05$) when compared to wild-type Nisin A samples. Plate count analysis of samples after 24 h revealed that samples containing M21V and S29A plus EDTA demonstrated recovery of *Cronobacter* and an increase in bacterial numbers.

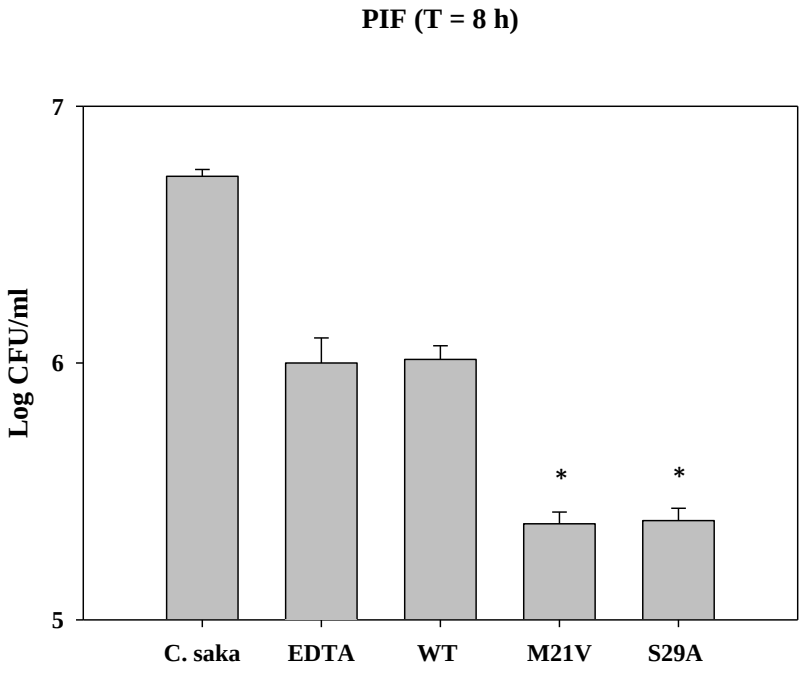


Fig. 6 Growth of *lux*-tagged (1 x 10^5 CFU/ml) *C. sakazakii* in reconstituted PIF, in PIF plus 20 mM EDTA, in PIF plus 48 µM wild-type Nisin and 20 mM EDTA, in PIF plus 48 mM Nisin V and 20 mM EDTA, in PIF plus 48 mM Nisin S29A and 20 Mm EDTA, at room temperature and measured after 8 h**.** Error bars represent the standard deviations of triplicate experiments. (*) represents Nisin variants statistically enhanced against the wild-type control ($p < 0.05$).

Discussion

Ensuring a safe food supply is a major challenge for the food industry. Consumers are continually demanding safe, minimally processed ready to eat convenience foods which will cater for all demographics and life-styles. A plethora of new methods and preservatives aimed at reducing microbial food spoilage have been identified and developed (Devlieghere *et al.*, 2004). These include high hydrostatic pressure, pulsed electric fields, modified atmosphere packaging, natural antimicrobial compounds and bacteriocinogenic and non-bacteriocinogenic cultures. In terms of natural preservatives, antimicrobial agents which are food grade and therefore acceptable for use in the food industry are hugely important. The use of preservatives such as Nisin provide a useful preservation technology which can replace older techniques leading to food products which retain their flavour, nutrition and most of all are safe and natural for the consumer (Devlieghere *et al.*, 2004). With Nisin increasingly being used as a preservative in a variety of foods, (permitted for use in over 50 countries) the potential for Nisin variants as preservatives in specific foods in the future is enormous (Delves-Broughton, 2005). The fact that these variants often differ from the wild-type Nisin A by only one amino acid suggests that these variants are more likely to become legally accepted preservatives in the future (Field *et al.*, 2010b).

To date, Nisin A has been used in a wide variety of foods (reviewed by Delves-Broughton *et al.*, 2005) and previous studies have shown that Nisin A can be influenced by a wide range of factors within foodstuffs including fat content, proteolytic degradation, partitioning into polar or nonpolar food components and sodium chloride concentrations. For example, Chollet and co-workers investigated the antimicrobial effect of sodium chloride, fat and proteolytic enzymes on the

concentration of Nisin in Emmental cheese and its subsequent antimicrobial activity (Chollet *et al.,* 2008). Agarose-based assays determined that increased anhydrous milk concentrations caused a decrease in Nisin concentrations by a factor of 1.6 and a subsequent significant decrease in Nisin activity. Increasing sodium chloride concentrations was found to increase Nisin bioactivity. This work indicated that Nisin interacts with fat globules in the cheese matrix and was not affected by proteases. Another study by Jung *et al*. (1992) investigated the influence of fat and emulsifiers on the efficacy of Nisin at inhibiting *L. monocytogenes* in fluid milk (Jung *et al.,* 1992). Results showed that Nisin activity (50 U/ml) decreased by about 33% when it was added to skim milk and by more than 88% when added to milk containing 12.9% fat. In half-and-half milk or milk containing 12.9% milk fat, Nisin was far less effective at inhibiting *L. monocytogenes*. A review by Galvez *et al*. (2007) suggests that the efficacy of bacteriocins in food is in fact much lower when compared to results obtained in culture media. In some instances, up to ten-fold higher bacteriocin concentrations must be added to foods in order to achieve the same inhibitory effect seen in culture media (Gálvez *et al.*, 2007). This is due to a number of limiting factors such as the target bacteria and food related factors such as pH, storage, processing conditions and the presence of enzymes or additives (Gálvez *et al.*, 2007). The microbiota of the food is also an important factor. In the presence of a complex food microbiota, the efficacy of bacteriocins may be reduced due to the antimicrobial binding to insensitive bacteria such as Gram negative bacteria (Gálvez *et al.*, 2007). During the course of this study, more than ten times the MIC for Nisin V (48 µM) was used in food trials involving Gram negative bacteria. Despite this high concentration no inhibitory affect was observed. By comparison, much less

peptide was needed to confer an antimicrobial effect on the Gram positive bacterium *Listeria*.

To date, two studies have investigated the efficacy of Nisin variants in model food systems (Rouse *et al.*, 2012, Field *et al.*, 2010b). The study by Rouse *et al*. (2012) used two Nisin variants (SVA and NAK) to investigate whether the enhanced activity observed in carrageenan based agar but not in MIC based assays could be replicated in a carrageenan containing food containing 10^5 or 10^4 CFU/ml of *L. monocytogenes*. Results showed that both peptides dramatically outperformed Nisin A with respect to controlling *L. monocytogenes* in commercially produced chocolate milk containing carrageenan when stored at 22 °C and 4 °C for 3 days. Similarly, a study by Field *et al*. (2010b) used Nisin variants V and T to investigate whether either peptide showed enhanced activity against 10^7 CFU/ml of *lux*-tagged *L. monocytogenes* in frankfurter meat over a 1 h. Results revealed that in the presence of Nisin A and T, bioluminescence increased whereas the corresponding nisin V treated sample showed a marked decrease in bioluminescence following 1 hour incubation at 37 °C. In the present study, we found that both Nisin V and for the first time S29A, significantly reduced growth of *L. monocytogenes* in a model food system using a low initial inoculum (1×10^5 CFU/ml) and storage at ambient temperature over 24 h and 3 h respectively. It is evident from results obtained that the enhanced potency of Nisin V can be maintained for up to 24 h within a complex and high-fat food such as frankfurter meat. The performance of the Nisin V peptide under these 'abusive' conditions suggests that were it to be combined with good manufacturing processes and other hurdle technologies, this derivative could be a very effective anti-*listerial* food preservative. Although it is also evident that S29A can control *L. monocytogenes* growth initially and for up to 5 h, the antimicrobial

effect is not maintained for as long as that of M21V. However, we should recognise that levels of contamination in real food systems are usually much lower than the levels used in these experiments.

Given that S29 derivatives displayed superior antimicrobial activity to Nisin A when assessed against a range of Gram negative food-associated pathogens (Field *et al.*, 2012), we investigated whether the enhanced activity of either V or S29A in broth could translate to liquid-based foods such as reconstituted PIF, apple juice or liquid whole egg. Despite the activity observed when Nisin was added to laboratory media, no antimicrobial effect was observed in PIF, apple juice or liquid egg for Nisin A, Nisin V or S29A despite the fact that almost 13-fold the concentration of Nisin V or S29A needed for MICs in broth was applied.

The various limiting factors previously discussed combined with the fact that Gram negative bacteria have a more resistant cell envelope, may offer an explanation as to why Nisin has not been found to inhibit Gram negative pathogens in food matrices. One possible solution to this problem could involve the use of chemical substances or other microbial substances in conjunction with Nisin. Galves *et al*. (2007) states that chelators and other compounds can permeabilise the outer membrane of Gram negative bacteria by extracting Ca^{2+} and Mg^{2+} ions that stabilise the lipopolysaccharide part of the structure, thereby allowing certain bacteriocins to reach the inner cytoplasmic layer (Gálvez *et al.*, 2007). Oshima *et al*. (2012) investigated a variety of anti-microbial agents alone and in combination to determine their bacteriostatic or bactericidal effect on *C. sakazakii*. Results showed that the addition of Nisin (at a concentration of 8000 AU ml¯1) had no inhibitory effect when present as a single compound, however the combination of Nisin with the lactoperoxidase system (LPOS) inhibited outgrowth of *C. sakazakii* at 37 °C for 8 h

(Oshima *et al.*, 2012). In the present study, we found for the first time that both Nisin V and S29A, when combined with EDTA, significantly reduced growth of *C. sakazakii* in reconstituted PIF over an 8 h period. These results demonstrate that the addition of Nisin variants alone had no inhibitory effect when present as a single compound, however combinations of Nisin variants with EDTA significantly reduced *C. sakazakii* levels for 8 h. Further food trials would be necessary to determine where this combined effect would be observed in other liquid-based food models and whether other antimicrobial agents or chelators could be used along with Nisin variants to exhibit an enhanced antimicrobial effect on bacteria. It has also been suggested that prior exposure of cells to sub-lethal heat, osmotic shock or freezing in conjunction with Nisin use may overcome the difficulties encountered by bacteriocins when trying to traverse the Gram negative bacterial cell wall (Gálvez *et al.*, 2007). However, further investigations would be necessary to determine which treatments or combinations of antibacterial agents could be used along with Nisin A variants to inhibit Gram negative pathogens in liquid-based food matrices.

Experiments carried out in this chapter highlight the importance of bioluminescence-based assays to the food industry. These experiments show it is possible to monitor the impact of various antimicrobial agents on a number of bacteria in many food systems over the time course of an assay. Bioluminescent imaging is a faster and less labour intensive method of monitoring strain growth than conventional methods. However, this work has also identified limitations of bioluminescent imaging. Low acid environments such as those in apple juice do not allow light to be emitted and so bioluminescence can not be used successfully in all types of food systems. Similarly, *lux*-tagged microorganism must emit high levels of light over long time periods in order for luminescences to be sucessfully detected.

Conclusion

In summary, we have examined the activity of Nisin derivatives V and S29A against foodborne pathogens in laboratory media and selected foods. For the first time, we report the enhanced bioactivity of Nisin V against Gram negative bacteria in agarose-based assays and laboratory media. We have also demonstrated the enhanced bioactivity of Nisin S29A and V against *Listeria monocytogenes* in commercially produced frankfurter meat maintained under sub-optimal conditions showing that Nisin V outperforms both the wild-type Nisin and S29A with respect to controlling growth of *Listeria* over a 24 h period. We also report the enhanced antimicrobial effect of Nisin V and S29A in combination with EDTA, against *lux*-tagged *C. sakazakii* in reconstituted PIF over a 8 h period. This study highlights the usefulness of bioluminescence-based assays over conventional methods of monitoring bacterium growth. The study also confirms the importance of rigorously testing novel antimicrobial compounds in model food systems and under a variety of conditions to examine their effectiveness in more 'real life' situations.

Thesis summary

Bioluminescence technology has many potentially beneficially uses in the area of food microbiology. *Lux*-based assays offer a sensitive and real-time method for monitoring the presence, quantity and the metabolic state of bacteria in laboratory media as well as complex food systems.

In particular, bioluminescence assays can aid in the development of Powdered Infant Formula (PIF) and provide information on *Cronobacter* infections associated with ingestion of contaminated PIF. The risk of *Cronobacter* infections poses a serious threat to PIF manufacturers and attempts are constantly being made to not only understand the factors affecting its growth and survival in PIF, but also to develop more effective control strategies during production. With this in mind, chapter *II* of this thesis examines the potential of using a *lux*-tagged *C. sakazakii* strain to monitor growth of the bacterium in various liquids. Our study demonstrates that bioluminescent imaging facilitates culture-independent real-time tracking of *C. sakazakii*, in opaque food matrices such as PIF, which are not conducive to optical density measurements. When the *lux*-labelled strain was examined under laboratory controlled conditions, growth of the tagged strain was found to be similar to that of the non–tagged counterpart and the integrated plasmid was found to be stable for up to 24 h. This suggests that the tagged strain is a suitable substitute for the wild-type. When examining the influence of added ingredients (e.g. antimicrobial agents) on the growth of *C. sakazakii*, it was found that the impact of these ingredients could be monitored using bioluminescence as a reporter.

Following on from this work, Flaherty *et al*. (2013) *lux*-labelled a further nine *Cronobacter* spp. and determined that their growth could also be easily monitored in real-time by means of light emissions. It was also determined that while all PIF's tested were able to support the growth of each of the *Cronobacter* strains, differences

were observed between the brands. Variations in light emissions were also noted for individual *Cronobacter* strains. The monitoring of light emissions by a combination of two strains that produced higher and lower than average RLU readings was found to be a good surrogate for the entire collection of *lux*-tagged strains (Flaherty *et al.*, 2013). Assays with mixes of strains demonstrated that a cocktail of two *lux*-tagged *Cronobacter* strains provides a good representation of the average growth of all ten strains in IMF. This means that rather than performing future growth analyses in IMF with all ten strains, the use of two strains would reduce the amount of work and samples required (Flaherty *et al*., 2013).

Oshima *et al*. (2012) also examined the potential use of bioluminescent *Cronobacter* spp. in high-throughput assays aimed at monitoring the effects of various PIF compositions on growth of the bacterium. In particular, Oshima *et al*. (2012) examined the efficacy of organic acids, bacteriocins and the lactoperoxidase system (LPOS) in inhibiting growth of *Cronobacter* spp. in rehydrated PIF. Results showed that in the presence of LPOS and Lacticin 3147, growth of *Cronobacter* spp. was inhibited for up to 12 h when PIF was rehydrated at 40 or 50°C. Each of these studies demonstrates the potential industrial applications of *lux* technology to the PIF industry.

Chapter *III* of this thesis involves investigation of a bioluminescence based 'early-warning' system for starter culture disruption by antimicrobial agents such as antibiotic residues. To achieve this, we developed a new *lux*-plasmid and successfully introduced this *lux* plasmid into *Lactococcus* strains to produce stable luminescent phenotypes. The strains were constructed using either conjugation or electroporation procedures and the physiological characteristics (growth, stability and acid resistance) of the strains were not negatively affected. The newly developed

plasmid pRH001*lux* was successfully transformed into several *L. lactis and L. cremoris* strains and all transformants exhibited the luminescent phenotype. Using these strains, we established an assay capable of rapidly monitoring the antimicrobial activity of selected antibiotics in milk, thereby making these strains useful biomonitoring reporters for the dairy industry. However, these strains were not suitable for bacteriophage detection assays.

Chapter IV examined the effect of bioengineered Nisin variants (Nisin V and S29A) on a number of luciferase-tagged foodborne pathogens, in vitro and in food models at room temperature (representative of a temperature abuse situation) when compared to the wild-type Nisin. Bioluminescent reporter strains and modified agarose-based assays were used to quantitatively assess how well each of the peptides performed against a range of foodborne pathogens under stressed conditions. For the first time, we observed the enhanced bioactivity of Nisin V against Gram negative bacteria (*Escherichia coli, Salmonella* Typhimurium and *Cronobacter sakazakii*) in agarose assays and laboratory media (*E. coli).* However, further analysis of Nisin V against Gram negative bacteria in PIF, apple juice and liquid whole egg revealed no inhibitory effect.

The enhanced bioactivity of S29A and Nisin V against *Listeria monocytogenes* in commercially produced frankfurter meat maintained under sub-optimal temperature is also reported. In this instance, we demonstrated that Nisin V outperforms both the wild-type Nisin and S29A, with respect to controlling growth of *Listeria* over a 24 hour period. The generation of Nisin variants in a food-grade manner provides an important industrial application of these peptides as enhanced food preservatives particularly in the area of minimally processed foods. From this work, we determined that the use of bioluminescence-based technology provides a useful method of

tracking the effectiveness of these food grade peptides in both laboratory media and liquid based food systems. Future work may involve investigating Nisin variants in combination with other antimicrobial peptides, acids and bacteriocins to identify combinations which would inhibit Gram negative bacteria in model food systems.

In conclusion, this body of work shows that *lux*-tagged microorganisms have numerous potential food industry-related applications and could offer many advantages over the existing traditional reporter methods. Bioluminescent strains may be of benefit to the area of food safety by examining the growth and survival of pathogens in various foods and under a variety of food storage conditions. Examining the effects of different food compositions on the ability of pathogens to cause disease can result in assays which optimise processing regimes. These assays could also lead to the identification of novel preventatives and control strategies which will in turn lead to a safer food supply. Bioluminescence-based assays may also have potential applications in the dairy industry, in particular during cheese production and the production of probiotics. The development of large banks of *lux*-tagged starter culture strains could help identify even more strains suitable for starter strain rotation strategies and prevent starter culture failure or the development of slow cheese production vats in the future.

Appendix

Supplementary Information for Chapter *II*

During this chapter we examined the potential of using a *lux*-tagged *Cronobacter sakazakii* strain to monitor growth of the bacterium in various liquids. The figures presented below represent a correlation curve of the *lux*-tagged *C. sakazakii* strain, an optical density curve comparing the wild-type and *lux*-tagged strain, a growth curve of the *lux*-tagged *C. sakazakii* at varying inoculums in PIF and a growth curve of the *lux*-tagged strain grown in the presence of competing bacteria.

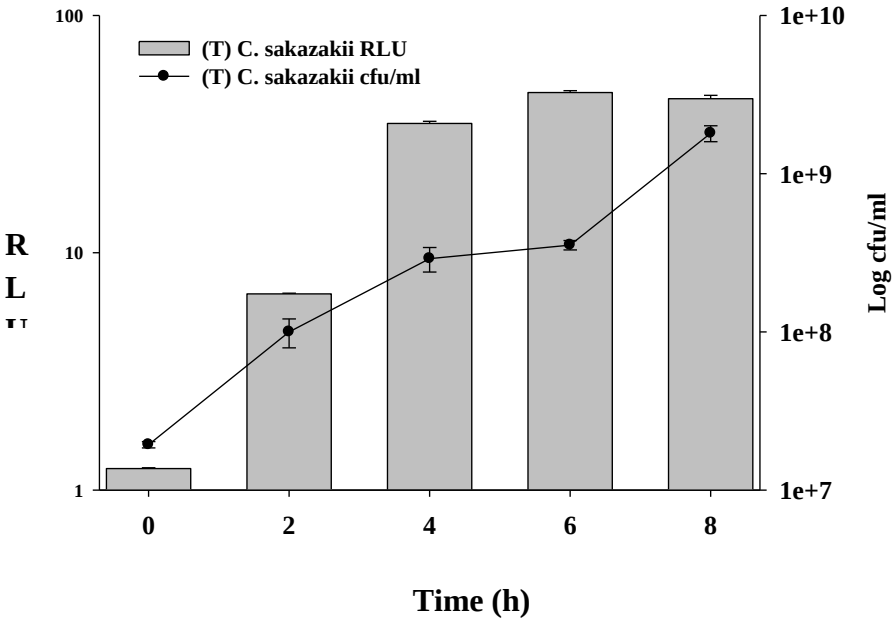


Fig. 1 Comparison of RLU (relative light units) and Log CFU per milliliter for *lux*-tagged *C. sakazakii*. (R^2 ~ 0.84)

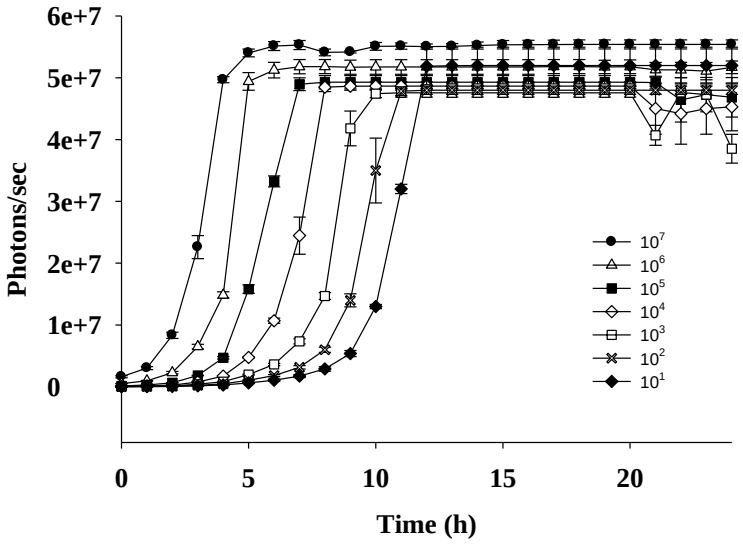


Fig. 2 Growth curve of *lux*-tagged *C. sakazakii* in PIF. Varying initial inoculums were used: 1 Log CFI/ml (♦), 2 Log CFU/ml (x), 3 Log CFU/ml (□), 4 Log CFU/ml (◊), 5 Log CFU/ml (■), 6 Log CFU/ml (Δ), and 7 Log CFU/ml.

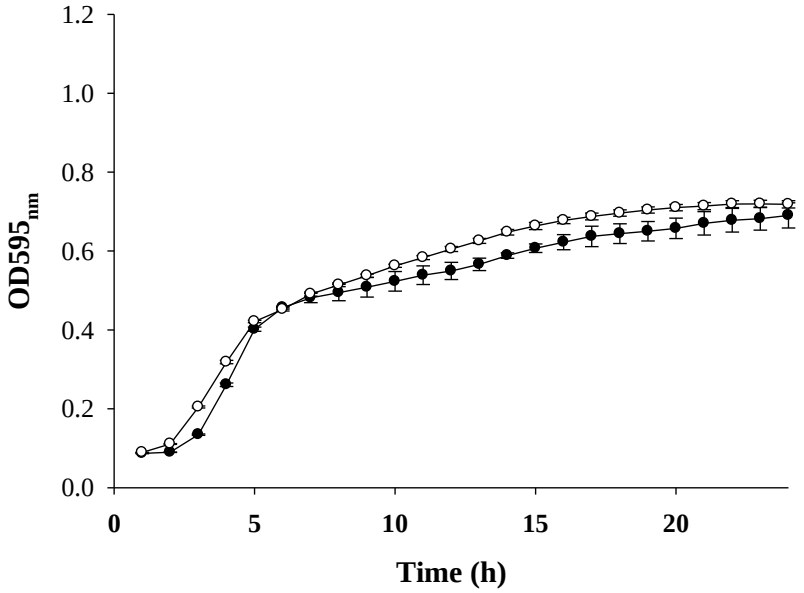


Fig. 3 Growth curve of *lux*-tagged *C. sakazakii* (●) and non-tagged *C. sakazakii* (o), in LB broth following 24 h of growth in the absence of antibiotic. Data are represented as OD595nm ± standard deviations.

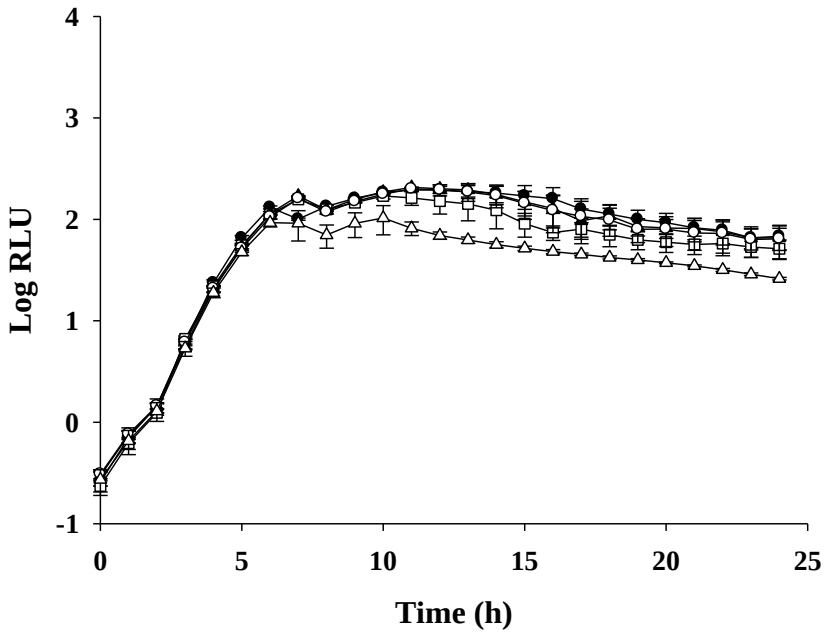


Fig. 4 Growth curve of *lux*-tagged *C. sakazakii* (●) plus *Escherichia coli* (▲), *Salmonella* Typhimurium (Δ), *Listeria monocytogenes* (o) and *E. coli*, *S.* Typhimurium and *L. monoctyogenes* all together (□) in LB broth during 24 h of growth in the absence of antibiotic. Data are represented as mean RLU's ± standard deviations.

Supplementary Information for Chapter *III*

The aim of this study was to construct a bank of bioluminescent *Lactococcus* starter culture strains using a conjugative vector which would then be able to monitor the action of antimicrobial agents in laboratory media and whole milk. The figures presented below represent a correlation curve of *lux*-tagged 5250 (measuring RLU's and CFU's), stability assays, growth curves of *lux*-tagged starter strains grown in PIF, growth curves of *lux*-tagged and non-tagged starter strains grown in GM17, growth curve of *lux*-tagged strains transformed by electroporation and conjugation and bioluminescence growth curves measuring the antimicrobial activity of antibiotics in whole milk.

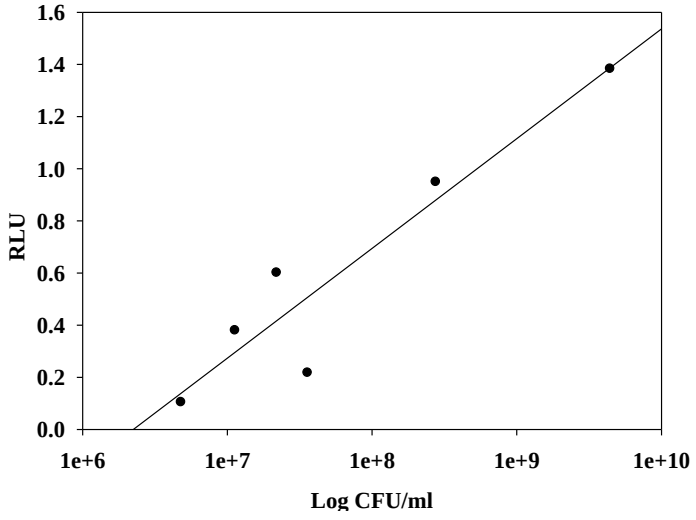


Fig. 1 Correlation of RLU (relative light units) and average Log CFU per milliliter (Log CFU/ml) for *lux*-tagged Starter culture 5250. (R^2 ~ 0.8885)

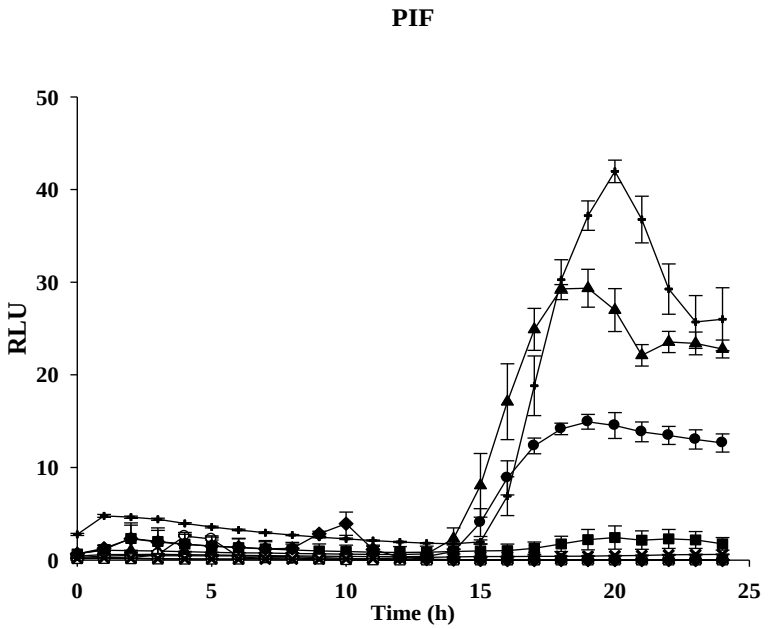


Fig. 2 Growth of each *lux*-tagged starter culture strain, 158 (●), 4272 (o), WM1 (■), 4935 (□), 5250 (+), 5101 (X), 745 (▲), 743 (Δ), 275 (♦), in reconstituted PIF broth at 30 °C. Bioluminescence was measured in RLU by a Luminoskan luminometer.

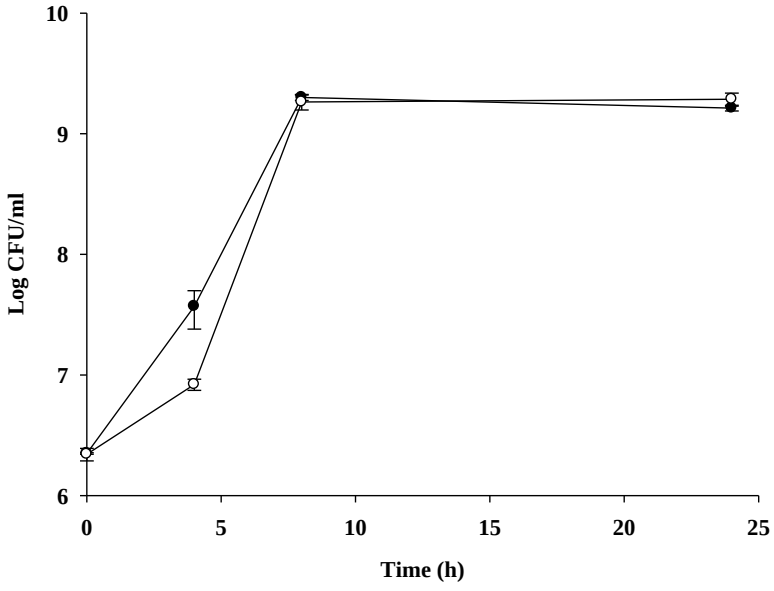


Fig. 3 Growth of *lux*-tagged (●) and non-tagged (o) starter strain *L. lactis* 745 in GM17 broth at 30 °C (electroporation). Data are represented as average Log CFU per millilitre ± standard deviations for three biological repeats.

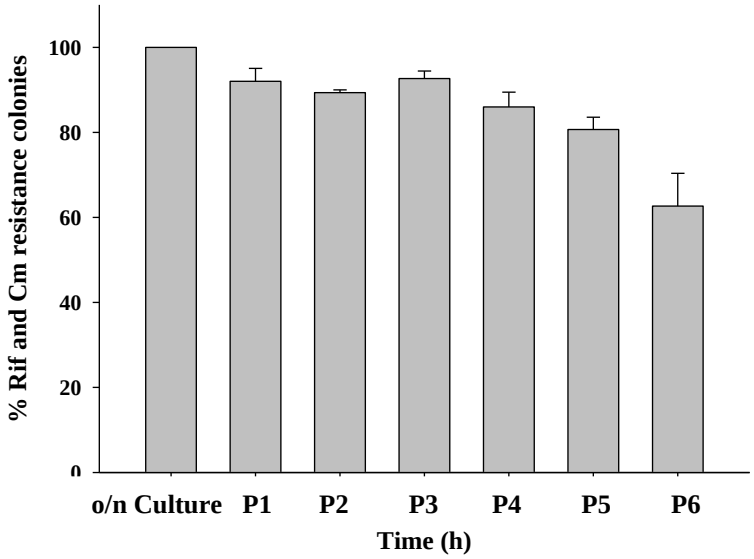


Fig. 4 Stability of pRH001*lux* in *L. lactis* 158 represented as a percentage of chloramphenicol resistant colonies following 24 h of growth in the absence of antibiotic (electroproration).

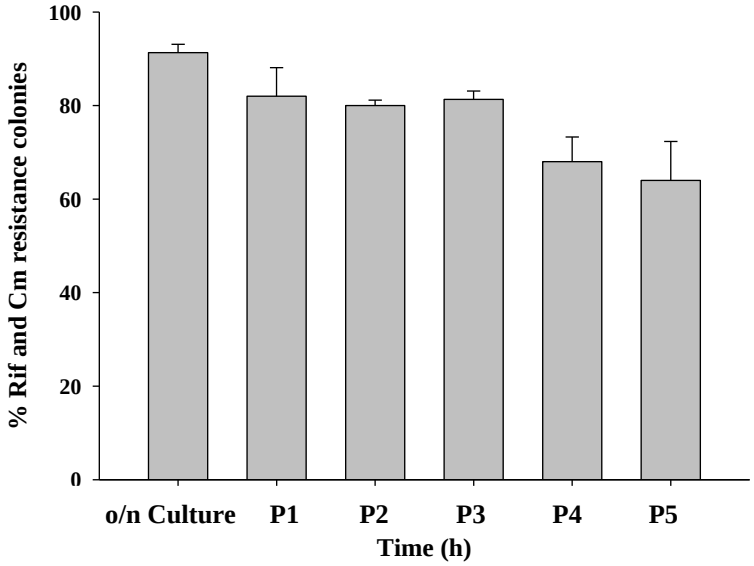
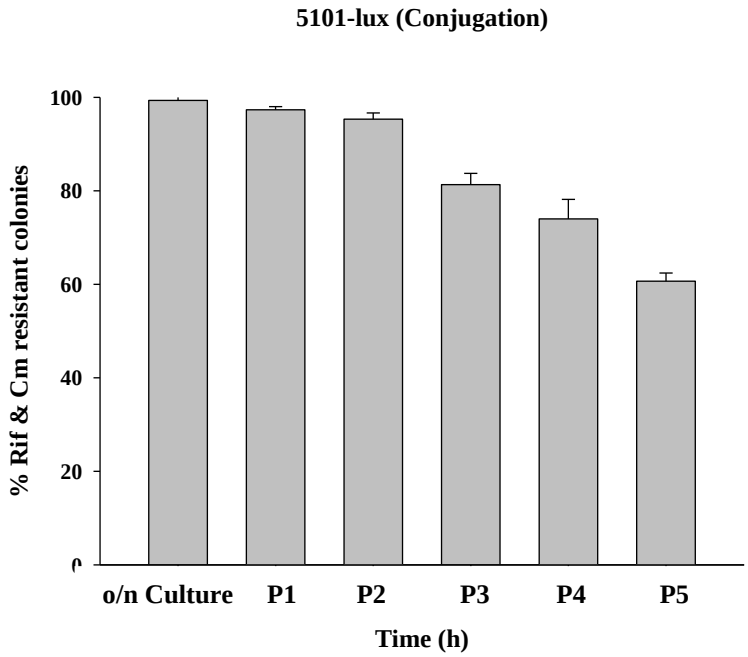


Fig. 5 Stability of pRH001*lux* in *L. lactis* 275 represented as a percentage of chloramphenicol resistant colonies following 24 h of growth in the absence of antibiotic (electroporation).



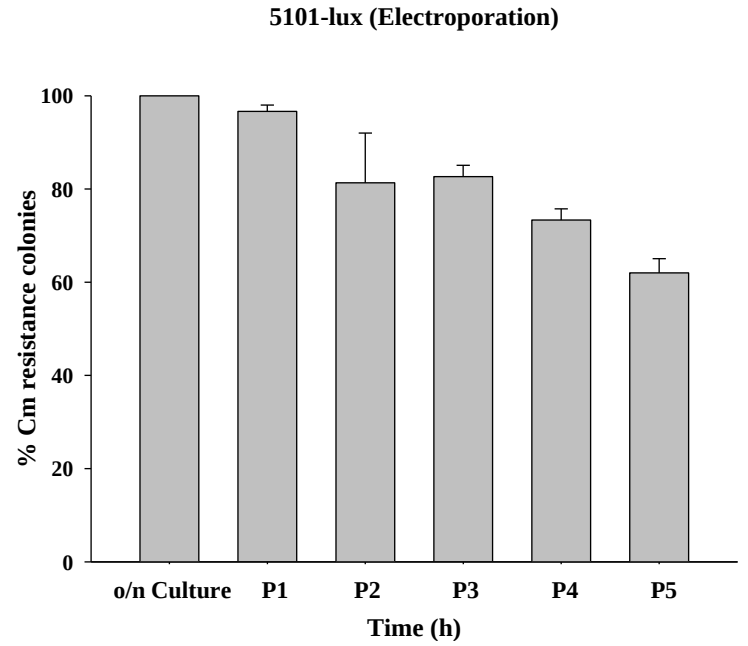
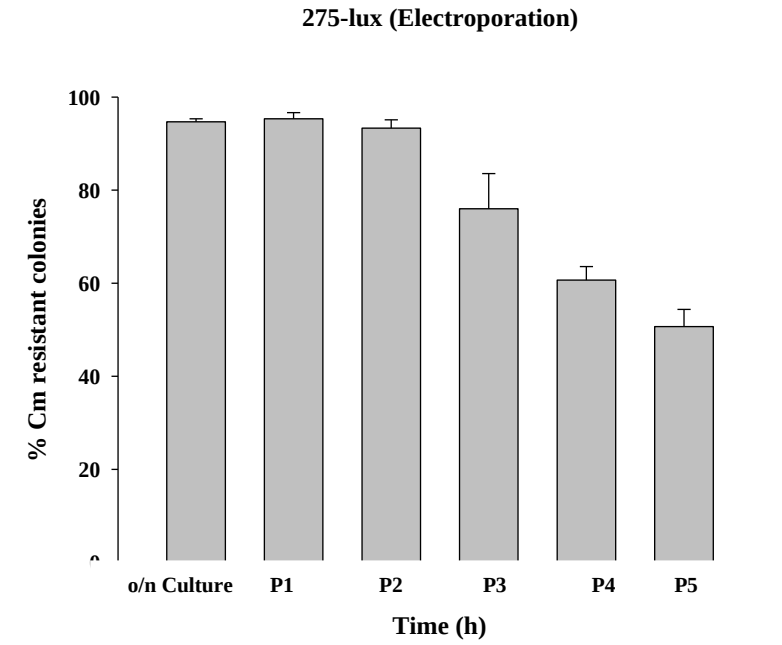


Fig. 6 & 7 Stability of pRH001*lux* in *L. lactis* 5101 transformed by conjugation (top) and by electroporation (bottom) represented as a percentage of chloramphenicol resistant colonies following 24 h of growth in the absence of antibiotic.



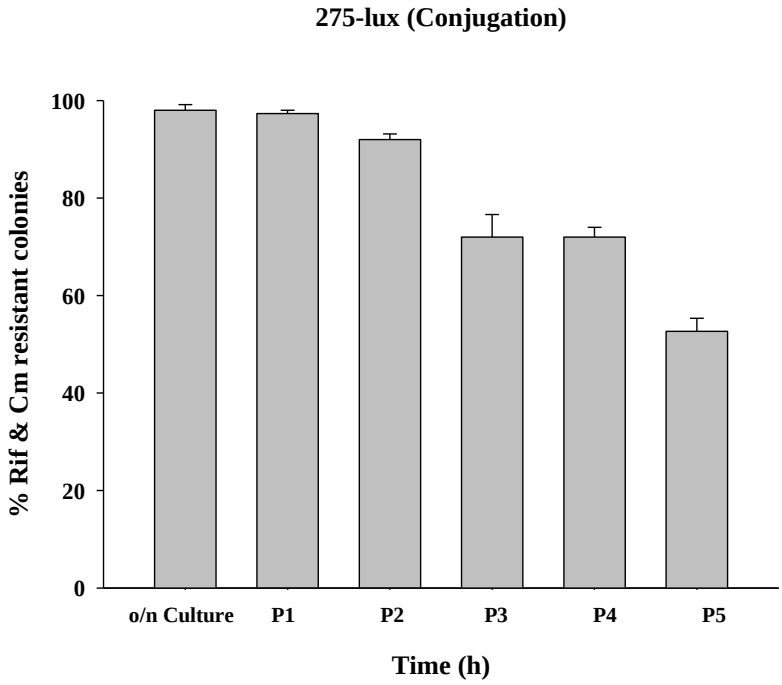
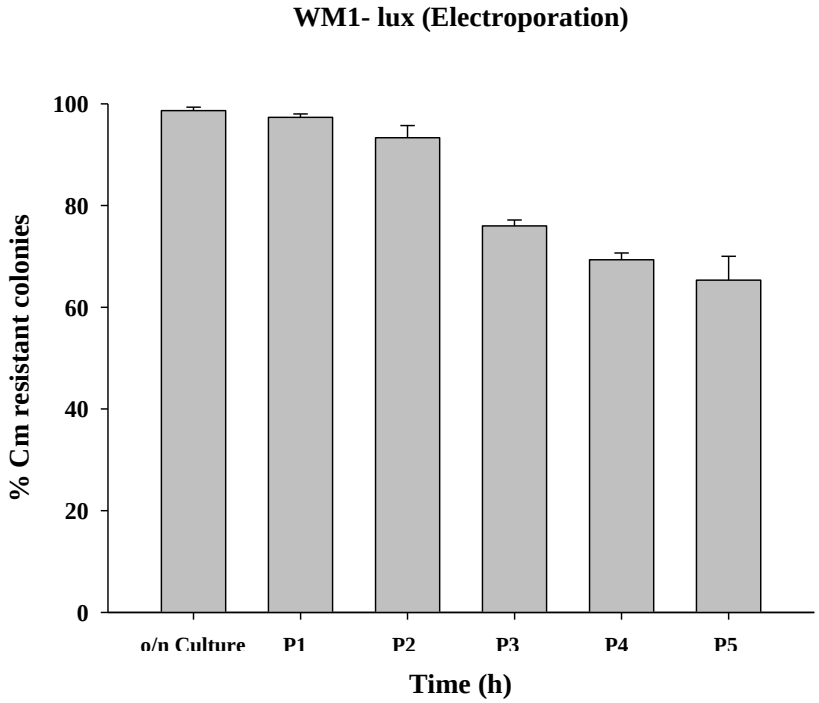


Fig. 8 & 9 Stability of pRH001*lux* in *L. lactis* 275 transformed by conjugation (top) and by electroporation (bottom) represented as a percentage of chloramphenicol resistant colonies following 24 h of growth in the absence of antibiotic.



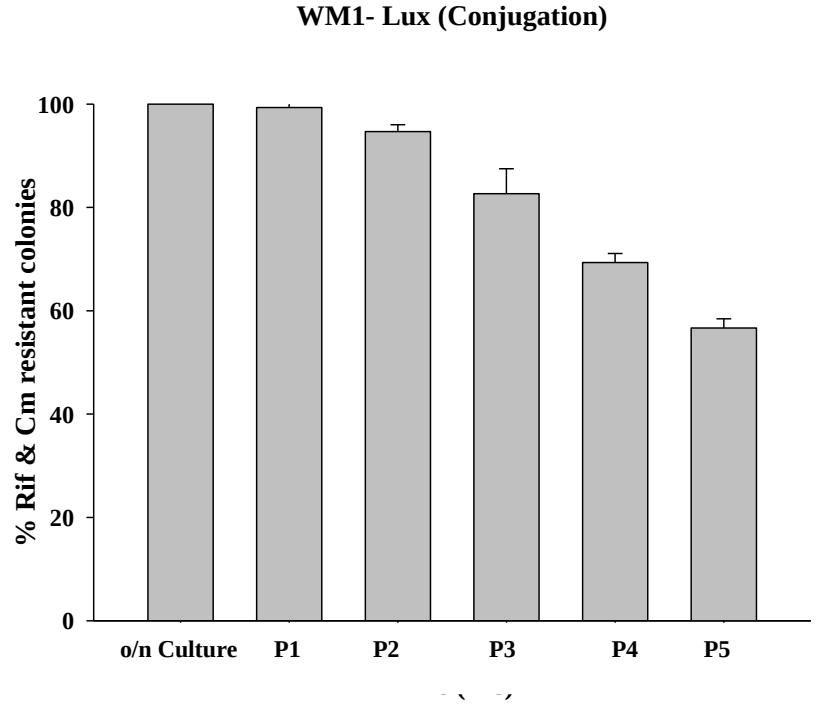


Fig. 10 & 11 Stability of pRH001*lux* in *L. lactis* WM1 transformed by electroporation (top) and *L. lactis* WM1 transformed by conjugation (bottom) represented as a percentage of chloramphenicol resistant colonies following 24 h of growth in the absence of antibiotic.

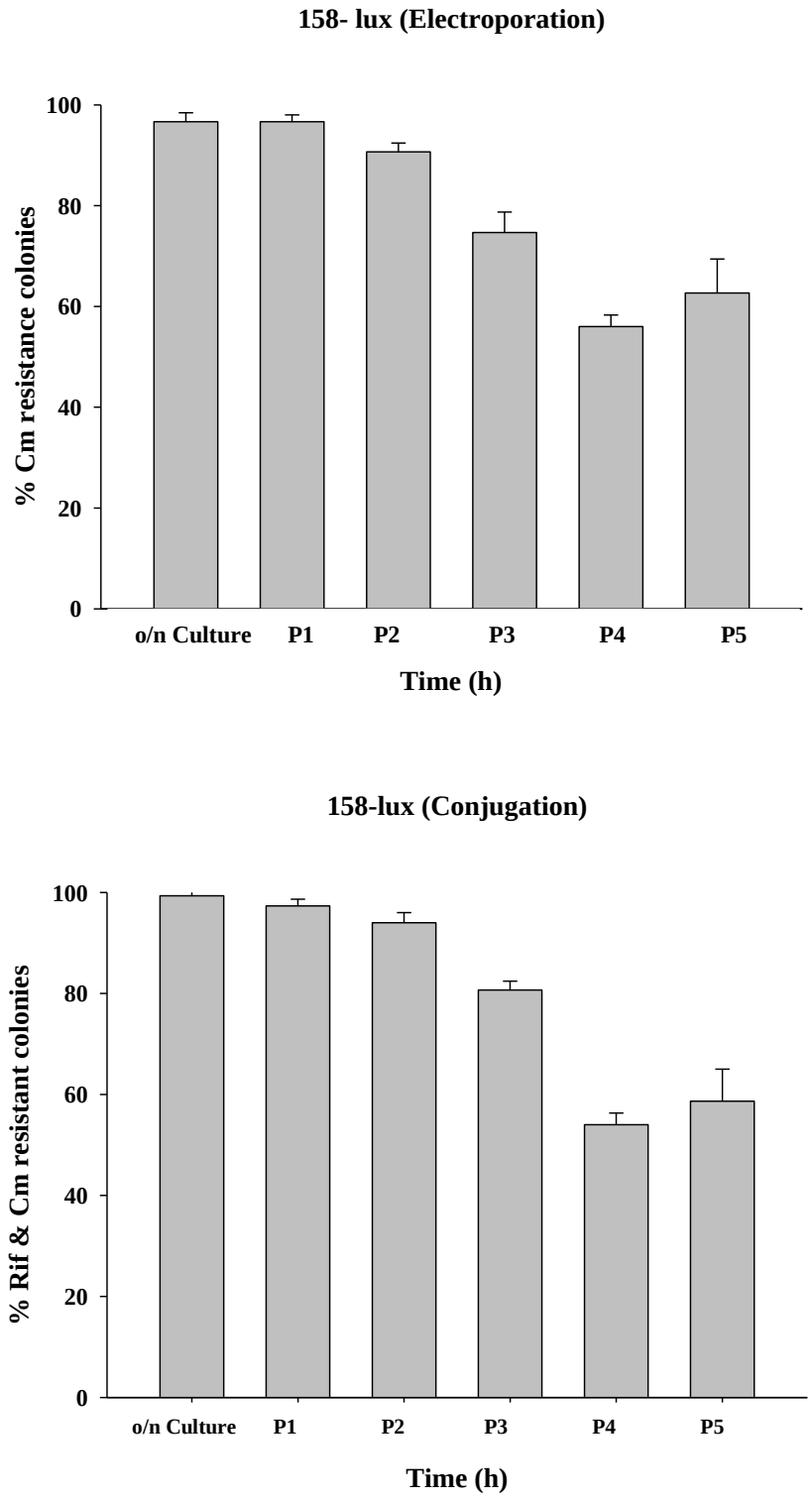


Fig. 12 & 13 Stability of pRH001*lux* in *L. lactis* 158 transformed by electroporation (top) and 158 transformed by conjugation (bottom) represented as a percentage of chloramphenicol resistant colonies following 24 h of growth in the absence of antibiotic.

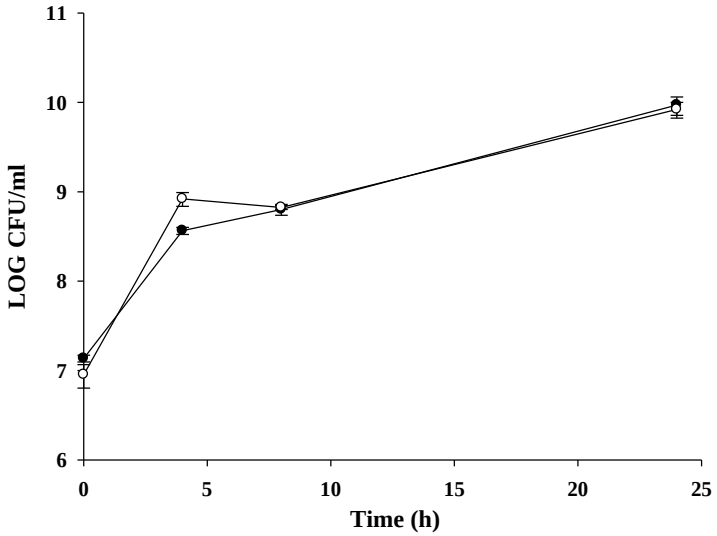


Fig. 14 Growth curve of *L. lactis* 275*lux* transformed by electroporation (●) and conjugation (o) in GM17 broth following 24 h of growth in the absence of antibiotic. Data are represented as average Log CFU per millilitre ± standard deviations ($p < 0.05$).

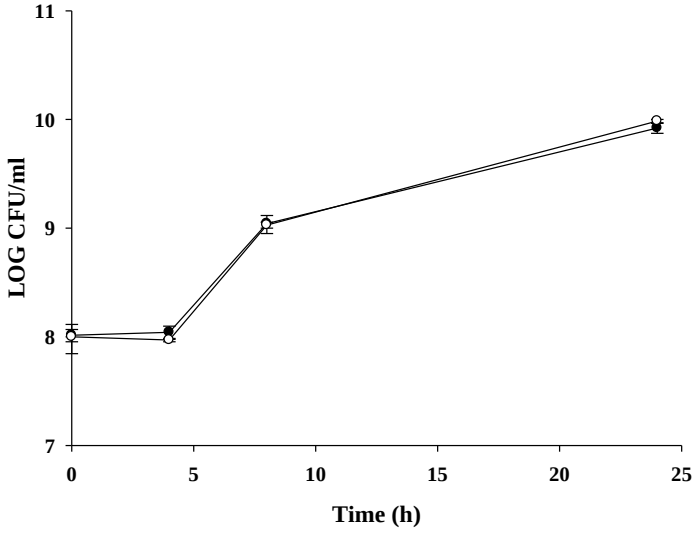


Fig.15 Growth curve of *L. lactis* 5250*lux* transformed by electroporation (●) and conjugation (o) in GM17 broth following 24 h of growth in the absence of antibiotic. Data are represented as average Log CFU per millilitre ± standard deviations

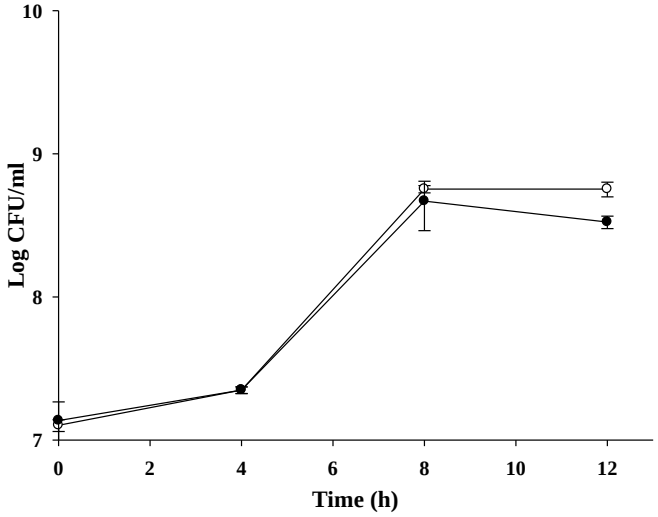


Fig. 16 Growth curve of *L. lactis* 158*lux* transformed by electroporation (●) and conjugation (o) in GM17 broth following 24 h of growth in the absence of antibiotic. Data are represented as average Log CFU per millilitre ± standard deviations

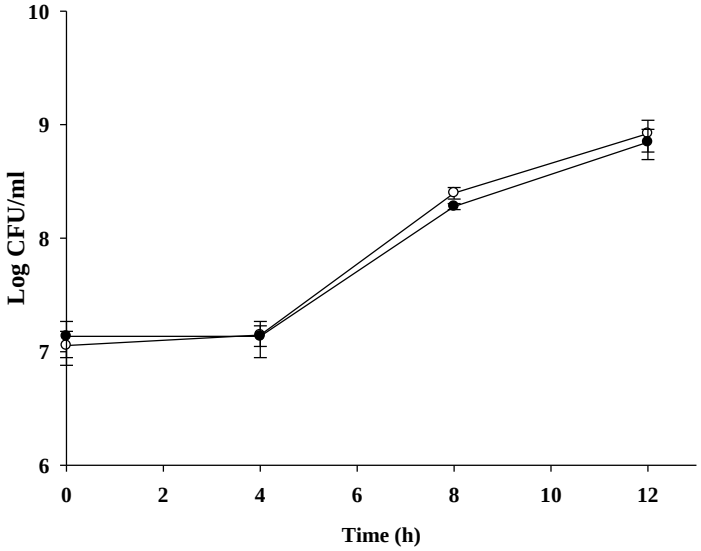


Fig. 17 Growth curve of *L. lactis* WM1*lux* transformed by electroporation (●) and conjugation (o) in GM17 broth following 24 h of growth in the absence of antibiotic. Data are represented as average Log CFU per millilitre ± standard deviations.

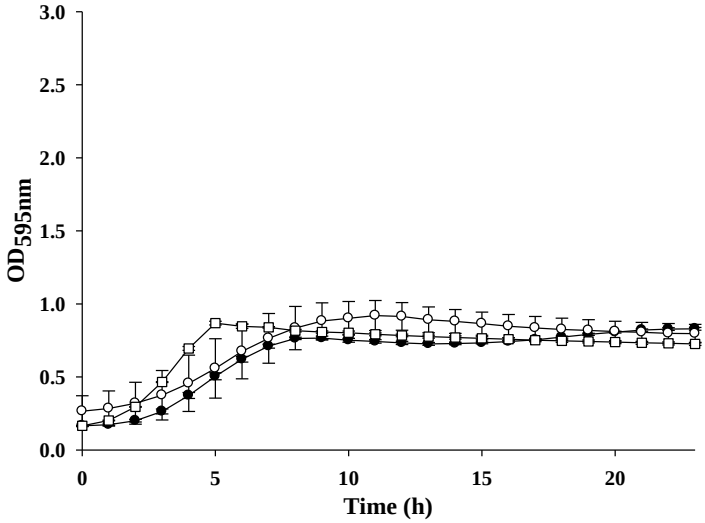


Fig. 18 Growth curve of *L. lactis* WM1*lux* alone (□), transformed by electroporation (●) and by conjugation (o) in GM17 broth following 24 h of growth in the absence of antibiotic. Data are represented as OD595nm millilitre ± standard deviations

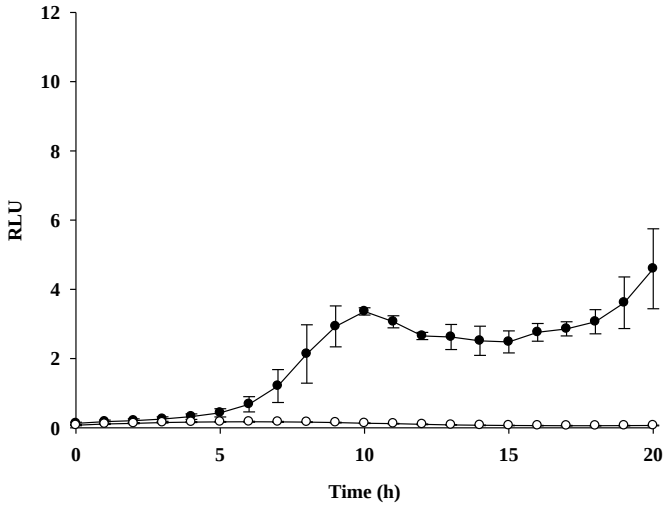


Fig. 19 Bioluminescence as a measure of the antimicrobial activity of Ampicillin in whole milk as determined by the luminoskan luminometer over 20 h. (●) represents 5250*lux*, (o) represents *L. lactis* 5250*lux* plus 5 µg/ml of Ampicillin. RLU = Relative Light Units.

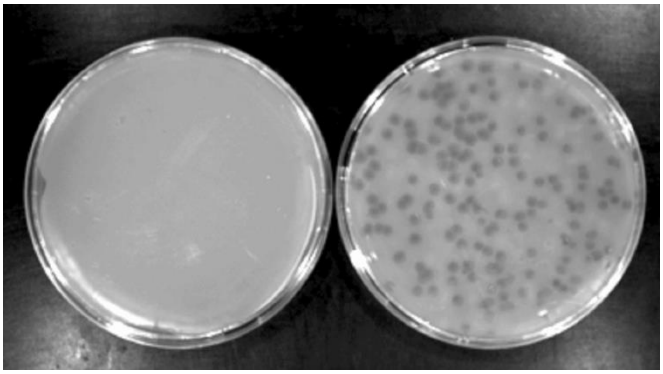


Fig. 20 Plaque assay demonstrating the effect of Tuc2009 (*left*) and c2 phage (right) on growth of the *lux*-tagged starter culture strain *L. lactis* 5250*lux* as determined using a conventional technique.

Supplementary Information for Chapter *IV*

Chapter *IV* of this thesis examines the activity of Nisin derivatives V and S29A against foodborne pathogens in laboratory media and selected foods. The figures presented below represent growth curves of *lux*-tagged *Listeria monocytogenes*, *Cronobacter sakazakii*, *Escherichia coli* and *Staphylococcus aureus* at various pH's and salt concentrations.

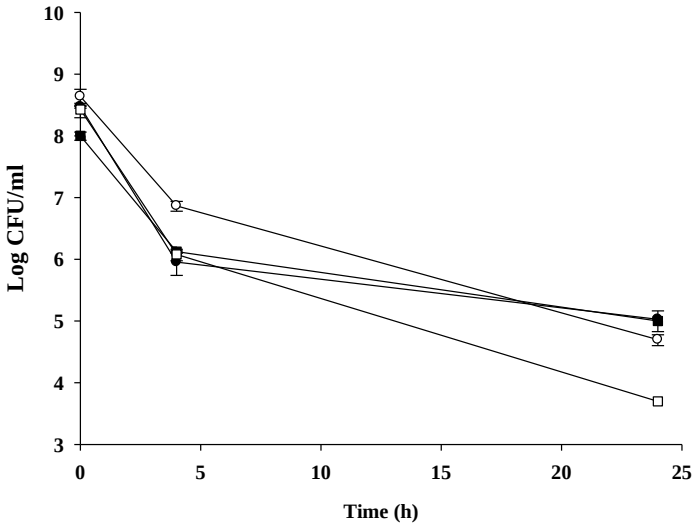


Fig. 1 Growth curve of *lux* tagged *L. monocytogenes* (●), *C. sakazakii* (o), *E. coli* (■) and *S. aureus* (□) in broth at pH 4 over a 24 h period. Data are represented as average Log CFU/ml ± standard deviations.

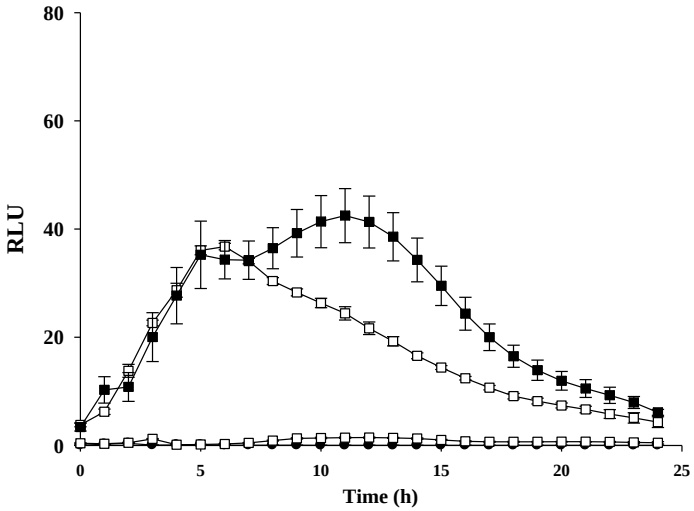


Fig. 2 Growth curves of *lux* tagged *L. monocytogenes* (●), *C. sakazakii* (o), *E. coli* (■) and *S. aureus* (□) in broth at pH 7 over 24 h of growth. Data are represented as average RLU ± standard deviations.

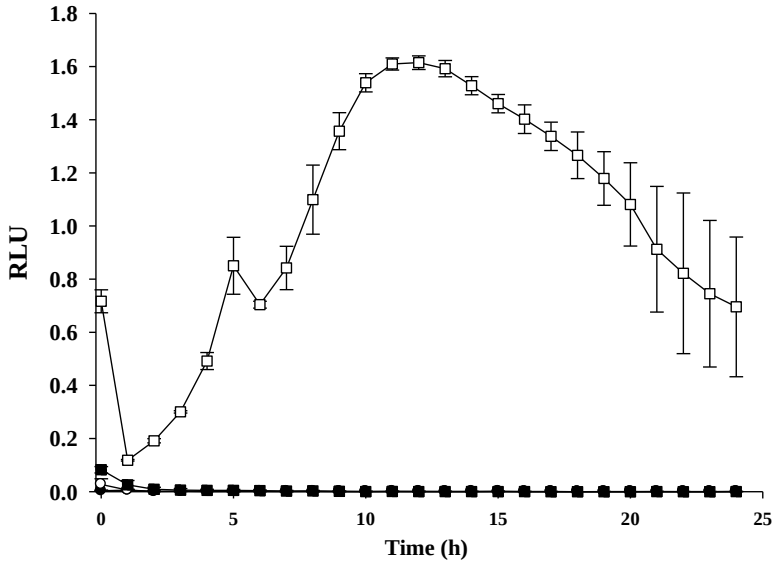


Fig. 3 Growth curve of *lux* tagged *L. monocytogenes* (●), *C. sakazakii* (o), *E. coli* (■) and *S. aureus* (□) in broth at pH 10 over 24 h of growth. Data are represented as average RLU ± standard deviations.

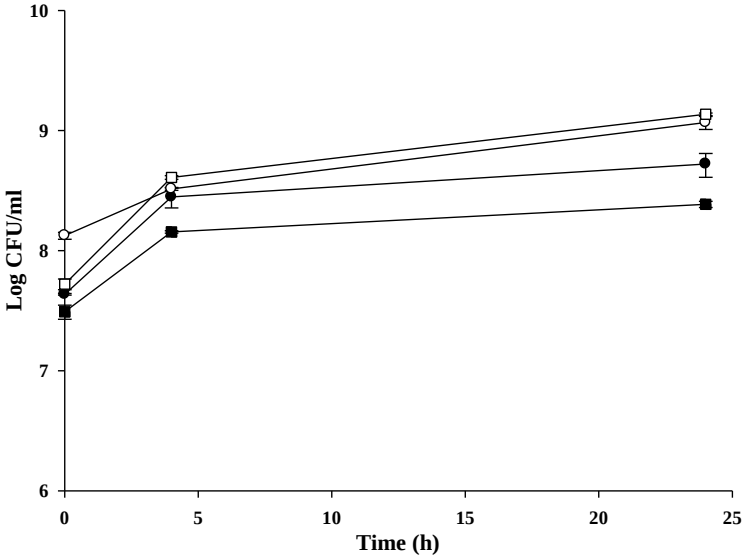


Fig. 4 Growth curve of *lux* tagged *L. monocytogenes* (●), *C. sakazakii* (o), *E. coli* (■) and *S. aureus* (□) in broth at pH 7 over 24 h of growth. Data are represented as average Log CFU/ml ± standard deviations.

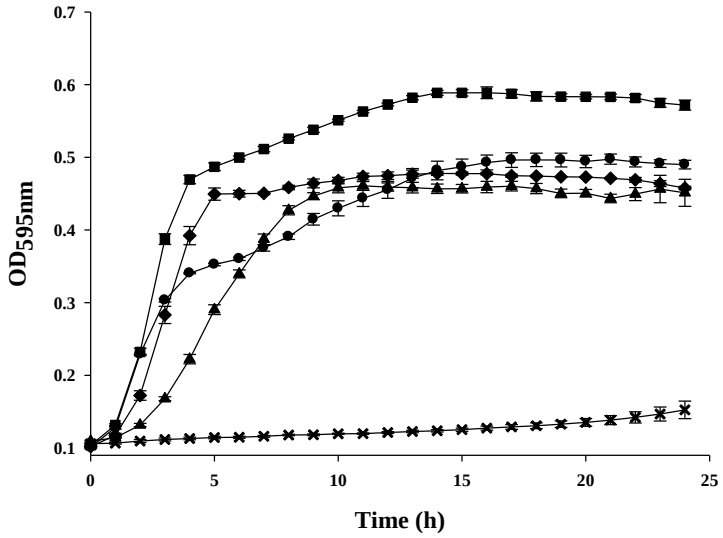


Fig. 5 Growth curve of *lux*-tagged *C. sakazakii* in LB broth (●), plus 2% salt (■), 4% (♦), 6% (▲) and 10% (x) over 24 h of growth. Data are represented as OD595nm millilitre ± standard deviations.

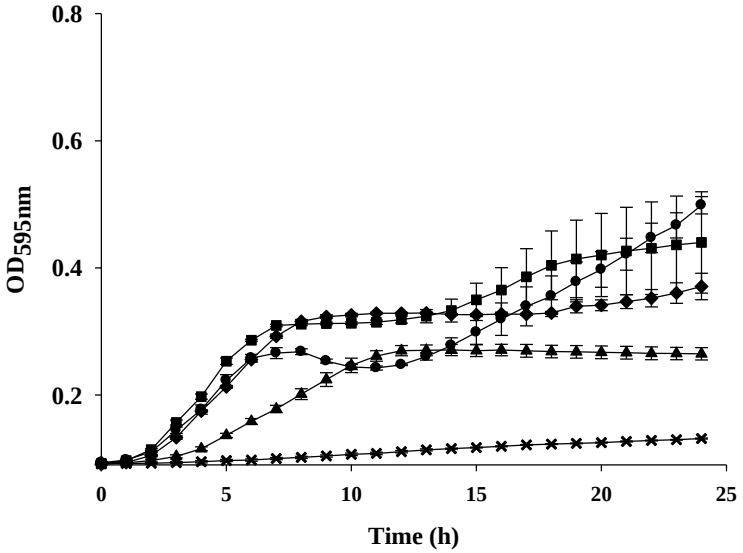


Fig. 6 Growth curve of *lux*-tagged *L. monocytogenes* in LB broth (●), plus 2% salt (■), 4% (♦), 6% (▲) and 10% (x) over 24 h of growth. Data are represented as OD595nm millilitre ± standard deviations.

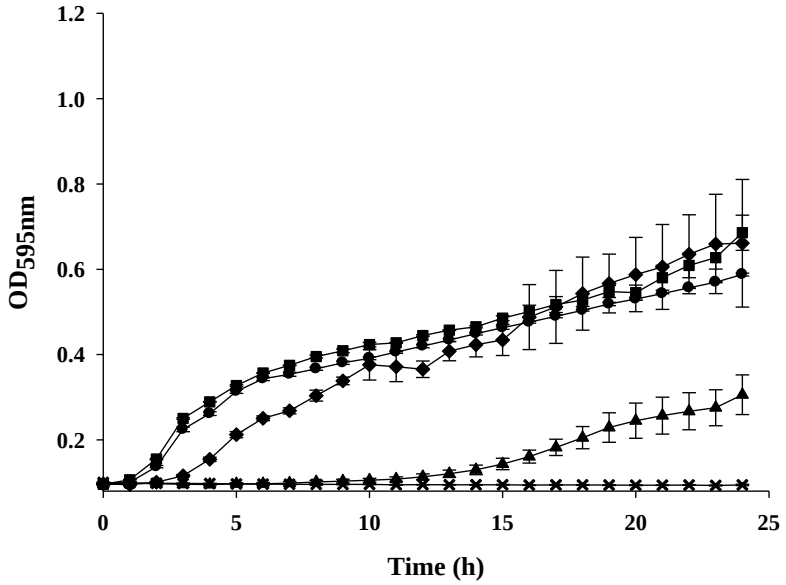


Fig. 7 Growth curve of *lux*-tagged *E. coli* in LB broth (●), plus 2% salt (■), 4% (♦), 6% (▲) and 10% (x) over 24 h of growth. Data are represented as OD595nm millilitre ± standard deviations.

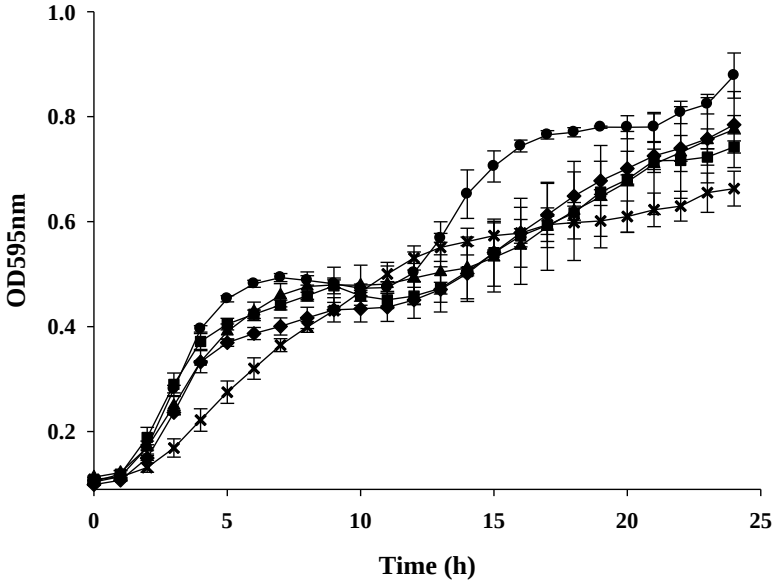


Fig. 8 Growth curve of *lux*-tagged *S. aureus* in LB Broth (●), plus 2% salt (■), 4% (♦), 6% (▲) and 10% (x) over 24 h of growth. Data are represented as OD595nm millilitre ± standard deviations.

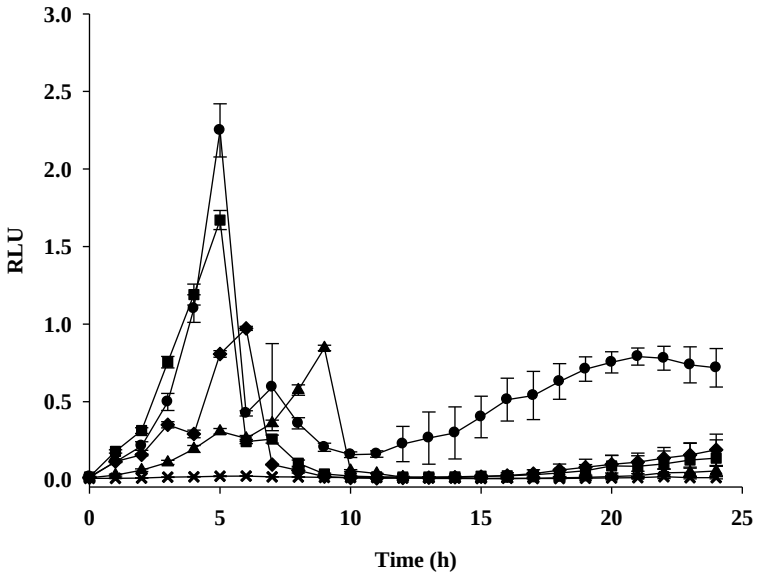


Fig. 9 Growth curve of *lux*-tagged *L. monocytogenes* in LB broth (●), plus 2% salt (■), 4% (♦), 6% (▲) and 10% (x) over 24 h of growth. Data are represented as RLU ± standard deviations.

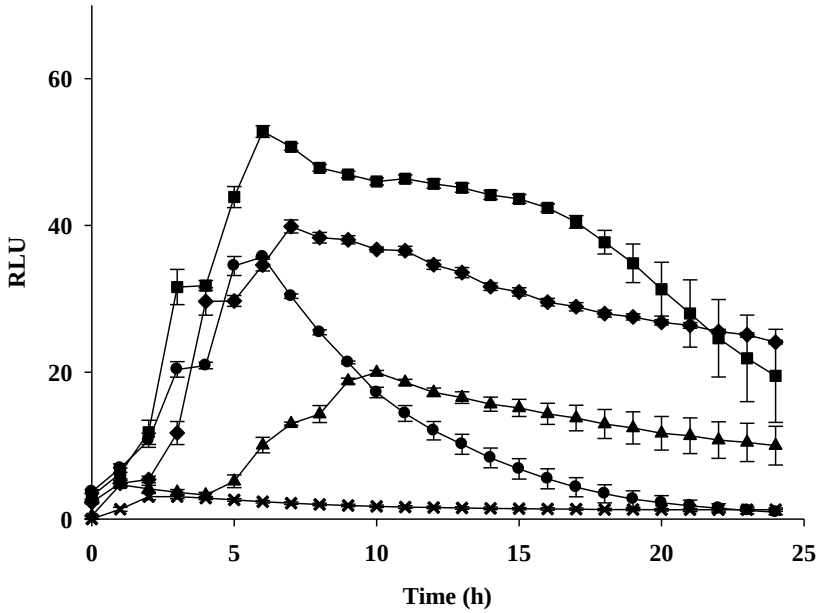


Fig. 10 Growth curve of *lux*-tagged *C. sakazakii* in LB broth (●), plus 2% salt (■), 4% (♦), 6% (▲) and 10% (x) over 24 h of growth. Data are represented as RLU ± standard deviations.

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