Microbially derived bioactive peptides to improve human health

A thesis presented to The National University of Ireland, Cork for the degree of Doctor of Philosophy

By

Robert Martin Kent, B.Sc.

Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork

School of Microbiology, University College Cork, Cork, Ireland

October 2014

Research Supervisors:

Prof. Paul Ross

Prof. Catherine Stanton

Prof. Gerald Fitzgerald

“Hey, what day is this?”

“It’s Wednesday, its Tuesday I think”

“I think the tide’s with us”

“Keep kickin”

“I used to hate the water”

“I can’t imagine why”

TABLE OF CONTENTS

DECLARATION

I hereby certify that this material, which I submit for assessment on the programme of study leading to the award of Ph.D., is entirely my own work and was not submitted for another degree, either at University College Cork or elsewhere.

Signed: _____

Student Number: 104061403

Date: _____

Publications

Kent, R. M., Guinane, C. M., O'Connor, P. M., Fitzgerald, G. F., Hill, C., Stanton, C. and Ross, R. P.. Production of the antimicrobial peptides Caseicin A and B by *Bacillus* isolates growing on sodium caseinate. *Letters Applied Microbiology*, 55, 2, 2012 141-148.

Guinane, C. M., Kent, R. M., Norberg, S., Fitzgerald, G. F., Hill, C., Stanton, C. and Ross, R. P.. Host Specific Diversity in *Lactobacillus johnsonii* as Evidenced by a Major Chromosomal Inversion and Phage Resistance Mechanisms. *PLOS One,* 6(4): e18740 2011.

Kent, R.M., Fitzgerald, G.M., Stanton, C. and Ross R.P.. Novel approaches to improve the intrinsic microbiological safety of powdered infant formula. *Nutrients* 2015, 7(2), 1217-1244; doi:10.3390/nu7021217

Kent, R. M., Guinane, C. M., Norberg S., O'Connor, P. M., Fitzgerald, G. F., Hill, C., Stanton, C. and Ross, R. P.. Generation of caseicin peptides from casein by hydrolysis with thermolysin enzymes. *International Dairy Journal* 2015, doi;10.1016/j.dairyj.2015.04.001

Kent, R.M., Stack H.M., O'Connor P.M., Hill, C. Fitzgerald, G.M., Seeley, R.J., Stanton, C. and Ross R.P.. Recombinant *Lactobacillus paracasei* strain producing active gut hormone Glucagon-Like peptide-1 analogue. Manuscript in preparation.

Abbreviations

ACE = Angiotensin-1-converting enzyme
ACT = Artemis comparison tool
AMP = Antimicrobial peptide
CAC = Codex Alimentarius Commission
CDC = Centres for disease control and prevention
DPP-IV = Dipeptidyl peptidase 4
CFU = Colony forming unit
CMP = Caseinmacropeptide
Da = Dalton
EFSA = European food safety authority
EU = European Union
FAO = Food and agricultural organisation of the United Nations
FDA = Food and drug administration
FOS = Fructooligosaccharide
FSAI = Food safety authority Ireland
FUF = Follow-up Formula
GOS = Galactooligosaccharide
GIT = Gastro-intestinal tract
GLP-1 = Glucagon-like peptide 1
GMP = Good manufacturing processes
GP-HPLC = Gel permeation-HPLC
GRAS = Generally regarded as safe
GUL = Guidance upper limit
HACCP = Hazard analysis critical control point
HGT = Horizontal gene transfer
HPLC = High-performance liquid chromatography
ICMFS = International commission on microbiological specification of foods
IMF = Infant milk formula
kDa = Kilodalton
LAB = Lactic acid bacteria
LF = Lactoferrin
LFcin = Lactoferricin
LB = Luria Bertani
LPS = Lipopolysaccharide
MALDI-TOF = Matrix assisted laser desorption/ionization-Time of flight
MGE = Mobile genetic element
MIC = Minimum inhibitory concentration
MLST = Multi-locus sequence typing
MPN = Most probable number
MRS = deMan, Rogosa and Sharpe
MW-SPPS = Microwave-assisted solid-phase peptide synthesis
NEC = Necrotizing enterocolitis
Npr = Neutral protease
Omp = Outer membrane protein
PCR = Polymerase chain reaction
PIF = Powdered infant formula
PFGE = Pulse-field gel electrophoresis
RSM = Reconstituted skim milk

RP-HPLC = Reverse phase-HPLC
RT-PCR = Real-time PCR
T6SS = Type-6 secretion system
WHO = World Health Organisation

Abstract

In recent times, increasing attention has been paid to bioactive peptides which positively modulate health. This thesis describes a study of various methods to produce such peptides. Initially, the generation of anti-*Cronobacter* spp. peptides by fermentation of milk protein is described. A porcine gut isolate, *Lactobacillus johnsonii* DPC6026 was initially used in an attempt to generate two previously described antimicrobial peptides. Phenotypic analysis indicated unsatisfactory casein hydrolysis. The genome of the strain was sequenced and annotated. Results showed a number of unique features present in the microorganism's genome, most notably a large symmetrical inversion of approximately 750kb in comparison with the human isolate *L. johnsonii* NCC 533. Genes indicating the presence of extracellular proteinases were not found. The data suggest significant genetic diversity and intra-species genomic rearrangements within the *L. johnsonii* species and indicates a host-specific divergence of *L. johnsonii* strains with respect to genome inversion and phage exposure.

Bacillus spp. are widely used in industrial fermentations and the generation of commercial protease enzymes. Some strains are also recognised as milk powder contaminants. For these reasons a bank of *Bacillus* strains was generated and investigated for caseinolytic ability and subsequent caseicin production. A number of strains were found to produce a high percentage of small peptides following fermentation. Two previously characterised antimicrobial peptides were generated following fermentation by specific *B. cereus* and *B. thuringiensis* strains. Peptides generated in this manner retained antimicrobial activity against a *Cronobacter* milk-powder isolate. In conclusion, a novel method to produce two antimicrobial peptides is described

which concurrently highlights the potential of *Bacillus* spp. to produce milk-derived bioactive peptides.

Due to the regulatory issues concerning the use of *Bacillus*-derived ingredients in infant foods, a sterile enzymatic method to generate antimicrobial peptides from casein is subsequently described. Bioinformatic tools were first used to predict candidate industrial enzymes capable of liberating antimicrobials from the milk protein casein. Following this, various industrial enzymes were experimentally tested against milk proteins. Resultant hydrolysates were examined and found to produce peptides with activity against *Cronobacter* spp.. This study establishes a potential industrial-grade method to generate antimicrobial peptides.

Research has indicated that administration of the incretin peptide GLP-1 leads to improvement of glycaemic control in type 2 diabetes patients. The generation of a recombinant lactic acid bacteria capable of producing an analogue of this peptide is described. *In-vivo* analysis confirmed predicted insulinotropic activity. The results indicate a therapy with potential to alleviate hyperglycaemia in type 2 diabetics and also illustrate a method using bacteriocin producing cellular machinery to generate fusion proteins for the improvement of human health.

In summary, this thesis describes the generation of bioactive peptides by bacterial fermentation, tailored enzymatic hydrolysis and recombinant bacterial methods. The resultant peptides possessed significant activity. Furthermore, the techniques described contribute to bioactive peptide research with regards novel methods of production and industrial scale-up.

Chapter 1

Novel approaches to improve the intrinsic microbiological safety of powdered infant milk formula.

Robert M. Kent, Gerald F. Fitzgerald, Catherine Stanton and R. Paul Ross

Nutrients 2015, 7(2), 1217-1244; doi:10.3390/nu7021217

1.1. Abstract

Human milk is recognised as the best form of nutrition for infants. However in instances where breast-feeding is not possible, unsuitable or inadequate, infant milk formulae are used as breast milk substitutes. These formulae are designed to provide infants with the optimum nutrition for normal growth and development and are available in either powder or liquid form. Powdered infant formula is widely used for convenience and economic reasons. Current manufacturing processes are not capable of producing a sterile powdered infant formula. Due to their immature immune systems and permeable gastro-intestinal tracts infants are more susceptible to infection via foodborne pathogenic bacteria than other age-groups. Consumption of powdered infant formula contaminated by pathogenic microbes can be a cause of serious illness. In this chapter, we first discuss the current manufacturing practices present in the infant formula industry, followed by the pathogens of greatest concern, *Cronobacter* and *Salmonella*. Finally, methods of improving the intrinsic safety of powdered infant formula via the addition of antimicrobials such as bioactive peptides, organic acids, probiotics and prebiotics are discussed.

1.2. Introduction

Breast milk is recognised as the gold standard (Vandenplas, 2002) in infant nutrition and is consistently recommended by national and international health organizations for the first year of life (Kent & Doherty, 2014; Riordan & Wambach, 2010). Breastfeeding provides all the nutrition required for normal infant growth and development, is optimum for infant digestive conditions and also increases the affective relationship between infant and mother. Breast milk contains a number of immuno-regulatory components that lowers an infant's risk of gastrointestinal and respiratory infections. Although the benefits of breastfeeding in providing the optimal balance of nutrients required for infant growth and development are widely accepted and human milk is the first choice for infants, infant milk formulae (IMF) play a vital role when breastfeeding is not sufficient, possible or desirable. Infant formula is intended as an effective substitute and it is formulated to mimic the nutritional constituents of breast milk. Newborn infants are particularly vulnerable to infection due to immature internal organs and the lack of a developed immune system (Lönnerdal, 2012). Also, commensal microorganism which can act as a barrier to infection in the digestive tract may yet be established at this early stage of life (Chap *et al.*, 2009). IMF requires very high levels of microbiological quality and must conform to national and international microbiological criteria (Codex Alimentarius Commsion CAC, 2008) due to the susceptibility of newborns to bacterial infection (Forsythe, 2005). Unfortunately, current manufacturing technologies mean the generation of a completely sterile product is impossible meaning intrinsic contamination of

IMF may be a cause of possibly serious illness in infants (Codex Alimentarius Commsion CAC, 2008).

Although production of a completely sterile powdered product is not feasible, every effort is taken to limit the possibility of contamination. High standards of hygiene are maintained throughout the production process and the microbiological quality of each batch is closely monitored. Unlike the powdered form, the liquid product, which can be in a concentrate or a "ready to feed" form, is sterile and so is generally recommended to infants who are at a high risk of infection (WHO, 2007). Powdered infant formula (PIF) is not a sterile product even if it has been manufactured to hygienic standards (WHO, 2007) and is not marketed as one (Jason, 2012). It is however a cheaper option than the liquid forms of feed and has a longer shelf life. From a manufacturers' perspective, it offers a greater scope for modifying the formula density should it be required during production (Agostoni *et al.*, 2004). Processing methods and concerns for the microbiological quality used for standard PIF also apply to formulae designed for ill children and also follow-up formula (FUF).

In this article, we review PIF and the problems associated with bacterial contaminations, particularly contamination by *Cronobacter* and *Salmonella*. There is an increasing interest in the development of natural effective antimicrobial agents for the preservation of foods due to safety concerns with synthetic compounds (Nair, Kumar, Joy, & Venkitanarayanan, 2004). Specifically, there is an increasing interest in the use of natural antimicrobials for use in PIF with direct action against *Cronobacter* (Harouna *et al.*, 2014). For this reason, we will also discuss the potential of natural additives capable of preventing the proliferation of disease causing pathogens in formula, and

how these strategies can be implemented with regard to the strict infant formula legislation in place.

1.3. Production of powdered infant formula

Bovine milk is the basis for most infant formulae but contains higher levels of fat, minerals and protein compared to human milk so it is skimmed and diluted, to more closely resemble human milk composition (Codex Alimentarius Commission CAC, 2007; Koletzko *et al.*, 2005). Soy based formulae are also available and are widely used but it has been recommended that their use be limited due to potentially harmful effects to the infant due to the presence of phytoestrogens in the formulae (Badger *et al.*, 2009; Bhatia & Greer, 2008) .

The composition of powdered infant formula (PIF) is strictly regulated and each manufacturer must follow established guidelines outlined to them by governing bodies. These values are set by the Codex Alimentarius Commission (CAC 1979), company-company agreements as well as "in-house" levels regarded as workable based upon prior experience (Forsythe, 2005). All the major components added to formula (protein, lipids, carbohydrates) have minimum and maximum values (Table 1.1.). Ionizing radiation is currently not permitted for any reason due to the organoleptic deterioration it would cause the product (Codex Alimentarius Commission CAC, 2007; FAO, 2007). Both whey and casein are acceptable sources of protein, and individual amino acids may be added to improve the formula's nutritional value. Only L forms of these amino acids are allowed. D Forms are not permitted for fear they may cause D-lactic acidosis (Connolly & Lönnerdal, 2004). Commercially

hydrogenated fats and oils are not allowed. Fructose is avoided due to hereditary fructose intolerance present and undiagnosed in some newborns, which may have fatal consequences. Many vitamins and minerals have a guidance upper level (GUL) amount for inclusion in PIF. These levels are designed for nutrients without enough information for a full rigorous risk assessment. These components must have an established history of safe use, and aid in the infant's nutrition (Codex Alimentarius Commission CAC, 2007).

PIF can be produced in one of three different ways, a Dry-mix, Wet-mix or combined process. In the Dry-mix process, the various formula components are received heat-treated separately from suppliers and blended together to ensure a uniform blend of nutrients throughout the powder. This process is less capital intensive than the Wet-mix process and importantly, allows the manufacturing line to be maintained in a dry state for prolonged periods of time, denying any pathogens present access to water and thereby minimizing the chance of bacterial growth. The microbiological quality of the finished product is determined by the individual component ingredients received by the manufacturer as in many cases the powder will not receive further in-house heat-treatments. For this reason PIF manufacturers who use the Dry-mix method try to develop and maintain close relationships with their suppliers. After large-scale blending of the individual components the final product undergoes a final check to ensure conformance to appropriate specifications. A Wet-Mix process involves blending, homogenising and pasteurizing all the components together. Due to the inclusion of a heat-treatment step the microbiological quality of the finished product is far less reliant on the base ingredients. This process also ensures the uniform

distribution of nutrients throughout each batch. The nature of this process requires that manufacturing equipment be routinely wet-cleaned. The presence of liquid in the manufacturing environment due to this cleaning can allow the growth and establishment of microorganisms. Manufacturers separate their plants into strict wet and dry areas and restrict movement of equipment and personnel in these areas in an effort to minimize cross-contamination. Following pasteurization heat-sensitive micronutrients are added. The microbiological quality of these micronutrients is important as further heat-treatments may not be applied to the product. The liquid mix is then spray-dried to form a powder. The Combined process involves drying a wet mixture of the major formula ingredients (fat, protein and carbohydrates), referred to as infant formula base powder. This base powder is then combined with pre-dried minor ingredients (vitamins and minerals) and mixed to produce the finished product. Regardless of the process used the resultant powder is packed in containers, flushed with inert gas, sealed with an airtight cap, coded and labelled. Each batch of formula is usually held until samples from it have undergone analysis for uniformity, nutritional content and microbiological safety (FAO, 2007).

Various food safety authorities regulate and monitor the components and manufacture of PIF and scientific committees advise on whether ingredients should be added/removed or whether the amounts of individual components should be altered with regard to new research. The Codex Alimentarius Commission (CAC) comprises 186 members (185 states and 1 organisation [European Union, EU]). As well as these countries over 200 non-governmental organisations and inter-governmental organisations are observers

of the CAC. The CAC develops and adopts food standards that serve as reference for the international food trade. In many cases Codex standards also serve as a basis for national legislation (Codex Alimetarius Commission CAC, 2013). For EU member states the EU directive 2006/141/EC (this directive was recently slightly amended with regard to protein content [Commission directive 2013/46/EU]) sets values and labelling protocols for the composition of infant formulae and follow-on formulae.

Of note is the fact even if perfectly sterile processes conditions were achieved in a factory setting, exposure is more likely to come from the formula preparer/caregiver (FAO, 2007). Extrinsic contamination can occur due to use of contaminated utensils and equipment in the preparation environment or from the preparer and can influence the microbiological safety of both powder and liquid infant formulae. In 2007 the World Health Organization (WHO) in collaboration with the Food Safety Authority of Ireland (FSAI) and the Food and Agricultural Organization of the United Nations (FAO) issued guidelines for the safe preparation, handling and storage of PIF. If the formula preparer follows such guidelines correctly the potential for infection will be low (WHO, 2007). However in certain instances, through carelessness or incompetence, appropriate guidelines may not be followed. The presence of a microbiological protectant in the formula could help maintain a low amount of a pathogenic organism following reconstitution and lower the risk of catastrophic infection. While microorganisms do not grow in dry PIF, following reconstitution it provides an excellent medium for bacterial growth particularly if it is not refrigerated. If held for an extended period of time certain microorganisms

present in the formula, either due to extrinsic or intrinsic contamination will rapidly grow to potentially harmful levels.

1.4. Pathogens in Infant Formula

1.4.1. Introduction

As previously mentioned PIF is not a sterile product. Milk feeds act as excellent media for bacterial multiplication so any pathogens which have survived processing or contaminate the product afterward may rapidly multiply if given the opportunity post rehydration (Agostoni *et al.*, 2004). A joint FAO/WHO consultation group (2004-2006) identified the primary microorganisms associated with PIF contamination as *Cronobacter* sp., *Salmonella enteritidis, Enterobacter agglomerans, Hafnia alvei, Klebsiella pneumoniae, Citrobacter koseri, Citrobacter freundii, Klebsiella oxytoca, Enterobacter cloacae, Escherichia coli, Serratia* sp., *Acinetobacter* sp., *Bacillus cereus, Clostridium difficile, Clostridium perfringens, Clostridium botulinum, Listeria monocytogenes* and *Staphylococcus* sp.. *Cronobacter* sp. and *Salmonella enterica* were identified as the pathogens of most concern in PIF. PIF contaminated with either of these organisms has been associated with serious illness and death (WHO, 2007).

1.4.2. *Cronobacter*

Cronobacter are gram-negative, motile, non-spore-forming peritrichous rods of the *Enterobacteriaceae* family (Farmer, Asbury, Hickman, & Brenner, 1980; FSAI, 2011). Previously referred to as *Enterobacter sakazakii*, the microorganism has recently been formally reclassified as a new genus, *Cronobacter*, currently comprising ten species, *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Cronobacter muytjensii*, *Cronobacter dublinensis*, *Cronobacter universalis*, *Cronobacter condimenti*, *Cronobacter helveticus*, *Cronobacter pulveris* and *Cronobacter zurichensis* (Holý & Forsythe, 2014; C. Iversen *et al.*, 2007; C. Iversen *et al.*, 2008; Carol Iversen *et al.*, 2008; Joseph *et al.*, 2012). Due to the relatively recent reclassification, there is uncertainty regarding the specificity of *Cronobacter* in publications prior to 2007. The species can be classified into two groups; Group 1 (*C. sakazakii* and *C. malonaticus*) and Group 2 (*C. turicensis* and *C. universalis*). From a healthcare perspective Group 1 species are of more importance and form the majority of clinical isolates (Holý & Forsythe, 2014).

Cronobacter infection represents a serious health risk in infants; particularly neonates, and death may occur in 40%-80% of cases (Drudy, Mullane, Quinn, Wall, & Fanning, 2006; Gurtler, Kornacki, & Beuchat, 2005; Nazarowec White & Farber, 1997). The microorganism has been implicated in cases of meningitis, septicemia, and enteritis and is found in sites normally sterile in healthy individuals such as blood, bone marrow and cerebrospinal fluid in infected individuals (Drudy *et al.*, 2006; Gurtler *et al.*, 2005; C. Iversen *et al.*, 2008). *Cronobacter* infection has also been implicated in cases of necrotizing enterocolitis (NEC) which affects 2-5% of all premature infants

and is the most common gastrointestinal emergency in neonates (Hunter *et al.*, 2008; Van Acker *et al.*, 2001). Infected infants are usually treated with antibiotics such as ampicillin-gentamicin or ampicillin chloramphenicol (Lai, 2001). Survivors often suffer from various neurological and/or gastrointestinal sequlae such as hydrocephalus, poor neural development (C. Iversen & Forsythe, 2003) and short gut syndrome (Hunter *et al.*, 2009). Since 2002 *Cronobacter* have been recognized as "Severe for restricted populations, life threatening or substantial chronic sequlae for long duration" by the International Commission on Microbiological Specification for foods (ICMFS) (Forsythe, 2005; ICMFS, 2002) An infectious dose of 10,000 Colony Forming Units (CFU) in a single feeding was proposed by the WHO/FAO in 2007 (WHO/FAO 2008). Although understanding of *Cronobacter* has increased considerably in recent years, there remains unresolved questions regarding its pathogenicity and few virulence factors have been identified to date.

It is likely that the infection route is via attachment and invasion of cells in the intestine following consumption. Indeed, gene clusters responsible for fimbriae have been identified in a number of *Cronobacter* genomes. *C. sakazakii* species possess genes which encode for Beta-fimbriae while other *Cronobacter* genomes encode for curli fimbriae (Joseph & Forsythe, 2011; Kucerova, Joseph, & Forsythe, 2011) which may reflect a host-influenced evolution (Holý & Forsythe, 2014). *C. sakazakii* alone also utilizes exogenous sialic acid, a substance present in breast-milk, mucin and gangliosides (glycosphingolipids associated with the central nervous system) which is added to infant formula due to its association with brain development (Joseph, Hariri, Masood, & Forsythe, 2013). Five putative type VI secretion system (T6SS)

gene clusters have been recognized in *Cronobacter* genomes although functional expression of these genes in *Cronobacter* is yet to be confirmed (C. Grim *et al.*, 2012; C. J. Grim *et al.*, 2013; Joseph & Forsythe, 2011). This system is recently described and may be involved in cytotoxicity, cell adherence/invasion and the ability to survive within the host. A number of studies have indicated that outer membrane proteins (omp) A and X have roles in invasion and translocation of brain microvascular endothelial cells which form the blood-brain barrier (Kim, Bae, & Lee, 2012; Mohan Nair, Venkitanarayanan, Silbart, & Kim, 2009). The mechanism(s) responsible for brain cell destruction are yet to be elucidated (Holý & Forsythe, 2014).

The natural reservoir of *Cronobacter* has not been confirmed and it has been isolated from both human and non-human sources. PIF is the food most commonly associated with infection. However the source of contamination of PIF by *Cronobacter* is an area of contention (Himelright *et al.*, 2002; Norberg *et al.*, 2012). The bacterium has been isolated from both home (García, Notario, Cabanás, Jordano, & Medina, 2012) and hospital environments (Flores, Medrano, Sánchez, & Fernandez-Escartin, 2011) as well as milk powder processing plants (Craven, McAuley, Duffy, & Fegan, 2010; Müller, Stephan, Fricker-Feer, & Lehner, 2013). *Cronobacter* has also been detected in previously unopened PIF products indicative of intrinsic contamination (FAO, 2007; WHO, 2006, 2007). Certain components of PIF such as starches and inulin have been considered as possible sources of contamination (Walsh *et al.*, 2011). Although it is generally agreed that *Cronobacter* are relatively thermotolerant organisms, they are incapable of surviving pasteurization (Strydom, Cawthorn, Cameron, & Witthuhn, 2012) indicating that PIF

becomes contaminated downstream of this process (Gurtler *et al.*, 2005; C. Iversen & Forsythe, 2003; NazarowecWhite & Farber, 1997) by additional ingredients, plant equipment or via asymptomatic carriage of *Cronobacte*r by workers in the processing plant. Indeed *Cronobacter* has been isolated from the faeces, skin and mouth of otherwise healthy individuals (Hägg, Kaveewatcharanont, Samaranayake, & Samaranayake, 2004; Kandhai *et al.*, 2010).

Regardless of how contamination occurs, once present it is estimated that some strains of *Cronobacter* can survive, dormant in PIF for at least two years (C. Iversen & Forsythe, 2003) and rapidly grow upon reconstitution (Barron & Forsythe, 2007). Tolerance of desiccated conditions for such an extended period of time can be attributed to certain aspects of the bacterium's physiology, perhaps most notably the propensity some strains possess to produce a capsule (Barron & Forsythe, 2007; Holý & Forsythe, 2014). *Cronobacter* is resistant to desiccation over a wide range of a_w (0.25-0.86). Over a 12 month storage period, the pathogen has been shown to survive better in dried formula possessing an a_w between 0.25–0.30 than at 0.69–0.82 (FSAI, 2011). This resistance to osmotic stress may be aided by the organisms ability to accumulates solutes such as trehalose which can help stabilize membrane components (Breeuwer, Lardeau, Peterz, & Joosten, 2003) As well as protection from desiccation, the capsule material may also protect *Cronobacter* from disinfectant agents (Beuchat *et al.*, 2009) used to clean processing plant equipment and preparatory utensils. Upon reconstitution, the organism is capable of growth at temperatures from as low as 6°C to as high as 47°C (C Iversen, Lane, & Forsythe, 2004). *Cronobacter* can adhere to

materials commonly used in food preparation utensils (e.g. silicone, stainless steel and polycarbonate) which may increase the chance of extrinsic contamination during formula preparation and feeding (Holý & Forsythe, 2014). Finally, the organism appears to tolerate a broad range of pH conditions (pH 4.5-10) which contributes to its survival under various acidic/basic conditions (Breeuwer *et al.*, 2003).

The rise in *Cronobacter* notoriety has prompted changes in the microbiological criteria for PIF and reconstitution procedures. Perhaps most notable is the FAO-WHO document (WHO, 2007) "Guidelines for the safe preparation, storage and handling of powdered infant formula" which was the result of two FAO-WHO meetings held in 2004 and 2006 (Norberg *et al.*, 2012). The document recommends reconstituting with water which has cooled to 70°C from boiling in order to destroy vegetative cells, reconstitute only the required amount and reduce the storage period prior to consumption as much as possible (WHO, 2007). The use of 70°C water is not adhered to in all countries and can be impractical for a number of reasons. For one the insertion of a thermometer into the liquid presents another route of possible contamination. For this reason caregivers are advised to boil water and let it cool for 30 minutes which can be impractical (particularly in cases where feeding may be required every two hours) and inexact due to extrinsic influences on temperature. The use of 70°C (or higher depending on the accuracy the caregiver practices) water may also affect the nutritional quality of the formula due to damage to heat sensitive ingredients such as some vitamins and probiotics (Holý & Forsythe, 2014).

Various microbiological tests are applied to samples from each PIF batch which are compared with microbiological criteria outlined by the CAC, "in-house" levels deemed workable due to prior experience and company agreements (Norberg *et al.*, 2012). Many infant food producers are legally-bound to implement good manufacturing processes (GMPs) or Hazard Analysis Critical Control Point (HACCP) principles into their control plan in order to reduce the risk of contamination (Hamrin & Hoeft, 2012). In the USA only the state of Minnesota requires reporting of incidences of *Cronobacter* infection (CDC, 2014; Jason, 2012). The incidence rate of infection for the USA at least are one per 100,000 infants which rises to 9.4 per 100,000 in infants of low birth weight (FAO-WHO, 2006). A 2002 FDA study which detected *Cronobacter* in 23 % of sampled PIF (Jason, 2012), coupled with three neonatal intensive (Himelright, 2002; Simmons, Gelfand, Haas, Metts, & Ferguson, 1989; Van Acker *et al.*, 2001) care outbreaks which strongly implicated PIF as the vehicle of transmission prompted the WHO to announce that PIF had strong links microbiologically and epidemiologically to *Cronobacter* infection in infants (FAO, 2007). The previous FDA method for analysis of PIF was based on the most probable number (MPN) approach which was then followed by a number of culturing steps which could take a week to produce a result. Presumptive *Cronobacter* isolates were then confirmed using biochemical profiling (API) and oxidase tests. In March 2012 the FDA replaced the chapter "Isolation and Enumeration of Enterobacter sakazakii from Dehydrated Powdered Infant Formula" with a chapter specifically for *Cronobacter* in their Bacteriological analytic manual which can be located at

(http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm289378.htm). The new method includes Real-Time Polymerase Chain Reaction (RT-PCR) analysis for rapid screening of isolates as well as more traditional culture-dependent ones. Positive RT-PCR should be validated using the cultural method (Strydom *et al.*, 2012). The FDA are currently implementing new rules for PIF manufacturers which include specific testing for *Salmonella* and *Cronobacter* (FDA, 2014).

Outbreaks of neonatal *Cronobacter* infections are hard to document due to the difficulty in proving that it was contaminated PIF that caused the illnesses. There currently exists no global active surveillance network for this pathogen. The FAO/WHO reported 120 incidences of *Cronobacter* in children younger than 3 years old between 1961 and 2008 (Jaradat, Al Mousa, Elbetieha, Al Nabulsi, & Tall, 2014). It is estimated that the actual level of infection is higher (Control & CDC, 2009). Specific incidences of infant and neonatal *Cronobacter* infection have been recently reviewed elsewhere (Norberg *et al.*, 2012). It should be noted that the outbreak (Himelright, 2002) which initiated the FAO-WHO risk assessment of *Cronobacter* and the WHO preparation of PIF guidelines was due to a formula product specifically for neonates and not regular PIF and as such, is not covered by any new legislation related to standard PIF (Holý & Forsythe, 2014). Also of note is the fact that in certain instances, children below 6 months of age receive follow-up formula (FUF) in error (Chap *et al.*, 2009). Changes to PIF legislation should accommodate this malpractice.

1.4.3. *Salmonella*

Salmonella diverged from a common ancestor with *Escherichia* 100-150 million years ago and evolved as an intracellular pathogen (Doolittle, Feng, Tsang, Cho, & Little, 1996). The taxonomy and nomenclature of *Salmonella* are controversial, complex and continually evolving. The *Salmonella* genus is composed of two distinct species, *Salmonella bognori* (a cold-blooded vertebrate commensal) and *Salmonella enterica*, which is divided into six sub-species. These sub-species are classified in to 50 serogroups based on the somatic (O) antigen, and further divided into over 2,400 serovars based on the flagellar (H) antigen. The majority of serovars associated with diseases in humans belong to *S. enterica* subsp. 1. This subspecies causes two types of diseases in humans due to ingestion of contaminated foodstuffs or water; enteric fever (typhoid) or gastroenteritis. Gastroenteritis is mainly caused by *S. enteritidis* and *S. enterica* serovar *Typhimurium* (Sabbagh, Forest, Lepage, Leclerc, & Daigle, 2010). Intrinsic contamination of PIF with non-typhoidal *Salmonella enterica* is an important cause of infection and illness in infants (FAO, 2007).

Rates of *Salmonella* infection are highest in infants less than a year old (Jones *et al.*, 2006) In the United States for example, incidence rates in infants are eight times higher that rates observed in the adult population. The higher incident rate may be due to a higher susceptibility in infants, or the fact that infants are more likely to receive medical attention than adults (WHO, 2006). Infections for the most part are sporadic (Olsen *et al.*, 2001) but outbreaks caused by the pathogen have been recorded (Angulo, Cahill, Wachsmuth, de Lourdes Costarrica, & Embarek, 2008; Boehmer *et al.*, 2009). Outbreak

detection is usually due an unusual serotype of *Salmonella*, possessing a trait facilitating their detection being responsible for illness (FAO/WHO, 2006). Non-typhoidal *Salmonella* infection can cause gastroenteritis leading to diarrhoea, fever and vomiting (Jones *et al.*, 2006) but in certain instances can lead to more serious conditions such bacteraemia, septic arthritis, pneumonia and meningitis. Due to their immature immune systems, infants infected by Non-typhoidal *Salmonella* are more likely to suffer these debilitating potentially fatal diseases (Boehmer *et al.*, 2009; Jones *et al.*, 2006). The severity of disease *Cronobacter* can cause in infants, coupled with its relatively recent emergence has caused it to overshadow concerns regarding contamination of infant formula with *Salmonella* (Angulo *et al.*, 2008). It should also be noted that in due to the nature of bacterial transmission which can occur when an infected individual practices poor hygiene, non-infant formula caused illness is far more likely than illness caused by intrinsically contaminated PIF. When intrinsic *Salmonella* contamination does occur, the bacteria generally enter infant formula due to contamination by ingredients which have not undergone sufficient heat-treatment, or contamination due to the processing environment post-thermal treatment (FAO, 2007).

Control measures implemented by PIF manufacturers are similar to those used by other dairy powder manufacturers and are based on 4 principles: (1) Avoidance of entry of *Salmonella* into the processing facilities and in particular the zones from drying to filling, considered as high-hygiene area (appropriate zoning and segregation); (2), avoidance of multiplication of *Salmonella* in case of entry (elimination of water); (3) hygienic design of high-

hygiene zones and the equipment located in such zones; and (4) use of dry-mix ingredients which are free of *Salmonella*.

Salmonella infections and outbreaks due to intrinsically contaminated PIF are usually only reported in instances where the causal strain is a rare one. At least five outbreaks related to intrinsic PIF contamination occurred between 1985 and 2005. An outbreak occurring in France in 2005 led to 141 confirmed cases of illness. A case controlled study conducted to find the source of the infection identified a particular infant formula brand as responsible and molecular identification techniques provided verification (Brouard *et al.*, 2007). This case highlights the importance of surveillance, which in France is based on a network of laboratories which voluntarily send isolates to a national reference centre to be serotyped. The rare serotype *S. enterica Agona,* was implicated and allowed the source of infection to be traced. In 2008, Spain's National Centre of Microbiology notified an increase in *S. kedougou* isolates from ill children leading to a product recall for the infant formula implicated. During the outbreak 31 children (>1 year) became ill and symptoms included fever and diarrhoea. Salmonella isolated from the patients showed indistinguishable pulse-field gel electrophoresis (PFGE) profiles and identical antibiotic resistance profiles. The author's results indicated that a particular brand of PIF was the cause of the outbreak and no further cases were detected following its recall. The strain implicated had previously been responsible for two outbreaks in the United Kingdom and Norway (meat products were implicated in those cases). The authors concluded that the increase in this serotype of *Salmonella* was only detected due to the low-expected frequency of it in Spain (Rodríguez-Urrego *et al.*, 2010).

Current Codex Alimentarius recommendations indicate a product is fit for consumption if sixty 25g samples test negative for microorganisms. *Salmonella* is rarely detected in PIF using this method (Rodríguez-Urrego *et al.*, 2010). In one outbreak in 1985, it was reported that *Salmonella* was present in very low numbers (1.6 organisms per 450g) (WHO, 2006). In most investigations, the epidemic strain is isolated from bulk samples. Outbreaks of salmonellosis due to contaminated PIF are likely underreported due to the difficulty in implicating a serotype without a rare trait, a lack of routine serotyping in an area or blaming an illness on another factor (WHO, 2006).

1.5. Antimicrobial agents

1.5.1. Bioactive Peptides

Bioactive peptides are small peptide sequences consisting of 3-50 amino acid residues (Benkerroum, 2010; H. Korhonen & Pihlanto, 2006). These peptides may possess a number of physiological properties including opiate, anti-thrombotic, anti-hypertensive and antimicrobial activity *in vivo* and *in vitro* (Clare & Swaisgood, 2000). These peptide sequences exist in a latent, encrypted state in a precursor protein which may be of animal, bacterial or plant origin (Smacchi & Gobbetti, 2000) and are released via direct enzymatic hydrolysis or fermentation (H. Korhonen & Pihlanto, 2006). To date, hundreds of bioactive peptides have been identified and this number continues to grow as novel methods of generating peptides are developed (Benkerroum, 2010; Rydengård *et al.*, 2008). Milk proteins have been recognised as one of the most valuable sources of bioactives (Gill, Lopez-Fandino, Jorba, & Vulfson, 1996; H. Korhonen & Pihlanto, 2006) and are generally assured to be safe and inexpensive (Benkerroum, 2010). Particular to this article, from a regulatory standpoint, the inclusion of dairy-derived bioactive peptides in PIF is not of major concern. Indeed, hydrolysed milk protein fragments are already present in formulae in the form of hypoallergenic infant formulas (Hannu Korhonen, Pihlanto-Leppäla, Rantamäki, & Tupasela, 1998).

Milk is composed of a number of proteins, the major two being bovine casein and whey with casein accounting for 80% of the total protein content (Silva & Malcata, 2005). There are 4 major types of casein proteins, αS1-, αS2-, ß- and κ-casein (Malkoski *et al.*, 2001). Whey protein consists of a number of globular proteins such as bovine serum albumin and ß-lactoglobulin, isolated

from the whey fraction of milk (Farrell *et al.*, 2004). Some whey proteins, such as lactoferrin possess inherent antimicrobial properties (Benkerroum, 2010; Tomita, Wakabayashi, Yamauchi, Teraguchi, & Hayasawa, 2002).

Antimicrobial peptides (AMPs) have been recognised as important elements of the innate immune system (Kamysz, Okroj, & ukasiak, 2003). These peptides are diverse and ancient molecules and are present in various sources. Regardless of the source, antimicrobial peptides show similar recurrent structural and functional characteristics. These peptides interact with bacterial membranes causing disruption leading to rapid cell death (López Expósito & Recio, 2006). Most AMPs can adopt an amphipathic conformation allowing for interaction with hydrophilic and hydrophobic interfaces on the bacterial membrane. Peptides also contain a positively charged domain which aides disruption of the negatively charged bacterial membrane (López-Expósito, Amigo, & Recio, 2008). It is thought that AMPs could also target intracellular components of bacteria such as nucleic acids or intracellular proteins (Kragol *et al.*, 2001). To date, physiologically active peptides such as those possessing angiotensin-1-converting enzyme (ACE)-inhibitory activity have been the subject of more scrutiny than antimicrobial peptides (Benkerroum, 2010). In the past, healthcare industries had an initial lack of interest in antimicrobial peptides due to cost and efficacy in comparison with conventional antibiotics (Lahov & Regelson, 1996). However, due to a consumer preference for lightly processed foods containing low-levels of artificial preservatives and chemicals, AMPs are now being investigated as possible natural bio-preservatives (Al-Nabulsi *et al.*, 2009). Although current antimicrobials may not be potent enough to use directly as a replacement for

traditional antibiotics, the potential to act as a natural prophylactic protectant in many foods cannot be underestimated (Benkerroum, 2010).

One of the best known AMPs is Lactoferrin (LF), which is an 80 kda iron-binding molecule (Baldi *et al.*, 2005; López Expósito & Recio, 2006) found in neutrophils and most other biological exocrine secretions. It is present in the whey component of milk of a number of mammals (Facon & Skura, 1996). LF is resistant to proteolysis in the gut and can be found intact in the faeces of breastfed neonates signifying that initially, it may not have a large role to play in infant nutrition (L. A. Davidson & Lönnerdal, 1987; Kuwata *et al.*, 2001). The concentration of LF in human milk is 2 mg/ml (although this varies during lactation; at points up to 10 mg/ml in colostrum (Actor, Hwang, & Kruzel, 2009)) compared 0.02-0.2 mg/ml in bovine milk (Shimazaki, 2000) indicating it's importance to infants. The iron-binding activity of LF allows it to inhibit the growth of bacteria, parasites and fungi (Farnaud & Evans, 2003). LF is also capable of binding to the surface of Gram-negative bacteria causing cell death due to the release of lipopolysaccharide (LPS) from the cell membrane (Ellison 3rd, Giehl, & LaForce, 1988) which occurs in a temperature dependent manner (Al-Nabulsi *et al.*, 2009). Apart from antimicrobial properties, the bioactivities of LF include anti-inflammatory, anti-cancer and immuno-modulating properties as well as the ability to promote the growth of *Bifidobacteria* (Al-Nabulsi & Holley, 2007).

A recent study evaluated the antimicrobial activity of LF (of bovine origin) on *Cronobacter*. The authors used both native and iron-saturated LF in their antimicrobial assays which were carried out in phosphate buffer, bovine whey and bovine skim milk. The results suggest that binding of iron is

important for antibacterial activity against the pathogen as native LF was the only form which reduced growth (concentration and incubation time-dependent) in all media tested. The study also demonstrated the protective effect that skim milk and whey have for the bacteria in comparison to the phosphate buffer which is of importance with regards to PIF. The authors also concluded that bovine LF is highly resistant to most standard forms of pasteurization (Harouna *et al.*, 2014). Another study demonstrated the anti-adhesive effect that LF had against a *Cronobacter* strain originally isolated from PIF (*C. sakazakii* 4603) as intestinal adherence is an important initiator of bacterial pathogenesis (Casadevall & Pirofski, 2001; Isaacson, 1982). The results indicated that adherence could be significantly reduced (80-99 %) *in-vitro* at a concentration of 10 mg/ml LF. The inclusion of an oligosaccharide did not increase anti-adhesive activity (Quintero-Villegas, Wittke, & Hutkins, 2014). Of note, a study by Al-Nabulsi *et al.* demonstrated that PIF may have an inhibitory effect on LF antimicrobial activity, possibly due to the high concentration of divalent cations in the powder (Al-Nabulsi *et al.*, 2009) which has been shown to reduce the antimicrobial activity of LF against other pathogens (Ellison, Giehl, & LaForce, 1988). The authors found that 2.5 mg/ml of LF was able to inactivate 4 $\log^{10}$ CFU/ml of undessicated *Cronobacter* cells when suspended in peptone water at 37° C. However, there was no detectable antimicrobial activity in PIF under the conditions tested. The authors also concluded that the high concentration of iron in reconstituted PIF may have negated the iron binding ability of LF (Al-Nabulsi *et al.*, 2009). As well as the antimicrobial effects exhibited by LF, studies have shown it can have a positive effect on outcomes of respiratory disease and levels of red blood cells in

circulation when added to infant formula (Francescato, Mosca, Agostoni, & Agosti, 2013; King Jr *et al.*, 2007). Recently, an EFSA panel announced that bovine LF is safe for inclusion in infant formula at specific concentrations (EFSA, 2007).

In 1992, Bellamy *et al.*, 1992 identified an N-terminal region within LF that was antimicrobial. Pepsin digestion of both human and bovine LF, leads to the release of the molecule lactoferricin human (LFcinH) and lactoferricin bovine (LFcinB) respectively. Like LF, LFcin possesses antimicrobial activity against a variety of Gram-positive and Gram-negative microbes. Indeed, it is suggested that the molecule's smaller size allows better access to bacterial membranes increasing potency (Meisel, 1998). In this respect, it can directly cause bacterial death complementing the iron chelating-mediated antimicrobial activity associated with intact LF (Jenssen & Hancock, 2009). LF has shown antimicrobial activity against a broad range of pathogenic bacteria, either via iron sequestering or direct bacterial membrane interference (García-Montoya, Cendón, Arévalo-Gallegos, & Rascón-Cruz, 2012). Methods for the production and pasteurization of both LF and LFcin have been patented and both have been added to a number of foods including infant formula to improve safety (Tomita *et al.*, 2002). A third protein, within the LF parent sequence, lactoferrampin has also shown broad spectrum activity against both Gram-positive and Gram-negative bacteria and the yeast *Candida albicans*. Lactoferrampin is found within the N1 domain of bovine lactoferrin and appears to be crucial for the candidacidal activity of the parent protein (Van der Kraan *et al.*, 2005).

The αS1-casein component of casein has been shown to be a source of a number of AMPs. A notable and early example is isracidin, produced by the chymosin-mediated hydrolysis of bovine casein. The peptide possesses bactericidal activity against a broad range of pathogenic Gram-positive and Gram-negative bacteria. The Weizmann Group, the discoverers of isracidin had previously isolated and patented some of the earliest AMPs, the casecidins. Like isracidin, the casecidins had a broad spectrum of activity, particularly against Gram-positive bacteria (Lahov & Regelson, 1996). The casecidins required high concentrations *in vitro* in order to be effective and so were not seen as effective antimicrobials (Benkerroum, 2010; Lahov & Regelson, 1996). Similarly, isracidin also requires high concentrations *in vitro* (Hayes, Ross, Fitzgerald, Hill, & Stanton, 2006). *In vivo* experiments with the peptide have shown it to be more effective however. Isracidin was shown to be non-toxic and effective in preventing illness due to *S. aureus* infection in a range of animals and an effective prophylactive anti-mastitis treatment over a long period similar to an innate immune-response (Lahov & Regelson, 1996). The isracidin case highlights differences in potency of antimicrobial peptides in *vivo* and *in vitro* and the importance of possible indirect modes of action which help prevent illness in the body (Benkerroum, 2010; Kolb, 2001; Lahov & Regelson, 1996).

Hayes *et al.* (2006) described the generation of 3 AMPs also from αS1-casein. These peptides, namely caseicin A, B and C were generated by the proteolytic action of a LAB strain. Caseicin A and B share homology with isracidin consisting of 9 and 8 amino acid residues respectively, found within it. Results from *in vitro* assays indicated that these peptides (particularly

caseicin A) had potencies similar to that of isracidin against *E.coli* and were also effective against other Gram-negative bacteria but less so against Gram-positives. Caseicin A possesses a +2 positive charge and has a lower minimum inhibitory concentration (MIC) value than the neutral caseicin B. Caseicin C is the least potent of the three described (Hayes *et al.*, 2006). A second study by Hayes *et al.* (2009) investigated the possibility of producing a safe antimicrobial agent capable of inhibiting the growth of *Cronobacter* in infant formula. The study used a filtered (3kDa) lactic acid bacteria (LAB) fermentate derived from sodium caseinate containing both caseicin A and B peptides and added it to PIF at various concentrations. At 0.2% (wt/vol) the antimicrobial agent had a bacteriostatic effect on the pathogen and at higher concentrations, a bactericidal one. As the organisms and materials used in the production of this antimicrobial ingredient have generally regarded as safe (GRAS) status, the research demonstrated a viable and feasible approach to inhibit the growth of *Cronobacter* in PIF (Hayes *et al.*, 2009).The work described in Chapters 3 and 4 of this thesis expand on these particular peptides. Chapter 3 describes the generation of caseicin A and B via *Bacillus*-mediated hydrolysis of casein. Results indicated that the enzyme complement responsible for caseicin generation is a common feature of a number of closely related *Bacillus* species. Chapter 4 of this thesis describes the use of *Bacillus* derived enzymes to generate caseicin A. *Bacillus/Bacillus* enzyme generated caseicin peptides maintained antimicrobial activity.

Casocidin-1 is a 39 amino acid sized fragment originating from bovine αS2 casein characterised in 1995 which possesses antimicrobial activity against a range of bacteria including *Bacillus*, *E. coli* and *Staphylococcal* strains

(Zucht, Raida, Adermann, Magert, & Forssmann, 1995). It was proposed that this peptide could be used in a number of food products including infant formula as a preservative with antibacterial properties. It has not been used commercially yet however due to the difficulty in producing a pure product. It has been proposed that a crude milk preparation containing casocidin-1 would be a better alternative to a pure product in order to ease production (Benkerroum, 2010). In fact it was reported that various fractions of a crude preparation possessed some antimicrobial activity suggesting a synergistic effect between a number of factors (Zucht *et al.*, 1995).

κ-casein has also been a source for antimicrobial bioactives, most notably kappacin, also known as caseinomacropeptide (CMP). CMP exists in a number of variations but is active only in a nonglycosyltated, phosphorylyated form (Malkoski *et al.*, 2001). Kappacin was patented (Reynolds, 2009) and has been used commercially as an oral hygiene product in its pure form and also in a combination with zinc (which enhances its antibacterial activity). It has been proposed that kappacin could also be used as a preservative due to its broad antimicrobial activity and the history of safe use of κ-casein. Kappacin also possesses higher antimicrobial activity in foods with high calcium contents (Benkerroum, 2010). PIF has a minimum of ~50 mg/ml (CAC 2007) of calcium per serving which would serve to increase the potency of this antimicrobial peptide.

A considerable number of peptides isolated from milk have been reported with various activity and possibilities to prevent the growth of contaminants in PIF (Benkerroum, 2010). Some may be better candidates for PIF supplementation than others but for all bioactive peptides there are number

of hurdles that must be overcome. The natural concentration of peptides in milk is low. Financially viable processes that lead to stable peptides which give reproducible results are needed. To date the commercial production of bioactives has been restricted due to a lack of large scale technologies. Following hydrolysis, milk is often filtered to concentrate the peptides and sometimes fractionated to produce pure peptides (H. Korhonen & Pihlanto, 2007). As highlighted by the case of casocidin-1, lengthy and difficult production methods can affect the success of a product. Production difficulties may be offset by potent antimicrobials which can be added in minute amounts to batches of PIF or by generating powders containing antimicrobials that have not undergone the same level of processing (filtering, fractionation) and maintain the ability to inhibit pathogen growth (Hayes *et al.*, 2009). The case of isracidin where interest waned due to variations in results indicates the importance of batch stability for a successful product. Isracidin also demonstrates the differences between *in vitro* and *in vivo* assays when working with bioactive peptides as they can stimulate the immune system indirectly increasing efficacy in ways not yet understood (Kolb, 2001; Lahov & Regelson, 1996). As peptides designed to prevent the growth of pathogens such as *Cronobacter* would have served their intended purpose before ingestion, stability against stomach enzymes would not be an issue. Metal cations in certain foods can adversely affect cationic peptides though so reactivity with other components present in formula would be have to addressed (H. Korhonen & Pihlanto, 2006). Of course, any additive to PIF must be completely safe and not interfere with the organoleptic qualities of the food product.

An AMP generated from a cheap safe source like milk which meets the criteria would prevent the post-rehydration growth of contaminants when added to PIF. Keeping these bacteria at a low level could prevent illness and subsequent deaths and morbidities. An AMP, alone or in synergy with another compound may be the best method to increase the safety of PIF.

1.5.2. Organic Acids

As a group, organic acids primarily include the saturated straight-chain monocarboxylic acids as well as their respective derivatives (phenolic, hydroxylic, unsaturated and multicarboxylic versions) (Cherrington, Hinton, Mead, & Chopra, 1991). They have a long history of use in food products as additives and preservatives for prevention of microbial and fungal contamination in food production, processing and storage (Ricke, 2003). In products for human consumption, acetic, sorbic, benzoic and propionic acid constitute the most commonly used acid preservatives due to good solubility, taste and their low toxicity (Cherrington *et al.*, 1991). Although the mechanisms of antimicrobial activities of organic acids have yet to be fully elucidated they are capable of exerting a bacteriostatic or bactericidal effect. These effects are influenced by the physiochemical state of both the organism in question and the surrounding environment. pH is considered the primary determinant given the weak acidic nature of most of these compounds (Back, Jin, & Lee, 2009; Ricke, 2003). It is assumed that undissociated forms of organic acids can penetrate the cell membrane and once internalised into the neutral pH of the cytoplasm, disassociate into anions and protons effecting the function of macromolecules within the cell (Back *et al.*, 2009; P. Davidson, Doyle, Beuchat, & Montville, 2001; Ricke, 2003). Exporting excess protons requires ATP and may result in a depletion of cellular energy (P. Davidson *et al.*, 2001).

Direct acidification of infant formula with lactic acid was reported to be an effective method to prevent the rapid growth of a number of pathogenic bacteria. Infant formula was first fermented with a lactic acid bacteria which

negatively influenced the growth of certain pathogens inoculated into the formula. To determine if this effect was due to pH or the presence of the fermenting bacteria, the authors compared the fermented formula with unfermented lactic acid acidified formula. They concluded that the formula acidified with lactic acid had similar bacteriostatic properties as the fermented formula (Joosten & Lardeau, 2005). Due to the increasing interest in anti-*Cronobacter* strategies for infant formula there has been a recent increase in the number of studies investigating the potential of organic acids as protectants.

Recently, nine organic acids were investigated for anti-*Cronobacter* activity. Five of them, namely malic, formic, propionic and citric acid inhibited growth of one or more of the strains investigated on laboratory media. Agar disc diffusion assays showed that propionic acid was the most effective against the 71 strains of *Cronobacter* investigated, producing zones of inhibition (1.5 cm diameter) for all strains except one. From these assays, the authors concluded that the order of inhibition of the organic acids they used against the *Cronobacter* strains was propionic acid > acetic acid > malic acid ≥ citric acid > formic acid. Further assays using the most effective acids, propionic and acetic in a number of food systems, including a nutrition shake designed for infants (PediaSure®, ARLA FOODS) showed a bacteriostatic or bactericidal effect at 10 mM and 100 mM, respectively. In this study, hydrochloric acid (pH 4) did not inhibit *Cronobacter* in antimicrobial assays while organic acids at the same pH did indicating that inhibition of growth was not primarily due to pH (Back *et al.*, 2009).

In a study published in 2013, Choi *et al.* demonstrated that combinations of caprylic acid, citric acid and vanillin had a destructive effect on a number of

Cronobacter and *S. Typhimurium*. In their study, the authors spiked reconstituted PIF with the bacteria before adding the antimicrobial compounds. Results indicated that there was a significant synergistic action by the antimicrobials against the pathogens. The authors validated their initial findings by using desiccated cells in order to replicate the type of contamination usually seen in real-world applications. Once again, viability of the *Cronobacter* and *S. Typhimurium* strains was not maintained under the conditions examined. Flow cytometry and electron microscopy indicated plasmolysis and membrane disintegration led to bacterial death (Choi, Kim, Lee, & Rhee, 2013).

A recent study determined the effect of a number of organic acids on *Cronobacter* growth in both reconstituted PIF and laboratory media as well as the bacteriostatic effect of PIF, slightly acidified with acetic acid (pH 6.0) in combination with a simulated gastric model. The growth characteristics of 30 *Cronobacter* strains at various pH conditions in laboratory medium was first investigated, the results of which indicated that the majority of strains investigated (86 %) were resistant to pH 5.0, with zero growth inhibition observed over a 24 h period. The remaining four strains that were sensitive to pH 5.0 were deemed acid sensitive and used in the antimicrobial assays. In laboratory medium acetic, butyric and propionic acids were most inhibitory. The strains investigated were capable of growth at pH 5.5 except when exposed to these acids. The inhibitory effect of these acids in laboratory media was not observed in reconstituted PIF alone but had a synergistic effect when combined with simulated infant gastric conditions as demonstrated by a significant delay in growth of the acid-sensitive *Cronobacter* strains (Acetic

acid used to acidify the PIF) (Zhu, Schnell, & Fischer, 2013). The benefits of milk acidification for infants has previously been demonstrated (Carrion & Egan, 1990). However, as strong acid acidification is not always well tolerated, interest in the use of organic acids compounds in PIF to control and inhibit the growth of contaminants is rising (Ricke, 2003). Previously, it has been reported that *Cronobacter* strains pre-exposed to highly acidic conditions have a higher tolerance against subsequent acidic exposures (Kim *et al.*, 2012). Hence, directly acidifying formula to a lower pH may not be the best option in terms of microbial protection. Zhu *et al.* concluded that milder acidification of formula, in conjunction with infant gastric acid may achieve a protective function similar to direct acidification to a lower pH and will also lead to a product more readily accepted by the infant (Zhu *et al.*, 2013).

With regards to *Cronobacter*, one cause for concern with infant formula acidification would be variation in intra-genus susceptibility to the compounds, which has been previously demonstrated (Back *et al.*, 2009; Zhu *et al.*, 2013). Currently propionic and acetic acids are recognized as among the most potent anti-*Cronobacter* acids (Back *et al.*, 2009; Kim *et al.*, 2012; Oshima *et al.*, 2012; Ricke, 2003; Zhu *et al.*, 2013) possibly due to the greater proportion of acid molecules in an undisassociated form relative to other acids, which can increase levels of cytoplasmic acidification in bacteria (P. Davidson *et al.*, 2001).

From a regulatory perspective, although propionic and acetic acid have GRAS status they are not currently included in Codex standards for infant formula. Organic acids such as these may have application for the improved

safety of PIF alone or in combination with another antimicrobials following further research.

1.5.3. Probiotics and prebiotics

Probiotics ('Live microorganisms (bacteria or yeasts), which when ingested or locally applied in sufficient numbers confer one or more specified demonstrated health benefits for the host' (FAO/WHO., 2001)) are increasingly being added to infant formula due to health benefits with which they are associated (Mugambi, Musekiwa, Lombard, Young, & Blaauw, 2012; Shah, 2007). Prebiotics ('non-digestible carbohydrates which beneficially influence the growth of a specific group of bacteria in gastro-intestinal tract' (Boehm *et al.*, 2005)) such as Fructooligosaccharide (FOS) and Beta-galacto-oligosaccharide (GOS) inclusion in PIF has also become commonplace due to the purported effect they have on the growth of beneficial bacteria (Kent & Doherty, 2014). Various oligosaccharides have been investigated to confirm their prebiotic potential, such as acidic and neutral (GOS) and from pectin hydrolysis, short-and long chain FOS, inulin, and combinations of these substances (Francescato *et al.*, 2013). The term "Synbiotic" is used when both probiotics and prebiotics are administered together. Early colonization of the gut by commensal microorganisms is associated with a number of beneficial outcomes during infancy, including nutrient provision to the host via hydrolysis of non-digestible food components, modulation of mucosal immunity and the bacteria-mediated production of metabolites such as short chain fatty acids. Of relevance to this article is the fact that the presence of commensal bacteria in the gastrointestinal tract (GIT) is associated with a

decrease in pathogenic adherence and colonization of the gut (Zocco, Ainora, Gasbarrini, & Gasbarrini, 2007).

To date, the outcomes associated with probiotic and prebiotic PIF supplementation are associated with improving stool frequency/consistency, improving gastro-intestinal comfort and reducing the incidence of allergy (Braegger *et al.*, 2011; Mugambi *et al.*, 2012). While an increase in commensal bacteria in the GIT may indirectly lower the risk of infection due their effect on pathogenic adhesion, few studies investigating a direct inhibitory effect on *Cronobacter* have been reported. In one such study strains of *Lactobacillus acidophilus* and *Lactobacillus casei* initially isolated from infant stool samples were screened for antimicrobial activity against *C. sakazakii* strains isolated from infant formula. The authors demonstrated that bacteriocin production by the LAB strains (demonstrated by the use of a cell-free supernatant of the cells) had a significant inhibitory effect on the *C. sakazakii* isolates in reconstituted PIF. Activity was not observed following heat treatment or treatment with gastric enzymes indicating the bacteriocins were heat labile which could make their inclusion in powdered milk products difficult. The inclusion of live *L. acidophilus* and *L. casei* strains in reconstituted PIF also had an antimicrobial effect on certain strains of *C. sakazakii* while the CFU/ml of the LAB strains increased by about 2 logs (over a 6 h period). The authors concluded that further research was required to decide the best way to incorporate either the antimicrobial metabolites generated by the LAB strains, or the strains themselves into a PIF product (Awaisheh, Al-Nabulsi, Osaili, Ibrahim, & Holley, 2013).

A number of studies have indicated that prebiotic compounds can influence bacterial adhesion to the GIT (Hickey, 2012; Lane, Mehra, Carrington, & Hickey, 2010). The ability of an oligosaccharide to block adhesion depends on a structural similarity between carbohydrate binding sites usually recognised by pathogenic bacteria and the prebiotic. The prebiotic then binds to adhesins on the pathogen thereby inhibiting adherence to host epithelial cells (Shoaf-Sweeney & Hutkins, 2008). With specific regard to *Cronobacter* adhesion inhibition, a study by Quintero *et al.* found that either singly or in combination GOS and polydextrose (PDX) significantly reduced the levels of adherence *in-vitro* on two cell-lines representing the gut epithelium. Interestingly, both GOS and PDX are two oligosaccharides commercially used in infant formulae. It should be noted that the effects *in-vivo* were not investigated and the study used levels of GOS and PDX at higher concentrations than that found in infant formula.

Although initial results are promising, further studies are required to confirm if probiotics and/or prebiotics could effectively reduce *Cronobacter* infections related to PIF through adhesion inhibition or a direct antimicrobial action. Due to the current interest in *Cronobacter* further studies may lead to the discovery of GRAS strains with potent anti-*Cronobacter* activity. These strains would have to be able to survive the harsh processing and shelf-life conditions associated with PIF. If this inhibitory action was due to bacteriocin production, efficacy would have to be confirmed *in-vivo* as expression of bacteriocins by bacteria can differ from *in-vitro* conditions (Awaisheh *et al.*, 2013). Economic factors would also be an issue particularly with regard to prebiotic oligosaccharides. For example, even if a human milk oligosaccharide

was found to inhibit *Cronobacter* adhesion, its commercial use would be difficult to implement.

1.5.4. Alternate methods of PIF sterilization under research

Current knowledge dictates that total sterilization of PIF is only possible using irradiation, but as previously mentioned, this is forbidden by the Codex Alimentarius guidelines on PIF production. In PIF, *Cronobacter* is present in a dessicated state and the high doses required to inactivate it would have severe adverse effects on the organoleptic quality of the product (Codex Alimentarius Commission CAC, 2007; FAO-WHO, 2006; FAO, 2007). Currently the use of technologies such as magnetic fields and ultra-high pressure are at early stages of development and their effectiveness has not yet been established (Strydom *et al.*, 2012). The use of microwave technology on reconstituted PIF has proven effective at reducing numbers of *Cronobacter* cells in what is believed to both a non-thermal electromagnetic radiation effect and a direct thermal effect on the bacteria. In one trial, microwaving reconstituted PIF for 90s at 93°C effectively reduced an inoculum of 100 CFU/ml *Cronobacter* cells to zero (Kindle, Busse, Kampa, Meyer-König, & Daschner, 1996). However, the effect of this treatment on the nutrient value of the product would likely be an issue. There would also likely be adherence issues on the part of caregivers with this treatment.

1.6. Conclusions and Future perspectives

The microbiological integrity of PIF has been re-examined over the past decade in part due to the emergence of *Cronobacter*. A number of guidelines and regulatory changes have been published (Codex Alimentarius Commsion CAC, 2008; WHO, 2007) in order to aid caregivers and manufacturers of PIF and PIF ingredients reduce the chance of catastrophic contamination by pathogenic bacteria. Strict adherence to these guidelines will reduce incidences of PIF mediated infection (Norberg *et al.*, 2012). However, it is likely that on certain occasions these recommendations will not be followed either through negligence or incompetence. When this occurs the inclusion of antimicrobial solutions may help prevent illness. Natural antimicrobials, in particular those with GRAS status are of interest due to consumer concerns regarding chemical additives.

With specific regard to *Cronobacter*, a significant challenge is the identification of specific virulence factors related to particular species or strains (Strydom *et al.*, 2012). However, the recent increase in research on this genus has led to greatly improved detection methods and knowledge of important characteristics of individual species. Molecular methods are increasingly used to reliably and rapidly trace sources of infection and to study diversity between bacterial genomes (Holý & Forsythe, 2014). Increasingly, Multilocus sequence typing (MLST) is used to understand the diversity and evolution of pathogenic bacteria. A MLST scheme for *Cronobacter* (Jolley & Maiden, 2010) is available online (http://pubmlst.org/cronobacter/) and currently contains ~900 isolates. Researchers group sequence types elucidated by MLST into clonal complexes decided by similarities to a central allelic profile. The *C. sakazakii*

clonal complex ST4 has been frequently isolated from PIF processing plants, milk powder processing facilities and PIF itself (Holý & Forsythe, 2014; Joseph & Forsythe, 2011; Müller *et al.*, 2013; Power, Yan, Fox, Cooney, & Fanning, 2013) and is associated with cases of meningitis. Control of the ST4 lineage could reduce infant exposure to particularly virulent strains (Holý & Forsythe, 2014). Information derived from the study of strains from this clonal complex may also allow researchers to develop specific methods to prevent growth of virulent strains in various environments.

A multi-directional approach may be the most effective. For PIF producers, continued research into effective decontamination methods coupled with improved training and education of staff and strict implementation of good manufacturing principles are required. Efforts to educate medical staff and infant caregivers regarding proper handling and storage of the product should be increased (Strydom *et al.*, 2012). Consumers should also receive clear and accurate information regarding the risk of infection associated with various formulae so that they may make informed decisions regarding how to feed the infant (Jason, 2012). Finally, the inclusion of antimicrobials either singly, or in synergy with another compound or process will lower incidences of infection.

1.7. Acknowledgements

This work was funded by an Enterprise Ireland commercialisation fund CFTD/07/116.

1.8. References

Actor, J. K., Hwang, S.-A., & Kruzel, M. L. (2009). Lactoferrin as a natural immune modulator. *Current Pharmaceutical Design, 15*(17), 1956.

Agostoni, C., Axelsson, I., Goulet, O., Koletzko, B., Michaelsen, K. F., Puntis, J. W. L., . . . Turck, D. (2004). Preparation and handling of powdered infant formula: a commentary by the ESPGHAN Committee on Nutrition. *Journal of Pediatric Gastroenterology and Nutrition, 39*(4), 320.

Al-Nabulsi, A. A., & Holley, R. A. (2007). Effects on *Escherichia coli* O157: H7 and meat starter cultures of bovine lactoferrin in broth and microencapsulated lactoferrin in dry sausage batters. *International Journal of Food Microbiology, 113*(1), 84-91.

Al-Nabulsi, A. A., Osaili, T. M., Al-Holy, M. A., Shaker, R. R., Ayyash, M. M., Olaimat, A. N., & Holley, R. A. (2009). Influence of desiccation on the sensitivity of *Cronobacter* spp. to lactoferrin or nisin in broth and powdered infant formula. *International Journal of Food Microbiology, 136*(2), 221-226.

Angulo, F. J., Cahill, S. M., Wachsmuth, I. K., de Lourdes Costarrica, M., & Embarek, P. K. B. (2008). Powdered infant formula as a source of *Salmonella* infection in infants. *Clinical Infectious Diseases, 46*(2), 268-273.

Awaisheh, S. S., Al-Nabulsi, A. A., Osaili, T. M., Ibrahim, S., & Holley, R. (2013). Inhibition of *Cronobacter sakazakii* by Heat Labile Bacteriocins Produced by Probiotic LAB Isolated from Healthy Infants. *Journal of food science*, *78*(9), M1416-M1420.

Back, S.-Y., Jin, H.-H., & Lee, S.-Y. (2009). Inhibitory effect of organic acids against *Enterobacter sakazakii* in laboratory media and liquid foods. *Food Control*, *20*(10), 867-872.

Badger, T. M., Gilchrist, J. M., Pivik, R. T., Andres, A., Shankar, K., Chen, J.-R., & Ronis, M. J. (2009). The health implications of soy infant formula. *The American journal of clinical nutrition*, *89*(5), 1668S-1672S.

Baldi, A., Ioannis, P., Chiara, P., Eleonora, F., Roubini, C., & Vittorio, D. O. (2005). Biological effects of milk proteins and their peptides with emphasis on those related to the gastrointestinal ecosystem. *Journal of Dairy Research*, *72*(S1), 66-72.

Barron, J. C., & Forsythe, S. J. (2007). Dry stress and survival time of *Enterobacter sakazakii* and other Enterobacteriaceae in dehydrated powdered infant formula. *Journal of Food Protection*, *70*(9), 2111-2117.

Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K., & Tomita, M. (1992). Identification of the bactericidal domain of lactoferrin. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, *1121*(1-2), 130-136.

Benkerroum, N. (2010). Antimicrobial peptides generated from milk proteins: a survey and prospects for application in the food industry. A review. *International Journal of Dairy Technology*, *63*(3), 320-338. doi: 10.1111/j.1471-0307.2010.00584.x

Beuchat, L. R., Kim, H., Gurtler, J. B., Lin, L.-C., Ryu, J.-H., & Richards, G. M. (2009). *Cronobacter sakazakii* in foods and factors affecting its survival, growth, and inactivation. *International Journal of Food Microbiology*, *136*(2), 204-213.

Bhatia, J., & Greer, F. (2008). Use of soy protein-based formulas in infant feeding. *Pediatrics*, *121*(5), 1062-1068.

Boehm, G., Stahl, B., Jelinek, J., Knol, J., Miniello, V., & Moro, G. E. (2005). Prebiotic carbohydrates in human milk and formulas. *Acta Paediatrica*, *94*(s449), 18-21.

Boehmer, T. K., Bamberg, W. M., Ghosh, T. S., Cronquist, A., Fornof, M. E., Cichon, M. K., . . . Vogt, R. L. (2009). Health care–associated outbreak of *Salmonella Tennessee* in a neonatal intensive care unit. *American journal of infection control, 37*(1), 49-55.

Braegger, C., Chmielewska, A., Decsi, T., Kolacek, S., Mihatsch, W., Moreno, L., . . . Szajewska, H. (2011). Supplementation of infant formula with probiotics and/or prebiotics: a systematic review and comment by the ESPGHAN committee on nutrition. *Journal of Pediatric Gastroenterology and Nutrition, 52*(2), 238-250.

Breeuwer, P., Lardeau, A., Peterz, M., & Joosten, H. (2003). Desiccation and heat tolerance of *Enterobacter sakazakii*. *Journal of Applied Microbiology, 95*(5), 967-973.

Brouard, C., Espié, E., Weill, F.-X., Kérouanton, A., Brisabois, A., Forgue, A.-M., . . . de Valk, H. (2007). Two consecutive large outbreaks of *Salmonella enterica* serotype *Agona* infections in infants linked to the consumption of powdered infant formula. *The Pediatric infectious disease journal, 26*(2), 148-152.

CAC, (2007). Standards for infant formula and formulas for special medical purposes intended for infants. *CODEX STAN 72-108*.

CAC, (2008). CAC/RCP 66-2008 Code of hygienic practice for powdered infant formula for infants and young children.

CAC, (2013). Codex Members and Observers.

Carrion, V., & Egan, E. A. (1990). Prevention of neonatal necrotizing enterocolitis. *Journal of Pediatric Gastroenterology and Nutrition, 11*(3), 317-323.

Casadevall, A., & Pirofski, L. a. (2001). Host-pathogen interactions: the attributes of virulence. *Journal of Infectious Diseases, 184*(3), 337-344.

Centers for Disease Control and preventionCDC. (2014). *Cronobacter* Expanded Information. from http://www.cdc.gov/cronobacter/technical.html

Chap, J., Jackson, P., Siqueira, R., Gaspar, N., Quintas, C., Park, J., . . . Hartantyo, S. (2009). International survey of *Cronobacter sakazakii* and other *Cronobacter* spp. in follow up formulas and infant foods. *International Journal of Food Microbiology*, *136*(2), 185-188.

Cherrington, C., Hinton, M., Mead, G., & Chopra, I. (1991). Organic acids: chemistry, antibacterial activity and practical applications. *Advances in microbial physiology*, *32*, 87-108.

Choi, M., Kim, S., Lee, N., & Rhee, M. (2013). New decontamination method based on caprylic acid in combination with citric acid or vanillin for eliminating *Cronobacter sakazakii* and *Salmonella enterica* serovar Typhimurium in reconstituted infant formula. *International Journal of Food Microbiology, 166*(3), 499-507.

Clare, D. A., & Swaisgood, H. E. (2000). Bioactive milk peptides: A prospectus. *Journal of Dairy Science, 83*(6), 1187-1195.

Connolly, E., & Lönnerdal, B. (2004). D-lactic acid producing bacteria Safe to use in infant formulas. *NUTRA foods, 3*(3), 37-49.

Control, C. f. D., & CDC, P. (2009). *Cronobacter* species isolation in two infants-New Mexico, 2008. *MMWR. Morbidity and mortality weekly report, 58*(42), 1179.

Craven, H., McAuley, C., Duffy, L., & Fegan, N. (2010). Distribution, prevalence and persistence of *Cronobacter* (*Enterobacter sakazakii*) in the nonprocessing and processing environments of five milk powder factories. *Journal of applied microbiology, 109*(3), 1044-1052.

Davidson, L. A., & Lönnerdal, B. O. (1987). Persistence of Human Milk Proteins in the Breast Fed Infant. *Acta Pædiatrica, 76*(5), 733-740.

Davidson, P., Doyle, M., Beuchat, L., & Montville, T. (2001). Food microbiology–fundamentals and frontiers. *Chemical Preservatives and Natural Antimicrobial Compounds"(Eds. Doyle, MP*, 593-627.

Doolittle, R. F., Feng, D.-F., Tsang, S., Cho, G., & Little, E. (1996). Determining divergence times of the major kingdoms of living organisms with a protein clock. *Science, 271*(5248), 470-477.

Drudy, D., Mullane, N. R., Quinn, T., Wall, P. G., & Fanning, S. (2006). *Enterobacter sakazakii*: An emerging pathogen in powdered infant formula. *Clinical Infectious Diseases, 42*(7), 996-1002.

EFSA. (2007). Scientific opinion on bovine lactoferrin. *EFSA Journal 2012, 10*(5). doi: 10.2903/j.efsa.2012.2701

Ellison 3rd, R. T., Giehl, T. J., & LaForce, F. M. (1988). Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin. *Infection and Immunity, 56*(11), 2774.

Ellison, R. d., Giehl, T. J., & LaForce, F. M. (1988). Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin. *Infection and Immunity, 56*(11), 2774-2781.

Facon, M. J., & Skura, B. J. (1996). Antibacterial activity of lactoferricin, lysozyme and EDTA against *Salmonella enteritidis*. *International Dairy Journal, 6*(3), 303-313.

FAO-WHO. (2006). Expert meeting on *Enterobacter sakazakii* and *Salmonella* in powdered infant formula. Rome.

FAO. (2007). *Enterobacter sakazakii* and other microorganisms in powdered infant formula

FAO/WHO. (2001). Report on Joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria: FAO/WHO Cordoba.

Farmer, J. J., Asbury, M., Hickman, F., & Brenner, D. J. (1980). *Enterobacter sakazakii*: a new species of "Enterobacteriaceae" isolated from clinical specimens. *International Journal of Systematic Bacteriology, 30*(3), 569-584.

Farnaud, S., & Evans, R. W. (2003). Lactoferrin--a multifunctional protein with antimicrobial properties. *Molecular immunology, 40*(7), 395-405.

Farrell, H. M., Jimenez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K., . . . Swaisgood, H. E. (2004). Nomenclature of the proteins of cows' milk - Sixth revision. *Journal of Dairy Science, 87*(6), 1641-1674.

FDA. (2014). FDA Consumer Health Information Retrieved 15.8.14, from http://www.fda.gov/downloads/ForConsumers/ConsumerUpdates/UCM400238.pdf

Flores, J. P., Medrano, S. A., Sánchez, J. S., & Fernandez-Escartin, E. (2011). Two cases of hemorrhagic diarrhea caused by *Cronobacter sakazakii* in hospitalized nursing infants associated with the consumption of powdered infant formula. *Journal of Food Protection®, 74*(12), 2177-2181.

Forsythe, S. J. (2005). *Enterobacter sakazakii* and other bacteria in powdered infant milk formula. *Maternal and Child Nutrition, 1*(1), 44-50.

Francescato, G., Mosca, F., Agostoni, C., & Agosti, M. (2013). The ideal formula for healthy term infants. *Early Human Development, 89*, S126-S128.

FSAI. (2011). Guidance Note No. 18 Validation of Product Shelf-Life (Revision 1).

García-Montoya, I. A., Cendón, T. S., Arévalo-Gallegos, S., & Rascón-Cruz, Q. (2012). Lactoferrin a multiple bioactive protein: an overview. *Biochimica et Biophysica Acta (BBA)-General Subjects, 1820*(3), 226-236.

García, F., Notario, M., Cabanás, J., Jordano, R., & Medina, L. (2012). Incidence of bacteria of public health interest carried by cockroaches in different food-related environments. *Journal of medical entomology, 49*(3), 1481-1484.

Gill, I., Lopez-Fandino, R., Jorba, X., & Vulfson, E. N. (1996). Biologically active peptides and enzymatic approaches to their production. *Enzyme and Microbial Technology, 18*(3), 162-183.

Grim, C., Kothary, M., Gopinath, G., Jarvis, K., Beaubrun, J. J.-G., McClelland, M., . . . Franco, A. (2012). Identification and characterization of *Cronobacter* iron acquisition systems. *Applied and environmental microbiology, 78*(17), 6035-6050.

Grim, C. J., Kotewicz, M. L., Power, K. A., Gopinath, G., Franco, A. A., Jarvis, K. G., . . . Hu, L. (2013). Pan-genome analysis of the emerging foodborne pathogen *Cronobacter* spp. suggests a species-level bidirectional divergence driven by niche adaptation. *BMC genomics, 14*(1), 366.

Gurtler, J. B., Kornacki, J. L., & Beuchat, L. R. (2005). *Enterobacter sakazakii*: A coliform of increased concern to infant health. *International Journal of Food Microbiology, 104*(1), 1-34. doi: 10.1016/j.ijfoodmicro.2005.02.013

Hägg, U., Kaveewatcharanont, P., Samaranayake, Y., & Samaranayake, L. (2004). The effect of fixed orthodontic appliances on the oral carriage of Candida species and Enterobacteriaceae. *The European Journal of Orthodontics, 26*(6), 623-629.

Hamrin, P., & Hoeft, B. (2012). Quality control throughout the production process of infant food. *Annals of Nutrition and Metabolism, 60*(3), 208-210.

Harouna, S., Carramiñana, J., Navarro, F., Pérez, M., Calvo, M., & Sánchez, L. (2014). Antibacterial activity of bovine milk lactoferrin on the emerging foodborne pathogen *Cronobacter sakazakii*: effect of media and heat treatment. *Food Control*, 47, 520-525

Hayes, M., Barrett, E., Ross, R. P., Fitzgerald, G. F., Hill, C., & Stanton, C. (2009). Evaluation of an Antimicrobial Ingredient Prepared from a *Lactobacillus acidophilus* Casein Fermentate against Enterobacter sakazakii. *Journal of Food Protection, 72*(2), 340-346.

Hayes, M., Ross, R. P., Fitzgerald, G. F., Hill, C., & Stanton, C. (2006). Casein-derived antimicrobial peptides generated by *Lactobacillus acidophilus* DPC6026. *Applied and environmental microbiology*, *72*(3), 2260.

Hickey, R. M. (2012). The role of oligosaccharides from human milk and other sources in prevention of pathogen adhesion. *International Dairy Journal*, *22*(2), 141-146.

Himelright, I. (2002). *Enterobacter sakazakii* infections associated with the use of powdered infant formula-Tennessee, 2001. *J. Am. Med. Assoc.*, *287*, 2204-2205.

Himelright, I., Harris, E., Lorch, V., Anderson, M., Jones, T., Craig, A., . . . Jensen, B. (2002). From the Centers for Disease Control and Prevention. *Enterobacter sakazakii*, 2204-2205.

Holý, O., & Forsythe, S. (2014). *Cronobacter* spp. as emerging causes of healthcare-associated infection. *Journal of Hospital Infection*, *86*(3), 169-177.

Hunter, C. J., Singamsetty, V. K., Chokshi, N. K., Boyle, P., Camerini, V., Grishin, A. V., . . . Prasadarao, N. V. (2008). *Enterobacter sakazakii* enhances epithelial cell injury by inducing apoptosis in a rat model of necrotizing enterocolitis. *The Journal of infectious diseases, 198*(4), 586.

Hunter, C. J., Williams, M., Petrosyan, M., Guner, Y., Mittal, R., Mock, D., . . . Prasadarao, N. V. (2009). *Lactobacillus bulgaricus* Prevents Intestinal Epithelial Cell Injury Caused by *Enterobacter sakazakii*-Induced Nitric Oxide both In Vitro and in the Newborn Rat Model of Necrotizing Enterocolitis. *Infection and Immunity, 77*(3), 1031-1043. doi: 10.1128/iai.01192-08

ICMFS,. (2002). Microrganisms in foods. 7. Microbiological testing in food safety management Kluwer Academic/Plenum Publishers, New York.

Isaacson, R. (1982). Bacterial adherence to mucosal surfaces: an attribute of virulence. *Bulletin europeen de physiopathologie respiratoire, 19*(2), 75-80.

Iversen, C., & Forsythe, S. (2003). Risk profile of *Enterobacter sakazakii*, an emergent pathogen associated with infant milk formula. *Trends in Food Science & Technology, 14*(11), 443-454.

Iversen, C., Lane, M., & Forsythe, S. (2004). The growth profile, thermotolerance and biofilm formation of *Enterobacter sakazakii* grown in infant formula milk. *Letters in Applied Microbiology, 38*(5), 378-382.

Iversen, C., Lehner, A., Mullane, N., Bidlas, E., Cleenwerck, I., Marugg, J., . . . Joosten, H. (2007). The taxonomy of *Enterobacter sakazaki*i: proposal of a new genus *Cronobacter* gen. nov and descriptions of *Cronobacter sakazakii* comb. nov *Cronobacter sakazakii* subsp *sakazakii*, comb. nov., *Cronobacter sakazakii* subsp *malonaticus* subsp nov., *Cronobacter turicensis* sp nov., *Cronobacter muytjensii* sp nov., *Cronobacter dublinensis* sp nov and Cronobacter genomospecies I. *Bmc Evolutionary Biology*, *7*, 11. doi: 64 10.1186/1471-2148-7-64

Iversen, C., Mullane, N., McCardel, B., Tal, B. D., Lehner, A., Fannin, S., . . . Joosten, H. (2008). *Cronobacter* gen nov, a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen nov, comb nov, *Cronobacter malonaticus* sp nov, *Cronobacter turicensis* sp nov, *Cronobacter muytjensii* sp nov, *Cronobacter* dublinensis sp nov, *Cronobacter* genomospecies 1, and of three subspecies, *Cronobacter dublinensis* subsp *dublinensis* subsp nov, *Cronobacter dublinensis* subsp *lausannensis* subsp nov and *Cronobacter dublinensis* subsp *lactaridi* subsp nov. *International Journal of Systematic and Evolutionary Microbiology, 58*, 1442-1447. doi: 10.1099/ijs.0.65577-0

IJaradat, Z. W., Al Mousa, W., Elbetieha, A., Al Nabulsi, A., & Tall, B. D. (2014). *Cronobacter*, an opportunistic food borne pathogen; a review of its virulence and environmental adaptive traits. *Journal of medical microbiology*, jmm. 0.073742-073740.

Jason, J. (2012). Prevention of invasive *Cronobacter* infections in young infants fed powdered infant formulas. *Pediatrics*, *130*(5), e1076-e1084.

Jenssen, H., & Hancock, R. E. (2009). Antimicrobial properties of lactoferrin. *Biochimie*, *91*(1), 19-29.

Jolley, K. A., & Maiden, M. C. (2010). BIGSdb: Scalable analysis of bacterial genome variation at the population level. *BMC bioinformatics*, *11*(1), 595.

Jones, T. F., Ingram, L. A., Fullerton, K. E., Marcus, R., Anderson, B. J., McCarthy, P. V., . . . Wedel, S. (2006). A case-control study of the epidemiology of sporadic *Salmonella* infection in infants. *Pediatrics*, *118*(6), 2380-2387.

Joosten, H., & Lardeau, A. (2005). Enhanced microbiological safety of acidified infant formulas tested in vitro. *South African Journal of Clinical Nutrition*, *17*(3), 87-92.

Joseph, S., Cetinkaya, E., Drahovska, H., Levican, A., Figueras, M. J., & Forsythe, S. J. (2012). *Cronobacter condimenti* sp. nov., isolated from spiced meat, and *Cronobacter universalis* sp. nov., a species designation for *Cronobacter* sp. genomospecies 1, recovered from a leg infection, water and food ingredients. *International Journal of Systematic and Evolutionary Microbiology*, *62*(Pt 6), 1277-1283.

Joseph, S., & Forsythe, S. J. (2011). Predominance of *Cronobacter sakazakii* sequence type 4 in neonatal infections. *Emerging infectious diseases*, *17*(9), 1713.

Joseph, S., Hariri, S., Masood, N., & Forsythe, S. (2013). Sialic acid utilization by *Cronobacter sakazakii*. *Microbial informatics and experimentation*, *3*(1), 3.

Kamysz, W., Okroj, M., & ukasiak, J. (2003). Novel properties of antimicrobial peptides. *Acta Biochimica Polonica*, *50*(2), 461.

Kandhai, M., Heuvelink, A., Reij, M., Beumer, R., Dijk, R., van Tilburg, J., . . . Gorris, L. (2010). A study into the occurrence of *Cronobacter* spp. in The Netherlands between 2001 and 2005. *Food Control*, *21*(8), 1127-1136.

Kent, R. M., & Doherty, S. B. (2014). Probiotic bacteria in infant formula and follow-up formula: Microencapsulation using milk and pea proteins to improve microbiological quality. *Food Research International*, 2014, 64, 567

Kim, S.-J., Bae, Y.-M., & Lee, S.-Y. (2012). Stress response of acid-shocked *Cronobacter sakazakii* against subsequent acidic pH, mild heat, and organic acids. *Food Science and Biotechnology*, *21*(1), 205-210.

Kindle, G., Busse, A., Kampa, D., Meyer-König, U., & Daschner, F. (1996). Killing activity of microwaves in milk. *Journal of Hospital Infection*, *33*(4), 273-278.

King Jr, J. C., Cummings, G. E., Guo, N., Trivedi, L., Readmond, B. X., Keane, V., . . . de Waard, R. (2007). A double-blind, placebo-controlled, pilot study of bovine lactoferrin supplementation in bottle-fed infants. *Journal of Pediatric Gastroenterology and Nutrition*, *44*(2), 245-251.

Kolb, A. F. (2001). The prospects of modifying the antimicrobial properties of milk. *Biotechnology Advances, 19*(4), 299-316.

Koletzko, B., Baker, S., Cleghorn, G., Neto, U. F., Gopalan, S., Hernell, O., . . . Pencharz, P. (2005). Global standard for the composition of infant formula: recommendations of an ESPGHAN coordinated international expert group. *Journal of Pediatric Gastroenterology and Nutrition, 41*(5), 584-599.

Korhonen, H., Pihlanto-Leppäla, A., Rantamäki, P., & Tupasela, T. (1998). Impact of processing on bioactive proteins and peptides. *Trends in Food Science & Technology, 9*(8), 307-319.

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: production and functionality. *International Dairy Journal, 16*(9), 945-960.

Korhonen, H., & Pihlanto, A. (2007). Technological options for the production of health-promoting proteins and peptides derived from milk and colostrum. *Current Pharmaceutical Design, 13*(8), 829-843.

Kragol, G., Lovas, S., Varadi, G., Condie, B. A., Hoffmann, R., & Otvos Jr, L. (2001). The Antibacterial Peptide Pyrrhocoricin Inhibits the ATPase Actions of DnaK and Prevents Chaperone-Assisted Protein Folding†. *Biochemistry, 40*(10), 3016-3026.

Kucerova, E., Joseph, S., & Forsythe, S. (2011). The *Cronobacter* genus: ubiquity and diversity. *Quality Assurance and Safety of Crops & Foods, 3*(3), 104-122.

Kuwata, H., Yamauchi, K., Teraguchi, S., Ushida, Y., Shimokawa, Y., Toida, T., & Hayasawa, H. (2001). Functional fragments of ingested lactoferrin are resistant to proteolytic degradation in the gastrointestinal tract of adult rats. *Journal of Nutrition, 131*(8), 2121-2127.

Lahov, E., & Regelson, W. (1996). Antibacterial and immunostimulating casein-derived substances from milk: casecidin, isracidin peptides. *Food and Chemical Toxicology, 34*(1), 131-145.

Lai, K. K. (2001). *Enterobacter sakazakii* infections among neonates, infants, children, and adults - Case reports and a review of the literature. *Medicine, 80*(2), 113-122.

Lane, J. A., Mehra, R. K., Carrington, S. D., & Hickey, R. M. (2010). The food glycome: a source of protection against pathogen colonization in the gastrointestinal tract. *International Journal of Food Microbiology, 142*(1), 1-13.

Lönnerdal, B. (2012). Preclinical assessment of infant formula. *Annals of Nutrition and Metabolism, 60*(3), 196-199.

López-Expósito, I., Amigo, L., & Recio, I. (2008). Identification of the initial binding sites of [alpha] s2-casein f (183-207) and effect on bacterial membranes and cell morphology. *Biochimica et Biophysica Acta (BBA)-Biomembranes, 1778*(10), 2444-2449.

López Expósito, I., & Recio, I. (2006). Antibacterial activity of peptides and folding variants from milk proteins. *International Dairy Journal, 16*(11), 1294-1305.

Malkoski, M., Dashper, S. G., O'Brien-Simpson, N. M., Talbo, G. H., Macris, M., Cross, K. J., & Reynolds, E. C. (2001). Kappacin, a novel antibacterial peptide from bovine milk. *Antimicrobial agents and chemotherapy, 45*(8), 2309.

Meisel, H. (1998). Overview on milk protein-derived peptides. *International Dairy Journal, 8*(5-6), 363-373.

Mohan Nair, M. K., Venkitanarayanan, K., Silbart, L. K., & Kim, K. S. (2009). Outer membrane protein A (OmpA) of *Cronobacter sakazakii* binds fibronectin and contributes to invasion of human brain microvascular endothelial cells. *Foodborne pathogens and disease, 6*(4), 495-501.

Mugambi, M. N., Musekiwa, A., Lombard, M., Young, T., & Blaauw, R. (2012). Synbiotics, probiotics or prebiotics in infant formula for full term infants: a systematic review. *Nutr J, 11*(1), Article number 8.

Müller, A., Stephan, R., Fricker-Feer, C., & Lehner, A. (2013). Genetic diversity of *Cronobacter sakazakii* isolates collected from a Swiss infant formula production facility. *Journal of Food Protection®, 76*(5), 883-887.

Nair, M., Kumar, M., Joy, J., & Venkitanarayanan, K. S. (2004). Inactivation of *Enterobacter sakazakii* in reconstituted infant formula by monocaprylin. *Journal of Food Protection®, 67*(12), 2815-2819.

NazarowecWhite, M., & Farber, J. M. (1997). *Enterobacter sakazakii*: A review. *International Journal of Food Microbiology, 34*(2), 103-113.

Norberg, S., Stanton, C., Ross, R. P., Hill, C., Fitzgerald, G. F., & Cotter, P. D. (2012). *Cronobacter* spp. in powdered infant formula. *Journal of Food Protection®, 75*(3), 607-620.

Olsen, S. J., Bishop, R., Brenner, F. W., Roels, T. H., Bean, N., Tauxe, R. V., & Slutsker, L. (2001). The changing epidemiology of *Salmonella*: trends in serotypes isolated from humans in the United States, 1987–1997. *Journal of Infectious Diseases, 183*(5), 753-761.

Oshima, S., Rea, M. C., Lothe, S., Morgan, S., Begley, M., O'Connor, P. M., . . . Ross, R. P. (2012). Efficacy of organic acids, bacteriocins, and the lactoperoxidase system in inhibiting the growth of *Cronobacter* spp. in rehydrated infant formula. *Journal of Food Protection®, 75*(10), 1734-1742.

Power, K. A., Yan, Q., Fox, E. M., Cooney, S., & Fanning, S. (2013). Genome sequence of *Cronobacter sakazakii* SP291, a persistent thermotolerant isolate derived from a factory producing powdered infant formula. *Genome announcements, 1*(2), e00082-00013.

Quintero-Villegas, M. I., Wittke, A., & Hutkins, R. (2014). Adherence Inhibition of *Cronobacter sakazakii* to Intestinal Epithelial Cells by Lactoferrin. *Current microbiology*, 1-6.

Reynolds, E. C., Dashper, S.G., O'Brien-Simpson, N.M., Talbo, G.H., Malkosi, M. (2009). US 7588752 B2.

Ricke, S. (2003). Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. *Poultry science, 82*(4), 632-639.

Riordan, J., & Wambach, K. (2010). *Breastfeeding and human lactation*: Jones & Bartlett Learning.

Rodríguez-Urrego, J., Herrera-León, S., Echeita-Sarriondia, A., Soler, P., Simon, F., & Mateo, S. (2010). Nationwide outbreak of *Salmonella* serotype Kedougou associated with infant formula, Spain, 2008. *Eurosurveillance, 15*(22), 19582.

Rydengård, V., Shannon, O., Lundqvist, K., Kacprzyk, L., Chalupka, A., Olsson, A. K., . . . Schmidtchen, A. (2008). Histidine-rich glycoprotein protects from systemic Candida infection. *PLoS Pathog, 4*(8), e1000116.

Sabbagh, S. C., Forest, C. G., Lepage, C., Leclerc, J. M., & Daigle, F. (2010). So similar, yet so different: uncovering distinctive features in the genomes of *Salmonella enterica* serovars Typhimurium and Typhi. *FEMS microbiology letters, 305*(1), 1-13.

Shah, N. P. (2007). Functional cultures and health benefits. *International Dairy Journal, 17*(11), 1262-1277.

Shimazaki, K. (2000). Lactoferrin; a marvelous protein in milk? *Animal Science Journal (Japan)*.

Shoaf-Sweeney, K. D., & Hutkins, R. W. (2008). Adherence, Anti-Adherence, and Oligosaccharides: Preventing Pathogens from Sticking to the Host. *Advances in food and nutrition research, 55*, 101-161.

Silva, S. V., & Malcata, F. X. (2005). Caseins as source of bioactive peptides. *International Dairy Journal, 15*(1), 1-15.

Simmons, B. P., Gelfand, M. S., Haas, M., Metts, L., & Ferguson, J. (1989). *Enterobacter sakazakii* infections in neonates associated with intrinsic contamination of a powdered infant formula. *Infection Control and Hospital Epidemiology*, 398-401.

Smacchi, E., & Gobbetti, M. (2000). Bioactive peptides in dairy products: synthesis and interaction with proteolytic enzymes. *Food Microbiology, 17*(2), 129-141.

Strydom, A., Cawthorn, D.-M., Cameron, M., & Witthuhn, R. C. (2012). Species of *Cronobacter*–A review of recent advances in the genus and their significance in infant formula milk. *International Dairy Journal, 27*(1), 3-12.

Tomita, M., Wakabayashi, H., Yamauchi, K., Teraguchi, S., & Hayasawa, H. (2002). Bovine lactoferrin and lactoferricin derived from milk: production and applications. *Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire, 80*(1), 109-112.

Van Acker, J., De Smet, F., Muyldermans, G., Bougatef, A., Naessens, A., & Lauwers, S. (2001). Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. *Journal of Clinical Microbiology*, *39*(1), 293-297.

Van der Kraan, M. I., Nazmi, K., Teeken, A., Groenink, J., van't Hof, W., Veerman, E. C., . . . Nieuw, A. A. V. (2005). Lactoferrampin, an antimicrobial peptide of bovine lactoferrin, exerts its candidacidal activity by a cluster of positively charged residues at the C-terminus in combination with a helix-facilitating N-terminal part. *Biological chemistry*, *386*(2), 137.

Vandenplas, Y. (2002). Oligosaccharides in infant formula. *British Journal of Nutrition*, *87*, S293-S296. doi: 10.1079/bjn/2002551

Walsh, D., Molloy, C., Iversen, C., Carroll, J., Cagney, C., Fanning, S., & Duffy, G. (2011). Survival characteristics of environmental and clinically derived strains of *Cronobacter sakazakii* in infant milk formula (IMF) and ingredients. *Journal of applied microbiology*, *110*(3), 697-703.

WHO. (2006). *Enterobacter sakazakii* and *Salmonella* in powdered infant formula: meeting report: Food & Agriculture Org.

WHO. (2007). Safe preparation, storage and handling of powdered infant formula Guidlines.

Zhu, S., Schnell, S., & Fischer, M. (2013). Growth inhibition of *Cronobacter* spp. strains in reconstituted powdered infant formula acidified with organic acids supported by natural stomach acidity. *Food Microbiology*.

Zocco, M. A., Ainora, M. E., Gasbarrini, G., & Gasbarrini, A. (2007). *Bacteroides thetaiotaomicron* in the gut: Molecular aspects of their interaction. *Digestive and liver disease, 39*(8), 707-712.

Zucht, H. D., Raida, M., Adermann, K., Magert, H. J., & Forssmann, W. G. (1995). Casocidin-I-A casein Alpha S2 drivd pepide exhibits antibacterial activity *Febs Letters, 372*(2-3), 185-188.

Table 1.1.

Nutrient	Minimum	Maximum
Protein (g)	1.8	3
Taurine (mg)		12
Lipids (g)	4.4	6
Linoleic acid (mg)	300	1200
Phospholipids (g)		2
Carbohydrates (g)	9	14
Lactose (g)	4.5	
Sucrose *		>20 %
Glucose (g) *		2
Pre-cooked/gelatinised starch		30 % of total carb content
FOS/GOS **		0.8 g/100 ml
Sodium (mg)	20	60
Potassium (mg)	60	160
Chloride (mg)	50	160
Calcium (mg)	50	140
Phosphorous (mg)	25	90
Magnesium (mg)	5	15
Iron (mg)	0.3	1.3
Zinc (mg)	0.5	1.5
Copper (µg)	35	100
Iodine (µg)	10	50
Selenium (µg)	1	9
Manganese (µg)	1	100
Fluoride (µg)		100
Vit A (µg-RE)	60	180
Vit D (µg)	1	2.5
Vit K (µg)	4	25
Vit E (mg α-TE)	***	5
Vit C (ascorbic acid) (mg)	10	30
Vit B1 (thiamine) (µg)	60	300
Vit B2 (riboflavin) (µg)	80	400
Vit B6 (pyroxidine) (µg)	35	175
Vit B12 (µg)	0.1	0.5
Niacin (µg)	300	1500
Folic Acid (µg)	10	50
Pantothenic acid (µg)	400	2000
Biotin (µg)	1.5	7.5
Choline (mg)	7	50
Inositol (mg)	1	10
cytidine 5´-monophosphate (mg)		2.5
uridine 5´-monophosphate (mg)		1.75
adenosine 5´-monophosphate (mg)		1.5
guanosine 5´-monophosphate (mg)		0.5
inosine 5´-monophosphate (mg)		1

* only allowed in protein hydrolysate

** shall not exceed 90 % oligogalactoysyl-lactose and 10 % high molecular weight oligofructosyl-saccharose

*** 0.5 g of polyunsaturated fatty acids expressed as linoleic acid as corrected for the double bonds but in no case less than 0.5 mg per 100 available kcal

Chapter2

Host specific diversity in *Lactobacillus johnsonii* as evidenced by a major chromosomal inversion and phage resistance mechanisms

Robert M. Kent, Caitriona M. Guinane, Sarah Norberg, Colin Hill, Gerald F. Fitzgerald, Catherine Stanton and R. Paul Ross

PLoS ONE, 2011 Volume 6, Issue 4: e18740 2011.

2.1. Abstract

Genetic diversity and genomic rearrangements are a driving force in bacterial evolution and niche adaptation. We sequenced and annotated the genome of *Lactobacillus johnsonii* DPC6026, a strain isolated from the porcine intestinal tract. Although the genome of DPC6026 is similar in size (1.97mbp) and GC content (34.8%) to the sequenced human isolate *L. johnsonii* NCC 533, a large symmetrical inversion of approximately 750kb differentiated the two strains. Comparative analysis among 12 other strains of *L. johnsonii* including 8 porcine, 3 human and 1 poultry isolate indicated that the genome architecture found in DPC6026 is more common within the species than that of NCC 533. Furthermore a number of unique features were annotated in DPC6026, some of which are likely to have been acquired by horizontal gene transfer (HGT) and contribute to protection against phage infection. A putative type III restriction-modification system was identified, as were novel Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) elements. Interestingly, these particular elements are not widely distributed among *L. johnsonii* strains. Taken together these data suggest intra-species genomic rearrangements and significant genetic diversity within the *L. johnsonii* species and indicate towards a host-specific divergence of *L. johnsonii* strains with respect to genome inversion and phage exposure.

2.2. Introduction

The Gastro-intestinal (GI) tract is colonized by a vast and diverse community of microbes. Lactobacilli represent an important part of the natural gut microbiome of both humans and animals and have been extensively studied for their health promoting properties. *Lactobacillus johnsonii* is a member of the closely related "acidophilus complex" of lactobacilli and an autochthonous species of the gastro-intestinal tract. *L. johnsonii* strains are of interest due to the number of probiotic characteristics associated with this species, including immunomodulation, (Haller *et al.*, 2000; Ibnou-Zekri, Blum, Schiffrin, & von der Weid, 2003; Inoue, Otsuka, Nishio, & Ushida, 2007; Kaburagi *et al.*, 2007) attachment to epithelial cells (Granato *et al.*, 2004; Neeser *et al.*, 2000) and pathogen exclusion (Bernet, Brassart, Neeser, & Servin, 1994; La Ragione, Narbad, Gasson, & Woodward, 2004; Wegmann *et al.*, 2009).

For organisms commonly found in GI tract such *Lactobacillus acidophilus*, *Lactobacillus gasseri* and *L. johnsonii* there are a number of genome sequences available which have identified genetic traits that most likely function in gastric survival and promote interactions with the intestinal mucosa (E. Altermann *et al.*, 2005; Azcarate-Peril *et al.*, 2008; Pridmore *et al.*, 2004; Wegmann *et al.*, 2009). It has been proposed that GI-associated strains have adapted to their niche with a specialized set of metabolic and surface-related proteins (O'Sullivan *et al.*, 2009). In the *L. johnsonii* NCC 533 genome for example, large cell surface proteins were identified thought to be involved glycoprotein adhesion and persistence in the intestinal tract (Emmanuel Denou *et al.*, 2008; Pridmore *et al.*, 2004). A common trait documented also for this group of organisms is a general lack of genes encoding biosynthetic pathways

for amino acids, purine nucleotides and cofactors which may be reflective of their "symbiont" nature and an abundance of ABC transporters, peptidases and phosphotransferases (E. Altermann *et al.*, 2005; Azcarate-Peril *et al.*, 2008; Pridmore *et al.*, 2004).

Genomic heterogeneity within a bacterial species can be driven by the selective pressure of different environmental niches and can result from recombination events and the presence of mobile genetic elements (MGE), such as bacteriophage and IS elements. Genetic diversity and horizontal gene transfer (HGT) among closely related gut lactobacilli has been observed (Azcarate-Peril *et al.*, 2008; Berger *et al.*, 2007; Canchaya, Claesson, Fitzgerald, van Sinderen, & O'Toole, 2006; Pridmore *et al.*, 2004). Within the 'acidophilus complex' previous polyphasic analysis and comparative genomic analysis has indicated significant inter and intra-species diversity among MGE and at the region around the terminus of replication (Berger *et al.*, 2007). The possibility of genomic rearrangements at this region within *L. johnsonii* strains has also been previously suggested (E. Denou *et al.*, 2008) (Contribution by Pridmore D; (Klaenhammer *et al.*, 2002)).

Here we present the whole genome sequence of the porcine *L. johnsonii* isolate DPC6026 (previously named *L. acidophilus* DPC6026; (Hayes, Ross, Fitzgerald, Hill, & Stanton, 2006)) and explore the genetic content, the potential genomic rearrangements and diversity within the *L. johnsonii* species. This study also presents a number of MGEs novel to the *L. johnsonii* species and previously unidentified phage resistance mechanisms.

2.3. Materials and Methods

2.3.1. Bacterial strains, growth conditions

L. johnsonii DPC6026 was originally isolated from a porcine small intestine (Hayes *et al.*, 2006). This strain was previously identified as *L. acidophilus* DPC6026 however more refined 16S sequencing demonstrated that it belongs to the *L. johnsonii* species rather than the closely related *L. acidophilus*. All isolates used in this study are outlined in Table 2.1. Cultures isolated from faecal samples were as previously described (Casey *et al.*, 2007) and screened on Lactobacilli selective agar (LBS). *L. johnsonii* strains were cultured anaerobically in MRS (Difco) media at 37ºC.

2.3.2. Speciation of isolates

DNA was extracted from 10ml overnight cultures using the procedure previously described (Hoffman & Winston, 1987). The 16S rDNA were amplified from gDNA from each strain using species specific primers for *L. johnsonii, L. gasseri* and *L. acidophilus* as previously described (Walter *et al.*, 2000). Chosen isolates were confirmed by amplification using 16S Eubacterial primers (Takai & Horikoshi, 2000) and the 16S region was sequenced by conventional Sanger sequencing. The species was determined by nucleotide alignments (>98%) with deposited species in the NCBI database. Strains of the same species were confirmed to be different isolates by Pulsed-Field-Gel-Electrophoresis using the *apaI* enzyme (not shown).

2.3.3. Phylogenetic analysis

Reconstruction of evolutionary relationships were carried out using the MEGA 4 package (Tamura, Dudley, Nei, & Kumar, 2007). 16S rRNA sequence data was obtained from GenBank (*L. johnsonii* AE017198, *L. gasseri* CP000413, *L. acidophilus* CP000033, *Lactobacillus sakei* CR936503, *Lactobacillus reuteri* CP000705, *Lactobacillus fermentum* AP008937, *Lactobacillus brevis* HQ622718, *Lactobacillus plantarum* CP002222, *Lactobacillus salivarius* CP000233 and *Lactobacillus casei* FM177140) and was used to construct a consensus neighbour joining tree from 500 bootstrapping replicates.

2.3.4. Genome sequencing, assembly and comparative genomic analysis

Massively parallel 454 pyrosequencing with paired end tags of DPC6026 to a coverage of 23X was performed by 454 Beckmann Coulter Genomics (www.beckmancoulter.com) on a FLX sequencer followed by initial assembly in to 83 contigs using the Newbler program (***roche***-applied-science.com). Order and orientation of assembled contigs and predicted scaffolds was determined using the published genome sequences of *L. johnsonii* NCC533 (Pridmore *et al.*, 2004) and *L. johnsonii* FI9785 (Wegmann *et al.*, 2009). Primers were designed at gap edges using primer3 (Rozen & Skaletsky, 2000) for PCR amplification of gap regions using Platinum Hi-fidelity PCR Supermix (Invitrogen) or Kod DNA Polymerase (Novagen). Reactions were performed in a Biometra TGradient followed by directed sequencing of PCR products by primer walking, and whole genome assembly was performed using the PHRED-PHRAP-CONSED package (Ewing & Green,

1998; Gordon, 2003). Raw assembly reads were visualised and verified using the programme Hawkeye (Amos) (Schatz, Phillippy, Shneiderman, & Salzberg, 2007). Unmapped contigs were mapped using combinatorial PCR followed by primer walking. Frameshifts and ribosomal operons were annotated but not verified by conventional Sanger sequencing.

Coding regions were predicted using Glimmer 2 (Delcher, Harmon, Kasif, White, & Salzberg, 1999) and annotation was performed using GAMOLA (Eric Altermann & Klaenhammer, 2003). Complementary annotation data were provided by the SEED (Overbeek *et al.*, 2005) and the RAST annotation server (Aziz *et al.*, 2008). Data was manually curated (Oct 2010) using Artemis software V11 (Tim Carver *et al.*, 2008) where additional programmes were used including, PROSITE (www.expasy.ch) RBS finder (Delcher *et al.*, 1999) and GATU (Tcherepanov, Ehlers, & Upton, 2006). Comparative genomics was performed using the Artemis comparison tool (T. J. Carver *et al.*, 2005; Darling, Treangen, Messeguer, & Perna, 2007) and MAUVE software (Darling *et al.*, 2007). Circular maps were created using DNA plotter (T. Carver, Thomson, Bleasby, Berriman, & Parkhill, 2009).

2.3.4. Detection of novel features of DPC6026

Primers specific to the regions of genomic rearrangements were designed based on the genome sequences of DPC6026 and NCC533 (Table 2.2). Primers specific to 4 regions of the integrated prophage and the site of prophage integration were designed based on DPC6026. The primers used to detect the CRISPR elements and restriction modification systems were designed based on DPC6026 with at least 2 different specific combination of

primers used (Table 2.2). PCRs were performed on all strains (Table 2.1) to confirm genomic structure and elements using either Platinum Hi-fidelity PCR Supermix (Invitrogen) or Biotaq (Bioline).

2.3.5. Phage Induction

The induction of the prophage Φlj6026 was attempted by heat where the culture containing the phage was subjected to a thermal stress of 42°C for 1 hour or following the addition of mitomycin C (2-6µg/ml) (Sigma Chemical Co., St. Louis, MO). *L. johnsonii* was grown overnight in MRS broth at 37°C anaerobically. Fresh broth was inoculated with a 1% inoculum of the overnight strain and grown to $OD600_{nm}$ 0.1-0.3. The culture was centrifuged and the supernatant was filtered through a 0.45µm filter. The filtered supernatant was spotted on an overlay of a range of indicator strains and prophage release was determined by observing zones of lysis following incubation at 37ºC for 24h (Table 2.1).

2.3.6. Public data sources

The genome sequence of *L. johnsonii* DPC6026 is available from GenBank/EMBL under the accession number CP002464.

2.4. Results

2.4.1. General features of the genome of *L. johnsonii* DPC6026

The DPC6026 genome consists of a singular circular chromosome of 1.97mbp with an average G+C content of 34.8% and does not harbour any plasmids (Figure 2.1.). Overall, the genome of DPC6026 was highly similar to the previously sequenced members of the species *L. johnsonii* in size, G+C content and gene synteny (Pridmore *et al.*, 2004; Wegmann *et al.*, 2009). Total GC-skew analysis and the ORF orientation drift identified the *oriC* proximal to *dnaA* and the *terC* at ~1.05mb (Figure 2.1.). *In silico* analysis predicted 1795 protein coding genes.

Phylogenetic analysis based on the 16S rRNA gene sequences of *L. johnsonii* and other Lactobacilli revealed, in accordance with previous work (Zhang, Ye, Yu, & Shi), that *L. johnsonii* is closely related to other *L. acidophilus* complex members (Figure 2.2.). It is most related however to the gut bacterium *L. gasseri* as they occupy the same branch on the phylogenetic tree (Figure 2.2.).

2.4.2. Genetic homogeneity of the core genome of *L. johnsonii* sequenced isolates

Among the genes encoded in DPC6026, 150 genes (~9%) were not found in the human isolate NCC 533 (Pridmore *et al.*, 2004), 84 genes (5%) were novel to the *L. johnsonii* species and just 18 (1%) genes were not previously identified in the genus *Lactobacillus*. These results are in accordance with previous work by Berger *et al.*, (2007) which indicated a conservation of genes between *L. johnsonii* isolates to be between 83-92% with 5% strain specific

genes (Berger *et al.*, 2007). Genes novel to DPC6026 largely represented mobile DNA including genes encoding proteins with homology to phage related proteins, transposase and insertion elements.

The metabolic capabilities and biosynthetic pathways of DPC6026 are in accordance with the reliance of *L. johnsonii* on the surrounding environment for nutrients (Pridmore *et al.*, 2004). DPC6026 has a high number of PTS systems and ABC transporters enabling utilization of sugars available in the GI tract, similar to the closely related genomes of the 'acidophilus complex' (E. Altermann *et al.*, 2005; Azcarate-Peril *et al.*, 2008; Pridmore *et al.*, 2004). There were also 20 proteins with homology to peptidases annotated in the DPC6026 genome, including eight aminopeptidases, six dipeptidases and three endopeptidases. This is in agreement with the dependency of the *L. johnsonii* on exogenous amino acids for growth. The extracellular cell wall bound proteinase (LJ1840) that was annotated in NCC533 however was not found in the porcine strain. PCR analysis indicated that this was not present in any of the porcine isolates tested (not shown). This was surprising as the *L. johnsonii* DPC6026 strain was previously indicated to have proteolytic ability (Hayes *et al.*, 2006) and it was reported that DPC6026 generates antimicrobial peptides from casein in milk-based fermentations (Hayes *et al.*, 2006). However, our phenotypic analysis supports the genomic prediction that this strain alone cannot hydrolyse milk efficiently and further analysis to the possibility of indigenous microbiota from the fermentation substrates contributing to proteolysis and the liberation the antimicrobial peptides is ongoing.

The abundance of transport and regulatory proteins is also reflected in the genomes of *L. johnsonii* NCC533 (Pridmore *et al.*, 2004) and FI9785

(Wegmann *et al.*, 2009), however, there were differences in the genetic content of these proteins within each of the three genomes. These differences may be due to a differing GI environment among the disparate host species. Of note also is the differing complement of adhesion and cell surface proteins present in DPC6026 and in NCC533. Pridmore *et al.*, (2004) identified cell surface components (LJ0382, LJ0391, LJ1128, LJ1711, LJ1839) in the human isolate thought to be unique to NCC 533 and predicted to be secreted and attached to the cell surface. These proteins were all either absent or appeared to be fragmented (LJP0353, LJP0366, LJP0707 and LJP1463) in the porcine isolate. This could further indicate the importance of these proteins in colonisation of a human host.

2.4.3. Genome Architecture and Synteny

Despite a relatively conserved gene synteny between the sequenced *L. johnsonii* isolates, there is a large (~750kb) symmetrical inversion across the replication axis between the human isolate NCC 533 and the porcine isolate DPC6026 (Figure 2.3.). Whole genome alignments also indicate that the porcine isolate DPC6026 and poultry strain FI9785 share the same genomic arrangement (not shown).

Despite the large genomic inversion, the *ori* and *ter* regions do not appear to be disrupted based on the location of the inversion and on the GC-skew data. Indeed, while a slightly imbalanced replichore is evident, there is not a significant change in the replichore sizes of the two strains (Figure 2.1.). The existence of the inversion also did not lead to a significant difference in the growth rate of the strains (not shown). The inversion between DPC6026 and

NCC 533 was confirmed by site-specific PCR. Two primer pair sets were designed that overlap the left and right junction sites in DPC6026 and yield an amplicon in this strain but should not in NCC533 if this region had undergone an inversion. When the 2 primer sets are used in the combination (F/F) and (R/R), a PCR product is generated in NCC533 but not in DPC6026, thus confirming the differing genomic structures and an inversion event (Figure 2.6.). The genomic structure of 8 further porcine isolates and 2 human isolates of *L. johnsonii* was investigated using these primer sets. Results indicate all the porcine isolates harboured the same genomic structure as DPC6026. One human isolate, a type strain ATCC12088, harboured the same genomic arrangement as NCC533 (Figure 2.4.). The second human/type strain tested did not give a PCR product for either structure.

At both the right and left junction sites in NCC 533 a 1,460bp sequence of inverted repeats was identified including an insertion element ISLjo2 of the ISL3 family which may be responsible for the inversion event in NCC 533 or in an ancestral strain (Figure 2.6.). It has been documented that recombination involving direct repeats can lead to genomic inversions (Achaz, Coissac, Netter, & Rocha, 2003) and has been suggested previously as a possibility for the NCC 533 strain [18]. Differing genomic structures are also apparent on alignments of *L. johnsonii* strains with the closely related *L. gasseri* (Figure 2.2) (Azcarate-Peril *et al.*, 2008; Kaburagi *et al.*, 2007) indicating rearrangements in this group of bacteria can occur frequently and 'X-shaped' inversions across the replication terminus between species of the acidophilus group have been documented (Berger *et al.*, 2007).

Based on the comparative genomic PCR assays it is likely that the structure of DPC6026 is the more commonly found genomic structure of *L. johnsonii*. The repeat region and IS element present in NCC 533 was not present at this location in DPC6026 but was however at 4 other locations within the porcine genome. Given that this is a common element in *L. johnsonii* genomes it may be an indication of significant genome plasticity within the species.

2.4.4. Novel Mobile Genetic Elements of *L. johnsonii*

Acquisition of genes by HGT is considered a major driving force in bacterial evolution and can impact on genomic structure and stability. Laterally acquired DNA provides a readily available pool of genes for developing physiological properties that are helpful in a particular niche. A number of previously unidentified MGEs were identified in the DPC6026 genome (Figure 2.5a., 2.5b. and 2.5c.).

(i) Integrated prophage Prophages of *L. johnsonii* have been previously characterised (Ventura, Canchaya, Pridmore, Berger, & Brussow, 2003; Ventura, Canchaya, Pridmore, & Brussow, 2004) and are indicated to have large role in the diversification within the species (Canchaya, Fournous, & Brussow, 2004; E. Denou *et al.*, 2008). Genomic analyses revealed the presence of one complete prophage sequence, Φlj6026 (LJP0764-LJP0819), which is integrated next to tRNA loci at ~900kbp within the DPC6026 genome. Of note this prophage is within the region that is inverted relative to the human isolate, however is integrated in the opposite orientation (Figure 2.3.).

Φlj6026 is 43,608bp in length and encodes 58 proteins comprising the typical phage regions of integration, replication, packaging, structural and lysis domains (Figure 2a.). Φlj6026 phage shares an integration site with the NCC533 phage Φlj928 but most nucleotide identity with the NCC533 phage Φlj965 (Ventura *et al.*, 2004). We attempted to induce Φlj6026 by mitomycin C and heat treatments using the closely related *L. johnsonii*, *L. acidophilus* and *L. gasseri* strains as indicator organisms. Release of the prophage was not detected by the methods used. The apparent non-functionality of lj6026 is in accordance with previous work that has indicated that the related prophages Φlj965 and Φlj928 are not inducible (Ventura *et al.*, 2004). Distribution of Φlj6026 was investigated among *L. johnsonii* strains and strains of the closely related species *L. gasseri* and *L. acidophilus* (Figure 2.4.). Of the isolates tested only the porcine *L. johnsonii* isolate DPC6092, in addition to DPC6026, appeared to harbour the full phage. Partial matches were obtained with the human type strain DSM10533. Based on *in silico* analysis, the poultry isolate, FI9785 was found to also have a similar but not identical phage within the genome (Figure 2.4.).

(ii) IS Elements IS elements are recognisable by DNA recombination machinery and can play a large role in chromosomal rearrangements. The annotation of DPC6026 identified 51 gene features with similarity to either characterized or predicted transposases or to putatively truncated or degenerate transposase enzymes. The type of IS elements differed considerably among the *L. johnsonii* sequenced isolates. In DPC6026, insertion elements of the family IS1223 that had been identified in NCC 533 and FI9785 were found in addition

to copies of IS605 of in *L. acidophilus* NCFM (E. Altermann *et al.*, 2005) and ISLhe1 of *L. helveticus* DPC4571 (Callanan *et al.*, 2008).

(iii) Restriction Modification System Restriction Modification (RM) systems function to cleave foreign DNA and are the most common systems used to degrade incoming phage DNA. A novel restriction modification system was annotated on the genome of DPC6026. It is located at ~1.57mbp and consists of a restriction (LJP1436) and a methylase (LJP1437) component typical of the type III family of RM systems (Figure 2.5b.). This type III system has not been previously identified in *L. johnsonii* and, although it does share amino acid identity with the restriction component of *L. gasseri* (90%) (Azcarate-Peril *et al.*, 2008) and the modification component of *Lactobacillus fermentum* (55%) (Morita *et al.*, 2008), the complete system does not have a close homolog in any sequenced LAB. The type III R/M system is located in a ~15kb region that is absent from NCC533 (Figure 2.5b.). This region also contains a protein (LJP1446) with identity (30% amino acid) to abortive phage resistance proteins which suggest a combination of different phage defence mechanisms present. Comparative genomic analysis indicated that this element is not widely distributed as it was not found in any of the other strains tested in this study (Figure 2.4.).

2.4.5. Analysis of the CRISPR locus in DPC6026

Clustered regularly interspaced short palindromic repeats (CRISPR) represent a family of DNA repeats shown to provide acquired immunity against foreign genetic elements (Barrangou *et al.*, 2007; Horvath & Barrangou). A novel CRISPR-cas system of 6.1kb was identified in the

genome of the porcine isolate. This element is positioned at the centre of the region that is inverted relative to NCC 533. A slight alteration in GC content compared to the surrounding region suggests that this element was transferred by horizontal gene transfer (Figure 2.3c.).

CRISPR systems have been identified in nine *Lactobacillus* genomes to date (Horvath *et al.*, 2009), including closely related members of the acidophilus complex, *L. acidophilus* (E. Altermann *et al.*, 2005) and *L. helveticus* (Callanan *et al.*, 2008). Despite this, the content of the CRISPR loci (LJP1108-1110) in *L. johnsonii* was not identical when compared to elements in closely related organisms. Differences within the repeat region and in the CRISPR associated (Cas) proteins were also observed. The 36bp repeat 5`ATCTAAACCTTATTGATCTAACAACCATCTAAAAC3` is present 28 times with 27 unique spacer sequences. The three genes upstream of the repeats encode homologues for Cas proteins which are invariably associated with CRISPR repeats (Figure 2c.). This system does share some similarities with CRISPR loci in *L. salivarius* UCC118 (Claesson *et al.*, 2006) and *Lactobacillus casei* ATCC 334 (Cai, Thompson, Budinich, Broadbent, & Steele, 2009). Upstream of the first *cas* gene, remnant CRISPR repeats were also identified. This phenomenon has previously been reported in *Streptococcus thermophilus* (Horvath *et al.*, 2008) and *Bifidobacterium animalis* (Barrangou *et al.*, 2009) and *Bifidobacterium adolescentis* (Horvath *et al.*, 2009). The distribution of the element in other *L. johnsonii* strains was investigated and it was indicated by PCR analysis that only the *L. johnsonii* porcine isolates DPC6092 and DPC6214 contained a similar element indicating these elements may not be widespread in *L. johnsonii* strains (Figure 2.4.).

2.5. Discussion

The GI tract is a complex environment that provides a variety of ecological challenges. The significant differences presented in this study highlight strain specificity among the species of the gut. Importantly based on genomic structure analysis it is suggested that the human strain of *L. johnsonii* diverged from both animal and poultry isolates at some time, however, more representative strains of each species would need to be sequenced to shed more light on this.

The chromosomal inversion, a characteristic 'X-shaped' symmetrical rearrangement in this study occurs within strains of the same species and based on previous analysis on closely related species it would seem that inversions across the replication axis occurs frequently in this group of Lactobacilli during evolution (Berger *et al.*, 2007; Canchaya *et al.*, 2006). Large genomic inversions are generally not common among bacteria of the same species but have been described in a number of pathogens such as *E. coli* (Kotewicz, Jackson, LeClerc, & Cebula, 2007), *Salmonella* sp. (Liu & Sanderson, 1996), *Yersinia pestis* (Deng *et al.*, 2002), *Staphylococcus aureus* (Shukla, Kislow, Briska, Henkhaus, & Dykes, 2009) and also in the non-pathogenic *Lactococcus lactis* (Daveran-Mingot, Campo, Ritzenthaler, & Le Bourgeois, 1998). It has been indicated that inversions may not necessarily have a selective advantage or disadvantage or dramatic phenotypic effect (Daveran-Mingot *et al.*, 1998), however rearrangements have also been shown to have an effect on phenotype and cell fitness (Hill & Gray, 1988). Although both strains NCC 533 (Cai *et al.*, 2009) and DPC6026 (Figure 2.1.) appear to have a slightly unbalanced

replichore, it does not appear to have had a detrimental effect on the growth of the strains (not shown).

Despite the relative genetic homogeneity among the core regions of the sequenced *L. johnsonii* and the gene content reflecting a similar metabolic lifestyle in the GI tract, there are significant differences among adhesion proteins, mobile genetic elements and cell protection mechanisms. Notably, large differences between *L. johnsonii* isolates are in the phage complement and in putative phage resistance mechanisms. Phage integration within a replichore may influence genome stability leading to chromosomal inversions between highly conserved regions (Nakagawa *et al.*, 2003). *L. johnsonii* phages Φlj965, Φlj928 (Ventura *et al.*, 2003; Ventura *et al.*, 2004) and Φlj771 (E. Denou *et al.*, 2008) have been characterised and have been shown to contribute to strain diversity within the species (E. Denou *et al.*, 2008). Φlj6026 presented in this study is integrated within the region inverted to NCC533 and although it shares most homology with Φlj965 they are not integrated at the same site in the chromosome suggesting the phage was taken up separately by the strains and therefore it may have a particular advantage to the cell. However the functionality of this phage was not confirmed in this study. The existence of unique phage resistance mechanisms indicate that the DPC6026 genome may preferentially defend against foreign DNA integration using the CRISPR loci and/or the type III restriction modification system. As the particular elements were not found in many of the other strains tested, strain specific mechanisms for phage defence appear to be present.

It has been documented that the flora of the gut is thought to be largely modulated by the selective pressure imposed by the host and the other

microbiota present (Ley, Peterson, & Gordon, 2006). As a commensal of the GI tract, *L. johnsonii* appears to be a versatile and changing bacterium that can perhaps adapts to its niche by acquiring mobile genetic elements and through chromosomal recombination events.

2.6. Acknowledgements

This work was funded by an Enterprise Ireland commercialisation fund CFTD/07/116.

Caitriona Guinane assisted with genome assembly, comparison studies and manuscript preparation.

2.7. References

Achaz, G., Coissac, E., Netter, P., & Rocha, E. P. (2003). Associations between inverted repeats and the structural evolution of bacterial genomes. *Genetics, 164*(4), 1279-1289.

Altermann, E., & Klaenhammer, T. R. (2003). GAMOLA: a new local solution for sequence annotation and analyzing draft and finished prokaryotic genomes. *Omics A Journal of Integrative Biology, 7*(2), 161-169.

Altermann, E., Russell, W. M., Azcarate-Peril, M. A., Barrangou, R., Buck, B. L., McAuliffe, O., . . . Klaenhammer, T. R. (2005). Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. *Proc Natl Acad Sci U S A, 102*(11), 3906-3912. doi: 0409188102 [pii] 10.1073/pnas.0409188102

Azcarate-Peril, M. A., Altermann, E., Goh, Y. J., Tallon, R., Sanozky-Dawes, R. B., Pfeiler, E. A., . . . Klaenhammer, T. R. (2008). Analysis of the genome sequence of *Lactobacillus gasseri* ATCC 33323 reveals the molecular basis of an autochthonous intestinal organism. *Appl Environ Microbiol, 74*(15), 4610-4625. doi: AEM.00054-08 [pii] 10.1128/AEM.00054-08

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., . . . Zagnitko, O. (2008). The RAST Server: rapid annotations using subsystems technology. *BMC Genomics, 9*, 75. doi: 1471-2164-9-75 [pii] 10.1186/1471-2164-9-75

Barrangou, R., Briczinski, E. P., Traeger, L. L., Loquasto, J. R., Richards, M., Horvath, P., . . . Roberts, R. F. (2009). Comparison of the complete genome sequences of *Bifidobacterium animalis* subsp. *lactis* DSM 10140 and Bl-04. *J Bacteriol, 191*(13), 4144-4151. doi: JB.00155-09 [pii] 10.1128/JB.00155-09

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., . . . Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. *Science, 315*(5819), 1709-1712. doi: 315/5819/1709 [pii] 10.1126/science.1138140

Berger, B., Pridmore, R. D., Barretto, C., Delmas-Julien, F., Schreiber, K., Arigoni, F., & Brussow, H. (2007). Similarity and differences in the *Lactobacillus acidophilus* group identified by polyphasic analysis and comparative genomics. *J Bacteriol, 189*(4), 1311-1321. doi: JB.01393-06 [pii] 10.1128/JB.01393-06

Bernet, M., Brassart, D., Neeser, J., & Servin, A. (1994). *Lactobacillus acidophilus* LA 1 binds to cultured human intestinal cell lines and inhibits cell attachment and cell invasion by enterovirulent bacteria. *Gut, 35*(4), 483-489.

Cai, H., Thompson, R., Budinich, M. F., Broadbent, J. R., & Steele, J. L. (2009). Genome sequence and comparative genome analysis of *Lactobacillus casei*: insights into their niche-associated evolution. *Genome Biol Evol, 1*, 239-257. doi: 10.1093/gbe/evp019

Callanan, M., Kaleta, P., O'Callaghan, J., O'Sullivan, O., Jordan, K., McAuliffe, O., . . . Ross, R. P. (2008). Genome sequence of *Lactobacillus helveticus*, an organism distinguished by selective gene loss and insertion sequence element expansion. *J Bacteriol, 190*(2), 727-735. doi: JB.01295-07 [pii] 10.1128/JB.01295-07

Canchaya, C., Claesson, M. J., Fitzgerald, G. F., van Sinderen, D., & O'Toole, P. W. (2006). Diversity of the genus *Lactobacillus* revealed by comparative genomics of five species. *Microbiology, 152*(Pt 11), 3185-3196. doi: 152/11/3185 [pii] 10.1099/mic.0.29140-0

Canchaya, C., Fournous, G., & Brussow, H. (2004). The impact of prophages on bacterial chromosomes. *Mol Microbiol, 53*(1), 9-18. doi: 10.1111/j.1365-2958.2004.04113.x MMI4113 [pii]

Carver, T., Berriman, M., Tivey, A., Patel, C., Böhme, U., Barrell, B. G., . . . Rajandream, M.-A. (2008). Artemis and ACT: viewing, annotating and comparing sequences stored in a relational database. *Bioinformatics, 24*(23), 2672-2676.

Carver, T., Thomson, N., Bleasby, A., Berriman, M., & Parkhill, J. (2009). DNAPlotter: circular and linear interactive genome visualization. *Bioinformatics, 25*(1), 119-120. doi: btn578 [pii] 10.1093/bioinformatics/btn578

Carver, T. J., Rutherford, K. M., Berriman, M., Rajandream, M. A., Barrell, B. G., & Parkhill, J. (2005). ACT: the Artemis Comparison Tool. *Bioinformatics, 21*(16), 3422-3423. doi: bti553 [pii] 10.1093/bioinformatics/bti553

Casey, P. G., Gardiner, G. E., Casey, G., Bradshaw, B., Lawlor, P. G., Lynch, P. B., . . . Hill, C. (2007). A five-strain probiotic combination reduces pathogen shedding and alleviates disease signs in pigs challenged with *Salmonella enterica* Serovar Typhimurium. *Appl Environ Microbiol, 73*(6), 1858-1863. doi: AEM.01840-06 [pii] 10.1128/AEM.01840-06

Claesson, M. J., Li, Y., Leahy, S., Canchaya, C., van Pijkeren, J. P., Cerdeño-Tárraga, A. M., . . . Collins, J. K. (2006). Multireplicon genome architecture of *Lactobacillus salivarius*. *Proceedings of the National Academy of Sciences, 103*(17), 6718-6723.

Darling, A. E., Treangen, T. J., Messeguer, X., & Perna, N. T. (2007). Analyzing patterns of microbial evolution using the mauve genome alignment system. *Methods Mol Biol, 396*, 135-152. doi: 1-59745-515-6:135 [pii]

Daveran-Mingot, M. L., Campo, N., Ritzenthaler, P., & Le Bourgeois, P. (1998). A natural large chromosomal inversion in *Lactococcus lactis* is mediated by homologous recombination between two insertion sequences. *J Bacteriol, 180*(18), 4834-4842.

Delcher, A. L., Harmon, D., Kasif, S., White, O., & Salzberg, S. L. (1999). Improved microbial gene identification with GLIMMER. *Nucleic Acids Res, 27*(23), 4636-4641. doi: gkc675 [pii]

Deng, W., Burland, V., Plunkett, G., 3rd, Boutin, A., Mayhew, G. F., Liss, P., . . . Perry, R. D. (2002). Genome sequence of *Yersinia pestis* KIM. *J Bacteriol, 184*(16), 4601-4611.

Denou, E., Pridmore, R. D., Berger, B., Panoff, J.-M., Arigoni, F., & Brüssow, H. (2008). Identification of genes associated with the long-gut-persistence phenotype of the probiotic *Lactobacillus johnsonii* strain NCC533 using a combination of genomics and transcriptome analysis. *Journal of bacteriology, 190*(9), 3161-3168.

Denou, E., Pridmore, R. D., Ventura, M., Pittet, A. C., Zwahlen, M. C., Berger, B., . . . Brussow, H. (2008). The role of prophage for genome diversification within a clonal lineage of *Lactobacillus johnsonii*: characterization of the defective prophage LJ771. *J Bacteriol, 190*(17), 5806-5813. doi: JB.01802-07 [pii] 10.1128/JB.01802-07

Ewing, B., & Green, P. (1998). Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome research, 8*(3), 186-194.

Gordon, D. (2003). Viewing and editing assembled sequences using Consed. *Current protocols in bioinformatics*, 11.12. 11-11.12. 43.

Granato, D., Bergonzelli, G. E., Pridmore, R. D., Marvin, L., Rouvet, M., & Corthesy-Theulaz, I. E. (2004). Cell surface-associated elongation factor Tu mediates the attachment of *Lactobacillus johnsonii* NCC533 (La1) to human intestinal cells and mucins. *Infect Immun, 72*(4), 2160-2169.

Haller, D., Bode, C., Hammes, W. P., Pfeifer, A. M., Schiffrin, E. J., & Blum, S. (2000). Non-pathogenic bacteria elicit a differential cytokine response by intestinal epithelial cell/leucocyte co-cultures. *Gut, 47*(1), 79-87.

Hayes, M., Ross, R. P., Fitzgerald, G. F., Hill, C., & Stanton, C. (2006). Casein-derived antimicrobial peptides generated by *Lactobacillus acidophilus* DPC6026. *Appl Environ Microbiol, 72*(3), 2260-2264. doi: 72/3/2260 [pii] 10.1128/AEM.72.3.2260-2264.2006

Hill, C. W., & Gray, J. A. (1988). Effects of chromosomal inversion on cell fitness in *Escherichia coli* K-12. *Genetics, 119*(4), 771-778.

Hoffman, C. S., & Winston, F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene, 57*(2-3), 267-272.

Horvath, P., & Barrangou, R. CRISPR/Cas, the immune system of bacteria and archaea. *Science, 327*(5962), 167-170. doi: 327/5962/167 [pii] 10.1126/science.1179555

Horvath, P., Coute-Monvoisin, A. C., Romero, D. A., Boyaval, P., Fremaux, C., & Barrangou, R. (2009). Comparative analysis of CRISPR loci in lactic acid bacteria genomes. *Int J Food Microbiol, 131*(1), 62-70. doi: S0168-1605(08)00280-8 [pii] 10.1016/j.ijfoodmicro.2008.05.030

Horvath, P., Romero, D. A., Coute-Monvoisin, A. C., Richards, M., Deveau, H., Moineau, S., . . . Barrangou, R. (2008). Diversity, activity, and evolution of CRISPR loci in *Streptococcus thermophilus*. *J Bacteriol, 190*(4), 1401-1412. doi: JB.01415-07 [pii] 10.1128/JB.01415-07

Ibnou-Zekri, N., Blum, S., Schiffrin, E. J., & von der Weid, T. (2003). Divergent patterns of colonization and immune response elicited from two intestinal *Lactobacillus* strains that display similar properties in vitro. *Infect Immun, 71*(1), 428-436.

Inoue, R., Otsuka, M., Nishio, A., & Ushida, K. (2007). Primary administration of *Lactobacillus johnsonii* NCC533 in weaning period suppresses the elevation of proinflammatory cytokines and CD86 gene expressions in skin lesions in NC/Nga mice. *FEMS Immunol Med Microbiol, 50*(1), 67-76. doi: FIM233 [pii] 10.1111/j.1574-695X.2007.00233.x

Kaburagi, T., Yamano, T., Fukushima, Y., Yoshino, H., Mito, N., & Sato, K. (2007). *Effect of Lactobacillus johnsonii* La1 on immune function and serum albumin in aged and malnourished aged mice. *Nutrition, 23*(4), 342-350. doi: S0899-9007(07)00023-8 [pii] 10.1016/j.nut.2007.02.001

Klaenhammer, T., Altermann, E., Arigoni, F., Bolotin, A., Breidt, F., Broadbent, J., . . . Siezen, R. (2002). Discovering lactic acid bacteria by genomics. *Antonie Van Leeuwenhoek, 82*(1-4), 29-58.

Kotewicz, M. L., Jackson, S. A., LeClerc, J. E., & Cebula, T. A. (2007). Optical maps distinguish individual strains of *Escherichia coli* O157 : H7. *Microbiology, 153*(Pt 6), 1720-1733. doi: 153/6/1720 [pii] 10.1099/mic.0.2006/004507-0

La Ragione, R., Narbad, A., Gasson, M., & Woodward, M. (2004). In vivo characterization of *Lactobacillus johnsonii* FI9785 for use as a defined competitive exclusion agent against bacterial pathogens in poultry. *Letters in Applied Microbiology*, *38*(3), 197-205.

Ley, R. E., Peterson, D. A., & Gordon, J. I. (2006). Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell, 124*(4), 837-848. doi: S0092-8674(06)00192-9 [pii] 10.1016/j.cell.2006.02.017

Liu, S. L., & Sanderson, K. E. (1996). Highly plastic chromosomal organization in *Salmonella typhi*. *Proc Natl Acad Sci U S A, 93*(19), 10303-10308.

Morita, H., Toh, H., Fukuda, S., Horikawa, H., Oshima, K., Suzuki, T., . . . Hattori, M. (2008). Comparative genome analysis of *Lactobacillus reuteri* and *Lactobacillus fermentum* reveal a genomic island for reuterin and cobalamin production. *DNA Res, 15*(3), 151-161. doi: dsn009 [pii] 10.1093/dnares/dsn009

Nakagawa, I., Kurokawa, K., Yamashita, A., Nakata, M., Tomiyasu, Y., Okahashi, N., . . . Hamada, S. (2003). Genome sequence of an M3 strain of *Streptococcus pyogenes* reveals a large-scale genomic rearrangement in invasive strains and new insights into phage evolution. *Genome Res, 13*(6A), 1042-1055. doi: 10.1101/gr.1096703 13/6a/1042 [pii]

Neeser, J. R., Granato, D., Rouvet, M., Servin, A., Teneberg, S., & Karlsson, K. A. (2000). *Lactobacillus johnsonii* La1 shares carbohydrate-binding specificities with several enteropathogenic bacteria. *Glycobiology, 10*(11), 1193-1199.

O'Shea, E. F., Gardiner, G. E., O'Connor, P. M., Mills, S., Ross, R. P., & Hill, C. (2009). Characterization of enterocin- and salivaricin-producing lactic acid bacteria from the mammalian gastrointestinal tract. *FEMS Microbiol Lett, 291*(1), 24-34. doi: FML1427 [pii] 10.1111/j.1574-6968.2008.01427.x

O'Sullivan, O., O'Callaghan, J., Sangrador-Vegas, A., McAuliffe, O., Slattery, L., Kaleta, P., . . . Beresford, T. (2009). Comparative genomics of lactic acid bacteria reveals a niche-specific gene set. *BMC Microbiol, 9,* 50. doi: 1471-2180-9-50 [pii] 10.1186/1471-2180-9-50

Overbeek, R., Begley, T., Butler, R. M., Choudhuri, J. V., Chuang, H. Y., Cohoon, M., . . . Vonstein, V. (2005). The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. *Nucleic Acids Res, 33*(17), 5691-5702. doi: 33/17/5691 [pii] 10.1093/nar/gki866

Pridmore, R. D., Berger, B., Desiere, F., Vilanova, D., Barretto, C., Pittet, A. C., . . . Schell, M. A. (2004). The genome sequence of the probiotic intestinal bacterium *Lactobacillus johnsonii* NCC 533. *Proc Natl Acad Sci U S A, 101*(8), 2512-2517. doi: 101/8/2512 [pii]

Rozen, S., & Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol, 132*, 365-386.

Schatz, M. C., Phillippy, A. M., Shneiderman, B., & Salzberg, S. L. (2007). Hawkeye: an interactive visual analytics tool for genome assemblies. *Genome Biol, 8*(3), R34. doi: gb-2007-8-3-r34 [pii] 10.1186/gb-2007-8-3-r34

Shukla, S. K., Kislow, J., Briska, A., Henkhaus, J., & Dykes, C. (2009). Optical mapping reveals a large genetic inversion between two methicillin-resistant *Staphylococcus aureus* strains. *J Bacteriol, 191*(18), 5717-5723. doi: JB.00325-09 [pii] 10.1128/JB.00325-09

Takai, K., & Horikoshi, K. (2000). Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. *Applied and Environmental Microbiology, 66*(11), 5066-5072.

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol, 24*(8), 1596-1599. doi: msm092 [pii] 10.1093/molbev/msm092

Tcherepanov, V., Ehlers, A., & Upton, C. (2006). Genome Annotation Transfer Utility (GATU): rapid annotation of viral genomes using a closely related reference genome. *BMC Genomics, 7*, 150. doi: 1471-2164-7-150 [pii] 10.1186/1471-2164-7-150

Ventura, M., Canchaya, C., Pridmore, D., Berger, B., & Brussow, H. (2003). Integration and distribution of *Lactobacillus johnsonii* prophages. *J Bacteriol, 185*(15), 4603-4608.

Ventura, M., Canchaya, C., Pridmore, R. D., & Brussow, H. (2004). The prophages of *Lactobacillus johnsoni*i NCC 533: comparative genomics and transcription analysis. *Virology, 320*(2), 229-242. doi: 10.1016/j.virol.2003.11.034 S0042682203008663 [pii]

Walter, J., Tannock, G. W., Tilsala-Timisjarvi, A., Rodtong, S., Loach, D. M., Munro, K., & Alatossava, T. (2000). Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. *Appl Environ Microbiol, 66*(1), 297-303.

Wegmann, U., Overweg, K., Horn, N., Goesmann, A., Narbad, A., Gasson, M. J., & Shearman, C. (2009). Complete genome sequence of *Lactobacillus johnsonii* FI9785, a competitive exclusion agent against pathogens in poultry. *J Bacteriol, 191*(22), 7142-7143. doi: JB.01182-09 [pii] 10.1128/JB.01182-09

Zhang, Z. G., Ye, Z. Q., Yu, L., & Shi, P. Phylogenomic reconstruction of lactic acid bacteria: an update. *BMC Evol Biol, 11*, 1. doi: 1471-2148-11-1 [pii] 10.1186/1471-2148-11-1

Table 2.1. Strains used in this study.

Strain	Species	Source	Reference
[a]DPC6026	*L. johnsonii*	Porcine	(Hayes *et al.*, 2006)
DPC6092	*L. johnsonii*	Porcine	(Hayes *et al.*, 2006)
DPC6214	*L. johnsonii*	Porcine	(Hayes *et al.*, 2006)
DPC6560	*L. johnsonii*	Porcine	This study
DPC6561	*L. johnsonii*	Porcine	This study
DPC6562	*L. johnsonii*	Porcine	This study
DPC6563	*L. johnsonii*	Porcine	This study
DPC6564	*L. johnsonii*	Porcine	This study
DPC6565	*L. johnsonii*	Porcine	This study
NCC533	*L. johnsonii*	Human	(Pridmore *et al.* 2004)
DSM10533	*L. johnsonii*	Human /Type strain	[b]DSM
ATCC120883	*L. johnsonii*	Human/Type strain	[c]ATCC
LMG9433	*L. acidophilus*	Type strain	[d]LMG
ATCC4356	*L. acidophilus*	Human/Type strain	[c]ATCC
DPC6489	*L. gasseri*	Human	(O'Shea *et al.*, 2009)
LMG9203	*L. gasseri*	Type strain	[d]LMG

[a]DPC collection; Dairy Product Collection, Moorepark Food Research Centre, Fermoy, Co. Cork.

[b] DSM; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen

[c] ATCC; American Type Culture Collection

[d] LMG; BCCM/LMG Bacteria collection

Table 2.2. DPC6026 specific primers used in this study

Region	Primer	Sequence 5'-3'
6026 genomic structure	InversionF1	tatattttgggggcagttgg
	InversionR1	atgcttgcaaacaccaatga
	InversionF2	tcttgaactccacccgaatc
	InversionR2	tgcagaatgggttgcattta
Φlj6026-specific	IntegraseF	taggttttacattagtttccg
	IntegraseR	tggtggcagtgacaagagaa
	tRNA_F	cctaccttaaactgtaaagca
	tRNA_R	ctaaagttccacagtaaccac
	Lysin F	tttatgggttgccgcttatc
	Lysin R	gttccccacgggatatttct
	LJxxxF	tccagagccgtggctactat
	LJxxR	gaagcgacgaggcaaattac
Φlj6026-att_site	attL_F	ggccttagaaaatccgaagc
	attL_R	tggtggcagtgacaagagaa
	attR_F	cgcccgattaatttgagaaa
	attR_R	tttttgacaagtttatgatgcaa
CRISPR_system	Cas1_F	ggtctgtgataataacacagc
	Cas1_R	tcattcggtacctcatgaattagc
	Cas2_F	gctatggtatgcagataatcgc
	Cas2_R	cagtaccaatcaacaaggtcctgat
	Csn1_F	gtactgattcatgtggatgggtag
	Csn1_R	tgcattttctgagagtattacgcc
RM system	Res_F	ttggcttgctcaacactttg
	Res_R	ttgtttggcttccacaatca
	Mod_F	gaagcaatgcagagcgtaaa
	Mod_R	taagctgcatcacagcatcc

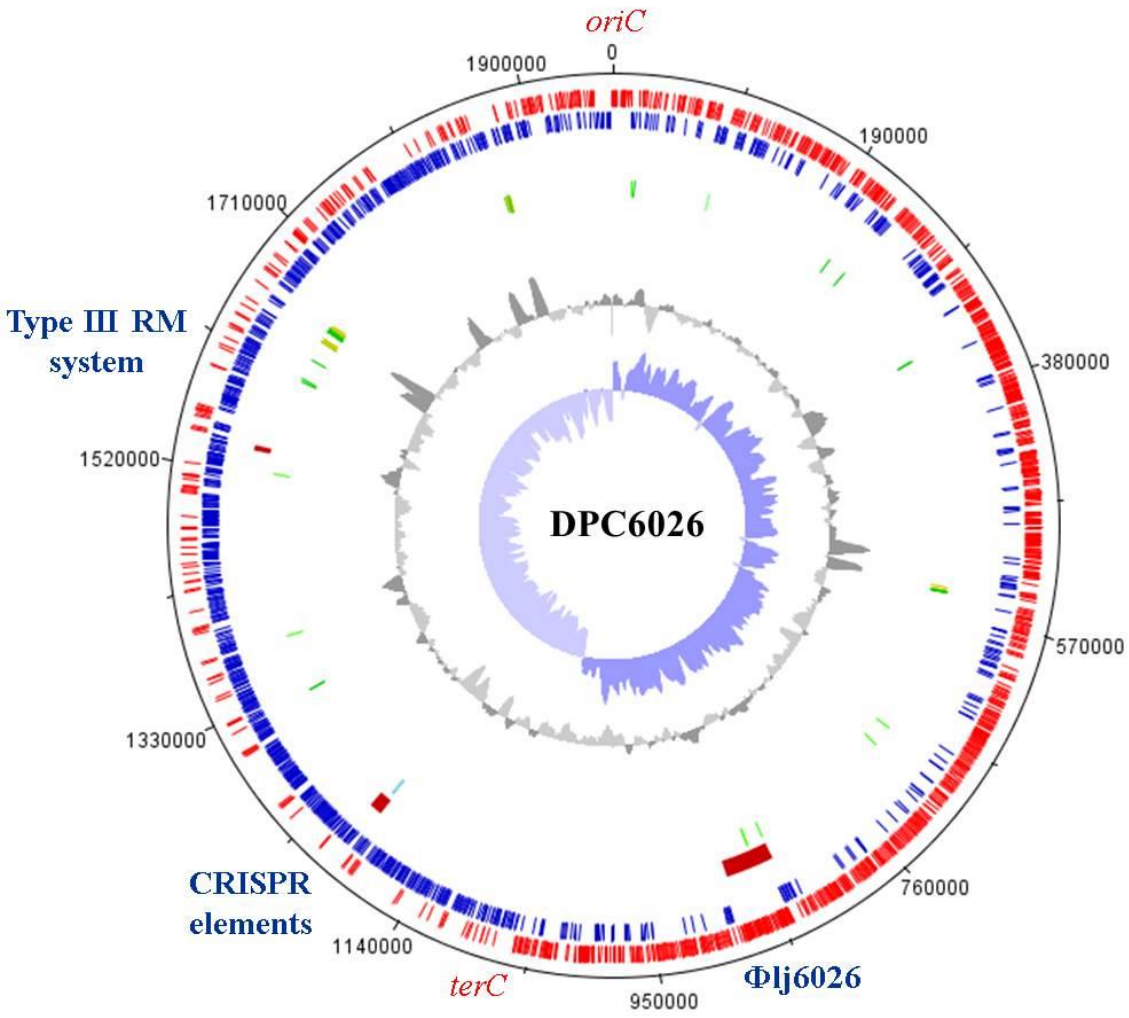


Figure 2.1. Genome Atlas of *L. johnsonii* DPC6026 The tracks from the outside represent 1. Forward CDS, 2. Reverse CDS, 3. Misc.features/MGE, 4. tRNA, rRNA 5. % GC plot 6. GC skew.

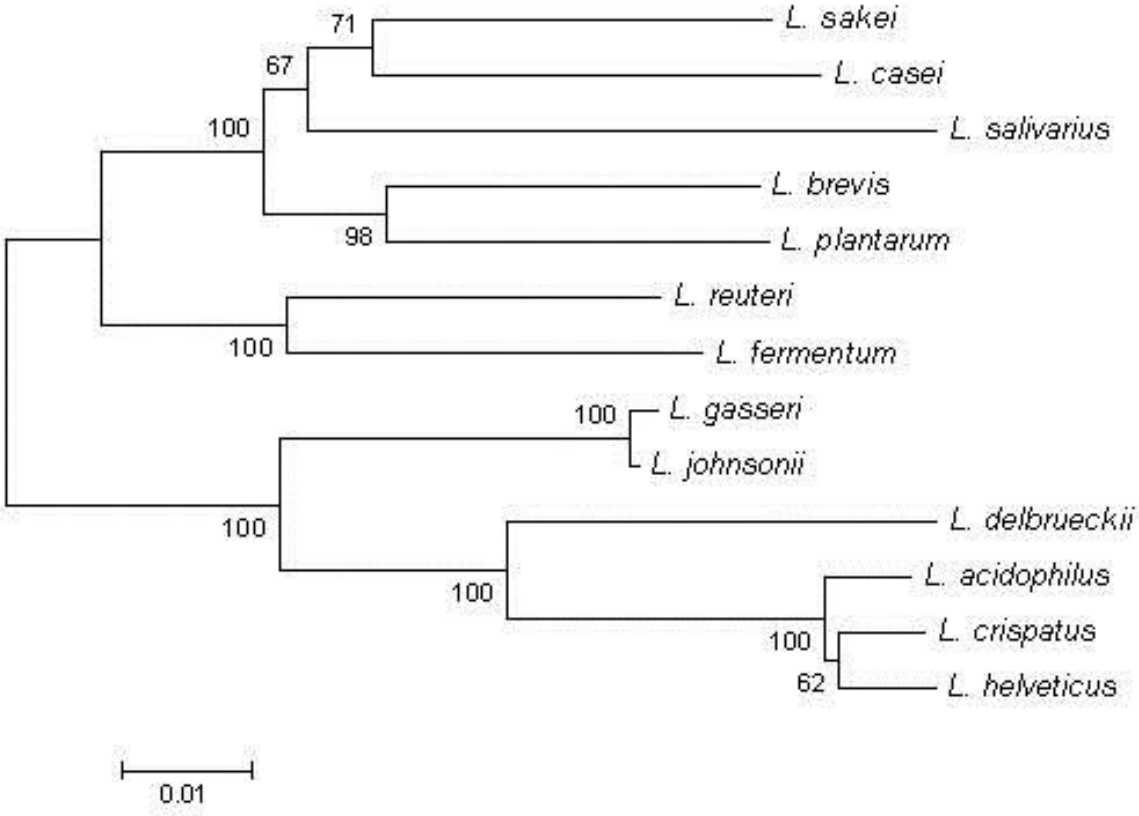


Figure 2.2. Phylogenetic tree based on the 16S rRNA gene sequences of *Lactobacillus* species

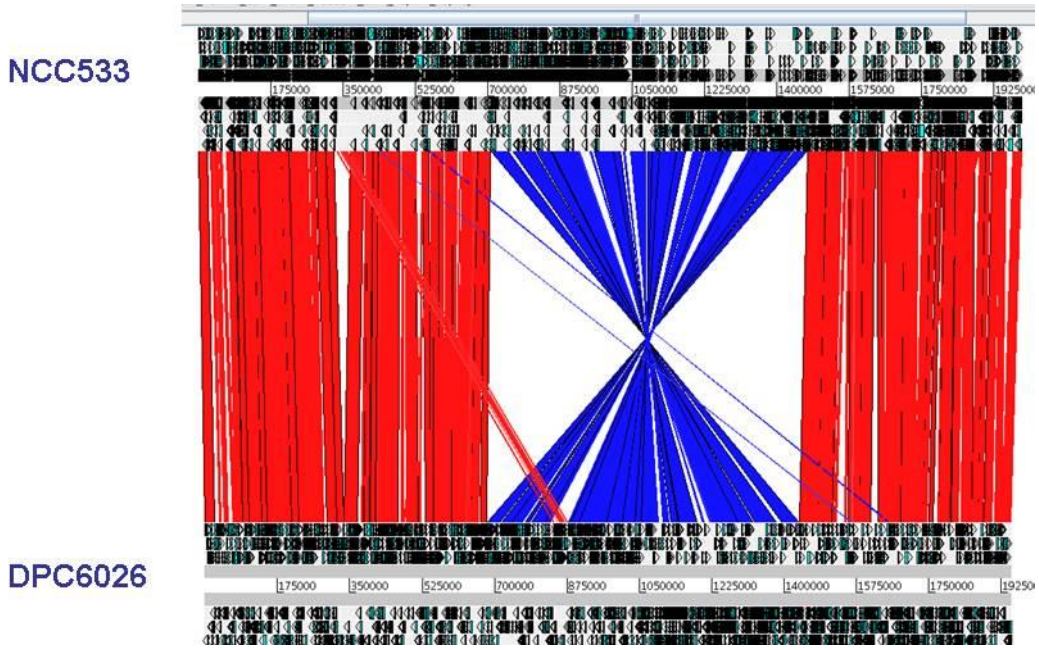


Figure 2.3. Pairwise comparison of the chromosomes *L. johnsonii* DPC6026 and NCC533 using ACT. The sequences have been aligned from the predicted replication origins (*oriC*). The colored bars separating each genome (red and blue) represent similarity matches identified by BlastN analysis, with a filter cutoff of 100. Red lines link matches in the same orientation; blue lines link matches in the reverse orientation.

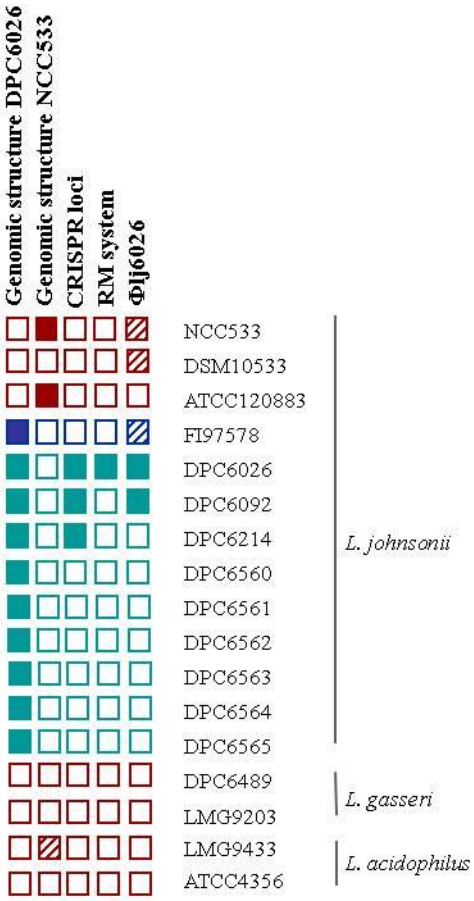
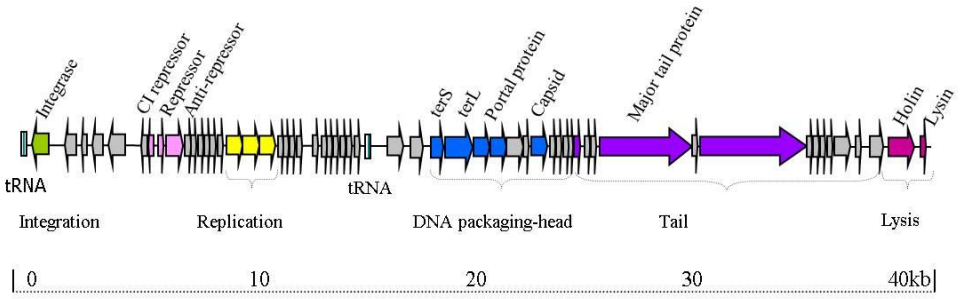
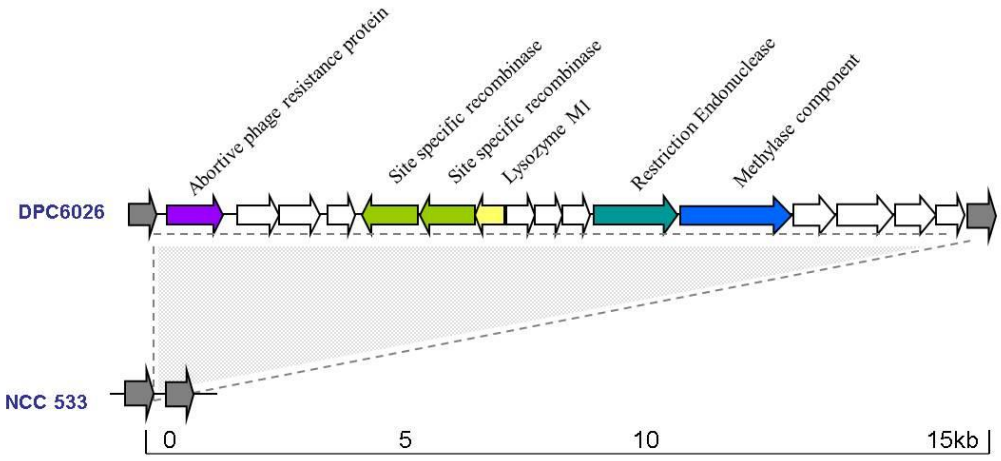


Figure 2.4. Comparative genomics of *Lactobacillus* strains. The distribution of the genomic inversion, the CRISPR loci, the type III restriction modification system and the integrated prophage among a panel of *L. johnsonii* isolates of human (red), poultry (blue) and porcine (green) origin, *L. gasseri* and *L. acidophilus* strains. A filled square indicates presence of the element, a hatched square indicates a partial element and an empty square indicates the element in absent.

(a)


Integrase
CI repressor
Repressor
Anti-repressor
terS
terL
Portal protein
Capsid
Major tail protein
Holin
Lysin
tRNA
tRNA
Integration
Replication
DNA packaging-head
Tail
Lysis
0
10
20
30
40kb

(b)


Abortive phage resistance protein
Site specific recombinase
Site specific recombinase
Lysozyme M1
Restriction Endonuclease
Methylase component
DPC6026
NCC 533
0
5
10
15kb

(c)

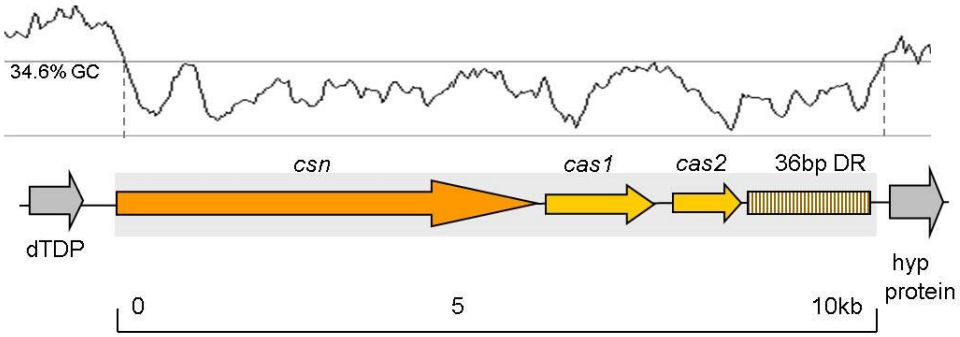


Figure 2.5. Graphic representation of the phage Φlj6026 and its demonstrated functionality (a), the graphical representation of the restriction modification (RM) system (b) and the CRISPR loci (c) in the genome of DPC6026. Genes within a mobile element that are annotated to have a similar function are coloured the same. The CRISPR (*csn*) gene is represented by dark orange arrow and CRISPR-associated genes (*cas1*, *cas2*) are represented by light orange arrows. Repeat/spacer region (36 DR; Direct Repeats) are represented by brown lines. The entire CRISPR associated region is represented by a filled grey rectangle corresponding to the lowered GC content as predicted by Artemis

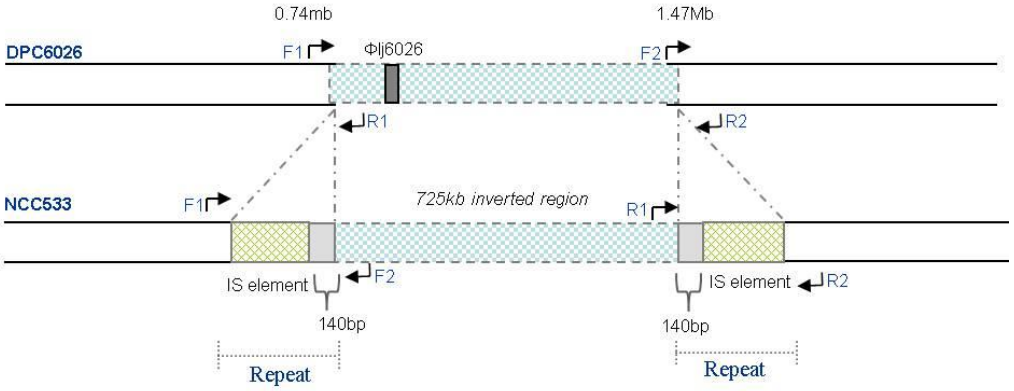


Fig 2.6. Schematic diagram of the genetic elements at the left and right junction sites in NCC533 with reference to DPC6026. In both junction sites, a transposase with an IS element (hatched box) and 140bp conserved sequence (filled grey box).

Chapter 3

Production of the antimicrobial peptides Caseicin A and B by *Bacillus* isolates growing on sodium caseinate

Robert M. Kent, Caitriona M. Guinane, Paula M. O'Connor, Gerald F. Fitzgerald, Colin Hill, Catherine Stanton and R. Paul Ross

Letters in Applied Microbiology, Volume 55, Issue 2, pages 141–148, 2012

3.1. Abstract

Aims: The aim of this study was to identify *Bacillus* isolates capable of degrading sodium caseinate and subsequently to generate bioactive peptides with antimicrobial activity.

Methods and results: Sodium caseinate (2.5% w/v) was inoculated separately with 16 *Bacillus* isolates and allowed to ferment overnight. Protein breakdown in the fermentates was analysed using gel permeation-HPLC (GP-HPLC) and screened for peptides (<3-kDa) with MALDI-TOF mass spectrometry. Caseicin A (IKHQGLPQE) and caseicin B (VLNENLLR), two previously characterised antimicrobial peptides, were identified in the fermentates of both *Bacillus cereus* and *Bacillus thuringiensis* isolates. The caseicin peptides were subsequently purified by RP-HPLC and antimicrobial assays indicated that the peptides maintained the previously identified inhibitory activity against the infant formula pathogen *Cronobacter sakazakii*.

Conclusions: We report a new method using *Bacillus* sp. to generate two previously characterised antimicrobial peptides from casein.

Significance and impact of the study: This study highlights the potential to exploit *Bacillus* sp. or the enzymes they produce for the generation of bioactive antimicrobial peptides from bovine casein.

3.2. Introduction

Bioactive peptides are generally small (3-50 amino acid residues) protein fragments that possess a number of physiological properties and are often "encrypted" in a larger protein (Benkerroum, 2010; Clare & Swaisgood, 2000). These peptides can be released by a number of methods including microbial fermentation with live cultures, enzyme hydrolysis using digestive enzymes and proteolysis by enzymes from fungal, bacterial or plant sources (Korhonen, 2009; Korhonen & Pihlanto, 2006). Milk proteins have been recognised as a rich source of bioactives as peptides generated from milk protein hydrolysis can exert a broad range of physiological and nutritional effects (Benkerroum, 2010; Gill, López-Fandiño, Jorba, & Vulfson, 1996; Korhonen & Pihlanto, 2006). From an evolutionary perspective, this may also make sense, given that it provides a mechanism to confer health potential benefits from mother to baby in the form of bioactive peptides released in the intestine via digestion (Newburg, 2005; Phadke *et al.*, 2005).

Milk-derived antimicrobial peptides could potentially be used as an intrinsic dairy-based protectant against pathogens in powder based food products (Hayes *et al.*, 2009). This is particularly true for infant formula which can be subject to *Cronobacter sakazakii* contamination. *C. sakazakii* is a Gram-negative opportunistic pathogen, rarely causing illness in adults but has been linked to cases of meningitis, bacteraemia and necrotizing enterocolitis in infants (Drudy, Mullane, Quinn, Wall, & Fanning, 2006; Gurtler, Kornacki, & Beuchat, 2005; NazarowecWhite & Farber, 1997). Powdered infant formula (PIF) has been implicated in outbreaks, and in sporadic cases of *C. sakazakii* related illness (Iversen & Forsythe, 2003).

The potential use of casein-derived peptides in an industrial setting is dependent on the economic feasibility, reproducibility of production, stability of the process and its permissive use in a final product. The generation of two antimicrobial casein-derived peptides, caseicin A and caseicin B by the fermentation of sodium caseinate with *Lactobacillus johnsonii* DPC 6026 (formerly *Lactobacillus acidophilus*) was previously described (Hayes, Ross, Fitzgerald, Hill, & Stanton, 2006). The peptides, derived from bovine α-s1 casein, caseicin A (αs1-CN f(21-29) IKHQGLPQE) and caseicin B (αs1-CN f(30-37) VLNENLLR) correspond to the amino acids 6 to 14 and 15 to 22 respectively, of the previously characterised antimicrobial peptide isracidin (Hill, Lahav, & Givol, 1974). Caseicin A and B peptides both demonstrated antimicrobial activity against the Gram-negative neonatal pathogen *Cronobacter* in lab media (caseicin A minimum inhibitory concentration (MIC): 0.05 mmol l^{-1}, caseicin B MIC: 0.22 mmol l^{-1}) (Hayes *et al.*, 2006). Further investigation confirmed the antimicrobial activity of chemically synthesised caseicin A and B albeit at higher concentrations of the peptide (McDonnell, Rivas, Burgess, Fanning, & Duffy, 2011; Norberg *et al.*, 2011). It was also demonstrated that caseicin A and B peptides can inhibit the growth of other important pathogens such as *E. coli*, *Listeria* sp., *Staphylococcus aureus*, *Salmonella* sp. and *Streptococcus mutans* (Hayes *et al.*, 2006; McDonnell *et al.*, 2011; Norberg *et al.*, 2011). Furthermore, filtered fermentates (≥3-kDa) containing the peptides were also successful in controlling *Cronobacter* levels in initial PIF trials (Hayes *et al.*, 2009).

Subsequent genomic analysis of the *L. johnsonii* DPC 6026 strain revealed however, the absence of a cell-wall proteinase (Guinane *et al.*, 2011)

and further experimental analysis indicated inefficient casein degradation by this strain itself (unpublished data). Furthermore, members of *L. johnsonii* and closely-related species are producers of D-lactic acid which is not permitted by Codex Alimentarius Commision (CAC) standards in infant formula (Connolly, Abrahamsson, & Björkstén, 2005) and therefore the use of these organisms would not be feasible without the inclusion of extra processing steps. Indeed, we have recently tested representatives from seven different *Lactobacillus* species in casein based fermentations but caseicin A and B were not recovered in any of the fermentates (unpublished data).

Bacillus species are the dominant bacteria in industrial fermentations for the generation of commercial proteases for food applications (Rao, Tanksale, Ghatge, & Deshpande, 1998). *Bacillus* cells have a number of industrially advantageous traits including short fermentation cycles, high growth rates and the ability to secrete a range of hydrolytic enzymes into the extracellular media (Gupta, Beg, Khan, & Chauhan, 2002; Schallmey, Singh, & Ward, 2004). These enzymes can be active over a wide range of temperatures and pH values and so have a broad range of uses in textile, food and beverage industries and detergent industries. The proteases of neutral *Bacillus* sp. are optimal near pH 7.0 and have previously been used to modify milk proteins (Schallmey *et al.*, 2004). Some species, such as *B. subtilis* have generally regarded as safe (GRAS) status with the Food and Drug Administration (FDA) (Zheng & Slavik, 1999) and others have reported caseinolytic properties (Mukherjee, Adhikari, & Rai, 2008; Ouoba *et al.*, 2003).

In this study, we investigated the proteolytic ability of various *Bacillus* sp. to degrade casein, including isolates recovered from dairy-based spray-dried powders. A number of isolates were found to produce a high percentage of small peptides (≥5-kDa) and subsequently were investigated for ability to produce caseicin A and B during fermentation of sodium caseinate or reconstituted skim milk.

3.3. Materials and Methods

3.3.1. Bacterial strains, media and growth conditions

The *Bacillus* isolates investigated in this study are outlined in Table 3.1. *Bacillus* sp. were cultured in BHI (Merck, Darmstadt, Germany) broth at 37^0C with agitation or on BHI agar. *B. cereus* DPC 6559 was isolated in this study as a contaminant from skim milk powder on Skim milk agar (Kerry Food Ingredients, Tralee, Ireland). The remaining *Bacillus* isolates investigated were obtained from various culture collections (Table 3.1.). *Cronobacter* (*Enterobacter*) *sakazakii* NCTC 8155 designated DPC 6440 was selected as a target organism for antimicrobial assays as it was originally isolated from a tin of milk powder (Farmer III, Asbury, Hickman, & Brenner, 1980) indicating an ability to contaminate dried milk products. This strain was propagated in LB broth at 37^0C with agitation or on LB agar.

3.3.2. Fermentation conditions

The proteolytic capability of bacterial strains was measured by gel permeation HPLC (GP-HPLC). Fermentations (5 mL - 8 L) were performed using sodium caseinate (2.5% w/v) (Kerry Food Ingredients, Tralee, Ireland) as a substrate. Strains were inoculated at 1% (v/v) and incubated at 37°C, with mild agitation for 17 h. The pH was measured before and after fermentation. Following fermentation, samples were diluted 1/10 and filtered through a 0.45 nm filter. Samples were analysed on the GP-HPLC using a Waters 626 pump, a 717 Autosampler, 996 photodiode array detector and 600s controller (Waters Corporation, Dublin, Ireland) and applied to a Grace (Grace, Illinois, U.S.A.) Nucleosil 100, C18, 5 µm column. Results were read at a wavelength of 280

nm. The mobile phase was 30% (v/v) acetonitrile with 0.1% (v/v) trifluroacetic acid (TFA). Unhydrolysed casein, subjected to the same processes was used as a control.

3.3.3. Detection and production of purified caseicin peptides from *Bacillus* isolates

Following fermentation with selected *Bacillus* isolates, fermentates were filtered through a size-exclusion 3-kDa centrifugal filter unit (Millipore Ltd., Hertfordshire, UK) and subsequent filtrates were analysed for caseicin peptide masses using Matrix-assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry. RP-HPLC was used to further purify *B. cereus* DPC 6559 and *B. thuringiensis* DPC 6431 3-kDa filtered fermentates. Briefly, *B. thuringiensis* DPC 6431 casein fermentates were filtered as described above. Filter permeates were applied to an analytical Jupiter Proteo RP-HPLC column (4.6 x 250 mm, 4 µ, 90 Å) running a 10% (v/v) to 35% (v/v) acetonitrile 0.1% (v/v) TFA gradient at a flow rate of 1 ml per minute over 40 min. Fractions were collected and analysed using MALDI-TOF mass spectrometry to determine where the caseicin peptides eluted. MALDI-TOF Tandem Mass spectrometry (MS/MS) in conjunction with Mascot search engine (http://www.matrix-science.com) was used to confirm the peptide sequences.

To generate larger quantities of caseicin A and B peptides 180 ml of *B. cereus* DPC 6559 3 kDa filtrate was lyophilised in a Genevac HT-4X evaporator (Genevac Ltd. Ipswich, UK). Dry permeate was resuspended in Milli Q water and applied to a Jupiter Proteo (250 x 10mm, 4u, 90 Å) RP-

HPLC column (Phenomenex, Cheshire, United Kingdom) running a 10-30% (v/v) acetonitrile 0.1% (v/v) TFA gradient over 40 min. Fractions were analysed for the relevant peptide peaks using MALDI-TOF Mass Spectrometry. Both caseicin A and caseicin B were further purified by pooling the relevant fractions and applying to a RP-HPLC C18 XBridge™ BEH300 (250 x 10mm, 5u, 300Å) column (Waters Corporation) running a 4-10% (v/v) isopropan-2-ol, 0.1% (v/v) TFA gradient over 40 min for caseicin A and a 10-18% (v/v) isopropan-2-ol, 0.1% (v/v) TFA over 40 min for caseicin B. Both peptides were further purified by applying fractions containing the peptide to a RP-HPLC Acclaim® (150 x 10 mm, 3µ, 300Å) column (Dionex Ireland Ltd., Dublin, Ireland) running a 20-35% (v/v) methanol 0% (v/v) TFA gradient over 40 min for caseicin A and a 33-46% (v/v) methanol 0.1% (v/v) TFA gradient over 40 min for caseicin B. Relevant fractions were once again pooled and dried.

3.3.4. Determination of antimicrobial activity

Antimicrobial assays were performed as described previously (Norberg *et al.*, 2011) with some modifications. Briefly, caseicin A and caseicin B were reconstituted in LB broth at varying concentrations (Fig. 3.2.) and added to a 96-well plate. *C. sakazakii* DPC 6440 was added to a final concentration 10^3 CFU ml^{-1} and incubated with varying dilutions of the relevant peptide at 37°C for 24 h in a microtiter plate reader with hourly readings recording the optical density (600 nm). Results are given as the mean values of three independent determinations ± SEM.

3.4. Results

3.4.1. Casein hydrolysis by *Bacillus* sp.

16 *Bacillus* isolates (Table 3.1.) were investigated for their ability to degrade casein and generate caseicin A and B from a casein substrate. Initially, the percentage of casein protein hydrolysed to below 5-kDa (Table 3.1.) was determined by GP-HPLC analysis. The results demonstrate that a number of the *Bacillus* isolates efficiently cleaved casein with 73-88% of casein hydrolysed to peptides <5-kDa compared to the untreated sodium caseinate. In contrast, 10 *Lactobacillus* strains representing seven different species were unable to significantly hydrolyse the casein with negligible or limited (~9%) hydrolysis to small peptides (data not shown). Strains capable of hydrolysing sodium caseinate grew to 10^7-10^8 log CFU ml^{-1} (Table 3.1.) and lowered the pH of the growth substrate slightly (pH 7.4 to pH 7.0-7.2). Not surprisingly, significant growth was not observed in strains unable to hydrolyse sodium caseinate.

3.4.2. Bacillus mediated production of caseicin A and caseicin B

Strains of *B. cereus*, *B. thuringiensis*, *Bacillus mycoides* and *Bacillus lichenformis* were capable of efficiently hydrolysing casein but only strains of the *B. cereus* and *B. thuringiensis* species were found to be able to generate the caseicin A and B peptides (Table 3.1.) following overnight fermentation of sodium caseinate. The correct peptide masses for caseicin A and B (1049-Da and 970-Da, respectively) were detected by MALDI-TOF Mass spectrometry on the filtered fermentates of the isolates. Representative strains of both *B. cereus* and *B. thuringiensis* were taken forward for further investigation. *B.*

thuringiensis DPC 6431 is a previously identified strain isolated from a human fecal sample and was found to produce the bacteriocin thuricin CD (Rea *et al.*, 2010). DPC 6559 was isolated in this study from milk powder and was determined by PCR analysis to have certain *B. cereus* associated toxins (data not shown). Following initial detection of correct peptide masses (inset of Fig. 3.1.), the peptides were purified by RP-HPLC and the amino acid sequence of the peptides were confirmed, by MS/MS, to be the caseicin A and B peptides identified in the previous study (Hayes *et al.*, 2006).

3.4.3. Antimicrobial assays

Much of the previous work on the caseicin peptides focused on the potential antimicrobial activity against the important PIF pathogen *Cronobacter sakazakii* (Hayes *et al.*, 2009; Hayes *et al.*, 2006; McDonnell *et al.*, 2011; Norberg *et al.*, 2011). We investigated in this study if peptides produced by *Bacillus* proteolysis also had similar antimicrobial activity against this pathogen. Both peptides produced by *Bacillus* sodium caseinate fermentations were indeed antimicrobial against *C. sakazakii* (Fig. 3.2.) in accordance with Hayes *et al.*, (2006) and Norberg *et al.*, (2011). Growth of *C. sakazakii* DPC 6440 was inhibited at concentrations of caseicin A of >1.0 mmol l^{-1} (1.049 mg ml^{-1}) compared to the growth of the strain without any added peptide (Fig. 3.2A.). Caseicin B also slowed at growth at concentrations >1.0 mmol l^{-1} (0.97 mg ml^{-1}) but at 3.0 mmol l^{-1} (2.91 mg ml^{-1}) complete inhibition of pathogen growth over the 24 hours was demonstrated.

3.5. Discussion

In this study, we compared different *Bacillus* species for their ability to degrade casein and release bioactive peptides with antimicrobial activity. To achieve this, a total of 16 different isolates were obtained from various different sources representing a broad spectrum of *Bacillus* isolates including those found to predominate spray-dried milk powders and other dairy environments. *Bacillus* species are ubiquitous in the dairy drying environment and can contaminate dairy products and powders during processing and handling. An array of *Bacillus* species are commonly isolated from such powders (Chen, Coolbear, & Daniel, 2004). This study demonstrated the ability of *B. cereus and B. thuringiensis* strains to degrade casein and produce the previously described antimicrobial peptides, caseicin A and B (Hayes *et al.*, 2006).

The *Bacillus* isolates investigated in this study sourced from dairy environments all efficiently hydrolysed sodium caseinate with the exception of *B. coagulans* LMG 6326 (Table 3.1.). Only members of *B. cereus* and *B. thuringiensis* species however generated the caseicin A and B peptides following fermentation of sodium caseinate. The processes used to generate bioactive peptides must be reproducible and efficient to be considered in an industrial context. The *Bacillus* isolates used in this study reliably produced the caseicin peptides during fermentation and did not require media supplementation, pH control, agitation or anaerobic conditions. The antimicrobial activity of the *Bacillus* produced caseicin A and B were also confirmed in this study (Fig. 3.2).

Due to the non-GRAS status of many *Bacillus* strains, the use of the fermentates directly generated in this study is not a feasible option (especially for PIF). In contrast, *Bacillus* species such as *B. subtilis* and *B. lichenformis* are considered GRAS organisms and possess many traits that make them attractive in an industrial setting and are seen as excellent hosts for the production of heterologous proteins (Schallmey *et al.,* 2004). Strains of these species however, were not successful in producing the particular antimicrobial peptides studied in this work. As caseicin A and B generation was found to be a common feature of the *B. cereus* and *B. thuringiensis* isolates investigated (Table 3.1), it is likely that this is as a result of the action of conserved proteolytic machinery present in these strains. *B. cereus* and *B. thuringiensis* are both group I bacilli, indistinguishable by 16S rRNA typing and therefore are very genetically closely related (Radnedge *et al.,* 2003). Genomic analysis of the *B. cereus* (Ivanova *et al.,* 2003) and *B. thuringiensis (*Zhu *et al., 2011)* genomes indicate an almost identical protease complement dominated by members of the neutral protease (npr) enzymes and thermolysin-like M4 family of peptidases. These enzymes have a number biotechnological applications (Adekoya & Sylte, 2009) and have previously been shown to generate bioactive peptides from casein (Mizuno, Nishimura, Matsuura, Gotou, & Yamamoto, 2004). Many species of the genus *Bacillus* generate a range of thermostable extracellular enzymes, a number of which have industrial applications (Schallmey *et al.*, 2004; Vecerek & Venema, 2000). If possible utilizing commercially available industrial enzymes in this manner is more cost effective than using an organism naturally producing an enzyme (Mizuno *et al.,* 2005). While the majority of casein-derived bioactive peptides have to

date been generated using gastrointestinal enzymes (Korhonen & Pihlanto, 2006), the use of *Bacillus* sp. enzymes could lead to the discovery of further novel bioactives. The results presented in this study demonstrated the potential to produce such bioactive peptides from Bovine casein using *Bacillus* species.

3.6. Acknowledgements

This project was funded by an Enterprise Ireland commercialisation fund CFTD/07/116.

Paula O'Connor provided technical assistance with MALDI-TOF MS.

3.7. References:

Adekoya, O. A., & Sylte, I. (2009). The thermolysin family (M4) of enzymes: therapeutic and biotechnological potential. *Chemical biology & drug design, 73*(1), 7-16.

Benkerroum, N. (2010). Antimicrobial peptides generated from milk proteins: a survey and prospects for application in the food industry. A review. *International Journal of Dairy Technology, 63*(3), 320-338. doi: 10.1111/j.1471-0307.2010.00584.x

Chen, L., Coolbear, T., & Daniel, R. M. (2004). Characteristics of proteinases and lipases produced by seven *Bacillus* sp. isolated from milk powder production lines. *International Dairy Journal, 14*(6), 495-504.

Clare, D. A., & Swaisgood, H. E. (2000). Bioactive milk peptides: A prospectus. *Journal of Dairy Science, 83*(6), 1187-1195.

Connolly, E., Abrahamsson, T., & Björkstén, B. (2005). Safety of D-lactic acid producing bacteria in the human infant. *J Pediatr Gastroenterol Nutr, 41*(4), 489.

Drudy, D., Mullane, N. R., Quinn, T., Wall, P. G., & Fanning, S. (2006). *Enterobacter sakazakii*: An emerging pathogen in powdered infant formula. *Clinical Infectious Diseases, 42*(7), 996-1002.

Farmer III, J., Asbury, M., Hickman, F., & Brenner, D. J. (1980). *Enterobacter sakazakii*: a new species of "Enterobacteriaceae" isolated from clinical specimens. *Int J Syst Bacteriol, 30*(3), 569-584.

Gill, I., López-Fandiño, R., Jorba, X., & Vulfson, E. N. (1996). Biologically active peptides and enzymatic approaches to their production. *Enzyme Microb Technol, 18*(3), 162-183.

Guinane, C. M., Kent, R. M., Norberg, S., Hill, C., Fitzgerald, G. F., Stanton, C., & Ross, R. P. (2011). Host Specific Diversity in *Lactobacillus johnsonii* as Evidenced by a Major Chromosomal Inversion and Phage Resistance Mechanisms. *PLoS ONE, 6*(4), e18740.

Gupta, R., Beg, Q. K., Khan, S., & Chauhan, B. (2002). An overview on fermentation, downstream processing and properties of microbial alkaline proteases. *Applied Microbiology and Biotechnology, 60*(4), 381-395. doi: 10.1007/s00253-002-1142-1

Gurtler, J. B., Kornacki, J. L., & Beuchat, L. R. (2005). *Enterobacter sakazakii*: A coliform of increased concern to infant health. *Int J Food Microbiol, 104*(1), 1-34. doi: 10.1016/j.ijfoodmicro.2005.02.013

Hayes, M., Barrett, E., Ross, R. P., Fitzgerald, G. F., Hill, C., & Stanton, C. (2009). Evaluation of an Antimicrobial Ingredient Prepared from a *Lactobacillus acidophilus* Casein Fermentate against *Enterobacter sakazakii*. *Journal of Food Protection, 72*(2), 340-346.

Hayes, M., Ross, R. P., Fitzgerald, G. F., Hill, C., & Stanton, C. (2006). Casein-derived antimicrobial peptides generated by *Lactobacillus acidophilus* DPC6026. *Appl Environ Microbiol, 72*(3), 2260.

Hill, R. D., Lahav, E., & Givol, D. (1974). A rennin-sensitive bond in alpha-S1 B-casein. *Journal of Dairy Research, 41*(1), 147-&.

Ivanova, N., Sorokin, A., Anderson, I., Galleron, N., Candelon, B., Kapatral, V., . . . Lapidus, A. (2003). Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. *Nature, 423*(6935), 87-91.

Iversen, C., & Forsythe, S. (2003). Risk profile of *Enterobacter sakazakii*, an emergent pathogen associated with infant milk formula. *Trends in Food Science & Technology, 14*(11), 443-454.

Korhonen, H. (2009). Milk-derived bioactive peptides: from science to applications. *J Funct. Foods, 1*(2), 177-187.

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality. *International Dairy Journal, 16*(9), 945-960. doi: 10.1016/j.idairyj.2005.10.015

McDonnell, M. J., Rivas, L., Burgess, C. M., Fanning, S., & Duffy, G. (2011). Inhibition of verocytotoxigenic *Escherichia coli* by antimicrobial peptides caseicin A and B and the factors affecting their antimicrobial activities. *Int J Food Microbiol*.

Mizuno, S., Matsuura, K., Gotou, T., Nishimura, S., Kajimoto, O., Yabune, M., . . . Yamamoto, N. (2005). Antihypertensive effect of casein hydrolysate in a placebo-controlled study in subjects with high-normal blood pressure and mild hypertension. *Brit J Nutr, 94*(1), 84.

Mizuno, S., Nishimura, S., Matsuura, K., Gotou, T., & Yamamoto, N. (2004). Release of Short and Proline-Rich Antihypertensive Peptides from Casein Hydrolysate with an *Aspergillus oryzae* Protease. *Journal of Dairy Science, 87*(10), 3183-3188.

Mukherjee, A. K., Adhikari, H., & Rai, S. K. (2008). Production of alkaline protease by a thermophilic< i> Bacillus subtilis</i> under solid-state fermentation (SSF) condition using< i> Imperata cylindrica</i> grass and potato peel as low-cost medium: Characterization and application of enzyme in detergent formulation. *Biochemical Engineering Journal, 39*(2), 353-361.

NazarowecWhite, M., & Farber, J. M. (1997). *Enterobacter sakazakii*: A review. *Int J Food Microbiol, 34*(2), 103-113.

Newburg, D. S. (2005). Innate immunity and human milk. *J Nutr, 135*(5), 1308.

Norberg, S., O'Connor, P. M., Stanton, C., Ross, R. P., Hill, C., Fitzgerald, G. F., & Cotter, P. D. (2011). Altering the composition of caseicin A and B as a means of determining the contribution of specific residues to antimicrobial activity. *Appl Environ Microbiol*, AEM. 02450-02410v02451.

Ouoba, L., Rechinger, K., Barkholt, V., Diawara, B., Traore, A., & Jakobsen, M. (2003). Degradation of proteins during the fermentation of African locust bean (Parkia biglobosa) by strains of Bacillus subtilis and Bacillus pumilus for production of Soumbala. *Journal of applied microbiology, 94*(3), 396-402.

Phadke, S. M., Deslouches, B., Hileman, S. E., Montelaro, R. C., Wiesenfeld, H. C., & Mietzner, T. A. (2005). Antimicrobial Peptides in Mucosal Secretions: The Importance of Local Secretions in Mitigating Infection. *J Nutr, 135*(5), 1289.

Radnedge, L., Agron, P. G., Hill, K. K., Jackson, P. J., Ticknor, L. O., Keim, P., & Andersen, G. L. (2003). Genome differences that distinguish *Bacillus anthracis* from *Bacillus cereus* and *Bacillus thuringiensis*. *Appl Environ Microbiol, 69*(5), 2755.

Rao, M. B., Tanksale, A. M., Ghatge, M. S., & Deshpande, V. V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiol Mol Biol R, 62*(3), 597.

Rea, M. C., Sit, C. S., Clayton, E., O'Connor, P. M., Whittal, R. M., Zheng, J., . . . Hill, C. (2010). Thuricin CD, a posttranslationally modified bacteriocin with a narrow spectrum of activity against *Clostridium difficile*. *Proc Natl Acad Sci USA, 107*(20), 9352.

Schallmey, M., Singh, A., & Ward, O. P. (2004). Developments in the use of *Bacillus* species for industrial production. *Canadian Journal of Microbiology, 50*(1), 1-17. doi: 10.1139/w03-076

Simpson, P., Fitzgerald, G., Stanton, C., & Ross, R. (2004). The evaluation of a mupirocin-based selective medium for the enumeration of bifidobacteria from probiotic animal feed. *J Microbiol Meth, 57*(1), 9-16.

Vecerek, B., & Venema, G. (2000). Expression of the neutral protease gene from a thermophilic *Bacillus* sp BT1 strain in *Bacillus subtilis* and its natural host: Identification of a functional promoter. *J Bacteriol, 182*(14), 4104-4107.

Zheng, G., & Slavik, M. (1999). Isolation, partial purification and characterization of a bacteriocin produced by a newly isolated Bacillus subtilis strain. *Letters in applied microbiology, 28*(5), 363-367.

Zhu, Y., Shang, H., Zhu, Q., Ji, F., Wang, P., Fu, J., . . . Zheng, J. (2011). Complete genome sequence of *Bacillus thuringiensis* serovar finitimus strain YBT-020. *J Bacteriol, 193*(9), 2379-2380.

Table 3.1. *Bacillus* strains screened for caseicin A and B production following overnight fermentation

Isolates	Log CFU ML^{-1}	% Casein Hydrolysed < 5-Kda	Caseicin A/B production	Source
Bacillus thuringiensis NCIMB 701132	$2.5x10^7$	82.69±0.80	Yes	Unknown
Bacillus thuringiensis DPC 6431[1]	$3.5x10^7$	84.84±0.62	Yes	Human Fecal Sample
Bacillus thuringiensis NCIMB 701157	$4.3x10^7$	74.06±0.84	Yes	Unknown
Bacillus cereus NCIMB 700577	$7.2x10^7$	85.04±0.59	Yes	Milk
Bacillus cereus NCIMB 700579	$4.6x10^7$	88.00±0.78	Yes	Milk
Bacillus cereus DPC 3805[2]	$4.3x10^7$	84.91±0.67	No	Milk powder
Bacillus cereus DPC 6559	$8.6x10^7$	87.85±1.3	Yes	Milk powder
Bacillus coagulans LMG 6326	$6.3x10^4$	No Hydrolysis	No	Milk powder
Bacillus firmus LMG 7125	$3.2x10^4$	4.37±0.89	No	Soil
Bacillus lichenformis DPC 3803	$4.6x10^7$	82.57±0.94	No	Milk powder
Bacillus lichenformis DPC 6337	$3.2x10^7$	86.95±0.73	No	Processed cheese
Bacillus mycoides DPC 6335	$5.8x10^7$	88.83±0.64	No	Processed cheese
Bacillus stearothermophilus DPC 3802	$3.1x10^7$	83.86±1.49	No	Milk powder
Bacillus stearothermophilus DPC 3804	$5.9x10^7$	86.35±1.48	No	Milk powder
Bacillus atrophaeus LMG 8198	$5.0x10^4$	No Hydrolysis	No	Unknown
Bacillus subtilis BGSC 168	$2.0x10^4$	22.42±0.53	No	Soil

[a] NCIMB; The National Collection of Industrial, Marine and Food Bacteria
[b] DPC collection; Dairy Product Collection, Moorepark Food Research Centre, Fermoy, Co. Cork
, Ireland
[c] LMG; BCCM/LMG Bacteria collection
[d] BGSC; Bacillus Genetic Stock Centre
[1] (Rea *et al.*, 2010) [2] (Simpson, Fitzgerald, Stanton, & Ross, 2004)

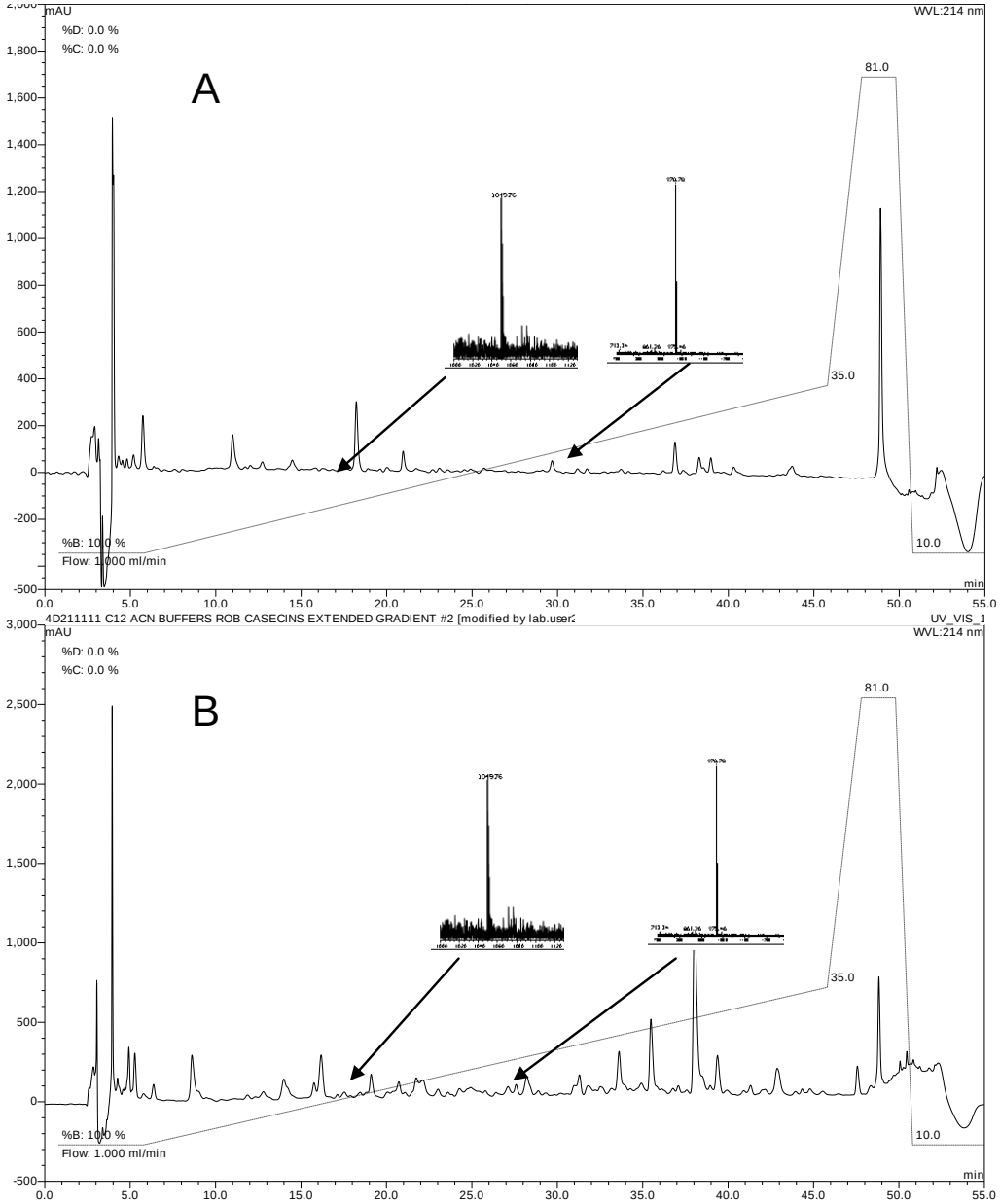
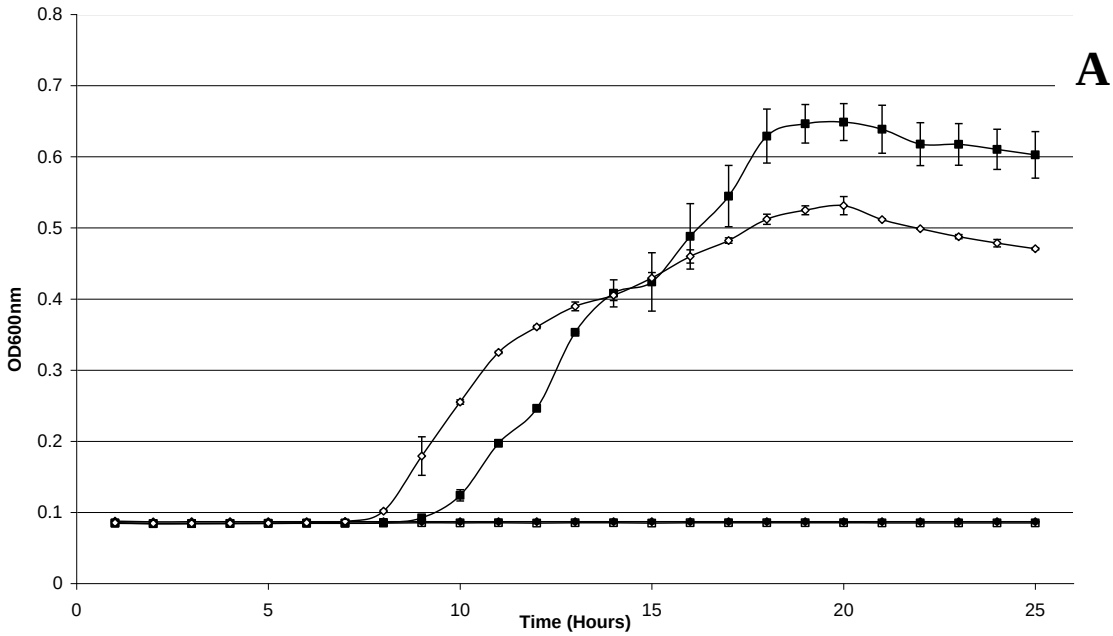


Figure 3.1. RP-HPLC chromatogram of sodium caseinate incubated with *B. cereus* DPC6559

for 17 h (A) and RP-HPLC chromatogram of sodium caseinate incubated with

B. thuringiensis DPC 6431 for 17 h (B) . Arrows indicate location of the caseicin peptides.

The inserts shows the MALDI-TOF spectrum of the peptides.


A
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD600nm
0
5
10
15
20
25
Time (Hours)

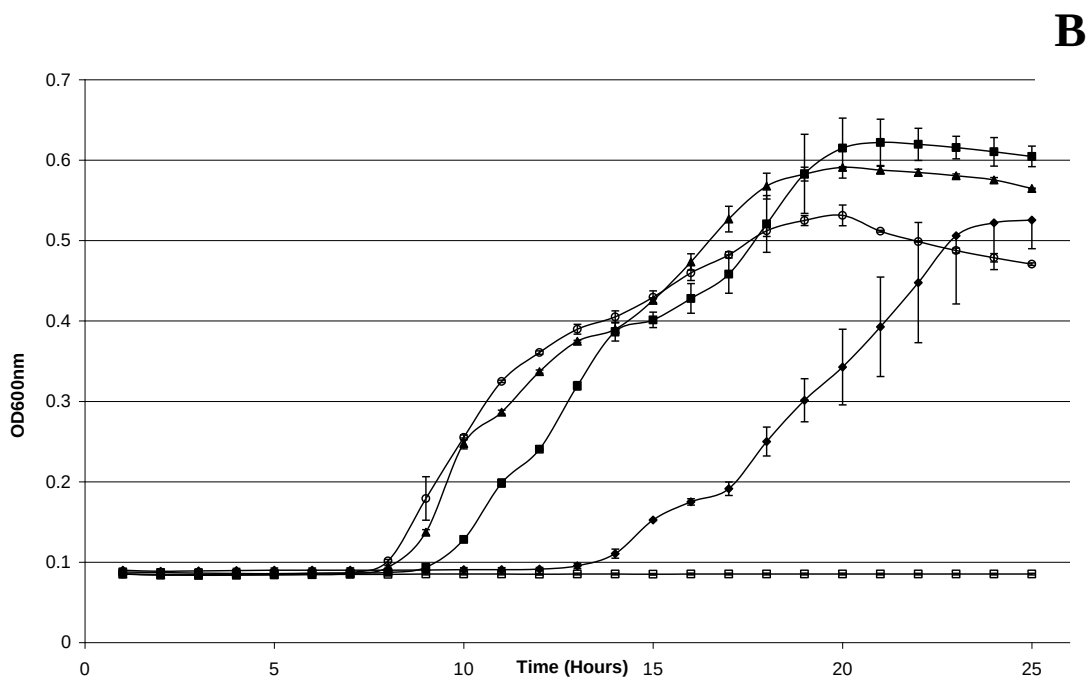

B
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD600nm
0
5
10
15
20
25
Time (Hours)

Figure 3.2. (A) Effect of caseicin A at various concentrations on the growth of *C. sakazakii* DPC 6440 in LB broth at 37°C. Symbols: ♦, 2.0 mmol l^{-1} caseicin A; ■, 1.0 mmol l^{-1} caseicin A; ▲, 0.75 mmol l^{-1} caseicin A;○, *C. sakazakii* DPC 6440 and LB broth only; □, LB broth only. (B) Effect of caseicin B at various concentrations on the growth of *C. sakazakii* DPC 6440 in LB broth at 37°C. Symbols: ♦, 3.0 mmol l^{-1} caseicin B; ■, 1.0 mmol l^{-1} caseicin B; ◊, *C. sakazakii* DPC 6440 and LB broth only; □, LB broth only.

Chapter 4

Generation of caseicin peptides from casein by hydrolysis with thermolysin enzymes

Robert M. Kent, Caitriona M. Guinane, Sarah Norberg, Paula M. O'Connor, Colin Hill, Gerald F. Fitzgerald, Catherine Stanton and R. Paul Ross

International Dairy Journal 2015, doi;10.1016/j.dairyj.2015.04.001

4.1. Abstract

The potential of producing the antimicrobial peptide caseicin A (IKHQGLPQE) from a casein substrate using a commercial proteolytic enzyme was assessed *in silico* and confirmed *in vitro*. Thermolysin (EC 3.4.24.27) and thermolysin-like enzymes (EC 3.4.24.28) were predicted by bioinformatic-aided analysis to be the likely candidates to liberate the bioactive peptide from α-S1-casein. Industrial sources of the thermolysin enzyme were then experimentally proven, under various conditions of hydrolysis, to liberate the 1049 Da caseicin A peptide from milk substrates at both lab and pilot scale. The antimicrobial ability of the filtered hydrolysates (<3kDa membrane) to reduce pathogen numbers spiked in infant formula was subsequently confirmed. During this process a second peptide, generated after hydrolysis with a specific enzyme, duplicate in size to caseicin A was identified and isolated. A synthetically generated peptide of identical amino acid sequence to this novel peptide, caseicin T (VFGKEKVNE), proved to have antimicrobial activity against a number of *Cronobacter* strains. In conclusion, this study presents the potential development of an industrial-grade method to produce milk-derived bioactive peptides.

4.2. Introduction

Milk proteins provide a natural source of bioactive peptides with potential health benefits and applications in the food industry. The release of these peptides from milk components is achieved either by hydrolysis using digestive proteases (Clare & Swaisgood, 2000) through food processing or by microbial enzymes in fermented products (Agyei & Danquah, 2011; Fitzgerald, 2006). Peptides derived from milk proteins, in particular, can exert numerous physiological benefits and milk proteins are seen as an important reservoir for bioactive peptides (Benkerroum, 2010; Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011). These bioactive peptides are known to contribute to the efficacy of functional foods in a number of specific cases (Gu, Majumder, & Wu, 2011). In order for bioactive peptides to become an industrially feasible option, peptides need to be released efficiently, economically and at sufficiently high enough quantities for application (Ross, Stanton, Hill, & Fitzgerald, 2007). Enzymatic hydrolysis is seen as a favourable method to generate milk bioactive peptides due to the lack of residual organic solvents or toxic chemicals in the end products. Bacterially-derived enzymes, in particular, are seen as advantageous due to the relatively lower costs. The majority of proteases generated by microorganisms are produced on the cell membrane making their harvesting and purification relatively economic (Agyei & Danquah, 2011; Zambrowicz, Timmer, Polanowski, Lubec, & Trziszka, 2012).

Previous studies from our group identified two casein-derived antimicrobial peptides, caseicin A (IKHQGLPQE) and caseicin B (VLNENLLR), with potential applications as dairy-based protectants against pathogens in powdered foods (Hayes *et al.*, 2009; Hayes, Ross, Fitzgerald,

Hill, & Stanton, 2006). The caseicin A and B peptides correspond to f(21-29) and f(30-37) respectively of bovine αS1-casein and together represent amino acids 6-23 of the characterised antibacterial peptide isracidin (Hayes *et al.*, 2006; Hill, Lahav, & Givol, 1974). Both peptides were found to inhibit the neonatal Gram-negative pathogen *Cronobacter sakazakii* in lab media and as part of fermentates in powdered infant formula (PIF) trials (Hayes *et al.*, 2009; Hayes *et al.*, 2006). Further work demonstrated their ability to inhibit other important Gram-negative pathogens including Salmonella and *Klebsiella* and the Gram-positive pathogen *Staphylococcus aureus* (McDonnell, Rivas, Burgess, Fanning, & Duffy, 2011; Norberg, Connor, *et al.*, 2011).

The enzymatic reactions responsible for the release of caseicin A and B from bovine sodium caseinate were predicted to be as a result of the combined action of a cell wall proteinase and 3 endopeptidases as a result of fermentation with the *Lactobacillus johnsonii* (previously *Lactobacillus acidophilus*) DPC 6026 strain (Hayes *et al.*, 2006). More recent work however, demonstrated that a common feature of *Bacillus cereus* and *Bacillus thuringiensis* strains is their ability to hydrolyse casein to produce both the caseicin A and B peptides following an overnight fermentation (Kent *et al.*, 2012). Members of *Bacillus* sp. are well documented to be efficient in hydrolysing casein and enzymes from many sources of *Bacillus* are exploited at an industrial scale and form key components of industrial biotechnology (Rao BM, 1998). The use of *Bacillus* cells or their lysates to directly produce the caseicin A and B antimicrobial peptides, however, is not an industrially feasible option (Kent *et al.*, 2012). A potential food-grade and cost effective solution is to employ already available

commercial *Bacillus*-derived enzymes that can liberate the antimicrobial peptides.

Computer aided design can theoretically predict the proteolytic cleavage sites among target proteins and therefore determine the possible bioactive peptides that can be released from complex systems (Minikiewicz, 2008). Previously Gu *et al.* demonstrated the potential of computer aided design by using an *in silico* approach to evaluate the suitability of various protein substrates as precursors of ACE inhibitory peptides (Gu *et al.*, 2011). The aim of this study was to use genomic and enzyme analysis to select commercial enzymes that could be used to release the caseicin peptides from casein as a basis to generate powders sufficient to contribute to the biosafety of infant milk formula. The antimicrobial activity of the powders to inhibit pathogen numbers was also demonstrated.

4.3. Materials and Methods

4.3.1. Bacterial strains and culture conditions.

C. sakazakii DPC6440 (NCTC 8155), *Salmonella typhimurium* LT2 and *S. aureus* SA113 were used as indicator organisms in powdered food trials. Other *Cronobacter* isolates used in synthetic caseicin T minimum inhibitory concentration (MIC) assays are listed in Table 4.1. Cultures were maintained at 37°C in Luria-Bertani (LB) media (Oxoid; Basingstoke, UK) under aerobic conditions.

4.3.2. Bioinformatic analysis.

The genome sequences of *Bacillus cereus* Q1 (CP0000227), *B. thuringiensis str. Al Hakam* (CP0000485) *and B. thuringiensis* BMB171 (CP0001903) were used for analysis of their protease complement. Protease enzymes were analysed using http://www.uniprot.org/, BRENDA (Chang, Scheer, Grote, Schomburg, & Schomburg, 2009) at http://www.brenda-enzymes.org/ and using the MEROPS database (Rawlings & Barrett, 1999; Rawlings, Morton, Kok, Kong, & Barrett, 2008) at http://merops.sanger.ac.uk/. The cleavage specificity of the thermolysin enzyme was analysed using peptide cutter http://expasy.org/tools/peptidecutter and the BIOPEP database (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008) http://www.uwm.edu.pl/biochemia.

4.3.3. Substrates and enzymes.

The proteases used were Thermolysin, Neutrase 0.8L, Protamex, Flavourzyme 500L (Sigma-Aldrich, St. Louis, MO), Thermoase PC10F,

Protease N, Newlase F, Protin NY100, Protease A, Protease M, Protease P, Acid Protease A (Amano Enzyme, Nagoya, Japan), and Protex 14L (Genencor, Rochester, NY, USA). Casein substrates included sodium caseinate and reconstituted skim milk (RSM) (Dairygold, Cork, Ireland) to 5 % and 10 % w/v respectively.

4.3.4. Enzymatic hydrolysis.

Enzymes were added to the casein substrates at varying concentrations (0.1 -2% w/v) and incubated at 37-65°C for 6-20 h to optimise hydrolysis. The pH was determined following hydrolysis. The hydrolysates were heated to 95^0C for 20 mins to inactivate the enzyme and then filtered through a size-exclusion 3kDa spiral cartridge filter (Millipore Ltd., Hertfordshire, UK). The filtrates were then taken forward for Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) Mass spectrometry (MS) and Reverse phase-high-performance liquid chromatography (RP-HPLC) analysis. Pilot-scale trials were determined at 10 L as above and subsequently spray-dried (Fox, 2003) to produce a bioactive powder.

Gel permeation-HPLC (GP-HPLC) as described previously (Kent *et al.*, 2012) was used to determine the optimum conditions for casein hydrolysis using the PC10F thermoase enzyme. Briefly, samples were diluted 1/10 following enzymatic treatment under relevant conditions and filtered through a 0.45 nm filter. Analysis on the GP-HPLC were performed using a Waters 626 pump, 600s controller and a 996 photodiode array detector (Waters Corporation, Dublin, Ireland) and were applied to a Grace Nucleosil 100, C18, 5-µm column (Grace, Il., USA). The mobile phase was 30% (v/v) acetonitrile

with 0.1% (v/v) trifluroacetic acid (TFA). Results were read at a wavelength of 280 nm. Sodium caseinate substrate was assayed at a concentration of 2.5%, 5% and 10%. Each percentage was treated with the enzyme at 2.5 mg/ml, 5mg/ml or 10 mg/ml. Each of these was in turn incubated at either 37°C, 50°C or 60°C. The degree of hydrolysis was measured at T0, T1, T2, T4, and T6h.

4.3.5. Mass Spectrometry (MS) and Reverse Phase (RP)-HPLC analysis of hydrolysates.

The permeates containing peptides of <3kDa were applied to a Jupiter Proteo (250 x 10mm, 4u, 90 Å) column (Phenomenex, Cheshire, United Kingdom) running a 10-30% acetonitrile 0.1% TFA gradient over 40 minutes. Fractions were analysed for the relevant peptide peaks using MALDI-TOF MS. Caseicin A was further purified by pooling the relevant fractions and running on a HPLC fitted with a RP C18 XBridge™ BEH300 (250 x 10mm, 5u, 300Å) column (Waters Corporation) running a 4-10% isopropan-2-ol, 0.1% TFA gradient over 40 minutes. Further purification if necessary was completed by applying fractions containing the peptide to a RP_HPLC Acclaim® (150 x 10 mm, 3µ, 300Å) column (Dionex Ireland Ltd., Dublin, Ireland) running a 20-35 % methanol 0.1% TFA gradient over 40 minutes. The caseicin A peptide was then lyophilised in a Genevac HT-4X evaporator (Genevac Ltd. Ipswich, UK). The amino acid sequence of a second peptide, caseicin T was verified by MS/MS. The MS/MS positive ion, averaged data +/- 0.8 Da was searched using the Mascot engine (http://www.matrix-science.com) with the following parameters: Database: NCBInr database or Swiss Prot, taxonomy: mammalian,

Enzyme thermolysin, one missed cleavage and variable modifications including methionine modified by oxygen.

4.3.6. Peptide Synthesis

The caseicin peptides were chemically synthesized using microwave-assisted solid-phase peptide synthesis (MW-SPPS) with a Liberty CEM microwave peptide synthesizer as previously described (Norberg, Connor, *et al.*, 2011). Caseicin A was made on H-Glu(OtBu)-HMPB-Chematrix resin and purified using RP-HPLC. Caseicin T was made on H-Glu(OtBu)-Wang-Matrix Innovation resin. Fractions containing the relevant peptide were identified using MALDI-TOF MS. Peptides were pooled and lyophilized as described earlier (Norberg, O'Connor, *et al.*, 2011).

4.3.7. MIC determination.

The Minimum inhibitory concentration (MIC) was determined for *C. sakazakii* DPC6440 using purified thermolytic caseicin A as previously described (Norberg, Connor, *et al.*, 2011; Wiedemann, 2006). Synthesized caseicin A was also assayed to compare to the enzyme-liberated caseicin A. Briefly, serial two-fold dilutions of caseicin A were made in LB broth and added to a 96 well plate. *C. Sakazakii* DPC6440 were cultured in LB broth to a final OD_{600} of 0.5. and then added to plates at a final concentration 10^5 CFU/mL. After incubation for 16 h at 37°C the MIC was read as the lowest peptide concentration that prevented visible growth. Results are given as the mean values of three independent determinations. Antimicrobial assays were performed as described previously (Kent *et al.*, 2012; Norberg, Connor, *et al.*,

2011) with some modifications. Briefly, caseicin T was reconstituted in LB broth at varying concentrations and added to a 96-well plate. Pathogenic strains (Table 4.1.) were added to a final concentration 10^3 CFU ml-1 and incubated with varying dilutions of the relevant peptide at 37°C for 24 h in a microtiter plate reader with hourly readings recording the optical density (600 nm). Results are given as the mean values of three independent determinations ± SEM.

4.3.8. Infant formula trials.

Infant formula trials were performed as previously described (Hayes *et al.*, 2009) with some minor modifications. Briefly, freeze-dried filtrates were reconstituted in water at 50 % w/v. A commercially available brand of infant milk was purchased. 25.5 g of infant formula was reconstituted in 180 ml of sterile, distilled water and pasteurized at 63ºC for 30 min in a water bath and subsequently cooled to room temperature. Pasteurised infant formula was dispensed in to 6-ml volumes and the sodium caseinate-thermoase hydrolysate was added to the formula at a final concentration of 10 % (wt/v). Controls employed included infant milk formula to which no caseicin powder was added, formula containing an unhydrolysed casein powder (heat-treated and not heat-treated) and or formula containing casein hydrolysed with a randomly selected proteases (data not shown). Pathogenic microbes were added to the infant formula to a final concentration of 10^1 – 10^2 CFU/ml. Samples were incubated at 37ºC and aliquots were taken and plated at T0, T1 and T6. All infant formula trials were carried out in triplicate.

4.4. Results

4.4.1. Identification of an enzyme that liberates caseicin A from α-S1-casein.

Previous work indicated that a conserved feature of the *B. thuringiensis* and *B. cereus* group is the ability to cleave casein to liberate the caseicin A IKHQGLPQE and B VLNENLLR peptides (Kent *et al.,* 2012). Other *Bacillus* species including *Bacillus subtilis, Bacillus firmus, Bacillus licheniformis, Bacillus stearothermophilus, Bacillus coagulans* and *Bacillus mycoides* were tested but were not found to produce the peptides from milk (Kent *et al.*, 2012).

In this study, *in silico* analysis of the available genome sequences from *B. cereus* (Xiong *et al.*, 2009) and *B. thuringiensis* (Challacombe *et al.*, 2007) were used to determine the potential protease gene candidates that may be responsible for the proteolytic nature of these species. The M4 family of peptidases which most resemble bacillolysin (EC 3.4.24.28) and thermolysin-like enzymes (EC 3.4.24.27) were predicted to be the most likely group of enzymes to have a role in casein degradation (Fig. 4.1a). These enzymes are neutral metalloproteases that show specificity for cleavage at hydrophobic amino acids but generally have broad substrate specificity. The Neutral protease (Npr) enzymes from *Bacillus* sp. have been well studied for industrial production and for elucidation of enzymatic mechanisms (Helmann, 1995; Imanaka, Shibazaki, & Takagi, 1986; Vecerek & Venema, 2000). The cleavage specificities for *Bacillus*-derived enzymes on bovine α-S1 casein were analysed using the ExPASy peptide cutter software (Gasteiger E., 2005) and the BIOPEP (Minkiewicz *et al.,* 2008) database. Although a perfect match to a full intact caseicin A peptide was not identified, based on this analysis,

thermolysin was the most likely candidate chosen as to liberate caseicin A from casein (Fig. 4.1b).

4.4.2. Hydrolysis of casein with commercial enzymes.

Commercial sources of EC 3.4.24.27 and EC 3.4.24.28 including thermolysin and bacillolysin enzymes were obtained in addition to other related proteases (EC 3.4.24.4) and selected unrelated commercial proteases from both bacterial and fungal sources (Table 4.2). All enzymes were tested for proteolytic activity and for the production of caseicin A and B from casein substrates. Although all enzymes could degrade casein, only sources of the thermolysin enzyme could produce caseicin A of 1049Da (IKHQGLPQE) following hydrolysis (Table 4.2.; Fig. 4.2a.). Commercial sources of the closely related bacillolysin enzymes (Neutrase 0.5L and Protease N) were unsuccessful in cleaving casein to produce caseicin A under the conditions tested despite the genetic loci identified in the *B. cereus* and *B. thuringiensis* genomes most resembling the bacillolysin group. Other proteases including enzymes both related (EC 3.4.24.4) and unrelated to thermolysin were also tested but none were found to release either of the caseicin peptides under the conditions of hydrolysis tested (Table 4.2.). Interestingly, a fungal source of an enzyme of the thermolysin family (EC 3.4.24.27; Protease A) also did not produce caseicin A following casein hydrolysis indicating the specificity of the *Bacillus*-derived form of the enzyme to produce the peptide (Table 4.2.). The caseicin B peptide of 970 Da (VLNENLLR) was not detected in any of filtrates. As the BIOPEP database analysis indicates that caseicin B is hydrolysed by the thermolysin enzyme (Fig. 4.1b.), it is therefore likely that

within the *Bacillus* cells a separate enzyme or mechanism is responsible for caseicin B production. Further analysis would be required to determine this.

GP-HPLC indicated that thermoase PC10F efficiently hydrolysed caseinate at all temperatures and concentrations investigated (data not shown). However, peptides of 1049 Da were only visible by MALDI-TOF MS after an incubation >4 h.

4.4.3. Caseicin peptide A production with industrial sources of thermolysin.

Additional investigation by MS and RP-HPLC with two industrial sources of thermolysin; Thermoase PC10F (Amano Enzymes) and Protex 14L (Genencor) revealed that caseicin A could be liberated from casein under varying conditions of hydrolysis including temperature (37-65^{0}C), time of hydrolysis (6-24h) and using different casein substrates (5% w/v Sodium caseinate and 10% RSM). An enzyme to substrate ratio of 0.5% w/v was optimal for caseicin A production. RP-HPLC (Fig. 4.2a. and 4.2b.) analysis revealed a yield significantly higher (~9-11 times greater) than achieved by bacterial fermentation (R. Kent *et al.*, 2012)

Ten litre pilot-scale trials were then performed to investigate if caseicin A was produced in sufficient quantities on scale up and if the bioactivity was stable. Hydrolysates were heat treated at 95^{0}C to inactivate the enzyme and subsequently spray dried to create a bioactive powder. MS and RP-HPLC analysis on resuspended powders demonstrated the presence of caseicin A in the spray dried powders (not shown).

Caseicin T generation was only apparent with Hydrolysis by Thermoase PC10F.

4.4.4. Antimicrobial activity of thermolytic-released caseicin A

The 1049Da caseicin A peptide IKHQGLPQE was purified by RP-HPLC and MICs were performed as previously described (Norberg, Connor, *et al.*, 2011) against a known sensitive strain, *Cronobacter sakazakii* DPC 6440 (Hayes *et al.*, 2006; Norberg, Connor, *et al.*, 2011). Purified sources of the peptide were obtained from 3 kDa hydrolysates that had been subjected to heat treatment to inactivate the thermolysin enzyme and a control which had not been heat treated. As the thermolysin enzyme is ~36 kDa it would also be removed by filtration. MIC data indicate that the both heat-treated and non-heat treated thermolytic-liberated caseicin A resulted in an MIC of 0.625mM which is identical to the MIC achieved with the synthesised peptides in this study and in previous work (Norberg, Connor, *et al.*, 2011).

4.4.5. Infant formula trials

Freeze-dried <3kDa Thermoase PC10F hydrolysates were generated using a Virtis Advantage freeze-dryer (SP Industries, 935 Mearns Road, Warminster, PA, USA) and were added to infant formula preparations at 0-12.5% w/v and preparations were spiked with the pathogens *C. sakazakii, S. aureus* and *S. typhimurium* and trials were performed as previously described (Hayes *et al.*, 2009). A total of four thermoase powders were produced to determine if a 3 kDa filtration and/or heat treatment would influence

antimicrobial activity. The function of the heat treatment step is to inactivate the thermolysin enzyme as it cannot be present in a final product. The size of the thermolysin enzyme is 36 kDa so if a filtering step is included (~3 kDa); it removes the thermolysin from the hydrosylate powders however. If the 3 kDa filtration step is omitted, then it would have to be heat treated to ensure that the enzyme was not active. Heat treatment at 95ºC for 20 min is documented to inactivate the thermolysin enzyme (Matthews BW, Weaver LH & Kester WR, 1974). A 3 kDa filtration step was used to purify and concentrate the active peptide. The milk was spiked with *S. aureus* SA113, 8325-4, Newman, *S. typhimuirum* LT2 and *C. sakazakii* DPC6440 each at a level of 10^1-10^2 CFU/ml and incubated for 6 h at 37˚C.

The thermoase powder exhibited similar activity levels to those observed for the reconstituted skim milk trials. While results varied slightly between strains, a 10^2-10^3 CFU/ml reduction in growth was observed with the thermoase hydrosylate powders. Interestingly, *C. sakazakii* DP6440 was completely inhibited following 6 hr incubation with the thermoase treated hydrosylate powder which had not been filtered or heat treated. The growth of the other organisms were all affected in a similar manner, with a 10^2 CFU/ml - 10^3 CFU/ml reductions in growth being observed (Fig 4.3.).

4.4.6. Antimicrobial activity of synthetic caseicin T

The antimicrobial activity of synthetically generated caseicin T was assayed against a number of pathogens including four *Cronobacter* strains. The peptide completely restricted growth in the *Cronobacter* strains assayed at a concentration of 2.5 mM (2.625 mg ml^{-1}). Caseicin T also prevented the

growth of a Salmonella strain at the same concentration. The peptide did not have an inhibitory effect on a *Staphylococcus aureus* strain (Fig. 4.4.).

4.5. Discussion

Milk-derived peptides with antimicrobial activity have much industrial potential as they have the advantage of being derived from a safe and economical source. Their large-scale manufacture and commercial exploitation however is still limited as industrial scale production is often not possible due to lack of suitable economical technologies. In general they also suffer from the disadvantage of having relatively high MIC values which are in the mM range compared to classical antimicrobial peptides such as defensins and bacteriocins with MIC value in the µM – nM range. Bioactive peptides may be produced either by *in vitro* enzymatic hydrolysis or by *in situ* microbial fermentation of food proteins. The use of enzymatic hydrolysis to produce natural bioactives is preferential to microbial fermentation for ease of scalability and as it not influenced by microbial growth parameters.

In this study, enzyme hydrolysis was used to liberate the caseicin A peptide (IKHQGLPQE) from casein previously described to be released as a result of microbial fermentation (Hayes *et al.*, 2006; R. Kent *et al.*, 2012) Thermolysin and thermolysin-like enzymes were predicted by bioinformatic analysis to be the likely candidates to release the peptide from α-S1 casein (Fig.4.1.). The *in silico* generation of a peptide of interest before wet-lab production allows the streamlining of the process (Agyei & Danquah, 2011; Wang, Zhang, Wang, Feng, & Shan, 2011). Improved protein and peptide databases increase the feasibility of predicting new and more potent bioactives prior to the beginning of time consuming laboratory synthesis. Experimental analysis using a number of commercial enzymes indicated that the peptide release was specific to the *Bacillus*-derived thermolysin enzyme (EC 3.4.24.27)

as the closely related bacillolysin enzymes (EC 3.4.24.28) or the *Aspergillus*-derived thermolysin were not successful in producing caseicin A under the conditions tested (Table 4.1.).

The generation of a previously unidentified antimicrobial peptide, caseicin T illustrates the fact that targeted production of a peptide can lead to unexpected bi-products. Although high concentrations of the peptide were required inclusion with another antimicrobial might allow for the creation of an effective hurdle system to prevent growth of contaminating bacteria. It should be noted that extensive hydrolysis of milk proteins, which was performed in this study leads to the generation of a large number of milk peptides. In this study a full screen of every resultant peptide was not undertaken and there is a possibility that numerous other peptides possess bioactivity. Indeed some unidentified peptides may also contribute to the antimicrobial activity observed in the infant formula trials described in this chapter. Caseicin T was further investigated due the peptides molecular size similarity with caseicin A.

Thermolysin is the best studied member of the zinc endopeptidase family and is currently used in the food industry as a peptide and ester synthetase in the production of the artificial sweetener aspartame (Ager *et al.*, 1998; Erbeldinger *et al.*, 2000; Ulijn *et al.*, 2001). Food-grade forms of thermolysin are available as partially purified sources as Thermoase PC10F (Amano enzyme) and Protex 14L (Genencor, Danisco) which were verified in this study or as an immobilised form (Chirax) which would allow enzymatic hydrolysis under milder and more controlled conditions. Sources of these enzymes are readily available in pilot and industrial scale which allows for easy scale up and are derived from *Bacillus thermoproteolyticus* which is a

food-grade organism. As the principal problem in bioactive peptide generation is the prohibitive cost of their manufacture (manufacturing costs can range from $50-400 per gram) (Marr, Gooderham, & Hancock, 2006) a streamlined enzymatic method shows increased feasibility at an industrial level.

The antimicrobial activity of the thermolytic-liberated caseicin A in a purified form and as part of powdered filtrates was also confirmed in this study. A major difficulty in development for industrial production however, is the need for a filtration step at present within the process. It is estimated that separation and purification stages account for up to 70 % of the capital and operating costs (Brady, Woonton, Gee, & O'Connor, 2008). Investigations into novel methods to isolate and purify bioactive peptides in a timely and economical manner are on-going (Agyei & Danquah, 2011).

Further trials with a range of powdered food products and pathogens are necessary to determine the full potential of the thermolysin generated powders described in this study. Although the powdered hydrolysates did not have a significant reduction in bacterial cells, growth was slowed indicating its ability to prevent the outgrowth of bacterial cells in food.

In conclusion, computer aided analysis identified in this study industrially available enzyme that can be potentially applied at pilot scale. This indicates the opportunity to tailor the release of specific peptides from complex sources by use of different known proteases singly or in combination for a greater diversity of potential functional food products.

4.6. Acknowledgements

This project was funded by an Enterprise Ireland commercialisation fund CFTD/07/116.

Paula O'Connor provided technical assistance with MALDI-TOF MS and MW-SPPS.

Infant formula trials were performed by Sarah Norberg

4.7. References

Agyei, D., & Danquah, M. K. (2011). Industrial-scale manufacturing of pharmaceutical-grade bioactive peptides. *Biotechnology advances*.

Benkerroum, N. (2010). Antimicrobial peptides generated from milk proteins: a survey and prospects for application in the food industry. A review. *International Journal of Dairy Technology, 63*(3), 320-338.

Brady, R., Woonton, B., Gee, M. L., & O'Connor, A. J. (2008). Hierarchical mesoporous silica materials for separation of functional food ingredients - A review. *Innovative Food Science & Emerging Technologies, 9*(2), 243-248. doi: 10.1016/j.ifset.2007.10.002

Challacombe, J. F., Altherr, M. R., Xie, G., Bhotika, S. S., Brown, N., Bruce, D., . . . Brettin, T. S. (2007). The complete genome sequence of Bacillus thuringiensis Al Hakam. *J Bacteriol, 189*(9), 3680-3681. doi: JB.00241-07 [pii] 10.1128/JB.00241-07

Chang, A., Scheer, M., Grote, A., Schomburg, I., & Schomburg, D. (2009). BRENDA, AMENDA and FRENDA the enzyme information system: new content and tools in 2009. *Nucleic Acids Res, 37*(Database issue), D588-592. doi: gkn820 [pii] 10.1093/nar/gkn820

Clare, D. A., & Swaisgood, H. E. (2000). Bioactive milk peptides: a prospectus. *J Dairy Sci, 83*(6), 1187-1195. doi: 10.3168/jds.S0022-0302(00)74983-6

Fitzgerald, R., Murray, BA., . (2006). Bioactive peptides and lactic fermentations. *International Journal of Dairy Technology, 59*, 118-125.

Fox, P. F. (2003). *Advanced dairy chemistry: proteins* (3 ed. Vol. 1): Elsevier.

Gasteiger E., H. C., Gattiker A., Duvaud S., Wilkins M.R., Appel R.D., Bairoch A.;. (2005). Protein Identification and Analysis Tools on the ExPASy Server. *(In) John M. Walker (ed): The Proteomics Protocols Handbook, Humana Press (2005).*

Gu, Y., Majumder, K., & Wu, J. (2011). QSAR-aided in silico approach in evaluation of food proteins as precursors of ACE inhibitory peptides. *Food Research International*.

Hayes, M., Barrett, E., Ross, R. P., Fitzgerald, G. F., Hill, C., & Stanton, C. (2009). Evaluation of an antimicrobial ingredient prepared from a Lactobacillus acidophilus casein fermentate against Enterobacter sakazakii. *J Food Prot, 72*(2), 340-346.

Hayes, M., Ross, R. P., Fitzgerald, G. F., Hill, C., & Stanton, C. (2006). Casein-derived antimicrobial peptides generated by Lactobacillus acidophilus DPC6026. *Appl Environ Microbiol, 72*(3), 2260-2264. doi: 72/3/2260 [pii] 10.1128/AEM.72.3.2260-2264.2006

Helmann, J. D. (1995). Compilation and analysis of Bacillus subtilis sigma A-dependent promoter sequences: evidence for extended contact between RNA polymerase and upstream promoter DNA. *Nucleic Acids Res, 23*(13), 2351-2360. doi: 5t0130 [pii]

Hill, R. D., Lahav, E., & Givol, D. (1974). A rennin-sensitive bond in alpha-s1 B-casein. *J Dairy Res, 41*(1), 147-153.

Imanaka, T., Shibazaki, M., & Takagi, M. (1986). A new way of enhancing the thermostability of proteases. *Nature, 324*(6098), 695-697. doi: 10.1038/324695a0

Kent, R.M., Guinane, C. M., O'Connor, P. M., Fitzgerald, G. F., Hill, C., Stanton, C., & Ross, R. P. (2012). Production of the antimicrobial peptides Caseicin A and B by Bacillus isolates growing on sodium caseinate. *Letters in Applied Microbiology*.

Marr, A. K., Gooderham, W. J., & Hancock, R. E. W. (2006). Antibacterial peptides for therapeutic use: obstacles and realistic outlook. *Current opinion in pharmacology, 6*(5), 468-472.

Matthews BW, Weaver LH and Kester WR. (1974). "The conformation of thermolysin". J. Biol. Chem. 249 (24): 8030–8044

McDonnell, M. J., Rivas, L., Burgess, C. M., Fanning, S., & Duffy, G. (2011). Inhibition of verocytotoxigenic *Escherichia coli* by antimicrobial peptides caseicin A and B and the factors affecting their antimicrobial activities. *International Journal of Food Microbiology*.

Minikiewicz, P., J. Dziuba, M. Darewicz, A. Iwaniak, M. Dziuba and D. Nalecz. (2008). Food Peptidomics. *Food Technology and Biotechnology, 46*(1), 1-10.

Minkiewicz, P., Dziuba, J., Iwaniak, A., Dziuba, M., & Darewicz, M. (2008). BIOPEP database and other programs for processing bioactive peptide sequences. *J AOAC Int, 91*(4), 965-980.

Norberg, O'Connor P.M., Stanton, C., Ross, R. P., Hill, C., Fitzgerald, G. F., & Cotter, P. D. (2011). Altering the composition of caseicins A and B as a means of determining the contribution of specific residues to antimicrobial activity. *Appl Environ Microbiol, 77*(7), 2496-2501. doi: 10.1128/AEM.02450-10

Rao BM, T. M., Ghatge SM, Deshpande VV (1998). Molecular and Biotechnological Aspects of Microbial Proteases: . *Microbiology and Molecular Biology Reviews, 62*(3), 597-635.

Rawlings, N. D., & Barrett, A. J. (1999). MEROPS: the peptidase database. *Nucleic Acids Res, 27*(1), 325-331. doi: gkc009 [pii]

Rawlings, N. D., Morton, F. R., Kok, C. Y., Kong, J., & Barrett, A. J. (2008). MEROPS: the peptidase database. *Nucleic Acids Res, 36*(Database issue), D320-325. doi: gkm954 [pii] 10.1093/nar/gkm954

Ross, P., Stanton, C., Hill, C., & Fitzgerald, G. (2007). Casein-derived antimicrobial peptides and Lactobacillus strains that produce them: WO Patent WO/2007/057,872.

Ryan, J. T., Ross, R. P., Bolton, D., Fitzgerald, G. F., & Stanton, C. (2011). Bioactive peptides from muscle sources: Meat and fish. *Nutrients, 3*(9), 765-791.

Vecerek, B., & Venema, G. (2000). Expression of the neutral protease gene from a thermophilic Bacillus sp. BT1 strain in Bacillus subtilis and its natural host: identification of a functional promoter. *J Bacteriol, 182*(14), 4104-4107.

Wang, Z. L., Zhang, S. S., Wang, W., Feng, F. Q., & Shan, W. G. (2011). A novel angiotensin I converting enzyme inhibitory peptide from the milk casein: Virtual screening and docking studies. *Agricultural Sciences in China, 10*(3), 463-467.

Wiedemann, I., *et al.*,. (2006). The mode of action of the lantibiotic lacticin 3147—a complex mechanism involving specific interaction of two peptides and the cell wall precursor lipid II. . *Molecular Microbiology, 61*, 285-296.

Xiong, Z., Jiang, Y., Qi, D., Lu, H., Yang, F., Yang, J., . . . Jin, Q. (2009). Complete genome sequence of the extremophilic Bacillus cereus strain Q1 with industrial applications. *J Bacteriol, 191*(3), 1120-1121. doi: JB.01629-08 [pii] 10.1128/JB.01629-08

Zambrowicz, A., Timmer, M., Polanowski, A., Lubec, G., & Trziszka, T. (2012). Manufacturing of peptides exhibiting biological activity. *Amino Acids*, 1-6.

Table 4.1. Bacterial strains challenged with synthetic caseicin T

Strain	**Culture Collection No.**	**Source**
C. sakazakii	DPC6440	Dried Milk powder
C. sakazakii	DPC6522	Blood
C. sakazakii	DPC6523	Cerebrospinal Fluid
C. sakazakii	DPC6528	Cerebrospinal Fluid
C. sakazakii	DPC6531	Brain Tissue
S. typhimurium	DT 104	Unknown
Staph. aureus	4330	Unknown

Table 4.2. Commercial and Food grade enzymes used in this study

Protease	**Source/Organism**	**Enzyme group**	**Caseicin A Peptide**
Thermolysin[a]	*Bacillus thermoproteolyticus*	EC 3.4.24.27	1049 Da IKHQGLPQE
Thermoase PC10F	*Bacillus thermoproteolyticus*	EC 3.4.24.27	1049 Da IKHQGLPQE
Protex 14L	*Bacillus thermoproteolyticus*	EC 3.4.24.27	1049 Da IKHQGLPQE
Protease N	*Bacillus subtilis*	EC 3.4.24.28	nd
Neutrase 0.5L[a]	*Bacillus subtilis*	EC 3.4.24.28	nd
Protin NY100	*Bacillus subtilis*	EC 3.4.24.4	nd
Protamex[a]	*Bacillus sp.*	EC 3.4.21.14	nd
Newlase F	*Rhizopus riveus*	EC 3.4.23.21	nd
Protease A	*Aspergillus oryzae*	EC 3.4.24.27	nd
Protease M	*Aspergillus oryzae*	EC 3.4.23.18	nd
Protease P	*Aspergillus melleus*	EC 3.4.21.63	nd
Acid protease	*Aspergillus niger*	EC 3.4.23.18	nd

[a] Lab-grade form of enzyme used
nd; No peptide detected

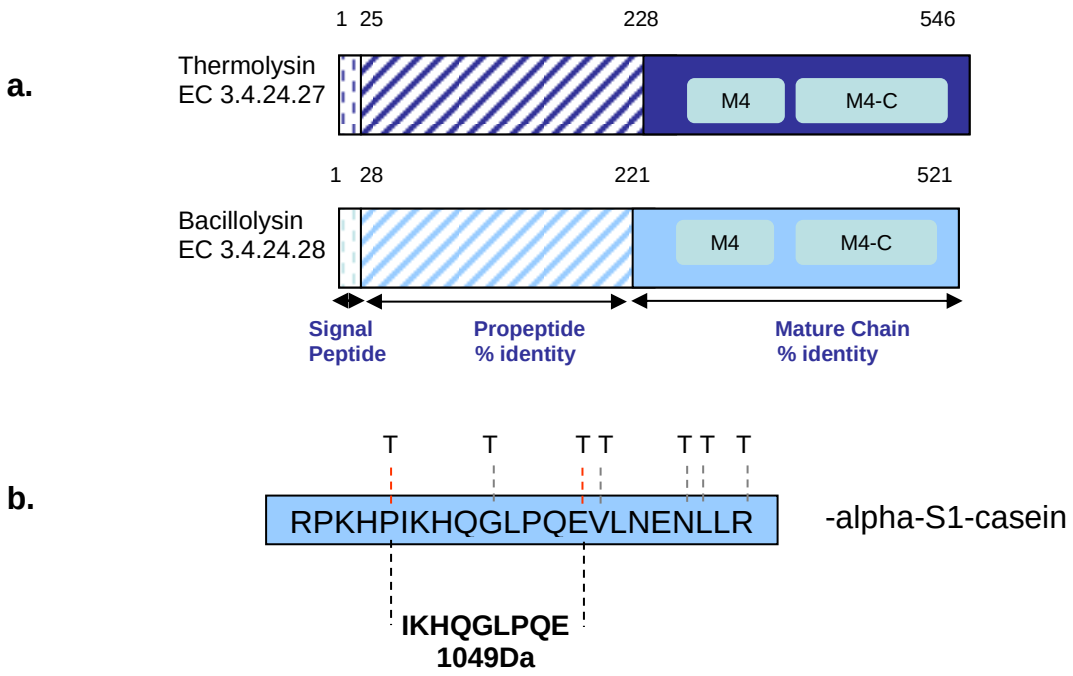
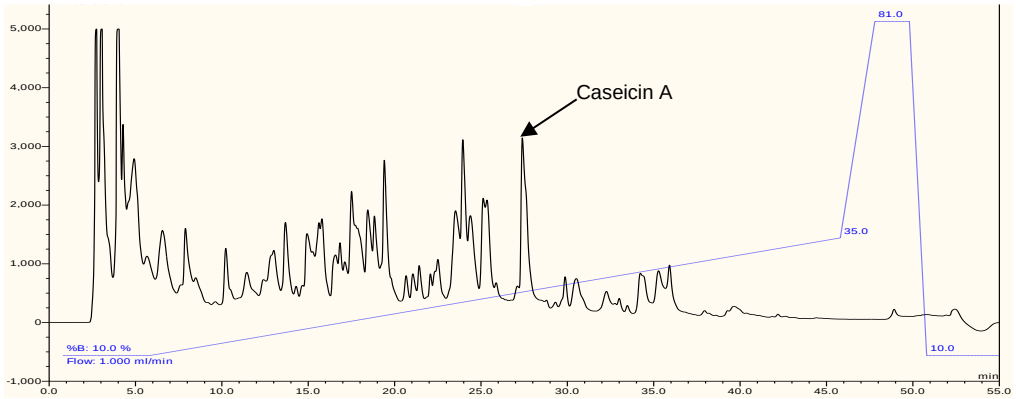


Figure 4.1. Protein representation of the thermolysin and bacillolysin enzymes with related functional domains (a) and predicted thermolysin cleavage sites on alpha-S1-casein (b).

a.



b.

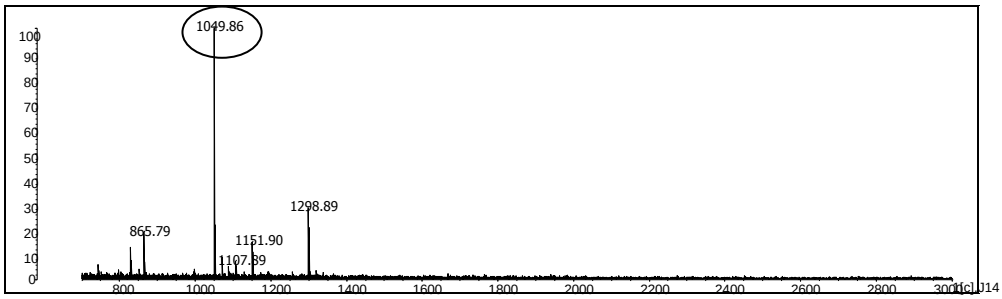
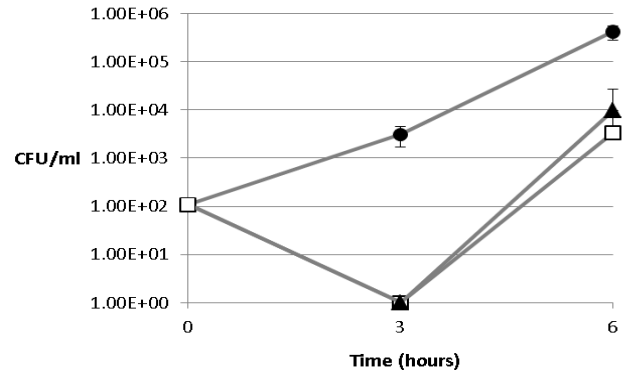
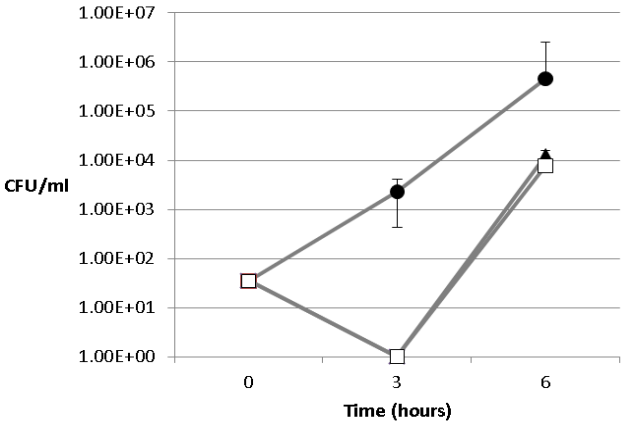


Figure 4.2. RP-HPLC of thermolysin produced caseicin A (b) and confirmed MS of the purified fraction (b).

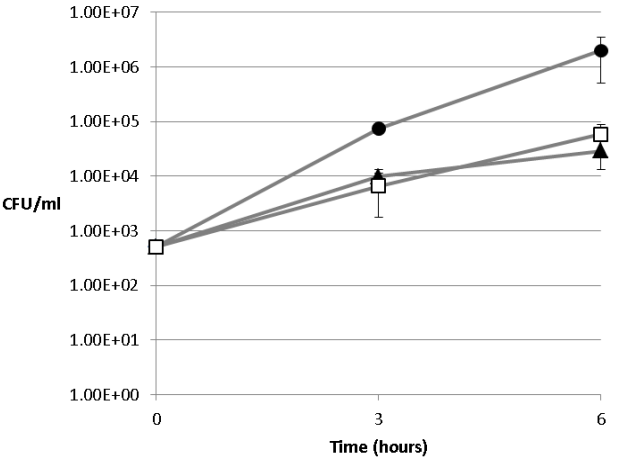
(a)


1.00E+06
1.00E+05
1.00E+04
CFU/ml 1.00E+03
1.00E+02
1.00E+01
1.00E+00
0
3
6
Time (hours)

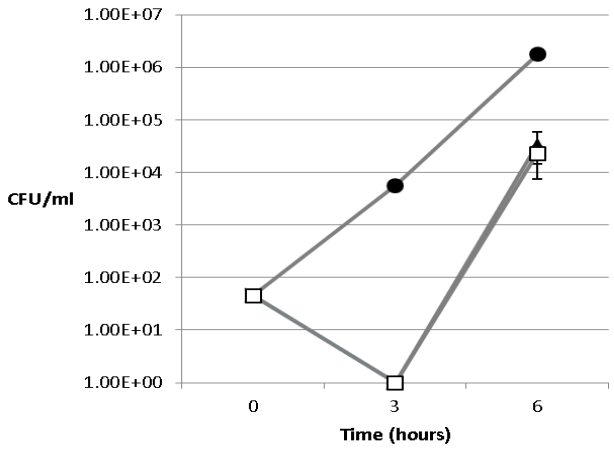
(b)


1.00E+07
1.00E+06
1.00E+05
1.00E+04
CFU/ml
1.00E+03
1.00E+02
1.00E+01
1.00E+00
0
3
6
Time (hours)

(c)


1.00E+07
1.00E+06
1.00E+05
1.00E+04
CFU/ml
1.00E+03
1.00E+02
1.00E+01
1.00E+00
0
3
6
Time (hours)

(d)



(e)

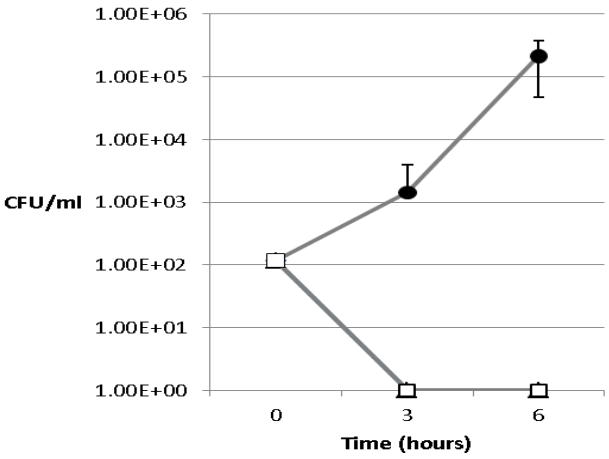
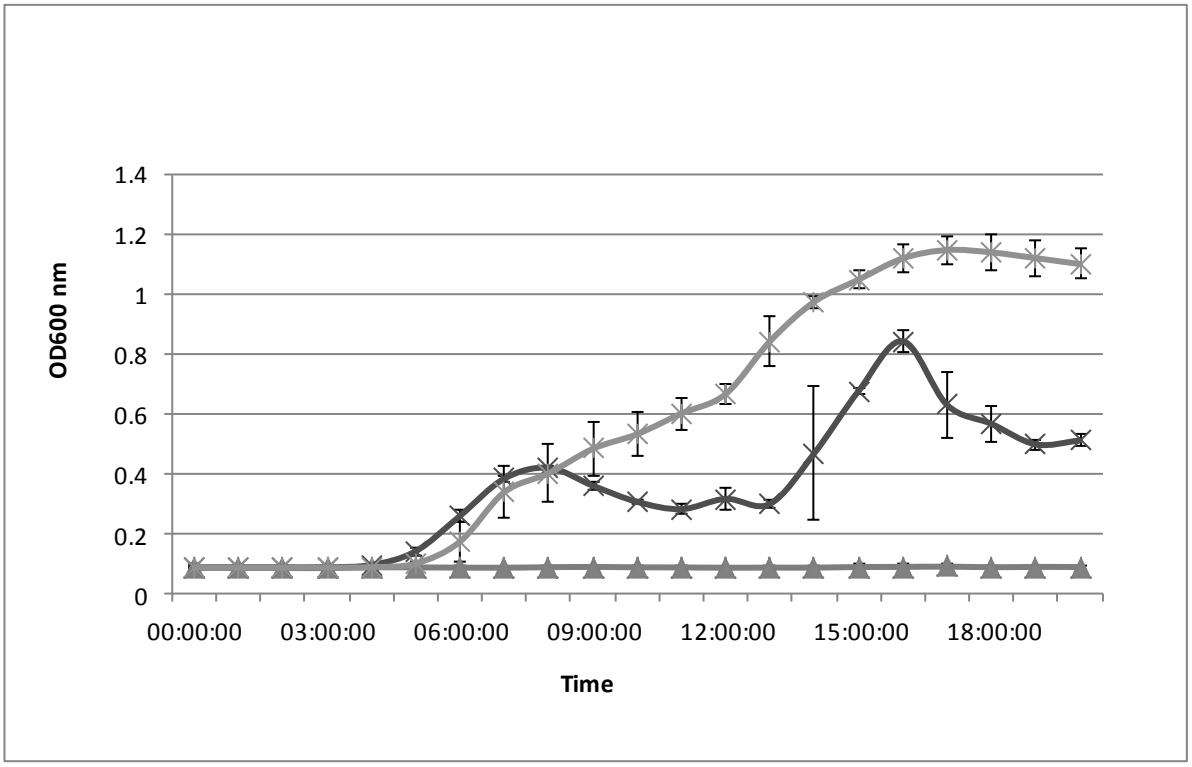
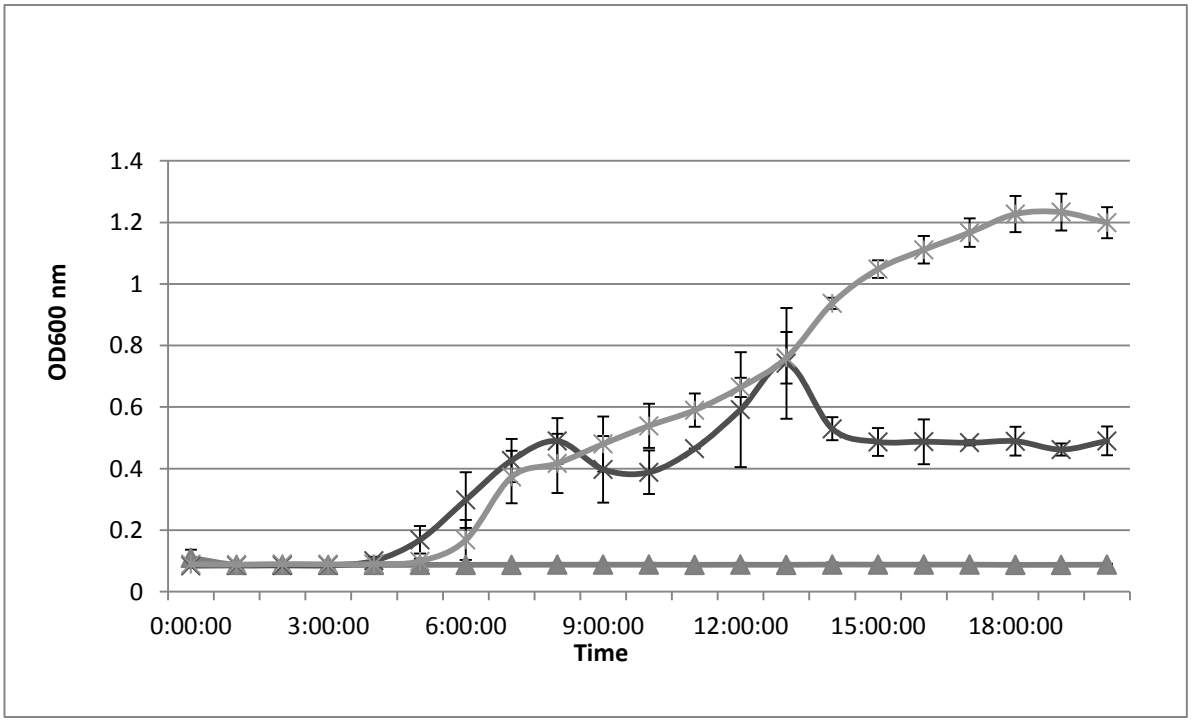


Figure 4.3. Effect of <3kDa thermolysin-hydrolysate in reconstituted infant formula at 37°C with unhydrolysed fermentate as a control against *C. sakazakii* DPC6440 (a), *Salmonella* LT2 (b) and *Staphylococcus aureus* 8325-4 (c), *S. aureus* Newman (d) and *S. aureus* SA113 (e). The indicator strains were incubated at 37°C for 6 h in the presence of 10% caseicin hydrosylate in reconstituted powdered infant formula. The mean of triplicate values were graphed and the standard deviations were also included. ●; represents the indicator incubated with unhydrolysed casein, □; represents the indicator incubated with 3Kda filtered casein powders hydrolysed with thermoase PC10F. ▲; represents the indicator incubated with casein powder hydrolysed with thermoase PC10F (unfiltered).

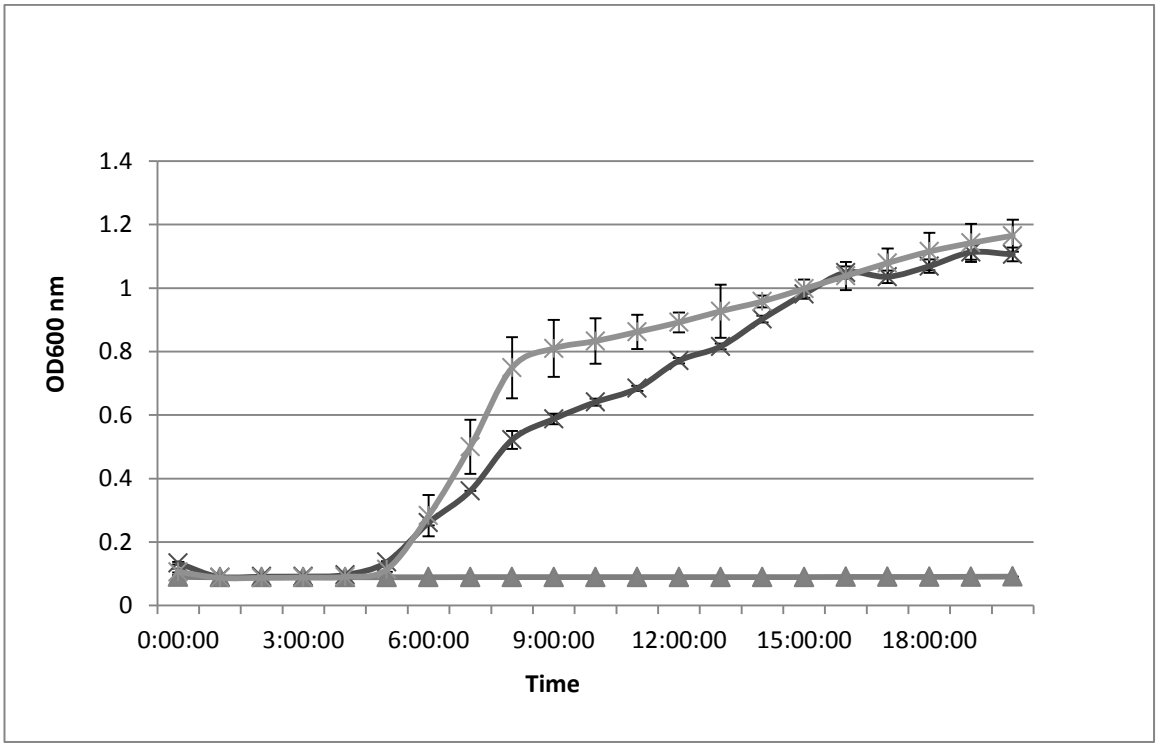
A


1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600 nm
00:00:00
03:00:00
06:00:00
09:00:00
12:00:00
15:00:00
18:00:00
Time

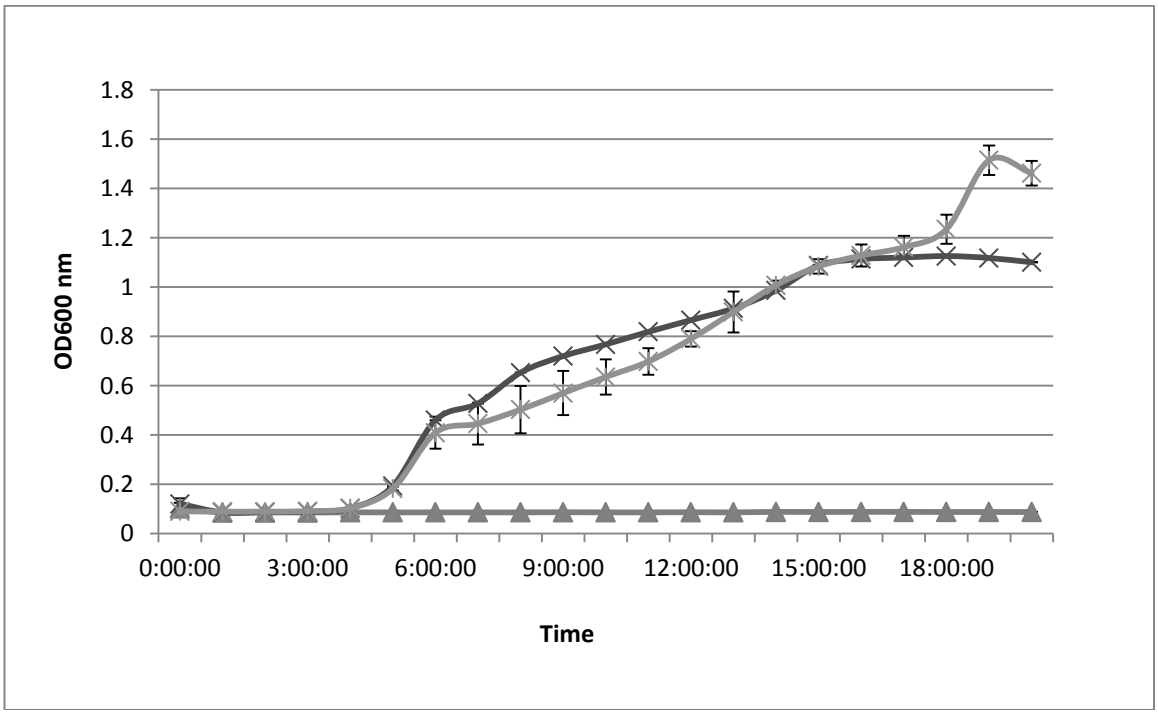
B


1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600 nm
0:00:00
3:00:00
6:00:00
9:00:00
12:00:00
15:00:00
18:00:00
Time

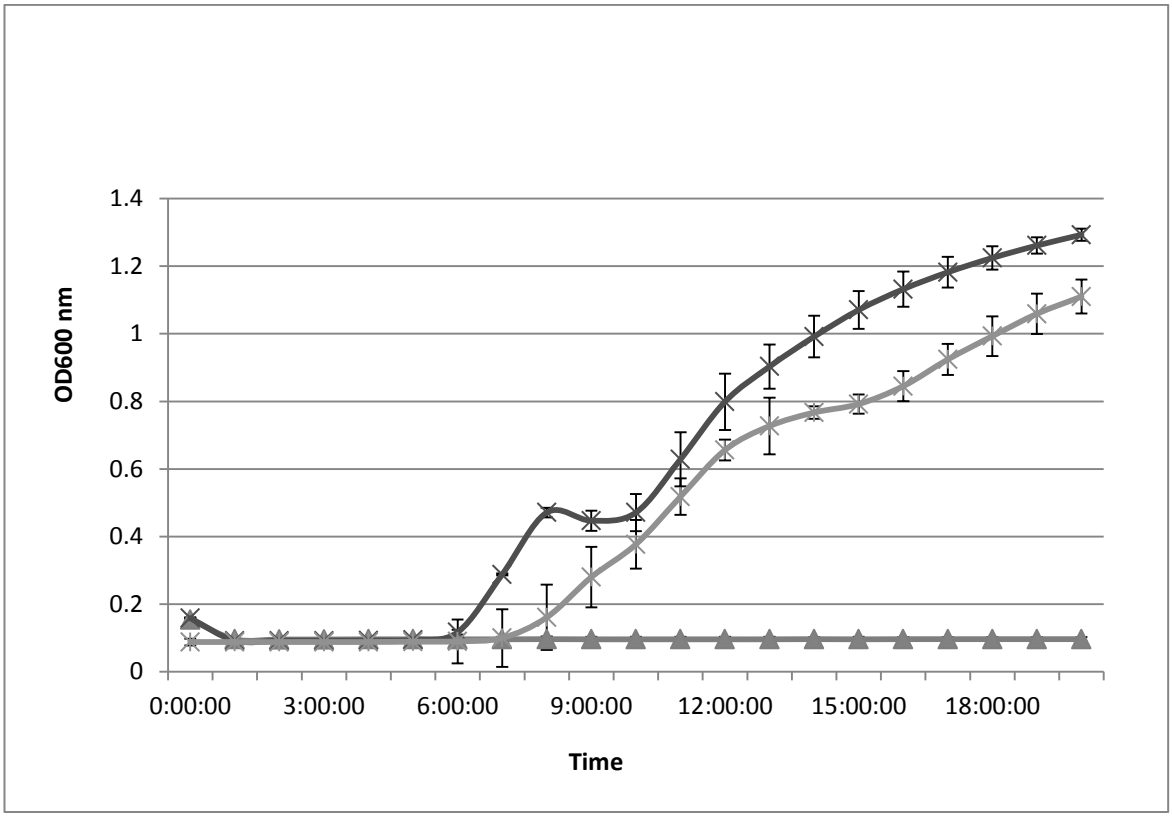
C


1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600 nm
0:00:00
3:00:00
6:00:00
9:00:00
12:00:00
15:00:00
18:00:00
Time

D


1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600 nm
0:00:00
3:00:00
6:00:00
9:00:00
12:00:00
15:00:00
18:00:00
Time

E


1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600 nm
0:00:00
3:00:00
6:00:00
9:00:00
12:00:00
15:00:00
18:00:00
Time

F

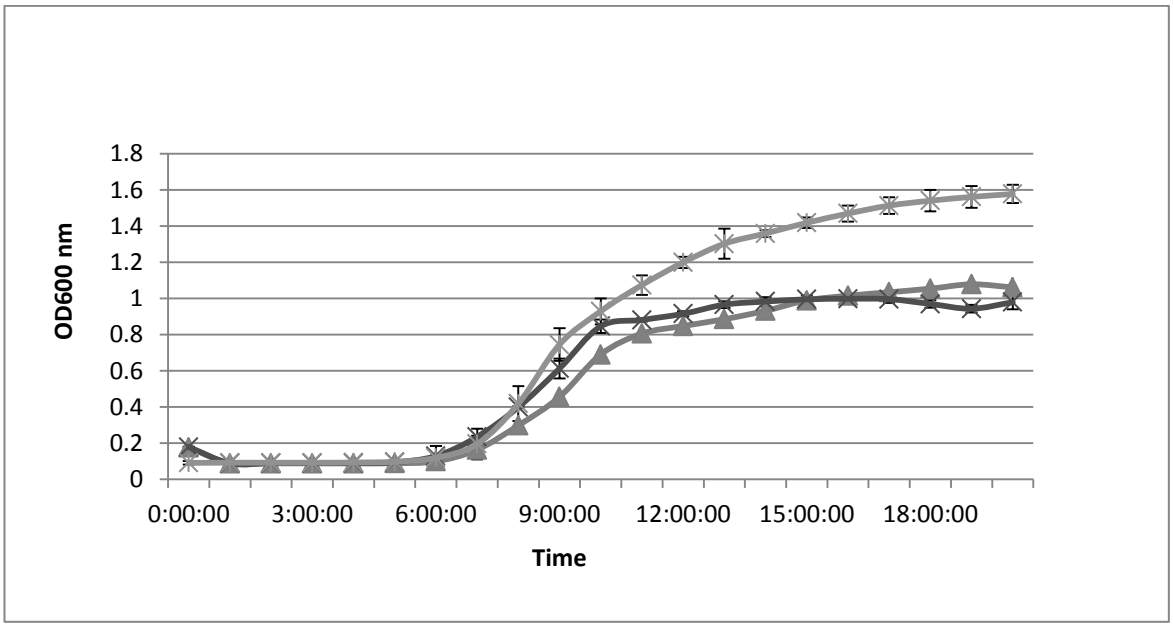

1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600 nm
0:00:00
3:00:00
6:00:00
9:00:00
12:00:00
15:00:00
18:00:00
Time

Figure 4.4. Effect of synthetic caseicin T on the growth of various strains in LB broth at 37°C. (A) *C. sakazakii* DPC6522, (B) *C. sakazakii* DPC6523 (C) *C. sakazakii* DPC628 (D) *C. sakazaki*i DPC6531 (E) *Salmonella* DT109 (F) MR *Staph. aureus* × represents the positive control; **X** represents caseicin T at a concentration of 1.25 mM ▲ represents caseicin T at 2.5 mM concentration.

Chapter 5

Recombinant *Lactobacillus paracasei* strain producing active gut hormone Glucagon-Like peptide-1 analogue

Robert M. Kent, Helena M. Stack, Paula M. O'Connor, Colin Hill, Gerald F. Fitzgerald, Seeley, R.J., Catherine Stanton and R. Paul Ross

5.1. Abstract:

Glucagon-like peptide-1 (GLP-1) is an important incretin hormone secreted by intestinal L-cells in response to nutrient ingestion. Administration of the peptide leads to improvement of glycaemic control in type 2 diabetes patients but therapeutic use of the peptide is limited as it has a short biological half-life due to the action of the aminopeptidase dipeptidyl peptidase IV (DPP IV) which is ubiquitously expressed throughout mammalian tissues. In this study, we investigated whether GLP-1 could be overexpressed by an intestinal *Lactobacillus* strain using the machinery for production and secretion of the bacteriocin pediocin. To achieve this, the operon required for pediocin production was first cloned into the expression vector pNZ44. The nucleotide sequence for KGLP-1, a DPP-IV resistance native GLP-1 analogue was then substituted for the pediocin structural gene using splicing overlap excision PCR. Both plasmid constructs were then electro-transformed into the *Lactobacillus paracasei* NCC 338 to generate two transformants, *Lactobacillus paracasei* DPC PED (pediocin producer) and *Lactobacillus paracasei* DPC 6770 (KGLP-1 producer). Transformants were screened for relevant heterologous peptide production using MALDI-TOF mass spectrometry. *Lactobacillus paracasei* DPC PED was screened for pediocin production using well diffusion assays. *Lactobacillus paracasei* DPC 6770 was screened for KGLP-1 production and *in-vitro* insulinotropic activity. Our results illustrate a method to generate recombinant strains capable of producing bioactive peptides with the potential to improve human health.

5.2. Introduction

The incretin effect is the term used to describe the greater insulin response induced by oral glucose load compared to the response induced by isoglycemic intravenous glucose infusion (Doyle & Egan, 2007; Elrick, Stimmler, Hlad, & Arai, 1964; Kazakos, 2011). This effect is principally attributed to two incretin hormones, namely glucose-dependent insulinotropic peptide (GIP) and Glucagon-like peptide-1 (GLP-1) (Doyle & Egan, 2007; Holst, 2007; Kazakos, 2011). Both hormones are post-prandially released and potentiate glucose-dependent insulin secretion by the pancreas (Drucker, 2006). The intensification of these hormones, especially GLP-1 has recently been the subject of extensive research in order to provide novel treatments for type 2 diabetes (Pontarolo *et al.*, 2013). The majority of GLP-1 is generated in enteroendocrine L-cells located in the distal gut after food intake and is found in two equipotent active forms, namely GLP-1 (7-37) and GLP-1 (7-36) amide (Drucker & Nauck, 2006). Although exogenous GLP-1 lowers post-prandial glucose levels in type 2 diabetes patients (Siegel *et al.*, 1999), the peptide is rapidly inactivated (2-3 min) by dipeptidyl peptidase IV (DPP-IV) (Baggio & Drucker, 2007; Mentlein, Gallwitz, & Schmidt, 1993). DPP-IV is a ubiquitous enzyme expressed throughout mammalian tissues and organs (Augustyns *et al.*, 1999) which selectively removes the N-terminal dipeptide from peptide sequences with alanine or proline in the second position (Lambeir, Durinx, Scharpé, & De Meester, 2003). In the case of GLP-1 DPP-IV cleaves the His^{7}-Ala^{8} dipeptide from the N-terminus of GLP-1 generating GLP-1 (9-36) amide. This truncated form of GLP-1 has no known biological activity and may act as a GLP-1 receptor agonist further inhibiting the action of intact GLP-1 (Knudsen & Pridal,

1996). Accordingly, the development of longer acting GLP-1 analogues, resistant to enzymatic cleavage would greatly improve the therapeutic potential of the peptide as a type 2 diabetes treatment (Chen *et al.*, 2007; Siegel *et al.*, 1999).

A number of strategies have been studied to overcome the therapeutic limitations caused by the short half-life of GLP-1 including the use of DPP-IV inhibitors, the generation of recombinant GLP-1-human serum albumin fusion proteins capable of prolonging biological half-life and designing structural analogues resistant to DPP-IV (Dou *et al.*, 2008). Previous research demonstrated that the addition of the amino acid lysine to native GLP-1 before the N-terminal histidine of the sequence conferred resistance to DPP-IV mediated cleavage. This analogue of GLP-1, designated KGLP-1 also maintained insulinotropic activity (Gao *et al.*, 2009). Previously, overlapping PCR technology was used to generate plasmid constructs that were subsequently transformed into *Pichia pastoris* strains capable of expressing a GLP-1 human albumin ((GLP-1A2G)2-HSA) fusion protein resistant to DPP-IV (Dou *et al.*, 2008).

In this study, two recombinant *L. paracasei* strains were generated, one containing the operon responsible for pediocin production and export, and one containing the same operon with the mature pediocin sequence replaced by the nucleotide sequence responsible for KGLP-1. Both strains were analysed for relevant peptide production via Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry and High performance liquid chromatography (HPLC). Bioactivity of the pediocin producing strain and the KGLP-1 producing strain was analysed by well diffusion assay and tissue culture using insulin producing RINm5F Beta cells, respectively.

5.3. Materials and Methods

5.3.1. Reagents

Standard GLP-1 (Product no.G9416) was from Sigma (St. Louis, MO, USA) and synthetic KGLP-1 (KHVEGTFTSDVSSYLEGQAAKEFIAWLVKGRG) was chemically synthesized in Teagasc Food Research Centre, Moorepark (Fermoy, Co. Cork, Ireland) using a Liberty CEM microwave peptide synthesizer as previously described (Norberg *et al.*, 2011) with minor alterations. Briefly KGLP-1 was generated on H-Gly-HMPB-Chematrix resin (PCAS Biomatrix Inc., Quebec, Canada) and purified using RP-HPLC. Cell culture media was obtained from LGC (LGC Standards, Queens Road, Teddington, Middlesex, TW11 0LY, England). Meso Scale Diagnostic (MSD) plates were supplied with reagents and were obtained from Meso Scale Discovery (Meso Scale Diagnostics, LLC, 9238 Gaither Road, Gaithersburg, MD 20877, USA). Remaining reagents used in this study were of analytical grade and are commercially available.

5.3.2. Bacterial strains and culture conditions

The strain *L. paracasei ssp. paracasei* NFBC 338 (*L. paracasei* NFBC 338) is a human gastrointestinal tract isolate obtained from University College Cork (UCC) under a restricted materials transfer agreement and was cultured at 1% (v/v) in de Man, Rogosa and Sharpe (MRS) broth (Difco) for ~ 17 h at 37°C under anaerobic conditions (anaerobic jars with Anaerocult A Gas Packs [Merck, Darmstadt, Germany]). *Pediococcus acidilactici* DPC 5492 (NCIMB 700993) was grown under similar anaerobic conditions at 30°C. *L. paracasei* DPC 6770

and *L. paracasei* DPC PED strains were grown using the conditions outlined for the wild-type strain. *Listeria innocua* DPC 3572 (Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland) was cultivated with brain-heart infusion (BHI) medium aerobically at 37°C.

5.3.3. Cell culture

RINm5F Insulinoma β-cells (*Rattus norvegicus*) (ATCC-CRL-11605) (LGC Standards, Queens Road, Teddington, Middlesex, TW11 0LY, England) were cultured in RPMI-1640 (LGC Standards) medium containing 10% (v/v) foetal bovine serum, and 1% (v/v) Penicillin-Streptomycin solution (Sigma: Cat. No. P0781). All cells were maintained in 75 cm^2 sterile tissue culture flasks (Corning Inc, NY, USA) at 37°C in an atmosphere in 5% CO_2. Cells were passaged when the confluency of the flasks was approximately 90%. For insulinotropic studies, cells were treated with trypsin and seeded into 6 well tissue culture plates (Sarstedt, Sinnottstown Lane, Drinagh, Co. Wexford, Ireland) at a density of 1 x 10^5 cells/well.

5.3.4. Molecular cloning procedures

The nucleotide sequence for KGLP-1 was synthesised using overlap extension PCR (OE-PCR) using primers 1, 2, 3 and 4 (Table 5.1.). DNA was extracted from *P. acidilacti* DPC 5492 and the pediocin leader (*ped lead*) sequence was amplified using primers 5 and 6 (Table 5.1.). A splicing overlap extension (SOE) PCR reaction using primers 2 and 5 (Table 5.1.) was used to combine the KGLP-1 sequence and pediocin leader. PCR reactions were carried out at 60°C using KOD polymerase (Merck Millipore). Mature pediocin was

generated using primers 6 and 7 (Table 5.1.). The *pedB*, (specifies immunity) *pedC* and *pedD* (gene products are membrane bound proteins required for peptide secretion) (Horn *et al.*, 1998) genes were amplified from *P. acidlilacti* DPC 5492 DNA using primers 7 and 8 (Table 5.1.). The restriction sites Pst1and Kpn1 were incorporated into the sequence to allow incorporation into the plasmid pNZ44 via a ligation reaction. Restriction polymerases and T4 DNA ligase were purchased from New England Biolabs (240 County Rd., Ipswich, MA, USA). Plasmid constructs were transformed into competent *E.coli* which was spread plated on LB agar containing 10 µg/ml chloramphenicol and incubated overnight. Successful transformants were treated with a Qiagen Plasmid Mini kit (Qiagen). Electrocompetent *L. paracasei* NFBC 338 cells were prepared using 3.5X SMEB (1M sucrose, 3.5mM $MgCL_2$), as previously described by (Luchansky 1988). Plasmids were electroporated into *L. paracasei* NFBC 338 which was plated as described earlier with the addition of 10 µg/ml chloramphenicol. Correct nucleotide sequences were confirmed by Beckman Coulter Genomics (Essex CM22 6TA, United Kingdom)

5.3.5. Peptide detection

Strains were grown under conditions outlined above for ~17 h. Each sample was centrifuged at 4,000 g for 15 min and subsequently filtered through a 0.22 nm syringe filter. Filter permeates were applied to a 3 ml, 200 mg C18 SPE column pre-equilibrated with methanol and water. The column was washed with 3 ml 30% (v/v) ethanol, followed by 3 ml of 70% (v/v) isopropan-2-ol (IPA) 0.1% trifluroacetic-acid (v/v) which was concentrated using a Centrivap Speedivac. Each sample was then applied to an analytical Jupiter Proteo RP-

HPLC column (4.6 x 250 mm, 4µ, 90Å) (Phenomenex, Cheshire, UK) running a 25% (v/v) to 55% (v/v) acetonitrile 0.1% (v/v) TFA gradient at 1 ml/min. Eluent was monitored at 214 nm and fractions were collected every minute. Relevant fractions were analysed for masses of interest using Matrix-assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry to visualize peptides of interest.

5.3.6. Activity of heterologously generated antimicrobial peptide

The agar well diffusion method (Ryan, Rea, Hill, & Ross, 1996) was used to determine the antimicrobial activity of *Lb. paracasei* NFBC 338 derived pediocin. Culture supernatants were adjusted to pH. 6.5 1 N NaOH and filter-sterilized (0.45 µm). Antimicrobial activity was assessed using the indicator strain *Listeria innocua* DPC 3572. Agar plates were incubated appropriately overnight prior to examination for zones of inhibition.

5.3.7. Evaluation of the bioactivity of *Lb. paracasei* DPC 6770 generated KGLP-1

Incretin hormone production was first assayed using a MSD Total GLP-1 plate (Cat. No. K150JVC-1) as per the manufacturer's instructions. Insulinotropic properties of *L. paracasei* DPC 6770 generated peptides were investigated using a method previously described (Salvucci, Neufeld, & Newsholme, 2013) with minor alterations. Briefly 2 ml of RINm5F β-cells were cultured as described in section 2.3. Cell monolayers were washed using KREBS-Ringer bicarbonate buffer (Sigma Aldrich) and starved for 40 min at basal glucose level via addition of 1 ml of KREBS buffer containing 1.1 mM

glucose (Sigma). Buffer was removed and the cells were stimulated with prepared filtrate from *L. paracasei* NFBC 338, DPC 6770 and DPC PED. Each sample was incubated with glucose at 2.2 mM, 4.4 mM or 8.8 mM glucose for 20 min. Sigma GLP-1 and synthetic KGLP-1 were also assayed at concentrations of 50 µmol, nmol and pmol for comparison. Following incubation samples were collected and treated with protease and phosphatase inhibitor (10 ul/ml) (Fisher Scientific UK Ltd, Bishop Meadow Road, Loughborough, UK.) (Cat. No. PN78443) before application to a MSD insulin plate (Cat. No. K152BZC-1). Insulin production was measured according to manufacturer's instructions using a MSD Sector 2400.

5.4. Results

5.4.1. Background

L. paracasei NFBC 338 was used to generate recombinant strains as it was intestinally derived and has been previously shown as safe for human consumption (McGee, Bakens, Wiley, Riordan, & Webster, 2011). *L. paracasei* NFBC 338 has also been commercialized as a probiotic culture in cheese (Collins, Fitzgerald, O'Sullivan, Ross, & Stanton, 2005) and therefore was deemed an appropriate candidate for our study. We investigate whether the production and excretion machinery for the bacteriocin pediocin would be suitable to generate the recombinants. Prior to functional studies, the presence of relevant DNA fragments in the recombinants by restriction endonucleases and subsequent gel electrophoresis was confirmed. Correct DNA sequences were validated by DNA sequencing (data not shown).

5.4.2. Expression of heterologous bioactive peptides

The molecular mass of KGLP-1 and pediocin as indicated by MALDI-TOF mass spectrometry showed peaks of 3512.68 Da and 4625.21 Da respectively, the appropriate molecular weights for both peptides (Fig. 5.1.). While GLP-1 has a molecular weight of approximately 3352.67-3358.67 Da, the amino acid sequence changes in KGLP-1 cause it to have a higher molecular weight. Cell lysis procedures were not required to detect the heterologous peptides in the cell-free supernatants indicating successful secretion by the recombinant *Lb. paracasei* strains. Well diffusion assays showed that the pediocin generated by *L. paracasei* DPC PED maintained antimicrobial activity against *L. innocua* comparable to that of *P. acidilactici* DPC 5492 (2560 mAU),

while the control strains did not inhibit growth of *L. innocua* (Fig. 5.2.). MSD Total GLP-1 plate demonstrated that *L. paracasei* DPC 6770 generated approximately 3854 pg/ml (40 µg/L) of KGLP-1 (Fig. 5.3.). Doubling the incubation time of *L. paracasei* DPC 6770 led to a lower level of detectable KGLP-1 peptide indicating some degradation over time. The controls strains did not produce any detectable levels of these peptides.

5.4.3. Evaluation of *in vitro* insulinotropic properties of heterologous KGLP-1

The insulinotropic effect of *L. paracasei* DPC 6770 generated KGLP-1 was analysed and the results are outlined in Figure 5.4.. KGLP-1 compared favourably with synthetic GLP-1 and KGLP-1 at levels of 50 pmol, higher than post-prandial levels of GLP-1 in circulation *in vivo* (Holst, 2007). Glucose concentration did not appear to have an effect on the *L. paracasei* DPC 6770 generated KGLP-1. Synthetic KGLP-1 compared favourably with Sigma Standard GLP-1 7-37.

5.5 Discussion

The therapeutic potential of GLP-1 has been recognised in recent years due to its spectrum of physiological actions particularly with regard to diabetes. As well as stimulating insulin secretion in the body, GLP-1 also stimulates insulin gene expression, inhibits glucagon secretion (Drucker & Nauck, 2006; Gao *et al.*, 2009) and reverses diabetes associated defects in the β cell within the pancreas (Egan, Bulotta, Hui, & Perfetti, 2003). The hormone also influences food intake, promoting gut satiety and delaying gastric emptying (Gao *et al.*, 2009; Gutzwiller *et al.*, 1999; Holst, 2007). Importantly, unlike other antihyperglycemic treatments GLP-1 does not induce hypoglycaemia which is an issue with some diabetic medications, as the actions of the hormone are glucose dependent (Gao *et al.*, 2009; Nathan, Schreiber, Fogel, Mojsov, & Habener, 1992). GLP-1 also possesses a glucagonostatic effect that is of therapeutic relevance (Ebinger, Jehle, Fussgaenger, Fehmann, & Jehle, 2000).

Modifications of the initial histidine in the GLP-1 amino acid sequence have previously been evaluated and most convey DPP-IV resistance. However, the biological activity of these modified peptides can vary significantly due to a reduced affinity for binding to the GLP-1 receptor. The Xaa-Ala and Xaa-Pro dipeptides at the N-terminus of GLP-1 are the recognising sequence(s) for DPP-IV. By extending the N-terminal of GLP-1 by attaching a Lysine to His^7 significant DPP-IV resistance was achieved whilst maintaining potent insulinotropic activity comparable to native GLP-1. In their study, Gao *et al.* (2009) encapsulated KGLP-1 in long acting sustained release micro-spheres which following injection into diabetic rats lowered glucose and led to insulin release for almost 10 days (Gao *et al.*, 2009). Sustained release of any GLP-R

agonist is important, as even if the agonist is resistant to DPP-IV degradation, renal clearance would still be an issue. In this present study, we used molecular cloning techniques to enable KGLP-1 and pediocin generation and secretion by an intestinally-derived *Lactobacillus*. Heterologous production of bacteriocins using recombinant microbial systems is seen as a way to improve production costs in instances where the natural producer generates an unsatisfactory yield or where the generation of synthetic peptides is cost prohibitive (Cintas, Herranz, & Hernández, 2011). Heterologous expression of bacteriocins can be achieved by exchanging leader peptides and/or dedicated ABC secretion and exporting systems as well as by adding signal peptides recognised by general secretory peptides (Richard, Drider, Elmorjani, Marion, & Prévost, 2004).

In our study, using PCR, OE-PCR, and SOE-PCR we were able to amplify the required genes from a *Pediococcus*, synthesize the nucleotide sequence responsible for KGLP-1 and combine the genes in a constitutive expression vector respectively. By incorporating the genes responsible for pediocin production and export into the pNZ44 expression vector and transforming into *L. paracasei* NFBC 338, we generated two strains capable of heterologous peptide production with potential benefits for human health. *L. paracasei* NFBC 338 was viewed as suitable candidate for transformation due to its probiotic, generally regarded as safe (GRAS) status, ability to survive spray-drying at an industrial level (Gardiner *et al.*, 2002) and its ability to survive the mammalian intestine and colonize the mammalian intestinal epithelium (McGee *et al.*, 2011; Stanton, Ross, Fitzgerald, & Collins, 2001). *In vitro* functional testing demonstrated the activity of both heterologously produced peptides. Recombinantly generated pediocin compared favourably to the natural peptide

against *L. innocua* in well-diffusion assays indicating no reduction in bacteriocin potency or production. Our results indicated that approximately 3854 pg/ml of KGLP-1 were produced by *L. paracasei* DPC 6770 which has previously been shown to be of biological significance *in vivo* when administered per kg of bodyweight (Gao *et al.*, 2009) . Functional testing using Rinm5f β-cells showed that *L. paracasei* DPC 6770 generated KGLP-1 led to significantly higher levels of insulin production compared to controls. Although the insulin production was not glucose dependent, Rinm5F β-cells, while seen as a good *in vitro* model of insulin production, have previously reported glucose insensitivity issues (Praz *et al.*, 1983; Skelin, Rupnik, & Cencic, 2010). Synthetic KGLP-1 had an effect on insulin production by the cells similar to the effect seen using Sigma native GLP-1 with both peptides stimulating insulin production in a dose dependant manner save for Sigma GLP-1 at a nanomolar concentration which may be due to assay sensitivity

Diabetes is one of the major chronic diseases affecting people today (Pontarolo *et al.*, 2013) and it's prevalence is expected to double to ~300 million within the next 20 years (Verspohl, 2012). Populations across all stages of social and economic development are observing this increase of diabetes. Various factors such as increased urbanization, decreased physical activity and high-calorie diets all contribute to higher frequencies of obesity, a major predisposing factor for diabetes (Mokdad *et al.*, 2003; Pontarolo *et al.*, 2013). The fact that diabetes has become the seventh leading cause of death in the U.S.A. illustrates the severity of this problem (Pontarolo *et al.*, 2013). Numerous pharmacological treatments have been developed to treat diabetes and have been reviewed sufficiently elsewhere (Corless, Kiely, McClenaghan, Flatt, & Newsholme, 2006;

Pontarolo *et al.*, 2013; Tahrani, Bailey, Del Prato, & Barnett, 2011; Verspohl, 2012). Of particular note to our research are the extensive recent investigations into the use of treatments based on incretin hormones capable of acting as GLP-1 receptor agonists. Exenatide a synthetic form of Gila monster derived exendin-4 shares -53% homology to endogenous GLP-1 and was the first approved GLP-1 receptor agonist. Current treatment requires twice daily subcutaneous administrations of 5 or 10 mg. A once weekly formulation has recently been approved for treatment by the FDA (Buse *et al.*, 2012). Liraglutide is another GLP-1 receptor agonist approved for treatment of type 2 diabetes. It shares 97% amino acid sequence identity with native GLP-1 and is 99% albumin bound, as free liraglutide is degraded by endogenous peptidase action *in vivo*. Liraglutide administered once daily enables significantly better glycaemic control compared to twice daily exenatide administration. Longer acting sustained release formulations are currently being researched to improve the convenience of treatments (Buse *et al.*, 2012; Pontarolo *et al.*, 2013).

The gut microbiota can exert profound effects on human health (Clemente, Ursell, Parfrey, & Knight, 2012). The methods we have outlined in our study could be used to tailor microbes with specific host interactions to improve overall health using bacteriocin export machinery to generate heterologous peptides, with *L paracasei* DPC 6770 being an example of a potential treatment for diabetes. Further analysis will have to be undertaken to confirm the efficacy of *L. paracasei* DPC 6770 *in vivo* although its ability to produce biologically-significant amounts of insulinotropic peptide and the ability of native *L. paracasei* NFBC 338 to survive industrial processing and gut passage make it a promising candidate for use as a diabetes treatment. *In vivo*

studies could confirm the levels of KGLP-1 produced by *L. paracasei* DPC 6770 in the gut, the amount of peptide crossing into the bloodstream and the effect upon the subject with regard to blood glucose levels. A treatment strategy using a recombinant probiotic microbe producing the incretin hormone avoids issues such as the expense incurred in peptide synthesis (Cintas *et al.*, 2011) and the difficulty caregivers have persuading patients to comply with therapies that involve multiple injections (Pontarolo *et al.*, 2013). Additionally the recombinant strain may also aid in a reduction in weight, which is particularly important in the early stages of diabetes treatment due to the appetite regulating activity of GLP-1.

In conclusion, we have generated a recombinant strain of an intestinal organism which produces a human gut hormone using bacteriocin machinery This indicates the potential for this method to be used to generate further recombinant strains capable of producing other bioactive peptides.

5.6. Acknowledgements

Robert Kent was in receipt of a Teagasc Walsh Fellowship, 2008-2012.

The Alimentary Pharmabiotic Centre is a research centre funded by Science Foundation Ireland (SFI). The authors and their work were supported by SFI CSET grant 07/CE/B1368

Helena Stack assisted with Molecular cloning procedures.

Paula O'Connor provided technical assistance with MALDI-TOF MS and MW-SPPS.

5.7. References:

Augustyns, K., Bal, G., Thonus, G., Belyaev, A., Zhang, X., Bollaert, W., . . . Haemers, A. (1999). The unique properties of dipeptidyl-peptidase IV (DPP IV/CD26) and the therapeutic potential of DPP IV inhibitors. *Current medicinal chemistry, 6*(4), 311-328.

Baggio, L. L., & Drucker, D. J. (2007). Biology of incretins: GLP-1 and GIP. *Gastroenterology, 132*(6), 2131-2157.

Buse, J. B., Nauck, M., Forst, T., Sheu, W. H., Shenouda, S. K., Heilmann, C. R., . . . Fineman, M. (2012). Exenatide once weekly versus liraglutide once daily in patients with type 2 diabetes (DURATION-6): a randomised, open-label study. *The Lancet*.

Chen, J., Bai, G., Yang, Y., Geng, P., Cao, Y., & Zhu, Y. (2007). Identifying glucagon-like peptide-1 mimetics using a novel functional reporter gene high-throughput screening assay. *Peptides, 28*(4), 928-934.

Cintas, L. M., Herranz, C., & Hernández, P. E. (2011). Natural and heterologous production of bacteriocins *Prokaryotic Antimicrobial Peptides* (pp. 115-143): Springer.

Clemente, J. C., Ursell, L. K., Parfrey, L. W., & Knight, R. (2012). The impact of the gut microbiota on human health: an integrative view. *Cell, 148*(6), 1258-1270.

Collins, J. K., Fitzgerald, G. F., O'Sullivan, G. C., Ross, R. P., & Stanton, C. G. (2005). Process for the manufacture of probiotic cheese: Google Patents.

Corless, M., Kiely, A., McClenaghan, N. H., Flatt, P. R., & Newsholme, P. (2006). Glutamine regulates expression of key transcription factor, signal transduction, metabolic gene, and protein expression in a clonal pancreatic β-cell line. *Journal of endocrinology, 190*(3), 719-727.

Dou, W. F., Lei, J. Y., Zhang, L. F., Xu, Z. H., Chen, Y., & Jin, J. (2008). Expression, purification, and characterization of recombinant human serum albumin fusion protein with two human glucagon-like peptide-1 mutants in *Pichia pastoris*. *Protein expression and purification, 61*(1), 45-49.

Doyle, M. E., & Egan, J. M. (2007). Mechanisms of action of GLP-1 in the pancreas. *Pharmacology & therapeutics, 113*(3), 546.

Drucker, D. J. (2006). The biology of incretin hormones. *Cell metabolism, 3*(3), 153-165.

Drucker, D. J., & Nauck, M. A. (2006). The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet, 368*, 1696-1705.

Ebinger, M., Jehle, D. R., Fussgaenger, R. D., Fehmann, H. C., & Jehle, P. M. (2000). Glucagon-like peptide-1 improves insulin and proinsulin binding on RINm5F cells and human monocytes. *American Journal of Physiology-Endocrinology And Metabolism, 279*(1), E88-E94.

Egan, J. M., Bulotta, A., Hui, H., & Perfetti, R. (2003). GLP-1 receptor agonists are growth and differentiation factors for pancreatic islet beta cells. *Diabetes/metabolism research and reviews, 19*(2), 115-123.

Elrick, H., Stimmler, L., Hlad, C., & Arai, Y. (1964). Plasma insulin response to oral and intravenous glucose administration. *Journal of Clinical Endocrinology & Metabolism, 24*(10), 1076-1082.

Gao, Z., Tang, Y., Chen, J., Bai, R., Zhang, Q., Hou, Y., . . . Bai, G. (2009). A novel DPP-IV-resistant analog of glucagon-like peptide-1 (GLP-1): KGLP-1 alone or in combination with long-acting PLGA microspheres. *Peptides, 30*(10), 1874-1881.

Gardiner, G. E., Bouchier, P., O'Sullivan, E., Kelly, J., Kevin Collins, J., Fitzgerald, G., . . . Stanton, C. (2002). A spray-dried culture for probiotic Cheddar cheese manufacture. *International Dairy Journal, 12*(9), 749-756.

Gutzwiller, J., Göke, B., Drewe, J., Hildebrand, P., Ketterer, S., Handschin, D., . . . Beglinger, C. (1999). Glucagon-like peptide-1: a potent regulator of food intake in humans. *Gut, 44*(1), 81-86.

Holst, J. J. (2007). The physiology of glucagon-like peptide 1. *Physiological reviews, 87*(4), 1409-1439.

Horn, N., Martínez, M. I., Martínez, J. M., Hernández, P. E., Gasson, M. J., Rodríguez, J. M., & Dodd, H. M. (1998). Production of pediocin PA-1 by Lactococcus lactis using the lactococcin A secretory apparatus. *Applied and environmental microbiology, 64*(3), 818-823.

Kazakos, K. (2011). Incretin effect: GLP-1, GIP, DPP4. *Diabetes Research and Clinical Practice, 93,* S32-S36.

Knudsen, L. B., & Pridal, L. (1996). Glucagon-like peptide-1-(9-36) amide is a major metabolite of glucagon-like peptide-1-(7-36) amide after in vivo administration to dogs, and it acts as an antagonist on the pancreatic receptor. *European journal of pharmacology, 318*(2), 429-435.

Lambeir, A.-M., Durinx, C., Scharpé, S., & De Meester, I. (2003). Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. *Critical reviews in clinical laboratory sciences, 40*(3), 209-294.

McGee, R. G., Bakens, A., Wiley, K., Riordan, S. M., & Webster, A. C. (2011). Probiotics for patients with hepatic encephalopathy. *Cochrane Database Syst Rev, 11*.

Mentlein, R., Gallwitz, B., & Schmidt, W. E. (1993). Dipeptidyl peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon like peptide 1 (7–36) amide, peptide histidine methionine and is responsible for their degradation in human serum. *European Journal of Biochemistry, 214*(3), 829-835.

Mokdad, A. H., Ford, E. S., Bowman, B. A., Dietz, W. H., Vinicor, F., Bales, V. S., & Marks, J. S. (2003). Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. *JAMA: the journal of the American Medical Association, 289*(1), 76-79.

Nathan, D. M., Schreiber, E., Fogel, H., Mojsov, S., & Habener, J. F. (1992). Insulinotropic action of glucagonlike peptide-I-(7–37) in diabetic and nondiabetic subjects. *Diabetes Care, 15*(2), 270-276.

Norberg, O'Connor, Stanton, C., Ross, R. P., Hill, C., Fitzgerald, G. F., & Cotter, P. D. (2011). Altering the composition of caseicins A and B as a means of determining the contribution of specific residues to antimicrobial activity. *Appl Environ Microbiol, 77*(7), 2496-2501. doi: 10.1128/AEM.02450-10

Pontarolo, R., Sanches, A. C. C., Wiens, A., Borba, H. H. L., Lenzi, L., & da Silva Penteado, S. T. (2013). Pharmacological Treatments for Type 2 Diabetes. doi: 10.5772/56456

Praz, G. A., Halban, P. A., Wollheim, C. B., Blondel, B., Strauss, A., & Renold, A. (1983). Regulation of immunoreactive-insulin release from a rat cell line (RINm5F). *Biochemical Journal, 210*(2), 345.

Richard, C., Drider, D., Elmorjani, K., Marion, D., & Prévost, H. (2004). Heterologous expression and purification of active divercin V41, a class IIa bacteriocin encoded by a synthetic gene in Escherichia coli. *Journal of bacteriology, 186*(13), 4276-4284.

Ryan, M. P., Rea, M. C., Hill, C., & Ross, R. P. (1996). An application in cheddar cheese manufacture for a strain of Lactococcus lactis producing a novel broad-spectrum bacteriocin, lacticin 3147. *Applied and environmental microbiology, 62*(2), 612-619.

Salvucci, M., Neufeld, Z., & Newsholme, P. (2013). Mathematical Model of Metabolism and Electrophysiology of Amino Acid and Glucose Stimulated Insulin Secretion: In Vitro Validation Using a β-Cell Line. *PLOS ONE, 8*(3), e52611.

Siegel, E. G., Gallwitz, B., Scharf, G., Mentlein, R., Morys-Wortmann, C., Fölsch, U. R., . . . Schmidt, W. E. (1999). Biological activity of GLP-1-analogues with N-terminal modifications. *Regulatory peptides, 79*(2), 93-102.

Skelin, M., Rupnik, M., & Cencic, A. (2010). Pancreatic beta cell lines and their applications in diabetes mellitus research. *Altex, 27*(2), 105-113.

Stanton, C., Ross, R., Fitzgerald, G., & Collins, K. (2001). *Application of probiotic bacteria to functional foods*: Teagasc.

Tahrani, A. A., Bailey, C. J., Del Prato, S., & Barnett, A. H. (2011). Management of type 2 diabetes: new and future developments in treatment. *The Lancet, 378*(9786), 182-197.

Verspohl, E. (2012). Novel Pharmacological Approaches to the Treatment of Type 2 Diabetes. *Pharmacological Reviews, 64*(2), 188-237.

Table 5.1. Oligonucleotides used in plasmid construct generation

Primer number	Nucleotide sequence 5'-3'
1	GGCCAATATCATTGGTGGTAAGCATGTGGAAGGCACCTTTACCAGCGATGTGAGCAG CTATCTGGAAGGCCAGGCGGC
2	CCGCTCGAGTTAGCCGCGGCCTTTCACCAGCCACGCAATAAATTCTTTCGCCGCCTG GCCTTCCAGATAGCTGCTCAC
3	AATCGGCGCCGGAAAG
4	TTCGTACACCTTCCGTGG
5	ACATCCATGGAAGAAGAAGGAGATTTTTG
6	GCACCTGCAGGCTAGCATTTATGATTAC
7	GCACCTGCAGCATTATGCTGAGCTGGCATC
8	GCACGGTACCGCGAGGATTTCACGG

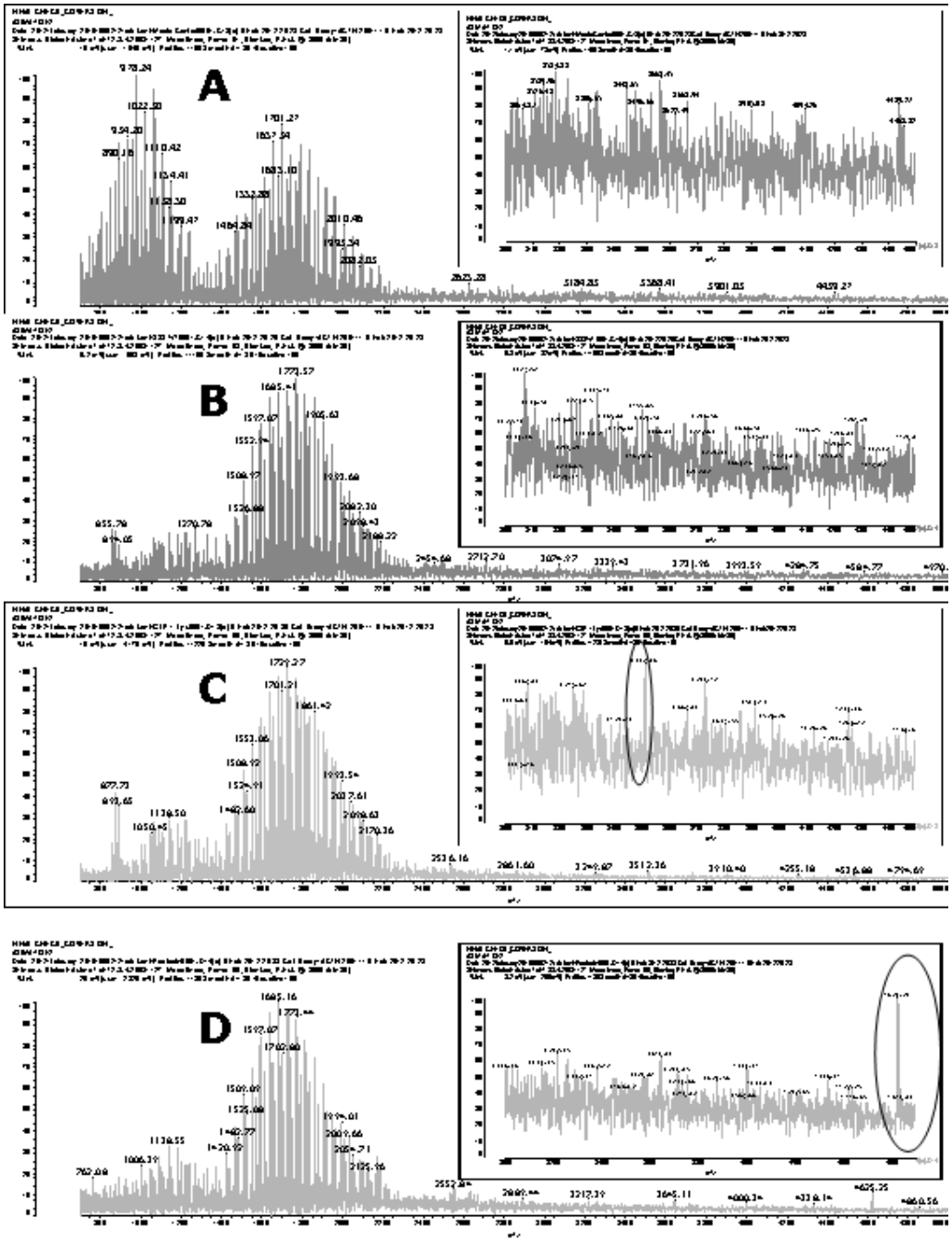


Figure 5.1. MALDI-TOF mass spectra indicating position of relevant peptide peaks. (A) MRS media control. (B) Wild-type *Lb. paracasei* NFBC 338 filtrate. (C) *Lb. paracasei* DPC 6770 filtrate; oval indicates position of KGLP-1 peptide. (D) *Lb. paracasei* DPC PED filtrate; oval indicates position of pediocin peptide.

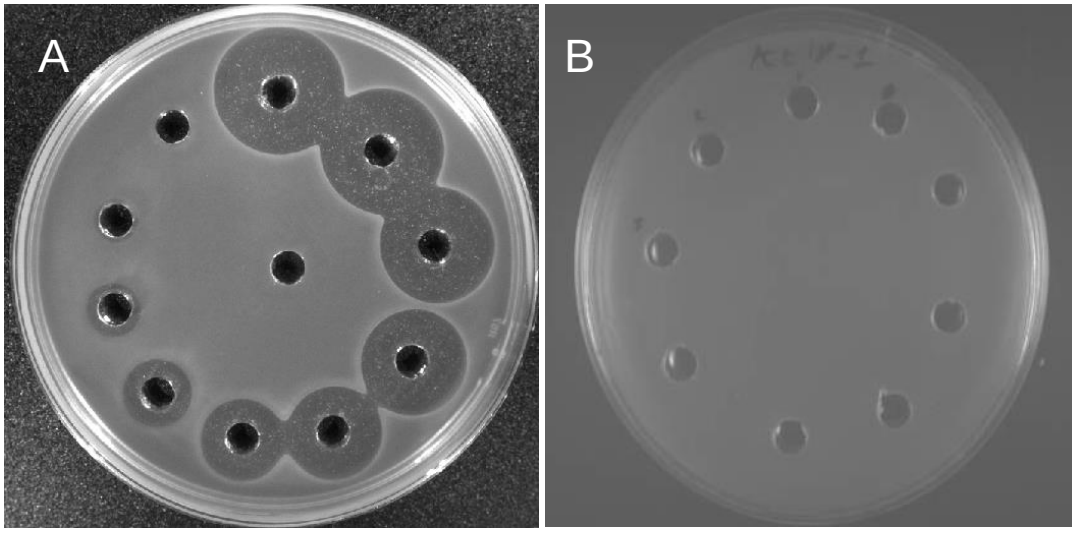


Figure 5.2. Well diffusion assay demonstrating pediocin production against *L. innocua* by *Lb. paracasei* DPC PED at 2560 mAU (A) and *Lb. paracasei* DPC 6770 control showing zero inhibition (B).

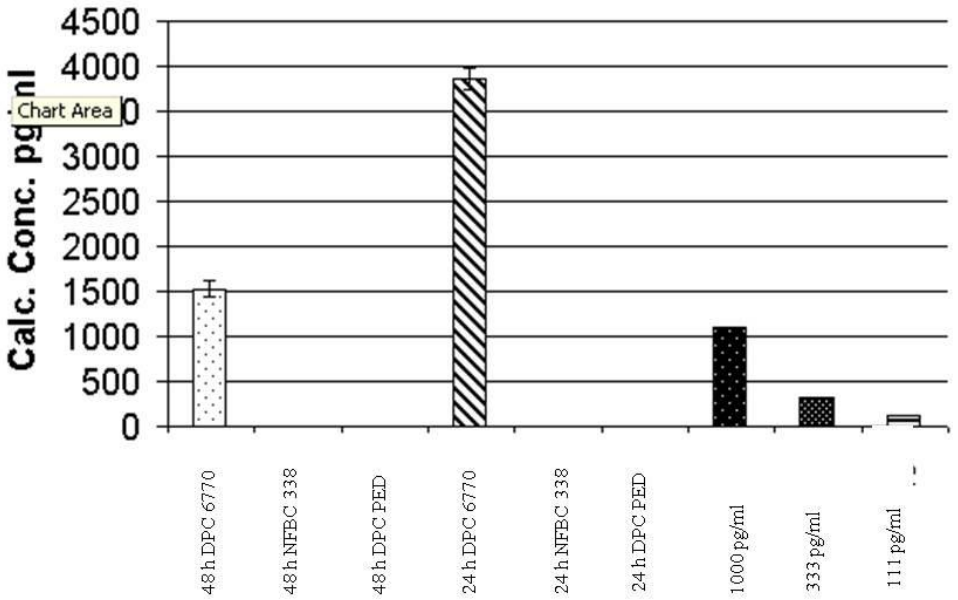


Figure 5.3. GLP-1 production as measured by a MSD Total GLP-1 plate. *Lb. paracasei* DPC 6770 production of GLP-1 after 24 and 48 h compared to control strains. 1000, 333 and 111 pg/ml are total GLP-1 standards supplied by kit manufacturer.

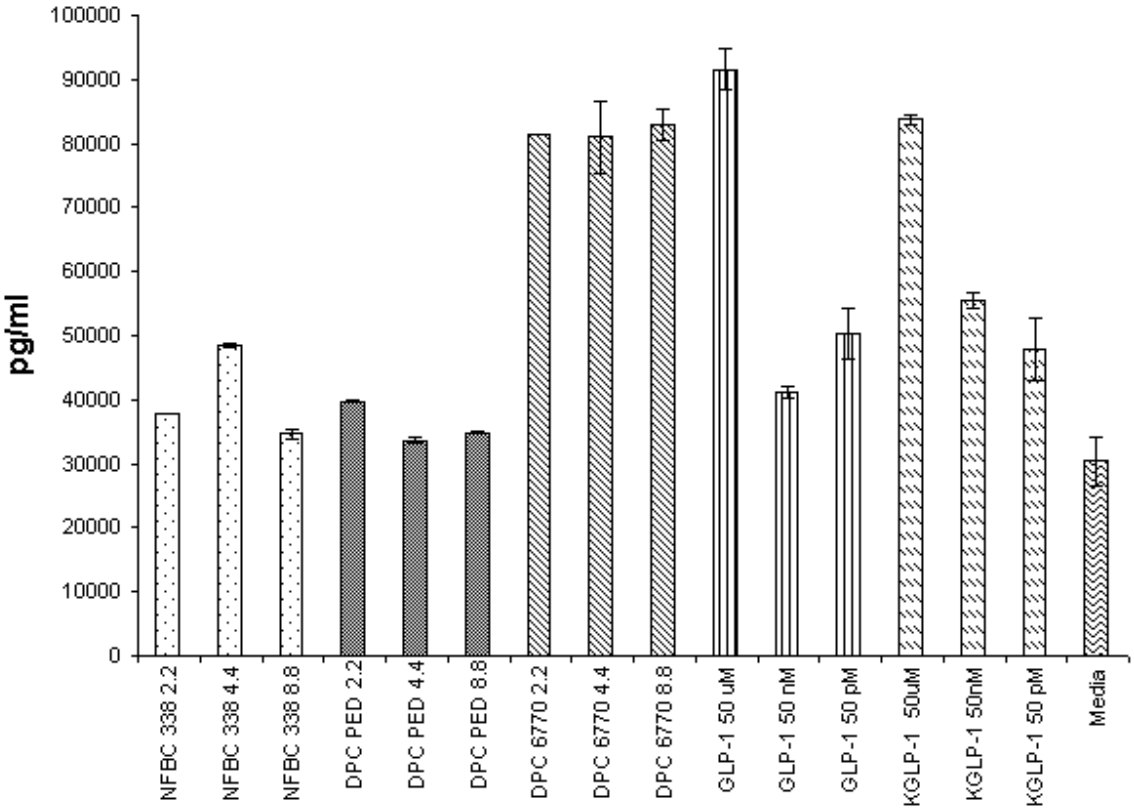


Figure 5.4. Insulin production (pg/ml) by Rinm5F-Beta cells treated with various cell filtrate preparations and purified KGLP-1 and GLP-1 at varying glucose concentrations with *Lb. paracasei* DPC 6770 compared to non-insulinotropic control strains and purified standards.

Chapter 6

General Discussion

Nutrition for growth and maintenance of the body have been considered the principal role of food since time immemorial. The fact that certain foods can cause further beneficial physiological effects post-consumption has led to an increase in research on the identification and characterisation of the specific components responsible for these benefits. Due to the extra benefits and various biological functions these food components may have, the term Functional Foods is often used to describe them. While a globally accepted definition has not yet been agreed on, Functional Food foods are broadly regarded as "generally consumed foodstuffs that (may) provide added health benefits following the addition/concentration of a beneficial ingredient, or the removal/substitution of an ineffective or harmful ingredient" (FSAI, 2007). The Functional Food market is currently estimated to be worth over $40 billion worldwide, and it is showing steady annual increases both in sales and new products launched (Valls *et al.*, 2013). Both probiotics and bioactive peptides represent an important component of this functional food market. Food-derived bioactive peptides can be generated via bacterial fermentation or enzymatic hydrolysis. Other methods may also be used to generate and deliver peptides with beneficial properties however. This thesis details the generation of peptides by bacterial fermentation, enzymatic hydrolysis and the use of recombinant molecular techniques highlighting industrially relevant ways to generate peptides.

The emergence of *Cronobacter* as an important pathogen has led to an increase in research on the genus. Since it's reclassification from *Enterobacter sakazakki* to the genus *Cronobacter*, molecular research has led to the classification of 10 separate species (Holý & Forsythe, 2014; Iversen *et al.*,

2007; C. Iversen *et al.*, 2008; Carol Iversen *et al.*, 2008; Joseph *et al.*, 2012). Investigations have to date identified particularly pathogenic strains of the organism and specific associated virulence factors (Holý & Forsythe, 2014; Stephan, Lehner, Tischler, & Rattei, 2011). These factors include proteolytic enzyme production, toxin production, adherence mechanism relevant to the gastro-intestinal tract, ability to cross intestinal and blood brain barriers and immune response evasion (Jaradat, Al Mousa, Elbetieha, Al Nabulsi, & Tall, 2014). The increase in knowledge of the genus, in particular, is allowing a better understanding of methods to prevent illness caused by *Cronobacter*. As infants are the group principally affected by infection, and powdered infant formula is the food most associated as the transmission vehicle, there is an urgent need for the development of natural and safe antimicrobials for use in this product. To date a number of candidates have arisen which could help improve the safety of PIF. These include bioactive peptides, organic acids and probiotic bacteria (Chapter 1). The initial aim of this thesis was the generation of milk-derived bioactive peptides with antimicrobial activity against *Cronobacter* strains. The thesis describes two novel methods to achieve this, and also expands by describing a third method of peptide generation, in which a recombinant lactobacilli produced a biologically active peptide.

The advent of high-throughput bacterial genome sequencing has significantly advanced the ease and cost with which it was once associated. This phenomenon can be exploited to rapidly analyse bacterial strains (Loman *et al.*, 2012). In Chapter 2 of this thesis the genome of *Lactobacillus johnsonii* DPC6026, an isolate from the porcine gastrointestinal tract (Hayes, Ross, Fitzgerald, Hill, & Stanton, 2006) was sequenced and annotated. This strain

was previously used to generate antimicrobial peptides via fermentation in bovine casein (Hayes, Ross, Fitzgerald, Hill, & Stanton, 2006). In this chapter, the strain was compared to other lactobacilli, particularly the to the human gut isolate *L. johnsonii* NCC533 (Pridmore *et al.*, 2004). The results indicated a number of differences between the strains, most noticeably a large symmetrical inversion of approximately 750kb across the replication axis. Eight other porcine *L. johnsonii* strains investigated in this study possessed the same genome structure as *L. johnsonii* DPC6026 while a single human isolate *L. johnsonii* ATCC12088, harboured the same genomic arrangement as *L. johnsonii* NCC533 with respect to the insertion. This study highlighted significant variation with regards cell protection mechanisms, adhesion proteins and putative phage resistant mechanisms reflecting host-specific divergence of *L. johnsonii* strains. There was however, a relatively conserved homogeneity in core regions of the genomes of the bacteria studied. *L. johnsonii* DPC6026 was found to possess a type III resistance modification system, which was not previously identified in *L. johnsonii*. This system coupled with a novel clustered regularly interspaced short palindromic repeats (CRISPR)-cas system suggests significant past exposure of the strain to phage in its ecological niche(s).

Chapter 3 describes a method to generate antimicrobial peptides (AMPs) capable of inhibiting *Cronobacter* growth from bovine casein using a *Bacillus* strain. *Bacillus* species are the predominant bacteria used during industrial fermentations and for commercial enzyme production (Rao, Tanksale, Ghatge, & Deshpande, 1998). In this study, 12 Of the 16 strains investigated demonstrated potent proteolytic activity when tested on a casein

substrate. Most of these strains were isolated from a dairy environment while four, which were incapable of breaking down casein, were isolated from a soil or unreported environment, save for *Bacillus coagulans* LMG6326 (a milk powder isolate). Interestingly, only strains from the species *Bacillus cereus* and *Bacillus thuringiensis* were capable of generating the antimicrobial peptides caseicin A and B. *B. cereus* and *B. thuringiensis,* along with *Bacillus anthracis* are members of the group I bacilli and are very closely related (Radnedge *et al.*, 2003). Previous investigations of *B. cereus* and *B. thuringiensis* have shown a very similar protease complement dominated by members of the thermolysin-like M4 family of peptidases and neutral protease (Npr) enzymes which may indicate why representatives of these two species generated the same peptides. As the main target for an antimicrobial would be infant formula, the strict regulatory conditions associated with the product should be taken into account. As milk proteins possess a long history of safe use and are a permissible ingredients in formula (CAC, 2007), they stand as an acceptable and natural source of antimicrobials (Benkerroum, 2010; Nagpal *et al.*, 2011). However, an issue here is the permissibility of the use of some *Bacillus* strains as a culture to generate them. For this reason, *Bacillus* species are used less frequently in fermentations to generate bioactive peptides when compared to Lactic Acid Bacteria (LAB). Although this study focused exclusively on the generation of caseicin A and B, further research examining various peptides generated via bacterial fermentation with proteolytic strains such as those utilized in this work may lead to the discovery of other bioactives. In order to be of use in an industrial context, the generation of bioactive peptides must be reproducible and cost efficient (Ross, Stanton, Hill, & Fitzgerald, 2007). The

use of strains such as those used in this study, which do not require complex media, pH control and an anerobic environment offer advantages over more fastidious bacteria from a cost perspective. Although this work focused exclusively on the generation of caseicin A and B, further research examining fermentates generated with other proteolytic *Bacillus* strains is warranted.

Due to the current regulatory concerns regarding the use of *Bacillus* strains to generate a preservative for infant formula, a food-grade technique to generate the caseicin peptides using *in-silico* methods and industrial enzymes was developed. Well maintained and updated online peptide databases have increased the potential and feasibility of predicating new bioactive peptides prior to the initiation of laboratory synthesis. Combining this technique with enzymatic hydrolysis is a sensible approach to peptide generation. The majority of known bioactives have been identified via hydrolysis with the gastric enzymes, trypsin and pepsin (Korhonen & Pihlanto, 2006). This makes sense from an evolutionary perspective as naturally consumed proteins are hydrolysed by these enzymes. The use of enzymes from other sources has been researched to a lesser degree however. In this study, the protease complement of *B. cereus* and *B. thuringiensis* were first examined through analyses of their genome sequences. The subsequent *in-silico* analysis of the enzymes revealed that the M4 family of peptidases were likely responsible for casein degradation (Chapter 4). Although a perfect match for caseicin production was not identified, the *in-silico* portion of this study removed the requirement to screen a vast bank of enzymes and focus on Enzyme Commission (E.C.) 3.4.24.28 (bacilliolysin) and EC 3.4.24.27 (thermolysin-like enzymes). These neutral metalloprotease enzymes display broad specificity but specifically cleave at

hydrophobic amino acids within the protein sequence (Večerek & Venema, 2000). Interestingly, every enzyme examined in this study efficiently hydrolysed the casein. Only enzymes derived from *Bacillus thermoproteolyticus* were capable of generating casein A. A thermolysin from a fungal source, although similar in structure, did not generate the peptide following exposure. Thermoase PC10F (Genencor, Rochester, NY, USA) hydrolysed casein into small peptides but 4 h incubation (37-65^{0}C) was required to allow detection of caseicin A peaks by Matrix-assisted laser desorption Time-of-Flight Mass Spectrophotometry indicating that some secondary hydrolysis was required. Reverse Phase High-Performance Liquid Chromatography indicated that the yield generated by enzymatic hydrolysis was 9-11 times greater than previously observed during bacterial fermentations (Chapter 2). The process was also increased to pilot (10 L) scale without a reduction in % yield. Thermolytic-liberated caseicin A was found to have an MIC of 0.625 mM which is identical to the MIC achieved with the synthesised peptides in this study and in previous work (Norberg *et al.*, 2012). A powder generated from a filtrate of the enzymatic hydrolysate also maintained antimicrobial activity, slowing the growth of the pathogenic strains assayed in infant formula. A major issue with bioactive peptide development is the prohibitive cost of manufacture (Marr, Gooderham, & Hancock, 2006). Research is on-going into ways to improve the feasibility of industrial production. A number of novel technologies applicable at such a scale, such as ion exchange chromatography and new methods of membrane separation are now being employed by the dairy ingredients industry (Korhonen, 2009; Korhonen & Pihlanto, 2006). These methods are seen as suitable ways to help

enrich bioactive peptide fractions of dairy hydrolysates. The methods outlined in this chapter in which the release of specific peptides is directed by prior *in-silico* investigations can streamline the generation of peptides and increase the amount of potential functional food products.

There currently exists a growing global trend of increasing obesity and diabetes levels. Indeed factors such as increased urbanization and high calorie diets are leading to a rise in the incidence of diabetes at all socio-economic levels across different populations (Ford, Giles, & Mokdad, 2004; Pontarolo *et al.*, 2013). Bioactive peptides are recognised as having a broad range of physiological activities *in-vivo*. Development of peptide based products which aid the management of factors such as hypertension, blood glucose levels and body mass indices (BMIs), which are associated with metabolic syndrome have tremendous potential to improve consumer health. Many diabetes sufferers use insulin to manage type-1 and type-2 diabetes mellitus (TODM and TTDM respectively) and currently there exists more than 20 different insulin products in the United States (http://www.diabetes.org/living-with-diabetes/treatment-and-care/medication/insulin/). There are a number of drawbacks to this treatment, such as overdose which can lead to life-threatening hypoglycaemia (Svingos, Fernandez, Reeder, & Parker, 2013) and poor adherence to therapy regimes by patients (Peyrot, Barnett, Meneghini, & Schumm-Draeger, 2012). In the healthy body, insulin secretion by the pancreas is potentiated principally by the action of two incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and Glucagon-like peptide-1 (GLP-1). GLP-1 in particular has been the subject of intense research as a novel therapy for TTDM (Pontarolo *et al.*, 2013). There are currently a number of GLP-1 based

therapies available and long-acting sustained release formulations are currently being developed (Buse *et al.*, 2013; Pontarolo *et al.*, 2013). In Chapter 5 of this thesis, molecular methods were used to generate two peptide generating recombinant lactobacilli. Our results indicated that the GLP-1 analogue generated by one of the recombinants possessed insulinotropic activity *in-vitro*. Although this study focused specifically on a treatment for diabetes, the method outlined can be used to incorporate other bioactive peptides into expression systems and possibly tailor treatments for specific medical conditions such as hypotension and improved cognitive functions.

Bioactive peptides are the subject of increasing interest for use in functional foods. However there is a need to demonstrate their efficacy in human clinical trials at doses that can be generated in a food vehicle economically. The study outlined in Chapter 3 highlights the potential of *Bacillus* to be used for bioactive peptide generation via fermentation. *Bacillus* species are often viewed as spoilage organism, particularly in the dairy industry. However, the inherent proteolytic ability possessed by many of these strains can be utilized to generate peptide fragments with potential bioactivities. Chapter 4 demonstrated the potential *in-silico* techniques have to streamline production of peptides. Moreover, the enzymatic method used in this chapter yielded a much higher amount of the antimicrobial of interest in comparison to bacterial fermentation. The findings of Chapter 5 describe not only a potential TTDM treatment, but also a method to generate probiotic bacteria producing physiologically relevant peptides. This thesis describes bioactive peptide generation via bacterial fermentation, targeted enzymatic

hydrolysis and recombinant bacteria contributing to the development of technologies which allow for peptide discovery and industrial-scale production.

Further investigations should be undertaken to elucidate the bioactive peptide generation capabilities of underutilized bacterial species such as *Bacillus*. This could be aided by or indeed be the starting point of studies like that describe in Chapter 4 of this thesis. The high level of proteolysis observed in casein protein during the investigations described in this work led to the generation of numerous peptides which may possess physiologically beneficial bioactivities. The techniques described could be utilized in with other protein sources, such as the milk protein whey, or protein from meat of plant source. The results observed in Chapter 5 of this work are promising with regards a TTDM therapy. However, *in-vivo* analysis, particularly human trials are required to fully validate the functionally and efficacy of the recombinant strain. As the peptide influences more than just insulin production, effects on aspects such as satiety would be investigated.

To conclude, the work described in this thesis specifically outlines two novel methods to generate anti-*Cronobacter* bioactive peptides as well the generation of a recombinant lactobacilli which produced an active insulinotropic peptide. In a broader sense, the thesis contributes to the field of bioactive peptide discovery by describing novel methods for their generation.

6.2 References:

Benkerroum, N. (2010). Antimicrobial peptides generated from milk proteins: a survey and prospects for application in the food industry. A review. *International Journal of Dairy Technology, 63*(3), 320-338. doi: 10.1111/j.1471-0307.2010.00584.x

Buse, J. B., Nauck, M., Forst, T., Sheu, W. H., Shenouda, S. K., Heilmann, C. R., . . . Fineman, M. (2013). Exenatide once weekly versus liraglutide once daily in patients with type 2 diabetes (DURATION-6): a randomised, open-label study. *The Lancet, 381*(9861), 117-124.

CAC, C. A. C. (2007). Standards for infant formula and formulas for special medical purposes intended for infants. *CODEX STAN 72-108*.

Ford, E. S., Giles, W. H., & Mokdad, A. H. (2004). Increasing prevalence of the metabolic syndrome among US adults. *Diabetes care, 27*(10), 2444-2449.

FSAI, F. S. A. I. (2007). *Functional Food*. Retrieved from http://www.fsai.ie/assets/0/86/204/667b54fe-972c-4c04-a6f8-9a0c5c92f886.pdf.

Hayes, M., Ross, R., Fitzgerald, G., Hill, C., & Stanton, C. (2006). Casein-derived antimicrobial peptides generated by *Lactobacillus acidophilus* DPC6026. *Applied and environmental microbiology, 72*(3), 2260-2264.

Holý, O., & Forsythe, S. (2014). *Cronobacter* spp. as emerging causes of healthcare-associated infection. *Journal of Hospital Infection, 86*(3), 169-177.

Iversen, C., Lehner, A., Mullane, N., Bidlas, E., Cleenwerck, I., Marugg, J., . . . Joosten, H. (2007). The taxonomy of *Enterobacter sakazakii*: proposal of a new genus *Cronobacter* gen. nov and descriptions of *Cronobacter* sakazakii comb. nov *Cronobacter sakazaki*i subsp *sakazakii*, comb. nov., *Cronobacter sakazakii* subsp *malonaticus* subsp nov., *Cronobacter turicensis* sp nov., *Cronobacter muytjensii* sp nov., *Cronobacter dublinensis* sp nov and *Cronobacter* genomospecies I. *Bmc Evolutionary Biology, 7*, 11. doi: 64 10.1186/1471-2148-7-64

Iversen, C., Mullane, N., McCardel, B., Tal, B. D., Lehner, A., Fannin, S., . . . Joosten, H. (2008). *Cronobacter* gen nov, a new genus to accommodate the biogroups of Enterobacter sakazakii, and proposal of *Cronobacter sakazakii* gen nov, comb nov, *Cronobacter malonaticus* sp nov, *Cronobacter turicensis* sp nov, *Cronobacter muytjensii* sp nov, *Cronobacter dublinensis* sp nov, *Cronobacter* genomospecies 1, and of three subspecies, *Cronobacter dublinensis* subsp *dublinensis* subsp nov, *Cronobacter dublinensis* subsp *lausannensis* subsp nov and *Cronobacter dublinensis* subsp *lactaridi* subsp nov. *International Journal of Systematic and Evolutionary Microbiology, 58*, 1442-1447. doi: 10.1099/ijs.0.65577-0

Jaradat, Z. W., Al Mousa, W., Elbetieha, A., Al Nabulsi, A., & Tall, B. D. (2014). *Cronobacter*, an opportunistic food borne pathogen; a review of its virulence and environmental adaptive traits. *Journal of medical microbiology*, jmm. 0.073742-073740.

Joseph, S., Cetinkaya, E., Drahovska, H., Levican, A., Figueras, M. J., & Forsythe, S. J. (2012). *Cronobacter condimenti* sp. nov., isolated from spiced meat, and *Cronobacter universalis* sp. nov., a species designation for *Cronobacter* sp. genomospecies 1, recovered from a leg infection, water and food ingredients. *International Journal of Systematic and Evolutionary Microbiology, 62*(Pt 6), 1277-1283.

Korhonen, H. (2009). Milk-derived bioactive peptides: From science to applications. *Journal of Functional Foods, 1*(2), 177-187.

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: production and functionality. *International Dairy Journal, 16*(9), 945-960.

Loman, N. J., Constantinidou, C., Chan, J. Z., Halachev, M., Sergeant, M., Penn, C. W., . . . Pallen, M. J. (2012). High-throughput bacterial genome sequencing: an embarrassment of choice, a world of opportunity. *Nature Reviews Microbiology, 10*(9), 599-606.

Marr, A. K., Gooderham, W. J., & Hancock, R. E. W. (2006). Antibacterial peptides for therapeutic use: obstacles and realistic outlook. *Current opinion in pharmacology, 6*(5), 468-472.

Nagpal, R., Behare, P., Rana, R., Kumar, A., Kumar, M., Arora, S., . . . Yadav, H. (2011). Bioactive peptides derived from milk proteins and their health beneficial potentials: an update. *Food & function, 2*(1), 18-27.

Norberg, S., O'Connor, P. M., Stanton, C., Ross, R. P., Hill, C., Fitzgerald, G. F., & Cotter, P. D. (2012). Extensive manipulation of caseicin A and B highlighting the tolerance of these antimicrobial peptides to change. *Applied and environmental microbiology*, AEM. 07312-07311.

Peyrot, M., Barnett, A., Meneghini, L., & Schumm-Draeger, P. M. (2012). Factors associated with injection omission/non-adherence in the Global Attitudes of Patients and Physicians in Insulin Therapy study. *Diabetes, Obesity and Metabolism, 14*(12), 1081-1087.

Pontarolo, R., Sanches, A. C. C., Wiens, A., Borba, H. H. L., Lenzi, L., & da Silva Penteado, S. T. (2013). Pharmacological Treatments for Type 2 Diabetes.

Pridmore, R. D., Berger, B., Desiere, F., Vilanova, D., Barretto, C., Pittet, A.-C., . . . Barrangou, R. (2004). The genome sequence of the probiotic intestinal bacterium *Lactobacillus johnsonii* NCC 533. *Proceedings of the National Academy of Sciences of the United States of America, 101*(8), 2512-2517.

Radnedge, L., Agron, P. G., Hill, K. K., Jackson, P. J., Ticknor, L. O., Keim, P., & Andersen, G. L. (2003). Genome differences that distinguish *Bacillus anthracis* from *Bacillus cereus* and *Bacillus thuringiensis*. *Applied and environmental microbiology, 69*(5), 2755-2764.

Rao, M. B., Tanksale, A. M., Ghatge, M. S., & Deshpande, V. V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiology and molecular biology reviews, 62*(3), 597-635.

Ross, P., Stanton, C., Hill, C., & Fitzgerald, G. (2007). Casein-derived antimicrobial peptides and *Lactobacillus* strains that produce them: WO Patent WO/2007/057,872.

Stephan, R., Lehner, A., Tischler, P., & Rattei, T. (2011). Complete genome sequence of *Cronobacter turicensis* LMG 23827, a food-borne pathogen causing deaths in neonates. *Journal of bacteriology, 193*(1), 309-310.

Svingos, R. S., Fernandez, E. M., Reeder, D. N., & Parker, J. J. (2013). Life-Threatening Hypoglycemia Associated with Intentional Insulin Ingestion. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy, 33*(3), e28-e33.

Valls, J., Pasamontes, N., Pantaleón, A., Vinaixa, S., Vaqué, M., Soler, A., . . . Gómez, X. (2013). Prospects of Functional Foods/Nutraceuticals and Markets *Natural Products* (pp. 2491-2525): Springer.

Večerek, B., & Venema, G. (2000). Expression of the neutral protease gene from a thermophilic *Bacillus* sp. BT1 strain in *Bacillus subtilis* and its natural host: identification of a functional promoter. *Journal of bacteriology, 182*(14), 4104-4107.

Acknowledgements

First and foremost I would like to thank Professors Paul Ross, Catherine Stanton and Gerald Fitzgerald for their guidance and support throughout my entire Ph.D. Thank you to the funding bodies listed throughout this thesis. I would also like to thank all the staff in the Biotechnology Department, Teagasc, Moorepark, particularly Paula O'Connor, Helen Slattery, Orla O'Sullivan, Helena Stack and Mary Rea for all their advice and assistance over the years.

A massive thank you has to go to Dr. Caitriona Guinane, for all her patience and support. Without Caitriona I would probably still be stuck at the start! I will forever be grateful.

My thanks to the many amazing friends I have made throughout the course of the Ph.D. In no particular order, Russell, Jeroen, DTS Barrett, The Hen, Rebecca, Mairead, Tobin, Mr. and Mrs. Coffey (a traffic spy legend), Bruen (your nothing but a …..), Dave Daly (live long and prosper), Buna, Felicia, Sheila, Casey (damn fine coffee), Calum, Skuse (orange is the new orange), James, Paul, Ruairi, Fodhla, Fergus, Jen, little Tom O' Callaghan, Dan Sully (Howzit?), Harsh, Brian, Kiera, Claire, Lainey (Sorry for "stealing" your coffee) and the many, many, many others.

Special thanks to JT and Shelly for all the support and advice especially near the end. Also thanks to JT for the running and banter. Thank you to Mammy O'Donnell too for the odd cake!

To all the people who had to suffer living with me during the thesis, a special thank you. Rob Mc thanks for Pink Floyd, whiskey and the road running (Shine on you crazy diamond). Devon, thanks for putting up with me, my garlic obsession and also whiskey. Dan B., thanks for all the craic and

advice. Big Man, thanks for all the support and for being a legend (also to your family for keeping me fed!!).

My family supported me so much throughout this process. Massive thank you to my brothers for everything they have done for me. My grandparents for their love and support, the odd plate of tripe and an occasional 20 euro note. Thanks too to Kieran, Evelyn and Ronnie for their support and Saturday dinners!

A huge thank you to my mother, the strongest person I know for everything she has done, for all the support and unconditional love. A scholar in her own right I could not have gotten through this without her.

Finally Fiona, my best friend. I can never thank you enough for your support, love and the occasional kick up the backside. Without you I would not have even started this thesis, let alone finished it. Thank you for brightening even the darkest days and always being there when I need you.