Discovery and Evaluation of Novel and Characterised Bacteriocins for Future Applications

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By

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

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Kevin Egan

List of publications:

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Thesis abstract

Bacteriocins are a heterogeneous group of small, ribosomal-synthesised, antimicrobial peptides produced by bacteria, capable of inhibiting bacteria both closely related or indeed those from other genera than the producer. These peptides are often active in the nanomolar range making them highly potent in low concentrations. This thesis expands the large body of research and knowledge that exists in the field of bacteriocins.

Firstly, a literature review examines the current and potential applications of bacteriocins to control spore-forming bacteria in food manufacturing. This review was the first to examine the mechanisms that underpin the anti-spore potential of bacteriocins and their viable use. A second literature review examined the efficacy of bacteriocin antibiotics as an alternative to current antibiotics and their lowered potential to induce microbiota dysbiosis during treatment. The first research chapter used conventional bacteriocin culture-based screening approaches in combination with whole genome *in silico* screening and peptide characterisation to discover new antimicrobial candidates in the genus *Geobacillus*. This resulted in the discovery of the potentially novel bacteriocin thermocin 458, who's amino acid sequence and molecular mass is unknown due to an interesting inability to generate enough peptide for in depth physicochemical characterisation. The second research chapter sought to identify the full potential of the genus *Geobacillus* as a reservoir for novel bacteriocin candidates using a bioinformatic approach. This ultimately resulted in the discovery of many potential bacteriocin gene clusters across a variety of bacteriocin classes that will likely ignite further *in vitro* characterisation as a result. The third and final research chapter of this thesis sought to advance bacteriocin mutagenesis

towards potential applications in cheese manufacture. Using a novel developmental approach, five new cheese starter cultures were created. Although three of the starter cultures produce nisin variants, they were created in a process that certifies their classification as non-genetically modified microorganisms (GMM) for contained use. This approach and its resultant non-GMM status is critical to future application and commercialisation of this technology.

This thesis seeks to drive and generate interest in bacteriocin discovery and application in both academia and industry. Furthermore, the studies contained provide direction for future development within this field and demonstrate the efficacy of bacteriocin use in both food and medicine applications.

Chapter Ia

Bacteriocins: Novel Solutions to Age Old Spore-Related problems?

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Abstract

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria, which have the ability to kill or inhibit other bacteria. Many bacteriocins are produced by food-grade lactic acid bacteria (LAB). Indeed, the prototypic bacteriocin, nisin, is produced by *Lactococcus lactis*, and is licensed in over 50 countries. With consumers becoming more concerned about the levels of chemical preservatives present in food, bacteriocins offer an alternative, more natural approach, while ensuring both food safety and product shelf life. Bacteriocins also show additive/synergistic effects when used in combination with other treatments, such as heating, high pressure, organic compounds, and as part of food packaging. These features are particularly attractive from the perspective of controlling sporeforming bacteria. Bacterial spores are common contaminants of food products, and their outgrowth may cause food spoilage or food-borne illness. They are of particular concern to the food industry due to their thermal and chemical resistance in their dormant state. However, when spores germinate they lose the majority of their resistance traits, making them susceptible to a variety of food processing treatments. Bacteriocins represent one potential treatment as they may inhibit spores in the post-germination / outgrowth phase of the spore cycle. Spore eradication and control in food is critical, as they are able to spoil and in certain cases compromise the safety of food by producing dangerous toxins. Thus, understanding the mechanisms by which bacteriocins exert their sporostatic/sporicidal activity against bacterial spores will ultimately facilitate their optimal use in food. This review will focus on the use of bacteriocins alone, or in combination with other innovative processing methods to control spores in food, the current knowledge and gaps therein with regard to bacteriocin-spore interactions and discuss future research

approaches to enable spores to be more effectively targeted by bacteriocins in food settings.

Introduction

Control and eradication of *Bacillus* and *Clostridium* spores is one of the most challenging aspects of microbial control faced by the modern food industry. Traditionally, spores have been controlled using extreme treatments such as high heat alone or in combination with chemical additives. However, modern consumers are more conscious than previous generations of the negative health effects associated with the consumption of certain chemical preservatives and of the significant effects of heat on the nutritional value and flavour of many foods. With ready-to-eat and minimally processed foods becoming a staple of the modern diet, the food industry is faced with an unprecedented challenge to provide food that is: (I) low in synthetic chemical additives, (ii) low in salt/sugar, (iii) nutritionally beneficial, and (iv) stable and safe, from a microbial perspective, over an extended period of time. As a result, the food industry is under pressure to employ innovative processing methods to meet consumer and regulatory demands. One potential innovation that has been intensively researched over the last number of decades, and is well positioned to provide a safe and effective alternative to existing processing technologies, involves the use of bacteriocins. This review will examine the efficacy of bacteriocins alone, and in combination with other processing technologies, to control spores in food.

The bacterial spore

Metabolically dormant spores of Gram-positive *Clostridium* and *Bacillus* species are formed during sporulation. This sporulation process is typically a response to cellular nutrient starvation and involves a complex cascade of enzyme reactions. This process of sporulation has been extensively described over the last number of

decades in the model spore former *B. subtilis* (see review by: Tan and Ramamurthi (2014). Spores consist of a core surrounded by a coat and/or endosporium. The spore core consists of DNA, enzymes, and dipicolinic acid (DPA). DPA plays a role in maintaining spore dormancy, providing resistance to DNA damaging substances and is usually bound to divalent cations such as Ca^{2+} at a 1:1 ratio in the core (Setlow, 2014b). The composition and structure of the metabolically inactive, dehydrated, spore confers resistance to changes in pH (Blocher and Busta, 1983), wet and dry heat, UV radiation, desiccation (Nicholson et al., 2000), and various toxic chemicals (Russell, 1990; Cortezzo and Setlow, 2005). A spore may be viable after extended periods of dormancy (Cano and Borucki, 1995), monitoring its environment for favourable growth conditions and when suitable, germination and outgrowth occur, ultimately resulting in a vegetative cell (Figure 1). Endospore-forming bacteria vary considerably with respect to genotype and phenotype and, with respect to phenotype, consist of aerobic, facultative anaerobic, and obligate anaerobic, psychrophilic, mesosphilic, thermophilic, psychotropic and thermotolerant strains (see review by: Doyle et al., 2015). This phenotypic heterogeneity of spore-forming bacteria means that virtually all types of food are potential targets for spore contamination and spore outgrowth, with potentially severe consequences with respect to food quality and safety.

There are many pathways via which spores can gain access to the food chain. Food products are composed of multiple ingredients, potentially from different international origins, each contributing their own specific quantity and diversity of spores into the final formulation. Factors such as microbial ecology, farming practices, the local climate, hygiene of the processing facility and animal feeding

practices determine the spore composition of an ingredient. Spores are also highly adhesive and may remain on the surfaces of equipment and contribute to problems long after their initial contamination of the facility. Reducing these initial spore loads is critical in avoiding problems downstream. However, it is important to note that spores are often selected for in food processing as their thermal resistance allows them to endure any heating steps (see review by: Carlin, 2011).

As early as 1956 (Stuy, 1956), the induction of spore germination was identified as a strategy that could facilitate spore eradication. When threshold levels of nutrients (such as amino acids, sugars, and nucleosides) are present, they bind to Ger complexes, located on the inner membrane of the spore. This strategy takes advantage of the loss of the resistance properties that a dormant spore possesses. It has been shown that once spores have germinated, they become more sensitive than dormant spores to: heat (Durban et al., 1970), X-Ray and UV radiation (Stuy, 1956; Munakata, 1974), and copper (Wheeldon et al., 2008). Interestingly the process of spore germination is not 100% efficient, due to the heterogeneity in germination rates among members of the spore population in response to a particular nutrient germinant. Previous studies have highlighted the specificity of germinant receptors (GRs): showing that GerA will respond to L-alanine and L-valine, while GerB and GerK will respond to a mixture of L-asparagine, D-glucose, D-fructose, and potassium ions (Moir et al., 1994; Atluri et al., 2006). The binding of the nutrients to their appropriate GRs results in the irreversible commitment of the spore to germination.

Commonly spores termed superdormant have been isolated from populations of *B. subtilis* following saturation with nutrient germinant. This super dormancy is attributed to the lag in initiation of the rapid loss of Ca^{2+}-DPA stage in spore germination. Following the initiation of rapid loss of Ca^{2+}-DPA from its core, the spore is no longer superdormant and its germination will proceed in a similar manner as dormant spores (Figure 1). This superdormancy may be an issue for antimicrobials (e.g. nisin) whose effect is only exhibited on those spores that have reached the end of stage II of germination (Figure 1; Chen et al., 2014). Superdormant spores may, however germinate, in response to an alternative germinant that utilizes an alternative GR. A different strategy, which can be used to increase germination of super dormant spores, is by using higher heat activation temperatures than is required for those non-superdormant spores (Ghosh et al., 2009). Treatment of spores with sublethal heat (also called heat activation) has been shown to increase the rate of germination of a number of spore species. Luu et al. (2015) suggested that although the main target of heat activation is the spore's GRs, this may only be indirect and that the sublethal heat is having a more direct effect on the inner membrane of the spore in which the GRs are situated, ultimately resulting in increased spore germination. Therefore decisive triggering of the spore germination process, will allow food processors to render spores sensitive to a variety of inactivation methods that are ineffective against highly resistant dormant spores.

Bacteriocins

Bacteriocins are a class of ribosomally synthesized antimicrobial peptides (AMPs) produced by bacteria. These small and naturally produced peptides can kill other

bacteria, which are closely (narrow spectrum) or distantly (broad spectrum) related to the producing bacteria (Cotter et al., 2005). It is hypothesized that the production of bacteriocins is a strategy to control competing bacteria in the hunt for nutrients and space in an environmental niche. Therefore, it is not surprising that it has been estimated that many bacteria produce at least one bacteriocin (Riley and Wertz, 2002), which may help them to influence the surrounding population dynamics. Although many bacteriocin-producing bacteria in the biosphere have been investigated, it is still the case that there remain many are that are still to be discovered (Yang et al., 2014). Indeed, bioinformatic mining of publically available genomes, along with other rapid techniques, are beginning to bridge this gap in initial discovery, by overcoming the previous dependence on the expensive, time consuming, culture-dependent nature of bacteriocin discovery and purification (Sandiford, 2015). BAGEL3 (BActeriocin Genome mining tooL) (van Heel et al., 2013) and antiSMASH 3.0 (antibiotics and Secondary Metabolite Analysis Shell) (Weber et al., 2015) are examples of web based genome mining tools that detect putative bacteriocin biosynthetic gene clusters. Liquid chromatography / mass spectrometry has also been used to rapidly detect bacteriocins in as little as 25 µl of culture supernatant and is sensitive enough to distinguish between variants of the same bacteriocin e.g., nisins A, Z, and Q (Zendo et al., 2008). High throughput, culture-based screens can also be valuable (Rea et al., 2010).

Bacteriocins from LAB are suitable for food preservation

Although there are many Gram-negative and Gram-positive microorganisms which produce bacteriocins, those produced by the lactic acid bacteria (LAB) are of particular interest to the food industry. Many of these bacteria already play a crucial

role in a variety of food fermentations by converting lactose to lactic acid, as well as producing a variety of additional antimicrobial molecules such as other organic acids, diacetyl, acetoin, hydrogen peroxide, antifungal peptides, and bacteriocins. The best known LAB genera are *Lactococcus, Streptococcus, Lactobacillus, Pediococcus,* and *Enterococcus,* though a number of other, generally regarded as more peripheral and less frequently applied from an industrial perspective, genera also exist. LAB offer several key properties which make their bacteriocins highly desirable for use in food: (i) the LAB are Generally Regarded As Safe (GRAS) and there are perceived by the public as having health promoting features, (ii) their bacteriocins are sensitive to digestive proteases such as pancreatin complex, trypsin and chymotrypsin, and thus don't impact negatively on the gut microbiota, (iii) they are non-toxic to eukaryotic cells (iv) they are often active across a range of pH values and are, in many cases, not temperature sensitive (Table 1), (v) they are gene encoded and therefore highly amenable to genetic manipulation where desired (Field et al., 2015), (vi) not all of the bacteriocins produced by the LAB have similar / the same mode of action, and (vii) they are active against a range of food pathogenic and spoilage bacteria.

Classification of bacteriocins produced by the LAB

LAB bacteriocins may be classified into two separate classes based on their modification status: Modified (class I), and minimally modified or cyclic (class II; Rea et al., 2011; Cotter et al., 2013).

Class I are comprised of all peptides that undergo post-translational modification during biosynthesis and include the subclass of lantibiotics among others. While

several other subclasses within class I have been described (Arnison et al., 2013; Cotter et al., 2013), this review will focus mainly on those with relevance to the food industry. The commercially important bacteriocin nisin is produced by *L. lactis* and is the prototypical member of the class I lantibiotics. Nisin is currently used in over 50 countries to improve food safety and extend shelf life. Other important members of this class include: the two peptide lantibiotic lacticin 3147 produced by *L. lactis* DPC 3147 (Suda et al., 2012), subtilin produced by *Bacillus subtilis* ATCC 6633 (Lee and Kim, 2011), and lacticin 481 produced by *L. lactis* CNRZ 481 (Piard et al., 1993). Lantibiotics undergo extensive post-translational modifications, resulting in the presence of unusual amino acids such as lanthionine, β-methyllanthionine, dehydrobutyrine, and dehydroalanine. Covalent bonds are formed between these non-standard residues, resulting in internal rings which are important for it's potent activity (Rink et al., 2007).

Class II bacteriocins are < 10 kDa, heat stable and non-modified peptides that can be further subdivided into four subgroups: IIa pediocin like, IIb two peptide bacteriocins, IIc cyclic bacteriocins, and IId single linear non-pediocin bacteriocins. Members of class IIa are *Listeria*-active peptides which contain a conserved amino acid consensus sequence across all members of this group: Y-G-N-G-V-X_1-C-X_2-K/N-X_3-X_4-C (where X is any amino acid) (Cui et al., 2012). This consensus sequence is often referred to as the "pediocin box" and is present at the N-terminal region of the class IIa bacteriocins. Class IIb bacteriocins are unmodified two peptide bacteriocins, which interact to give full activity; having little or no activity in isolation. Class IIc bacteriocins are covalently linked from their N to C termini during post-translational modification resulting in a circular backbone. Class IId are

a heterogeneous group, made up of bacteriocins which are linear, do not contain a pediocin box and do not require another peptide for full activity.

Using bacteriocins produced by *Enterococcus* in food

The bacteriocins produced by *Enterococcus* species are diverse, both in terms of their classification and inhibitory spectrum (Table 1). While most LAB are GRAS, and thus their associated bacteriocins can be considered for food applications, the status of enterococci is less clear. Indeed, many strains are clearly not food grade. Although *Enterococcus* species have been used as artisanal cultures in a variety of foods, their suitability for use in food is questionable as they have been sometimes associated with pathogenicity. Indeed, cases of urinary tract infections, bacteremia and endocarditis have been associated with *Enterococcus* species (Franz et al., 1999; Kayser, 2003). De Vuyst et al. (2003) suggested that *Enterococcus* species could be safely used in food if virulence genes are absent (cytolysin, vancomycin resistance, etc.). However, in a review by Franz et al. (2011), the ability of *Enterococcus* to acquire virulence and antibiotic resistance genes on mobile genetic elements was identified as a significant barrier to their use in food. Recently, Jaouani et al. (2015) examined the safety of previously identified bacteriocinogenic enterococci, by examining the presence of virulence and antibiotic resistance genes. Using these criteria, it was concluded that 22 / 55 of the strains tested were safe for use in food. Ultimately, *Enterococcus* are an important reservoir for bacteriocin discovery and therefore developing a comprehensive set of guidelines / considerations for their safe use would be highly valuable when considering their suitability for use in food.

Bacteriocin mode of action against vegetative cells

Mechanistically, bacteriocin molecules produced by the LAB act by one, or both, of two different mechanisms: (i) inhibition of cell wall biosynthesis, and (ii) pore formation.

At the cell envelope, lipid II plays a key role in the synthesis of peptidoglycan as it transports cell wall subunits across the bacterial cytoplasmic membrane. Lipid II delivers its peptidoglycan subunit cargo from the cytosol to an exterior multi-enzyme complex which is responsible for polymerization of that subunit into the peptidoglycan cell wall. The halting of cell wall biosynthesis by sequestering lipid II is a strategy employed by a number of antimicrobial compounds which results in cell death (see review by: Oppedijk et al., 2016). The important clinical antibiotic vancomycin also targets lipid II, though its lipid II binding site is distinctly different to the lantibiotic nisin. The alternative binding site for nisin results in the ability of nisin to kill bacterial cells which are resistant to vancomycin (Gut et al., 2011). Other bacteriocins that exert their bactericidal mechanism of action by inhibition of cell wall biosynthesis are mersacidin, which inhibits transglycosylation (Brötz et al., 1995), and lactococcin 972 which targets septum biosynthesis via lipid II (Martínez et al., 2008). While lipid II is an important receptor for certain bacteriocins, there are however other receptors to which bacteriocins bind on the Gram-positive cell such as: the mannose PTS system, the maltose ABC transporter, Zn-dependent metallopeptidase, and undecaprenyl pyrophosphatase phosphatase (see review by: Cotter, 2014). Indeed these bacteriocin-receptor complexes play an important role in specifying a bacteriocins spectrum of activity. The outer cell membrane of Gram-negative bacteria provides an effective barrier to bacteriocins

from binding their lipid II targets. However, Gram-negative bacteria can be sensitized toward bacteriocins if treated with agents or chemicals that destabilize the outer cell membrane (such as sodium phosphate buffer or EDTA).

Bacteriocins may also kill or damage cells by pore formation in the cell membrane. This pore formation is achieved by insertion of the bacteriocin into the cell membrane, forming a membrane pore. This pore results in depolarization of the membrane potential and diffusion of low molecular cytosolic compounds out of the cell; ultimately rendering the bacterial cell non-viable. Enterocin AS-48 is predicted to form aggregates which insert into the bacterial membrane, resulting in accumulation of positive charge along the cell surface, destabilizing the membrane potential, leading to pore formation and cellular leakage. Other bacteriocins that form pores include: streptococcin SA-FF22, lacticin F, and lactococcin A (Héchard and Sahl, 2002).

There are a number of members of the bacteriocins that exhibit dual modes of antimicrobial action by both: forming pores and inhibiting cell wall biosynthesis. The ability of such bacteriocins to act through two mechanisms of action can prevent the development of bacteriocin resistance. Moreover, it is worth noting that microorganisms that are resistant to antibiotics generally do not display cross-resistance to bacteriocins (Jordan et al., 2014). Nisin (Wiedemann et al., 2001), pediocin PA-1 (Diep et al., 2007), lacticin 3147 (Wiedemann et al., 2006), epidermin (Götz et al., 2014), and gallidermin (Götz et al., 2014) are examples of bacteriocins that display a dual mode of action, making their activity particularly potent against their targets.

Bacteriocin spore interactions

In comparison to the vast knowledge available with respect to bacteriocin interactions with vegetative cells, it is safe to say that there is considerably less known about bacteriocin / spore interactions. However, there are a small number of bacteriocins (Table 2) for which activity against a variety of bacterial spores has been demonstrated. Phase contrast microscopy can be utilized to determine at what stage in the spore cycle (Figure 1) the bacteriocin exhibits its anti-spore activity by combining the bacteriocins with dormant (phase bright) and germinated (phase dark) spores. Spore viability can then be examined following the treatment with bacteriocin to determine the bacteriocins effect on the spore. Two outcomes may ensue: the bacteriocin (i) does not require germination and will be sporicidal against dormant spores, or (ii) will be sporostatic to dormant and germinated spores but requires germination to inhibit spore outgrowth. Bacteriocins can also affect the germination rate of the spore, which can be examined by measuring the drop in absorbance (OD_{600nm}) of a dormant spore suspension as it transitions to a germinated spore suspension over a time period. These outcomes are however heterogeneous (Table 3), with differences occurring at species level where the same bacteriocin was used, and will be further discussed below.

Nisin

Previous studies have shown that for *B. anthracis* (Gut et al., 2008; 2011), *B. licheniformis* (Mansour et al., 1999), *C. difficile* (Nerandzic and Donskey, 2013), and *C. perfringens* (Udompijitkul et al., 2012), nisin had no impact on the process of germination, as it neither initiated, inhibited, or altered the rate of germination, as examined on the basis of spore refractility, with or without nisin. Conversely, the

presence of 25 μg/ml of nisin has been shown to have a progerminant activity for *C. botulinum* spores, as when it was present in the germination medium, the germination rate was doubled. However, the presence of nisin (125 μg/ml) has been shown to decrease the germination rate of *B. sporothermodurans* spores (Aouadhi et al., 2015).

With respect to anti-*B. anthracis* activity, it has been reported that nisin exerts its inhibitory effect after germination initiation, where nisin binds lipid II in the germinating spore and prevents it from becoming metabolically active by interfering with the establishment of a membrane potential and oxidative metabolism. Germination initiation is required for this lipid II binding to occur, as nisin is unable to associate with the dormant spore due to the absence of lipid II on the exterior of the spore (Gut et al., 2011). When investigating the effects of nisin on *C. perfringens* spores, it was observed that, as for studies involving *B. anthracis* and *C. butyricum*, nisin exhibited its inhibitory action during the stage of spore outgrowth (Udompijitkul et al., 2012). Using a truncated nisin derivative consisting of rings A, B and C (which could bind lipid II but not form pores) and fluorescently labeled unmodified nisin, it was shown that lipid II binding alone was insufficient to inhibit spore outgrowth. This was further investigated using the double mutants N20P/M21P and M21P/K22P, which were unable to form pores, but could bind lipid II. These nisin mutants were again shown to be unable to inhibit spore outgrowth. Through the use of the double mutant and truncated nisin, it is clear that pore formation is the essential mechanism by which nisin inhibits spore outgrowth, while lipid II is the target for nisin (acting as a receptor for nisin) to inhibit outgrowth in the germinating spore (Gut et al., 2008; 2011). While it has been shown that

truncated nisin consisting of rings A, B, and C does not inhibit spore outgrowth in *B. anthracis*, it has been reported elsewhere that this peptide does inhibit outgrowth of *B. subtilis* (Rink et al., 2007). While the mechanisms underlying these differing results have not yet been completely elucidated, some possible explanations given were (i) differences in outgrowth measurement methods and (ii) potential spore structure variations (Gut et al., 2011). Nisin however displays sporicidal activity against dormant *B. sporothermodurans* (Aouadhi et al., 2013), in contrast with the sporostatic activity against other targets described above.

The ability of microorganisms to develop resistance mechanisms to bacteriocins is a concern that could impede their widespread use in food (see review by: Draper et al., 2015). Nisin resistance has been reported for toxigenic spores of *C. botulinum* which had the ability to germinate and grow in levels of nisin that reduced levels of sensitive germinating spores by 7–8 $\log_{10}$ spores/ml (Mazzotta and Montville, 1999). The exact mechanism by which these spores exhibited nisin resistance is unknown but, interestingly it has been noted that nisin resistant strains have an altered fatty acid composition, which is consistent with a more rigid membrane. It has also been observed that nisin resistant strains of *C. botulinum* display cross-resistance to class II bacteriocins (Mazzotta et al., 1997).

Enterocin AS-48

Enterocin AS-48 produced by *Enterococcus faecalis* A-48-32 is a class IIc cyclic bacteriocin that is active against a number of *Bacillus* and *Clostridium* sp. (Table 1). Unlike nisin, the exact molecular mechanism by which enterocin AS-48 interacts with bacterial spores is unknown. It was observed that spores of *B. cereus* became

more sensitive to enterocin AS-48 gradually after germination and were sensitive to 25–50 μg/ml 10 min after germination initiation. The greatest effect of enterocin AS-48 was observed 90–120 min after germination initiation, when cellular growth occurred (Abriouel et al., 2002). Enterocin AS-48 has also been shown to be effective in inhibiting spore outgrowth using heat activated spores of *B. cereus*. In a boiled rice substrate, 25 μg/ml of enterocin AS-48 reduced heat activated spores incubated at 37 and 15°C, below the level of detection after 3 days, whereas at 6°C, this reduction took 14 days. A higher concentration of 35 μg/ml of enterocin AS-48, reduced the heat activated spores below the level of detection in rice gruel after 24 h at three different temperatures (6, 15, and 37°C) (Grande et al., 2006b).

Outgrowth inhibition of the important thermophilic spore-former *Geobacillus stearothermophilus* has also been shown using enterocin AS-48. *G. stearothermophilus* is regularly identified as a spoilage agent in low acid canned food, being highly heat resistant with a D_{121}°C value of 1 min, so its removal from canned products requires an extensive heat treatment (Durand et al., 2015). Viedma et al. (2009) tested the efficacy of enterocin AS-48 in inhibiting spore outgrowth of *G. stearothermophilus* using three food models, canned corn, canned peas and coconut milk, using a cocktail of two *G. stearothermophilus* strains. Here it was shown that AS-48, used at 1.75 μg/ml, reduced the viable counts of heat treated spores below the level of detection after 24 h. *B. licheniformis* was controlled in a commercial cider by AS-48 at a level of 5 μg/ml at 30°C. A significant reduction was observed in a population of germinated spores following treatment with AS-48 (Grande et al., 2006a).

The genus *Alicyclobacillus* has in recent years become a problem in the food industry. Members of this genus have an ability to grow at high temperatures (50–70°C), and at low pH values (3.0–3.5), which makes their eradication from certain foods problematic. *A. acidoterrestris* is a particular problem in acidic juice products such as apple, tomato and orange, amongst others (Steyn et al., 2011). Inhibition of *A. acidoterrestris* spores by enterocin AS-48 has been observed at concentrations as low as 2.5 μg/ml. At this concentration a reduction of 6 Log_{10} spores/ml was achieved. Using electron microscopy it was observed that the enterocin AS-48 treated spore structures sustained substantial damage supporting the hypothesis that the bacteriocin adsorbs to the spores negatively charged surface groups. This interaction with *A. acidoterrestris* would suggest a sporicidal rather than the sporostatic mechanism of action that is suggested for *B. cereus* (Grande et al., 2005).

Lacticin 3147

Lacticin 3147, produced by *L. lactis* subsp. *lactis* DPC3147, has been shown to inhibit spores of *C. tyrobutyricum* in milk. This *Clostridium* species is responsible for late blowing in hard cheese, as their spores can survive heat treatments and germinate in the ripening cheese. Previously nitrate was used to combat clostridia but has been banned by the European Food Safety authority (EFSA) in an effort to reduce nitrosamines in food (Bassi et al., 2015). When used at a concentration of 45 μg/ml, lacticin 3147 was also able to completely inactivate 4–5 Log_{10} spores/ml over a 24 h period. Additionally, when lacticin 3147 was added following a 24 h incubation of the spores, total inactivation 6 days post addition of the bacteriocin was observed. *In situ* production of lacticin 3147, in a model curd system, has also been shown to significantly reduce (3 Log_{10} spores/g) the number of *Clostridium* spores

after 13 days, when compared to a non-bacteriocin producing control. After 60 days of ripening, lacticin 3147 produced *in situ* was shown to be effective in reducing the levels of artificially contaminated clostridia (introduced prior to ripening) from 8 to 2 Log_{10} spores/g (Carmen Martínez-Cuesta et al., 2010).

Bificin C6165

Bificin C6165 produced by *Bifidobacterium animalis* subsp. *animalis* CICC 6165 was shown to inhibit species such as *Lactobacillus, Bifidobacterium, Enterococcus, Staphylococcus*, and *Alicyclobacillus acidoterrestris*. Indeed, from an anti-sporeformer perspective, it is notable that bificin C6165 inhibited 20/20 strains of *A. acidoterrestris* tested. Bificin C6165 could also reduce a population of *A. acidoterrestris* spores and was more effective as the concentration of the bacteriocin increased (Pei et al., 2013). Another important characteristic of bificin C6165 which makes it an ideal candidate for inhibition of *A. acidoterrestris* is its activity at acidic pH 3.5–6.5 (Pei et al., 2014).

Plantaricin TF711

Plantaricin TF711, produced by *Lactobacillus plantarum* TF711, is active over a broad pH range and is active against vegetative cells of *B. cereus* and *C. sporogenes* (Hernández et al., 2005). *C. sporogenes* acts as a research surrogate for proteolytic *C. botulinum* as these two species are closely related. This species has also been associated with late blowing of hard cheese (Bassi et al., 2015). Plantaricin TF711 was shown to reduce *C. sporogenes* spore counts significantly from 7 days onwards when introduced in the form of an adjunct culture producing the bacteriocin *in situ*. The bacteriocin was shown to be present at highest levels at day

21, after which its activity declined. This decline in activity could be due to loss of stability, depletion of the bacteriocin in the cheese, or reduced production of the bacteriocin (González and Zárate, 2015).

Thurincin H

Thurincin H produced by *B. thuringiensis* SF361 has been shown to be sporostatic against dormant *B. cereus* spores and sporicidal against germinated *B. cereus* spores. Similarly to other bacteriocins, thurincin H displays sporicidal activity after germination, while it was sporostatic to dormant spores. Although not an LAB bacteriocin, it has been suggested that Thurincin H may have potential for use in food (Wang et al., 2014).

Other bacteriocins active against bacterial spores

There are a number of other bacteriocins that have shown potential. Some of these are described here. Soria and Audisio (2014) revealed that heat activated *B. cereus* spores could be inhibited by the cell free supernatant of *E. faecium* SM21 containing an enterocin which produced a bacteriostatic effect at both pH 5 and pH 6. Bacteriocin production by *Streptococcus thermophilus* 580 was capable of inhibiting *C. tyrobutyricum* gas production in a ripening curd model for up to 14 days, when compared to controls which produced gas after 14 days (Mathot et al., 2003). Pentocin L and pentocin S, are produced by *Pediococcus pentosaceus* L and S, respectively. Both of these bacteriocins are inhibitory against a variety of vegetative *Bacillus* and *Clostridium* strains (Table 1). Furthermore, these bacteriocins were shown to be sporostatic by inhibiting the germination of three different strains of non-heat activated *B. cereus* spores. These

active proteins are larger than typical bacteriocins which suggests that these peptides may in fact be bacteriolysins (Yin et al., 2003).

Comparing the sensitivity of spore and vegetative cells of bacteriocins

To date there have been conflicting reports as to whether germinated spores are more or less resistant to bacteriocins than vegetative cells. Heat activated spores of *B. sporothermodurans* are less sensitive to nisin (1.25 μg/ml), than vegetative cells of *B. sporothermodurans* (Aouadhi et al., 2015). The Minimum Inhibitory Concentration (MIC) of nisin for vegetative cells of *C. butyricum, C. perfringens, C. sporogenes*, and *C. tyrobutyricum* was found to be 0.17, 0.75, 38.4, and 4.8 μg/ml, respectively. However, 23 μg/ml of nisin prevented outgrowth of heat activated *Clostridium* spores for up to 10 days. Unfortunately in this study it is unclear whether the vegetative cells were more or less resistant than their spores to the nisin treatment as no MIC for spores was carried out (Meghrous et al., 1999). Another study found that vegetative cells of *C. sporogenes* were less resistant to nisin than heat activated spores, yielding MICs of 0.23 and 1.11 μg/ml, respectively. In contrast, it was revealed that heat activated *C. beijerinckii* spores were less resistant with an MIC of 1.09 μg/ml while their vegetative cells exhibited an MIC of 1.3 μg/ml (Hofstetter et al., 2013). At odds with these findings, however, were the results obtained by Ávila et al. (2014), which compared the sensitivity of spores and vegetative cells of four clostridia: *C. tyrobutyricum, C. butyricum, C. beijerinckii*, and *C. sporogenes*. Using four representatives of each species, they showed that spores had a higher MIC, and thus were more resistant to nisin, than their vegetative counterparts in 15 of the 16 strains tested. The only exception was displayed by *C. tyrobutyricum* CET 4011 strain where the vegetative and spore MIC values were equal at 0.39 μg/ml. It is also important to

note that in this case all the MIC values were below the maximum permissible limit for nisin, which is 12.5 μg/ml in Europe.

Spores of *A. acidoterrestris* were found to be more sensitive to nisin than their vegetative cells. The MIC values for both spores and vegetative cells were carried out in mYPGA at two different pH values (pH 3.4 and pH 4.2). Interestingly, at pH 3.4, all spores were more sensitive (7/7) than their vegetative cells. However, at pH 4.2 (3/7) spores had equal MIC-values to their vegetative cells (Yamazaki et al., 2000). Whether this is due to the (i) enhanced activity of nisin at lower pH, (ii) negative effects of pH on the spore or (iii) a combined activity of both, has yet to be determined. These findings were further confirmed by Ruiz et al. (2013), who found the MIC of spores and vegetative cells of *A. acidoterrestris* to be 7.81 and 31.25 μg/ml, respectively.

Inhibition of spore outgrowth prevents toxin formation

Toxin formation is an important feature of a number of *Clostridium* and *Bacillus* species. There are two types of toxin with which *B. cereus* strains are frequently associated: (i) heat labile diarrheal enterotoxin and/or (ii) heat-stable emetic enterotoxin. Beuchat et al. (1997) showed that the production of diarrheal enterotoxin produced in beef gravy inoculated with *B. cereus* spores could be inhibited by addition of nisin. Enterotoxin production normally occurred after 3 and 9 days for heat activated *B. cereus* spores incubated at 15 and 8°C, respectively. Addition of 1 μg/ml of nisin inhibited enterotoxin production completely at 8°C, whereas a higher concentration of 5 μg/ml was needed to inhibit enterotoxin production at 15°C over a 14 day period. The levels of nisin required to prevent enterotoxin production from a spore inoculum also ensured that the final cell numbers did not exceed 4.03 and 6.23

Log_{10} CFU/g at 8 and 15°C, respectively. Without nisin, enterotoxin was produced when cell numbers exceeded 6.78 and 7.1 Log_{10} CFU/ml at 15 and 8°C, respectively. This is in agreement with the strategy of keeping the *B. cereus* population below ~7 Log_{10} CFU/g to prevent enterotoxin production (Christiansson et al., 1989). It would be interesting to see if the cell numbers in the presence of nisin were allowed to exceed these numbers would enterotoxin still be produced or would the enterotoxin production cease due to the presence of nisin.

Enterocin AS-48 was also shown to have an effect on enterotoxin production by psychrotrophic vegetative cells of *B. cereus*. Enterocin AS-48 completely inhibited enterotoxin production and bacterial growth for at least 72 h when used at 7.5 μg/ml. When enterocin AS-48 was used at subinhibitory concentrations (2.5 or 5 μg/ml) the growth of the cells were severely subdued and enterotoxin titres were 10-fold lower than non-bacteriocin treated controls (Abriouel et al., 2002).

Combining bacteriocins with other hurdles

Bacteriocins in combination with heating

The thermal resistance of bacterial spores makes their eradication from food by heat a major problem during food processing. Nisin at various concentrations has been shown to reduce the decimal reduction times (*D*-values) and thus the thermal resistance of bacterial spores. Therefore, nisin has been described as a compound with a "two-fold beneficial effect": (i) it enhances the heat sensitivity of the bacterial spore (Table 4) and (ii) it prevents the outgrowth of spores which survive the heat treatment (Komitopoulou et al., 1999). Pre-exposing heat activated *G. stearothermophilus* spores to nisin (50 μg/ml) at 4°C in chocolate milk for 15 and 24 h, resulted in significantly

reduced $D_{130°C}$ values of 20.5 and 25.1%, respectively, compared to those spores not pretreated with nisin. When the nisin pretreatment was raised to 100 μg/ml this did not cause a significant reduction over the lower concentration of 50 μg/ml (Beard et al., 1999). *B. amyloliquefaciens* spores were rapidly inactivated when treated with 90°C and 16 μg/ml of nisin, in contrast to the results when a 90°C treatment was used, alone, where there was no inactivation of spores (Hofstetter et al., 2013).

A reduction of 2 Log_{10} spores/ml was observed when spores of *C. sporogenes* spores were subjected to a heat treatment of 90°C for 2 h in the presence of 16 μg/ml nisin vs. a 90°C heat treatment without nisin. Additionally there was 30% greater DPA release when spores of *C. sporogenes* were heat treated at 90°C with nisin than those spores which were not treated in any way. However, when *C. beijerinckii* was subjected to the same conditions (90°C for 2 h and 16 μg/ml nisin), no increased inactivation was observed. The ability of nisin to increase the permeability of resting spores of *C. sporogenes* and *C. beijerinckii* was observed using DAPI staining. Fluorescence was observed after a treatment at 90°C with nisin, whereas a heat treated spore without nisin that did not fluoresce (Hofstetter et al., 2013). These findings are consistent with the hypothesis that nisin lowers the heat resistance of spores by permeabilizing their exterior.

Response surface technology (RSM) is an empirical modeling technique that can be used to examine and predict the relationship between the response variable and the test variable. RSM can be used to predict optimum processing conditions to achieve a pre-determined reduction in spores (Table 5).

Dormant *B. coagulans* spores were shown to be resistant to enterocin AS-48 in that use of 6 μg/ml bacteriocin resulted in an approximately one log reduction in the number of viable cells when dormant spores were treated with the bacteriocin in three food models: (i) tomato paste, (ii) syrup from canned peach, and (iii) juice from canned pineapple. However, using enterocin AS-48 at 3 and 6 μg/ml in combination with heat treatments (5 min at a minimum of 80°C) showed a significant reduction in the number of viable cells in both food models. When spores were incubated at 22°C for 48 h with bacteriocin, then heat treated at both 80 and 95°C, there was a significant difference in the number of viable cells obtained following both treatments relative to the non-heat treated controls or those that were heat treated without bacteriocin (Lucas et al., 2006). A relationship between heat temperature and survivors was observed, showing that viable counts in samples supplemented with bacteriocin decreased as the temperature was increased. This relationship was further evidenced by the significant reduction in viable counts obtained from bacteriocin treated spores heat treated at 95°C over those heat treated at 80°C. This relationship was observed in all three food models previously discussed (Lucas et al., 2006). Ultimately, this study nicely highlights the efficacy of bacteriocins to (i) reduce the severity of heat treatments and (ii) increase the effectiveness of heat treatments, when used to inactivate spores in food.

Another bacteriocin discussed previously, bificin C6165, has been shown to reduce the $D_{90°C}$ value of *A. acidoterrestris* as the bacteriocin concentration increased from 0 to 160 μg/ml. Addition of 80 and 160 μg/ml of bificin C6165 was shown to reduce the $D_{90°C}$ *A. acidoterrestris* CFD1 by 32.7 and 42.7%, respectively (Pei et al., 2014).

Bacteriocins in combination with pressure

High-pressure processing (HPP) is a "non-thermal" food preservation technique that inactivates harmful pathogens and vegetative spoilage microorganisms by using pressure rather than heat to effect pasteurization. HPP utilizes intense pressure (about 400–600 MPa or 58,000–87,000 psi) at chilled or mild process temperatures (< 45°C), allowing most foods to be preserved with minimal effects on taste, texture, appearance, or nutritional value. Microorganisms do however display a variability in their sensitivity to HHP in the order: Gram-negative bacteria > Gram-positive bacteria > bacterial spores. While HPP is an effective method used for the destruction of microorganisms in food, it is not sufficient alone to inactivate spores and therefore must be combined with other hurdles, such as bacteriocins, to increase its efficacy. Indeed, treating food with bacteriocins may be an excellent combination as HHP can induce germination, which can facilitate the germination-dependent sporicidal activity of bacteriocins. Black et al. (2008) showed that treatment of 8 Log_{10} spores/ml of *B. subtilis* with low pressure (100 MPa i.e., not HHP) at 40°C in milk resulted in germination and inactivation of 4 and 1 Log_{10} spores/ml, respectively. A similar level of germination, but without inactivation, was observed in milk when a higher treatment of 500 MPa was used. When spores were treated with a combination of HP (500 MPa) and nisin (12.5 μg/ml), spore germination and inactivation increased to 6 and 3 Log_{10} spores/ml, respectively. When cycled twice with nisin there was a further increase in spore germination and inactivation of 8 and 6 Log_{10} spores/ml, respectively. High pressure-induced germination is known not to require the presence of nutrient receptors and is characterized by a rapid release in DPA-Ca^{2+} from the core. Nisin can be characterized as a potent pro-germinant in the presence of germinants (naturally present in milk) such as L-alanine and L-cysteine.

Interestingly, nisin doubled the rate of germination in *C. botulinum* spores, while it had no effect on nisin resistant spores (Mazzotta and Montville, 1999). It was hypothesized that inactivation of spores by HPP and nisin could be due to (i) nisin and HP acting synergistically to inactivate spores or (ii) HP inducing germination after which nisin exerts its lethal effect on the germinated spore (Black et al., 2008). *C. sporogenes* spores were also shown to be inhibited rapidly by a treatment of nisin and 600 MPa at 90°C, relative to a treatment of 90°C alone (Hofstetter et al., 2013).

More recently, several studies have used response surface methodology (RSM) to test the effectiveness of high pressure, heat and nisin. Aouadhi et al. (2013) used RSM to investigate the effects of high pressure, in combination with moderate heat and nisin treatment, on *B. sporothermodurans* spores. The authors showed that spore inactivation was concentration dependent and that 1.25 and 125 μg/ml caused an inactivation of 0.4 and 4 Log_{10} spores/ml, respectively. Aouadhi et al. (2014) and Gao et al. (2011) showed that RSM (Table 5) could be effectively implemented to design an optimum treatment, involving multiple parameters to reduce spores loads by a predetermined amount.

Interestingly, superdormant spores of *B. cereus* and *B. subtilis* have been shown to germinate similarly to dormant spores when treated with pressure of 150MPa regardless of whether they were heat-activated or non-heat-activated. There have, however, been conflicting reports regarding the ability of pressure treatments to cause germination of an entire spore population. This uncertainty has impeded the widespread use of high pressure. It has been hypothesized that spores which remain

superdormant after high pressure may do so via a distinct mechanism from that involved in making some spores superdormant to nutrient germinants (Wei et al., 2010).

Bacteriocins in combination with pulsed electric field

Pulsed electric field (PEF) is an innovative food preservation method, which may be suitable for reducing spore loads in liquid food. One of the distinct advantages of PEF is that the thermal impacts on food are minimized as this treatment is relatively non-thermal. Any heat produced is directly influenced by the energy input of the treatment. While it is known that vegetative cells of *B. cereus* are sensitive to PEF and nisin (Pol et al., 2000) and that this combination is sporostatic, this treatment did not initiate germination nor did it affect the viability of the dormant spores. After germination, *B. cereus* immediately became sensitive to nisin (1.25 μg/ml) but it was longer (50 min) before outgrowing cells became sensitive to PEF (27 kV/cm, 302-μs pulses; flow rate, 10 ml/min). Unlike the synergistic activity of nisin and PEF against vegetative cells (Pol et al., 2000), when spores are treated with both PEF and nisin this synergistic activity was not observed as the reduction was comparable to nisin alone (Pol et al., 2001). While this combination is not synergistic against spores, food rarely contains spores alone but rather a mixed population of spores and vegetative cells. Therefore, this combination may still be an effective way of maintaining dormant spore numbers yet reducing the population of vegetative cells for increased food safety and shelf life.

Bacteriocins in combination with osmotic activation

Stimulation of dormant bacterial spore germination followed by subsequent inactivation, as previously discussed, is a promising method used for spore inactivation. Small, non-polar, hydrophobic solutes that permeate the plasma membrane have been shown to stimulate *B. cereus* germination (Preston and Douthit, 1984). Inhibition of non-heat activated *C. difficile* spores was significantly increased when treated with nisin and single osmotic activators (ammonium, glycerol, and Tris) compared to heat activated spores treated with nisin and solutes in a germination medium. For example, nisin in combination with heating resulted in a 1–2.5 $\log_{10}$ spores/ml decrease in viable spores but when nisin was combined with osmotic activators this increased to >3.5 $\log_{10}$ spores/ml (Nerandzic and Donskey, 2013). Using flow cytometry, it was observed that the membrane permeability of spores was significantly increased when treated with osmotic activators. Spores treated with both nisin and solute transitioned to phase dark (as spores germinate they appear phase dark using phase contrast microscopy), whereas those incubated with nisin and osmotic activators separately did not transition to phase dark (Nerandzic and Donskey, 2013). The proposed synergistic ability of nisin and osmotic activators to inhibit outgrowth was attributed to the osmotically induced loss of membrane integrity. Although *C. difficile* is of clinical importance, this use of osmotic activation could be used to overcome limitations of the germination dependent activity of bacteriocins with other food related strains of clostridia.

Bacteriocins in food packaging

The preservation of sausage casings of preserved intestines of animals has been practiced for centuries. However, this preservation method has been modernized to

suit modern consumer desires. Such a modernization is the binding of nisin to sausage casing in order to control *Clostridium* spore outgrowth. Wijnker et al. (2011) showed that nisin, at 100 μg/ml, when bound to casings and placed on agar plates seeded with *Clostridium* spores, produced zones whereas those casings with only 50 μg/ml did not. They also observed that addition of nisin at 50 μg/ml to the casings delayed *C. sporogenes* spore outgrowth between 1 and 8 days. Furthermore, at this concentration of 50 μg/ml, this sporostatic activity was observed for 30 days. In contrast, Meghrous et al. (1999) showed that a lower concentration of nisin, 23 μg/ml, delayed *clostridial* spore outgrowth by 10 days. It should also be noted that Wijnker et al. (2011) used 10^6 spores/ml whereas Meghrous et al. (1999) used 10^3 spores/ml. The reason that nisin at 50 μg/ml could inhibit outgrowth *in vitro* but not on the casings could be due to the irreversible binding of nisin to the collagen matrix of the casing wall. This would suggest that if outgrowth is to be prevented, the casings need to contain a higher concentration of nisin in order to overcome the deleterious effect of irreversible binding to the casing matrix.

Bacteriocins in combination with plant extracts

Plants contain innumerable constituents and are valuable sources of new and biologically active molecules possessing antimicrobial properties. The plant family *Piperaceae* are found in tropical and subtropical regions and are commonly used as to generate medicinal herbs. Ruiz et al. (2013) showed that a combination of nisin and *Piper aduncum* exhibited a strong antibacterial activity against spores of *A. acidoterrestris* and also exhibited a synergism (FIC = 0.24) against *A. acidoterrestris* vegetative cells. Prenylated chromone was identified as the active compound in this plant extract. *Piperaceae* extract is a natural food preservation

method that may be combined with nisin to lower (if any) heat treatment needed to reduce and inhibit spores outgrowth.

Bacteriocins in combination with fatty acid esters

Sucrose fatty esters are approved internationally for use as emulsifiers and these non-toxic molecules have also been reported to inhibit Gram-positive bacteria. A combination of nisin and the fatty acid ester, sucrose palmitate (P-1570), displayed synergism against spores of *B. cereus* whereas sucrose fatty acid esters alone caused no decrease in growth (Thomas et al., 1998). Total inhibition of *B. licheniformis* spore outgrowth was achieved when nisin (0.75 μg/ml) was combined with the fatty acid ester monolaurin (100 μg/ml) whereas when these treatments were used separately at higher concentrations they only partially inhibited outgrowth (Mansour et al., 1999).

Bacteriocins in combination with potassium sorbate

Sorbates are extensively used in the food industry, as they are able to inhibit, or delay growth of, spores and vegetative populations of bacteria. Although their mechanism of action is not full defined for bacterial spores, it is has been shown that potassium sorbate inhibits the growth of spores of *Bacillus* species (Oloyede and Scholefield, 1994). A combination of nisin (1.25 μg/ml) and potassium sorbate (2% w/v) has been shown to cause a synergistic reduction in the number of heat activated *B. sporothermodurans* spores. After 8 h there was ~3 Log_{10} spores/ml reduction. This reduction in spores continued albeit at a slower rate until 5 days where total inhibition of *B. sporothermodurans* spores occurred (Aouadhi et al., 2015). When tested separately at these levels, both nisin and potassium sorbate inhibited spore outgrowth. Nisin was not sporicidal but rather sporostatic, inhibiting

spore outgrowth. While potassium sorbate was not sporicidal, it did significantly perturb germination of *B. sporothermodurans* and inhibited the outgrowth of spores (Aouadhi et al., 2015). This ability of potassium sorbate to inhibit spore germination has previously been reported for spores of *B. cereus* and *C. botulinum* (Smoot and Pierson, 1981).

Discussion

While spores are a widely recognized problem in the food industry the majority of bacteriocin-related studies have focused on the elimination of vegetative cells from food. The removal of spores and inhibition of their outgrowth in food is important for (i) increasing shelf life and (ii) protecting the consumer from harmful pathogenic spore-formers. Although there are numerous bacteriocins which have been characterized as safe and effective molecules for use in food, to date, nisin is the only bacteriocin which is authorized for use as a food preservative. While this bacteriocin provides an effective and safe method to reduce spore outgrowth in food, it is important to recognize that this molecule has its limitations. Bacteriocins in food may be limited by: molecule specific solubility, the active pH range of the bacteriocin, inactivation by proteases in food, and the possible negative interactions that occur between certain bacteriocins and certain food components. One such limitation of nisin is its loss of activity as the pH of the food increases. There are a variety of bacteriocins which are more active than nisin at higher pH, such as gassericin A, pediocin AcM, and thermophilin T (Table 1), however they still need to be further characterized before their use in food may be authorized.

In the majority of cases nisin is only sporicidal against those spores in the outgrowth phase and therefore has no effect on those spores in the dormant phase. Although this model of nisin (and other bacteriocins) use in food suggests that germination is a prerequisite for its activity, it is important to note that there are relatively few studies which investigate bacteriocin/spore interactions. Furthermore, it should be recognized that the only detailed mechanism for bacteriocins/spore interaction is that of *B. anthracis*. Indeed, the limited number of existing studies highlights the need for

further research in this area. Understanding these interactions and mechanisms will ultimately lead to a more precise and optimal use of bacteriocins in food. Undeniably, the mode of action for a great many bacteriocins has yet to be elucidated and a better understanding of the methods by which bacteriocins kill bacteria will facilitate a solid basis for engineering new and more potent derivatives with optimized potency and stability. Given that spores must germinate to exert their adverse effects, future research should focus on stimulating spore germination to enable spores to be more effectively targeted by bacteriocins in food settings. Indeed, recent research provides stimulating evidence for using a germination step prior to spore destruction for promoting inactivation of *Bacillus* and *Clostridial* spores (Gut et al., 2008). Furthermore, although numerous components of the spore germination machinery are conserved between spore forming members of bacilli and clostridia, significant differences between the germination of spores of *Clostridium perfringens* and that of spores of a number of *Bacillus* species, both in the proteins and in the signal transduction pathways involved have been revealed (Abhyankar et al., 2014; Setlow, 2014a; Olguín-Araneda et al., 2015). Indeed, as the number of microbial genome sequences has increased dramatically, bioinformatics data contained in the large number of spore-forming Bacillales and Clostridiales genomes that have been sequenced and the information gained from their analysis, can be used to guide researchers to develop novel strategies to achieve a complete and permanent loss of the spore's ability to germinate and grow in food products.

Regardless of the specific bacteriocin of choice, it is clear that there is considerable evidence of the potential value of bacteriocins with respect to controlling sporeforming bacteria in food. In the case of spores, while this activity more

frequently tends to be sporostatic, there are also examples of sporicidal effects. As is the case for vegetative cells, the mechanisms via which bacteriocins inhibit spores may be heterogeneous but ultimately it is apparent that in general bacterial spores can be controlled using bacteriocins, and their application in combination with other novel non-thermal treatments makes their efficacy even greater. The use of the bacteriocins with other food processing hurdles, such as those previously described, thus has the potential to satisfy consumer demands for “clean label” products, enabling processors to produce foods of optimal quality and shelf life.

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Table 1. Bacteriocins that are active against vegetative cells of Gram-positive spore-forming bacteria.

[a] Mass estimated using SDS-PAGE

[b] Mass calculated based on amino acid sequence

[c] Mass obtained using mass spectrometry

ND Not determined

Bacteriocin	**Class**	**Producer**	**Size (Da)**	**Spectrum**	**Heat stability**	**Active pH**	**Sensitive Spore-formers**	**References**
Acidocin LCHV	IId	*Lactobacillus acidophilus* n.v. Er 317/402 strain narine	1158.2[c]	Broad	Heat stable	3 - 8	*B. cereus* *B. subtilis*	(Mkrtchyan et al., 2010)
Acidocin LF221A Acidocin LF221B	IIb	*Lactobacillus gasseri* LF221	3500-5000 [a]	Broad	Heat stable	2 - 9	*B. cereus* *Cl. sporogenes* *Cl. tyrobutyricum*	(Bogovič-Matijašić et al., 1998)
Bac217	IId	*Lactobacillus paracasei* subsp. *Paracasei* BGBUK2-16	7000 [a]	Broad	Heat stable	3 - 12	*B. cereus* *B. fragilis* *B. subtilis*	(Lozo et al., 2004)
BacC1	ND	*Enterococcus faecium* C1	10000 [a]	Broad	Heat stable	2 - 6	*B. cereus*	(Goh and Philip, 2015)
Bacteriocin L-1077	IIa	*Lactobacillus salvarius* 1077	3454	Broad	ND	ND	*C. perfringens*	(Svetoch et al., 2011)
Bifidocin B	IIb	*Bifidobacterium bifidum* NFBC 1454	4432.9[c]	Narrow	Heat stable	2 -10	*B. cereus*	(Yildirim and Johnson, 1998b; Yildirim et al., 1999)

Bificin C6165	ND	*Bifidobacterium animalis subsp. Animalis* CICC 6165	3395.1 [c]	Narrow	Moderate	3.5-6.5	*A. acidoterrestris*	(Pei et al., 2013)
Brevicin 925A	IId	*Lactobacillus brevis* 925A	ND	Narrow	Heat resistant	ND	*B. coagulans*	(Wada et al., 2009)
Divergicin 750	IId	*Carnobacterium divergens* 750	3447.7	Broad	ND	ND	*C. perfringens*	(Holck et al., 1996)
Duranicin TW-49M	IId	*Enterococcus durans* Q 49	5227.8 [c]	Narrow	Moderate	2 - 10	*B. coagulans* *B. circulans* *B. subtilis* *G. stearothermphilus*	(Hu et al., 2008)
Enterocin 7A/7B	IId	*Enterococcus faecalis* 710C	7A 5200.8[c] 7B 5206.65[c]	Broad	ND	ND	*C. butyricum* *C. botulinum* *C. perfringens* *C. sporogenes*	(Liu et al., 2011)
Enterocin A	IIa	*Enterococcus faecium* CTC492; *Enterococcus faecium* T136	3829[c]	Broad	Heat stable	2 - 10	*B. coagulans* *B. subtilis* *C. sporogenes* *C. tyrobutyricum*	(Aymerich et al., 1996; Casaus et al., 1997; Hu et al., 2010; Hu et al., 2014)
Enterocin AS-48	IIc	*Enterococcus faecalis* A-48-32	7140[c]	Broad	Heat stable	ND	*Alicyclobacillus* spp. *B. cereus* *B. coagualns* *B. lichenformis* *B. subtilis* *C. perfringens* *C. sporogenes* *C. tetani*	(Lucas et al., 2006b; Burgos et al., 2014)

							G. stearothermophilus *Paenibacillus* spp.	
Entrocin B	IId	*Enterococcus faecium* T136	5463[c]	Broad	Heat stable	ND	*B. coagulans* *B. subtilis* *C. sporogenes* *C. tyrobutyricum*	(Casaus et al., 1997; Hu et al., 2010)
Enterocin EJ97	IId	*Enterococcus faecalis* EJ97	5340[c]	Broad	Heat stable	2 - 9.5	*B. ciriculans* *B. coagulans* *B. macrolides* *B. megaterium* *B. moroccanus* *B. subtilis* *G. stearothermophilus* *Paenibacillus macerans*	(Galvez et al., 1998;Garcia et al., 2004)
Enterocin L50	IIb	*Enterococcus faecium* L50	A: 5190[c] B: 5178[c]	Broad	Heat stable	2 - 11	*B. cereus* *B. subtilis*	(Cintas et al., 1995; Basanta et al., 2010)
Enterocin IT	IId	*Enterococcus faecium* IT62	6390[c]	Narrow	ND	ND	*B. subtilis*	(Izquierdo et al., 2008)
Enterocin MR10	IIb	*Enterococcus faecalis* MRR10-3	A: 5201.6 [b] B: 5207.5[b]	Broad	Heat stable	4.6-9	*B. cereus* *B. lichenformis*	(Martin-Platero et al., 2006)
Enterocin NKR-5-3B	IIc	*Enterococcus faecium* NKR-5-3	6316.42[c]	Broad	Heat stable	2-10	*B. circulans* *B. coagulans* *B. subtilis*	(Himeno et al., 2015)
Enterocin RM6	IId	*Enterococcus faecalis* OSY-RM6	7.145[c]	Broad	ND	ND	*B. cereus*	(Huang et al., 2013)
Enterocin P	IId	*Enterococcus faecium* P13	4.493[b]	Broad	Heat stable	2 - 11	*B. cereus*	(Cintas et al.,

							C. botulinum *C. perfringens* *Cl sporogenes* *C. tryobutyricum*	1997)
Enterocin SE-K4	IIa	*Enterococcus faecalis* K-4	5356.2[c]	Narrow	Heat stable	3 - 11	*B. subtilis* *C. beijerinckii*	(Eguchi et al., 2001)
Gassericin A	IIc	*Lactobacillus gasseri* LA 39	3800[a]	Broad	Heat stable	2 - 12	*B. cereus*	(Nakamura et al., 2013)
Gassericin KT7	ND	*Lactobacillus gasseri* KT7	ND	Broad	Heat stable	2.5 - 9	*B. cereus* *B. subtilis* *C. botulinum* *C. perfringens*	(Zhu et al., 2000)
Garvieacin Q	IId	*Lactococcus* garvieae BCC 43578	5339[c]	Broad	Heat stable	2 - 8	*B. coagulans*	(Tosukhowong et al., 2012)
Lacticin 3147	I	*Lactococcus lactis* subsp. *Lactis* DPC3147	ltnA1: 3305[c] ltnA2: 2847[c]	Broad	Heat stable	5 - 9	*B. cereus* *B. subtilis* *C. sporogenes* *C. tyrobutyricum*	(McAuliffe et al., 1998; Martinez-Cuesta et al., 2010; Iancu et al., 2012)
Lacticin 481	I	*Lactococcus lactis* subsp. *lactis* CNRZ 481	2901[c]	Narrow	Heat stable	ND	*C. tyrobutyricum*	(Piard et al., 1990;Piard et al., 1993)
Lacticin LC14	ND	*Lactococcus lactis* BMG6. 14	3333.7[c]	Broad	Heat stable	2 - 10	*B. cereus* *B. thuringiensis*	(Lasta et al., 2012)
Lacticin Q	IId	*Lactococcus lactis* QU 5	5926.5[c]	Broad	Heat stable	2 - 10	*B. cereus* *B. circulans* *B. coagulans*	(Fujita et al., 2007)

Lacticin Z	IId	*Lactococcus lactis* QU 14	5968.9[c]	Broad	Heat stable	2 - 10	*B. subtilis* *B. circulans* *B. coagulans*	(Iwatani et al., 2007)
Lactococcin BZ	ND	*Lactococcus lactis* subsp. *lactis*	5500[a]	Broad	Heat stable	2-7	*B. cereus* *B. subtilis*	(Sahingil et al., 2011)
Lactococcin R	ND	*Lactococcus cremoris* subsp. *cremoris* R	2500[a]	Broad	Heat stable	2 - 9	*B. cereus* *B. subtilis* *C. perfringens* *C. sporogenes*	(Yildirim and Johnson, 1998a)
Leucocin H	IIb	*Leuconostoc* sp. MF215B	ND	Broad	ND	ND	*B. cereus* *C. perfringens*	(Blom et al., 1999)
Leucocyclicin Q	IIc	*Leuconostoc mesenteroides* TK41401	6115.59[c]	Broad	ND	ND	*B. cereus* *B. coagulans* *B. subtilis*	(Masuda et al., 2011)
Lactocyclin Q	IIc	*Lactococcus* sp. QU 12	6062[c]	Broad	Heat stable	3 - 9	*B. cereus* *B. coagulans* *B. subtilis*	(Sawa et al., 2009;Masuda et al., 2011)
Mesentericin ST99	ND	*Leuconostoc mesenteroides* ST99	ND	Broad	Heat stable	2 - 12	*B. subtilis*	(Todorov and Dicks, 2004)
Macedocin	I	*Streptococcus macedonicus*	2795[c]	Broad	Heat stable	4 - 9	*B. cereus* *B. subtilis* *C. sporogenes* *C. Tyrobutyricum*	(Georgalaki et al., 2002)
Macedovicin	I	*Streptococcus macedonicus* ACA-DC 198	3428.8[c]	Broad	ND	ND	*B. lichenformis* *C. sporogenes* *C. tyrobutiricum*	(Georgalaki et al., 2013)
Nisin	I	*Lactococcus lactis* subsp. *lactis*	3353.53[c]	Broad	Heat stable	2-6	*A. acidoterrerstris* *B. anthracis* *B. amyloliquefaciens*	(Meghrous et al., 1999; Pirttijarvi et

							B. cereus *B. coagualns* *B. fliexus* *B. lichenformis* *B. pumilus* *B. sporothemodurans* *C. beigerinckii* *C. butyricum* *C. perfringens* *C. sporogenes* *C. tyrobutyricum* *Paenbacillus jamilae*	al., 2001; Wijnker et al., 2011; Hofstetter et al., 2013; Oshima et al., 2014; Aouadhi et al., 2015)
Nisin Z	I	*Lactococcus lactis* NIZO 22186	3330.93	Broad	Heat stable	2-6	*B. cereus* *B. pumilus* *B. subtilis* *C. butyricum* *C. perfringens* *C. sporogenes* *C. tyrobutyricum*	(Rollema et al., 1995; Meghrous et al., 1999; Noonpakdee et al., 2003; Park et al., 2003; Rilla et al., 2003; Rumjuankiat et al., 2015)
Nisin Q	I	*Lactococcus lactis* 61-14	3327.5	Broad	Heat stable	ND	*B. circulans* *B. coagulans* *B. subtilis*	(Zendo et al., 2003)
Pediocin A	IIa	*Pediococcus pentosaceus* FBB61	80000[a]	Broad	Heat sensitive	ND	*B. cereus* *C. sporogenes*	(Piva and Headon, 1994)

							C. tyrobutyricum	
Pediocin AcH / PA-1	IIa	*Pediococcus acidilactici* PAC 1.0	4624[c]	Broad	Heat stable	2-10	*B. cereus* *C. butyricum* *C. perfringens* *C. sporogenes* *C. tyrobutyricum*	(Marugg et al., 1992; Meghrous et al., 1999; Rodriguez et al., 2002; Nieto-Lozano et al., 2010)
Pediocin AcM	IIa	*Pediococcus acidilactici* M	4618[c]	Broad	Heat stable	1 - 12	*B. cereus* *B. coagulans* *C. perfringens*	(Elegado et al., 1997)
Pediocin L50	IId	*Piococcus acidilactici* L50	5250[c]	Broad	Heat stable	2-11	*B. cereus* *C. botulinum* *C. perfringens* *C. sporogenes* *C. tyrobutyricum*	(Cintas et al., 1995)
Pentocin TV35b	ND	*Lactobacillus pentosus* TV35b	3930	Broad	Heat stable	1-10	*C. sporogenes* *C. tyrobutyricum*	(Okkers et al., 1999)
Plantaricin 163	IId	*Lactobacillus plantarum* 163	3553.2	Broad	Heat stable	2-10	*B. cereus*	(Hu et al., 2013)
Plantaricin 423	IIa	*Lactobacillus plantarum* 423; *Lactobacillus plantarum* LMG P-26358	3932[c]	Narrow	Heat stable	1-10	*B. cereus* *C. sporogenes*	(van Reenen et al., 1998; Mills et al., 2011)
Plantaricin C	Ì	*Lactobacillus plantarum* LL441	2880.3[c]	Broad	Heat stable	<8	*B. subtilis* *C. sporogenes* *C. tyrobutyricum*	(Gonzalez et al., 1994)
Plantaricin KL-1Y	IId	*Lactobacillus plantarum* KL-1	3497.97[c]	Broad	Heat stable	2-12	*B. cereus* *B. coagulans*	(Rumjuankiat et al., 2015)

							B. subtilis	
Plantaricin LP84	ND	*Lactobacillus plantarum* NCIM 2084	1000 - 5000[a]	Broad	Heat stable	ND	*B. cereus* *B. lichenformis* *B. subtilis*	(Suma et al., 1998)
Plantaricin PZJ5	IId	*Lactobacillus plantarum* ZJ5	2572.9[c]	Broad	Heat stable	2-6	*B. subtilis*	(Song et al., 2014)
Plantaricin S	IIb	*Lactobacillus plantarum* LPC010	α 2904[c] β 2873[c]	Broad	Heat stable	3-7	*C. tyrobutyricum*	(Soliman et al., 2011)
Plantaricin ST31	ND	*Lactobacillus plantarum* ST31	2755[c]	Broad	Heat stable	3-8	*B. subtilis*	(Todorov et al., 1999)
Plantaricin TF711	ND	*Lactobacillus plantarum* TF711	2500[a]	Broad	Heat stable	1-9	*B. cereus* *C. sporogenes*	(Hernandez et al., 2005; Gonzalez and Zarate, 2015)
Plantaracin UG1	ND	*Lactobacillus plantarum* UG1	3000-10000[a]	Narrow	Heat stable	3.5-8	*B. cereus* *C. perfringens* *C. sporogenes*	(Enan et al., 1996)
Plantaricin ZJ008	ND	*Lactobacillus plantarum* ZJ008	1334.77	Broad	Heat stable	2-8	*B. subtilis*	(Zhu et al., 2014)
Salivaricin D	I	*Streptococcus salvarius* 5M6c	3467.55	Broad	Heat stable	ND	*B. subtils* *C. butyricum* *C. bifermentans*	(Birri et al., 2012)
Thermophilin 1277	I	*Streptococcus thermophilus* SBTI1277	3700[a]	Broad	Heat stable	3 - 10	*B. cereus* *C. Butylicum* *C. Sporogenes* *C. tyrobutyricum*	(Kabuki et al., 2007)
Themophilin 13	IIb	*Streptococcus thermophilus* SFi13	5776[c]	Broad	ND	ND	*B. cereus* *B. subtilis* *C. botulinum*	(Marciset et al., 1997)

							C. tyrobutyricum	
Thermophilin T	ND	*Streptococcus thermophiles* ACA-DC 0040	2500[a]	Narrow	Heat stable	1 - 12	*C. sporogenes* *C. tyrobutyricum*	(Aktypis et al., 1998)
VJ13B	IIa	*Pediococcus pentosaceus* VJ13	4000[a]	Broad	Moderate	2 - 8	*B. cereus* *B. subtilis* *C. perfringens* *C. sporogenes*	(Vidhyasagar and Jeevaratnam, 2013)
Weissellicin Y	IId	*Weisella hellenica* Q13	4925[c]	Broad	Heat stable	3 - 11	*B. cereus* *B. circulans* *B. subtilis* *B. coagulans*	(Masuda et al., 2012)
Weissellicin M	IId	*Weisella hellenica* Q13	4968[c]	Broad	Moderate	3 - 11	*B. cereus* *B. circulans* *B. coagulans* *B. subtilis*	(Masuda et al., 2012)

Table 2. Bacteriocins that display inhibitory action against bacterial spores.

Bacteriocin	**Sensitive spores**	**Reference**
Nisin	*A. acidoterrestris, B. amyloliquefaciens B. anthracis, B. lichenformis, B. sporothermodurans , B. subtilis, B. cereus, G. stearothermophilus, C. perfringens, C. sporogenes, C. botulinum, C. difficile, C. beijerinckii*	(Komitopoulou et al., 1999; Mansour et al., 1999; Wandling et al., 1999; Pol et al., 2000; Black et al., 2008;Gut et al., 2008; Udompijitkul et al., 2012; Hofstetter et al., 2013; Nerandzic and Donskey, 2013; Aouadhi et al., 2015)
Enterocin AS-48	*A. acidoterrestris, B. cereus, B. lichenformis, G. stearothermophilus*	(Abriouel et al., 2002; Lucas et al., 2006a)
Bificin C6165	*A. acidoterrestris*	(Pei et al., 2014)
Lacticin 3147	*C. tyrobutyricum*	(Martinez-Cuesta et al., 2010)
Plantaricin TF711	*C. sporogenes*	(Gonzalez and Zarate, 2015)
Thurincin H	*B. cereus*	(Wang et al., 2014)

Table 3. Bacteriocin mode of action against bacterial spores is heterogeneous.

Bacteriocin	**Spore**	**Effect on germination rate**	**Effect on dormant spores**	**Requires germination to be active**	**Other remarks**	
Nisin	*B. anthracis*	None	None	Yes	Lipid II becomes available for nisin to bind following germination, followed by pore formation in the outgrowing spore.	(Gut et al., 2008; Gut et al., 2011)
	B. sporothermodurans	Decreased rate	None	Yes		(Aouadhi et al., 2015)
	B. lichenformis	None	None	Yes		(Mansour et al., 1999)
	C. butyricum		None	Yes		(Ramseier, 1960)
	C. botulinum	Increases rate	None	Yes		(Mazzotta and Montville, 1999)
	C. difficile	None	None	Yes		(Nerandzic and Donskey, 2013)
	C. perfringens	None	None	Yes		(Udompijitkul et al., 2012)
Enterocin AS-48	*A. acidoterrestris*		Sporicidal	No		(Grande et al., 2005)
	B. cereus	None	None	Yes		(Abriouel et al., 2002)
	B. coagulans		None	Yes		(Lucas et al., 2006a)
	B. lichenformis		None	Yes		(Grande et al., 2006a)
	G. stearothermophilus		None	Yes		(Viedma et al., 2009)
Thurincin H	*B. cereus*	None	None	Yes		(Wang et al., 2014)

Table 4. Nisin addition to food reduces spore *D* values.

The adition of nisin to food at various levels sensitises spores to heat, thereby reducing the time (*D* value) required to achieve a 1 Log_{10} spore/ml reduction in spore numbers.

Spore	**Nisin Conc µg/ml**	**Food model**	**$D_{x°C}$**	***D* values (minutes)**		**Reference**
				Non-nisin treated	**Nisin treated**	
B. cereus	25	Milk	$D_{80°C}$	26.5	15.9	(Vessoni and Moraes, 2002)
			$D_{90°C}$	9.9	8.3	
			$D_{97.8°C}$	1.8	0.97	
B. cereus	50	Milk	$D_{97°C}$	7.0	4.8	(Wandling et al., 1999)
			$D_{100°C}$	2.7	2.2	
			$D_{103°C}$	1.5	0.85	
G. stearothermophilus	100	Milk	$D_{130°C}$	16	12.5	(Wandling et al., 1999)
A. acidoterrestris	1.25	Apple juice	$D_{80°C}$	41.2	23.8	(Komitopoulou et al., 1999)

Table 5. RSM models can be used to predict a treatment to achieve a specific spore reduction in food.

Spore	**Predicted reduction**	**Treatment**				**Food model employed**	**Reference**
		Pressure (MPa)	**Temperature (**°C)	**Time (mins)**	**Nisin (µg/ml)**		
B. sporothermodurans	5 Log_{10} spore/ml		95	12	3.125	Water	(Aouadhi et al., 2014)
B. sporothermodurans	5 Log_{10} spore/ml		100	13	3.35	Milk	
B. sporothermodurans	5 Log_{10} spore/ml		100	15	3.375	Chocolate milk	
B. sporothermodurans	5 Log_{10} spore/ml	472	53	5	5.025	Water	(Aouadhi et al., 2013)
C. perfringens	6 Log_{10} spore/ml	654	74	13.6	8.2	UHT milk	(Gao et al., 2011)

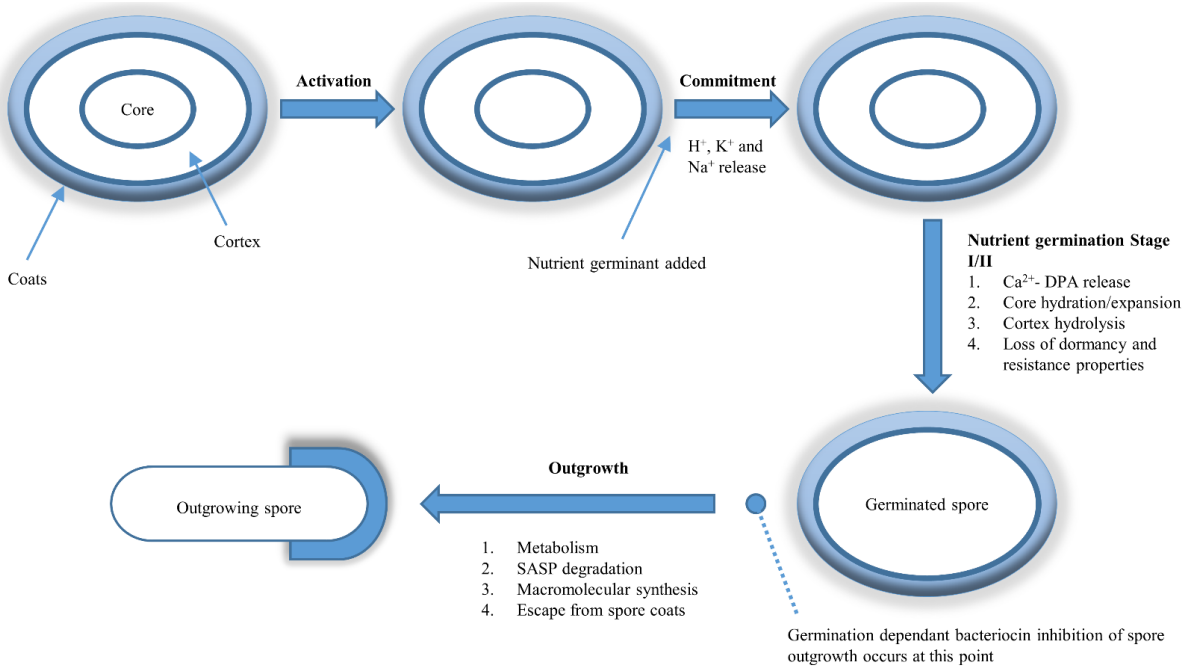

Figure 1. Germination dependent inhibition of spore outgrowth by bacteriocins.
Dormant spores may germinate after being activated by a variety of means; most commonly sub-lethal heat being used. Heat is believed to activate the dormant spores by making the germinant receptors (GR) more accessible to nutrient germinants. Once the GR-nutrient binding occurs, the spore is now committed to germination even if the germinant is removed. Stage 1 of germination consists of H^+, K^+ and Na^+ ion release followed by Ca^{2+}-DPA release. This release of Ca^{2+}-DPA triggers stage II of germination where the cortex is degraded, allowing the germ cell wall to expand and take up water. At the end of stage II the spore core is hydrated and has expanded along with the cortex. This rise in water content signals the end of stage II of germination and the beginning of the outgrowth phase. At this point bacteriocins that are not active against dormant spores become active, inhibit outgrowth and reduce viable counts from the germinated spore population. This figure is adapted from Setlow (2014a).

Chapter Ib

Bacteriocins: Antibiotics in the Age of the Microbiome

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Abstract

Antibiotics have revolutionised the treatment of infectious disease and improved the lives of billions of people worldwide over many decades. With the rise in antimicrobial resistance (AMR) and corresponding lack of antibiotic development, we find ourselves in dire need of alternative treatments. Bacteriocins are a class of bacterially produced, ribosomally synthesised, antimicrobial peptides that may be narrow or broad in their spectra of activity. Animal models have demonstrated the safety and efficacy of bacteriocins in treating a broad range of infections; however, one of the principal drawbacks has been their relatively narrow spectra when compared with small-molecule antibiotics. In an era where we are beginning to appreciate the role of the microbiota in human and animal health, the fact that bacteriocins cause much less collateral damage to the host microbiome makes them a highly desirable therapeutic. This review makes a case for the implementation of bacteriocins as therapeutic antimicrobials, either alone or in combination with existing antibiotics to alleviate the AMR crisis and to lessen the impact of antibiotics on the host microbiome.

Introduction

Antimicrobial resistance (AMR) has been recognised as one of the major threats to public health in the 21st century. In a report commissioned by the UK government in 2014, it was estimated that AMR could be responsible for 10 million deaths worldwide by 2050, with a global financial cost of $100 trillion (O'Neill, 2014). Meanwhile, the Centers for Disease Control and Prevention (CDC) estimates the annual cost of AMR in the USA to be about $20 billion in direct healthcare costs and $35 billion in additional costs to society due to lost productivity (Prevention, 2013). Apart from the human and financial costs associated with AMR, there are also ethical considerations that need to be addressed surrounding how we, as a society, respond and deal with the AMR crisis (Littmann and Viens, 2015). There are multiple reasons for the present AMR crisis, but significant factors include the incorrect/indiscriminate administration and use of antibiotics and a dry antibiotic development pipeline (Arias and Murray 2009; Riley et al., 2013). The CDC also recently estimated that, in the USA, ~50% of antibiotics are incorrectly prescribed. Moreover, the use of antibiotics in agriculture has continued, despite undeniable evidence that this practice adds to the AMR crisis. Resistance to a key ‘ last-resort’ antibiotic, colistin has been observed in the USA, Europe and Asia (Liu et al., 2016; Meinersmann et al., 2016; Ye et al., 2016). We have also seen the rapid spread of resistance to another ‘ last resort’ class of antibiotics, the carbapenems (Potter et al., 2016). With the emergence of these new resistant strains and the emergence of pan-resistant bacteria, it is safe to say we have truly arrived in the much-predicted post-antibiotic era (Fair and Tor, 2014).

It is important that we acknowledge that broad-spectrum antibiotic therapy has revolutionised the treatment of infectious diseases within the last century, but we must also admit to unintended consequences of antibiotic use, such as potentially negative effects on the host microbiome and their potential toxicity (Cotter et al., 2013; Riley et al., 2013). Although the field of microbiome research is in its infancy relative to that of antibiotic therapy, evidence strongly suggests that the composition of the microbiome can be an indicator of health and is likely to be involved in many aspects of human health and disease (Guinane and Cotter, 2013). Strides in DNA sequencing technology and bioinformatics have increased our understanding of the role of the microbiome in a variety of disease states. Indeed, the administration of antibiotics in early life and the subsequent disruption of the microbiota may contribute to the risk of obesity in later life (Petschow et al., 2013; Cox and Blaser, 2015). Furthermore, when subjected to broad-spectrum antibiotic therapy, non-target commensal microbes may evolve and/or acquire resistance mechanisms to evade the effects of the antibiotic, thereby contributing to the antibiotic resistance crisis.

Bacteriocins represent a class of powerful antimicrobial peptides that may provide at least part of a solution to the AMR crisis. We aim to demonstrate their efficacy in the treatment of infectious disease and their reduced impact on the host microbiome by comparison with broad-spectrum antibiotic therapy.

Bacteriocins: potent antimicrobial peptides

Many excellent reviews have been written about bacteriocins (Rea et al., 2011a; Cotter et al., 2013; Alvarez-Sieiro et al., 2016), but in brief they are a diverse group of peptides that may be classified into three distinct groups: class I (modified), class II (unmodified or cyclic) and class III (>10 kDa peptides). Apart from their potent antimicrobial activity (with minimum inhibitory concentrations [MICs] often in the nanomolar range), they have also been shown to have antiviral (Al Kassaa et al., 2014), anticancer (Kamarajan et al., 2015) and immunomodulatory properties (de Pablo et al., 1999). Bacteriocins typically have a narrow spectrum of activity, but broad-spectrum peptides are also present in this class of antimicrobials (e.g. nisin and lacticin 3147 inhibit a wide range of Gram-positive bacteria). As a result, these peptides may be suitable for treating infections of unknown aetiology, using broad-spectrum bacteriocins, or may allow more precise targeting of known infectious agents using highly active narrow-spectrum bacteriocins. Bacteriocins are gene-encoded, which makes them amenable to genetic alterations to improve functional characteristics. Furthermore, their toxicity is low and they may be administered as either purified peptide or produced in situ by bacteriocin-producing probiotic bacteria (Cotter et al., 2013). Bacteriocins are also known to interact with a variety of receptors, which are different from those targeted by antibiotics, making cross-resistance less likely (Cotter, 2014). Although a more targeted approach may still ultimately lead to resistance development in the infectious agent, it does reduce the likelihood of resistance development in commensal populations outside of the target range of the bacteriocin. Resistance mechanisms involving the class II receptors, the mannose phosphotransferase system, have been identified (Kjos et

al., 2011) along with a variety of resistance mechanisms to the class I lantibiotics (Draper et al., 2015).

The microbiota perspective

The term ‘ superorganism’ or ‘ holobiont’ has commonly been applied to describe the relationship that exists between humans and their commensal microbes and viruses (Goodacre, 2007). Understanding the role of the microbiota in health and protecting its diversity during the treatment of infectious disease is a key element of why bacteriocins may be suitable as alternatives to antibiotics.

The two-peptide sactibiotic bacteriocin Thuricin CD is a narrow-spectrum bacteriocin. Thuricin CD is highly active against one of the main causative agents of antibiotic-associated diarrhea (AAD), *Clostridium difficile*, which is responsible for 20– 30% of AAD cases (Rea et al., 2013). Briefly, AAD is caused by a disruption of the microbiota (often referred to as dysbiosis) following broad-spectrum antibiotic treatment and notably has a recurrence rate of 15– 60% (McFarland, 2008). Thuricin CD was shown to exhibit comparable activity to both vancomycin and metronidazole [two antibiotics used for the treatment of AAD which has progressed to *C. difficile*-associated disease (CDAD)]. Importantly, it showed almost no effect on microbial diversity when compared with both metronidazole and vancomycin in a distal colon model (Rea et al., 2011b). The modified R-Type bacteriocin Av-CD291.2 has also been shown to prophylactically inhibit colonisation of *C. difficile* in a mouse model without perturbing the microbiota (Gebhart et al., 2015). There are other broad-spectrum bacteriocins which are attractive therapeutic agents by virtue of their activity against *C. difficile*, but while the broad-spectrum lantibiotic lacticin 3147

is effective at killing *C. difficile*, it has a significant impact on the resident microbiome populations such as *Bifidobacterium*, *Lactobacillus* and *Enterococcus* species (Rea et al., 2007). It has also been shown that a commercially available product containing the lantibiotic nisin, Nisaplin®, can eliminate a *C. difficile* infection when added at a concentration of twenty times the MIC in a simulated human colon model. However, a significant decrease in the total microbiota count was observed, with Gram-positives being adversely affected (Le Lay et al., 2015).

Notably, in recent years, the emergence of vancomycin-resistant Enterococci (VRE) has become a great concern and therefore raises the issues surrounding the efficacy of treating CDAD with vancomycin if it presents a risk to the general population and the spread of antibiotic resistance. In this light, the treatment of CDAD with bacteriocins could be a valuable alternative to vancomycin. When VRE development has taken place, it has been shown that mice colonised with VRE can be decolonised through the use of an *Enterococcus* probiotic containing a conjugation defective plasmid which produces a bacteriocin named Bac-21 (Kommineni et al., 2015).

A defensin-like bacteriocin, bactofencin A, displays *in vitro* activity against *Listeria monocytogenes* and *Staphylococcus aureus* (O'Shea et al., 2009; O'Shea et al., 2013). Although one might expect this medium to broad-spectrum antimicrobial peptide to cause drastic changes in the host microbiome, this was in fact not the case. It was observed that the bactofencin peptide only subtly modulated an ex vivo host microbiome (distal colon model) when introduced as

a bacteriocin-producing probiotic or purified peptide (Guinane et al., 2016). While the purified peptide resulted in higher levels of beneficial microbes such as *Bifidobacterium*, it was also associated with lower levels of *Clostridium*, which has been linked to obesity and gut pathogenesis (Woting et al., 2014). Interestingly, although bactofencin does not show inhibitory activity *in vitro* against strains from the genera *Clostridium*, *Fusobacterium* and *Bacteroides*, the reduction in these populations in the bactofencin-treated faecal samples indicates that the consequence of bactofencin altering the overall microbiota structure affects, directly or indirectly, these normally insensitive populations when in the gut environment (Guinane et al., 2016).

It has also been shown, using bacteriocin-producing probiotic strains and their isogenic mutants, that the production of bacteriocins can aid the colonisation of a murine host (Umu et al., 2016). Sequencing data revealed that although bacteriocin production by the probiotics did not affect bacterial diversity at the phylum level, broad-spectrum bacteriocins (enterocins and garvicin ML) had a more significant impact on the genus/family diversity of the host microbiome than narrow-spectrum bacteriocins (sakacin A, plantaricins and pediocin PA-1).

Bacteriocins in animal models

Bacteriocins have been shown to be effective in the treatment of a variety of bacterial infections using two delivery methods, either as purified peptides (Table 1) or when delivered in situ by probiotics (Hegarty et al., 2016). It has been hypothesised that there are three mechanisms by which bacteriocins mediate their producers' probiotic properties (Dobson et al., 2012): (i)

competitive inhibition: bacteriocins may support colonisation of the host through competitive inhibition of the autologous microbiota; (ii) pathogen inhibition: bacteriocins may interact directly with a pathogenic target; or (iii) immunomodulation: bacteriocins may act as signaling peptides, recruiting other bacteria or recruiting immune cells to the site of infection to aid elimination of the pathogen (Figure 1).

Preventing infection

Oral disease is a widely recognized as a major public health issue worldwide, with dental caries in industrial countries affecting 60– 90% of school children and adults, making it the most prevalent human disease (Petersen, 2004; Simón-Soro and Mira, 2015). The concept of oral replacement therapy is an interesting example of prophylactic probiotic therapy, which may be used to treat dental caries and oral disease. The mutacin 1140 producing *Streptococcus mutans* BCS3-L1 may be suitable for replacement therapy as it has reduced cariogenic potential because it does not produce lactic acid, mediated through the removal of its entire lactic acid dehydrogenase operon (Hillman et al., 2007). Another interesting probiotic that has shown promise in the limitation of dental caries, plaque accumulation and acidification is *Streptococcus salivarius* M18. This strain has three plasmid and one chromosomally encoded bacteriocins, which is perhaps why it can colonise the oral cavity so effectively. It also produces two enzymes, urease and dextranase, which reduce the acidity of saliva and counteract plaque formation (Burton et al., 2013b). In a clinical trial, both the safety and efficacy of this strain's probiotic potential were demonstrated, and it was shown to significantly reduce plaque formation in subjects who received the

probiotic, over those who received the placebo (Burton et al., 2013a). Furthermore, the treatment of children who have a high risk of dental caries development, with an oral formulation of the *Streptococcus salivarius* M18 probiotic (Carioblis®), was shown to reduce the likelihood of new dental caries development (Di Pierro et al., 2015).

It has been demonstrated that dosing mice orally with the bacteriocin producer *Lactobacillus salivarius* UCC118 3 days prior to infection with *L. monocytogenes* resulted in a significant reduction in subsequent infection *by L. monocytogenes* (Corr et al., 2007). Nisin Z and pediocin AcH have also been shown to reduce and prevent the colonisation of a mouse model with VRE, where the bacteriocinogenic probiotic was administered 8 days prior to infection (Millette et al., 2008). It has also been demonstrated using a porcine model that *Salmonella enterica* serovar Typhimurium shedding is reduced and disease symptoms of infection are alleviated when a mixture of five probiotic strains was administered 6 days before infection (Casey et al., 2007). One of the probiotics, *L. salivarius*, produces salivaricin P, which can kill the other four strains in the probiotic mixture. Interestingly, this bacteriocinogenic strain dominated in the ileum (the primary attachment site of the infecting *Salmonella*), whereas it was only detected as a minor component in the faeces of the same animals. This suggests that bacteriocin production may play a role where colonisation can occur along the gastrointestinal tract (Walsh et al., 2008). The concept of using prophylactic probiotics to competitively colonise a pathogen' s niche could be an effective strategy in agriculture to reduce antibiotic usage. If, as expected, regulations limiting the use of antibiotics in agriculture come into force,

probiotics may be an invaluable alternative.

Acute otitis media (AOM) is a type of inflammatory disease of the middle ear, characterised typically by rapid inflammation, potential tympanic membrane perforation, along with fullness and erythema. It has been reported that the levels of normal α -haemolytic *Streptococcus* colonising the nasopharynx of otitis-prone children are much lower than those in healthy individuals and that recolonisation can significantly reduce the episodes of AOM (Roos et al., 2001;Marchisio et al., 2003). It has been demonstrated that treating otitis-prone children with a history of AOM with a nasal spray containing safe *Streptococcus salivarius* 24SMB (a strain which produces a bacteriocin-like substance) reduces the incidence rates of AOM compared with those of the placebo-treated group (Santagati et al., 2012).

Treating infection

Helicobacter pylori infection and colonisation results in a variety of disease states and may even lead to the development of gastric carcinoma. More recently, the prevalence of antibiotic-resistant *H. pylori* has been increasing, creating a need for a new therapeutic agent (Thung et al., 2016). It has been shown in mice that eradication of *H. pylori* was achieved using a bacteriocinogenic probiotic treatment of *Pediococcus acidilactici* BA28 (Kaur et al., 2014). Using a mixture of cranberry juice and the bacteriocin-producing probiotic culture *Lactobacillus johnsonii* str. La1 supernatant, the carriage of *H. pylori* was also reduced in children after 3 weeks of treatment (Gotteland et al., 2008).

One limitation to the use of bacteriocinogenic probiotics as therapeutics is their ability to survive gastrointestinal conditions and deliver bacteriocins to the site of infection. It has been shown that *P. acidilactici* UL5 and *Lactococcus lactis* ATCC 11454 can produce the bacteriocins pediocin PA-1 and nisin, respectively, *in situ* under simulated upper gastric conditions (Fernandez et al., 2014). Interestingly, the *in vitro* activity of a bacteriocin does not always correspond to the *in vivo* activity, where the bacteriocin is sometimes more or less active in an animal model, as is the case with mersacidin, which is more active *in vivo* than in *vitro* (Chatterjee et al., 1992).

Immunomodulation

Probiotic mediated immunomodulation has been described in various reviews (Erickson and Hubbard, 2000; Hardy et al., 2013), however, far less is known about bacteriocin-mediated immunomodulation (Figure 1). Walsh et al., (2008) described reduced CD25 induction on T-cells and monocytes, increased in CD4+ and CD8+ T cells, and increased IL-8 mRNA expression upon the administration of a five-strain bacteriocin producing probiotic. Furthermore it was seen through the use of gene-trait matching approach by Meijerink et al. (2010) that 6 bacteriocin genes were responsible for increased IL-10, IL-12p70 and TNF-alpha production by monocyte derived dendritic cells. IL-10, Gro-α and Mcp-1 induction was shown to be increased by nisin Z, pep5 and gramicidin in peripheral mononuclear blood cells. Mechanistic studies revealed that these bacteriocins act similarly to host immune defense peptides, initiating various signal transduction pathways (Kindrachuk et al., 2013).

Bacteriocins against Gram-negatives

Comparatively speaking, Gram-negative bacteria are relatively insensitive to bacteriocins compared with their Gram-positive counterparts, largely owing to their outer membrane, which acts as a physical barrier. Until recently, the treatment of Gram-negative infections with bacteriocins has not been favoured due to the efficacy of conventional antibiotics in the treatment of these infections. The rise of antibiotic-resistant Gram-negative bacteria to the last line of antibiotics (Liu et al., 2016) means that the treatment of these infections using bacteriocins can no longer be ignored.

Widespread use requires a solution to the relative insensitivity of Gram-negative microorganisms. One possibility is to use bacteriocins in combination with other antimicrobial agents, including conventional antibiotics. Although conventional antibiotics will have an impact on the host microbiota (as previously discussed), certain bacteriocin/antibiotic combinations can be synergistic (Naghmouchi et al., 2012; Draper et al., 2013; Naghmouchi et al., 2013; Rishi et al., 2014) and therefore lead to a reduced dose of both antimicrobial agents needed to treat an infection, thereby lowering the potential effect on the host microbiome and the cytotoxic effects on the host, and may potentially reduce the development of resistance. Success of antibiotics is also hindered by Gram-negative bacteria residing within biofilms, where they are highly resistant to antibiotic treatments. Bacteriocin/antibiotic combinations have shown great promise in overcoming biofilm-mediated resistance for important Gram-negative pathogens such as *Pseudomonas aeruginosa* (Field et al., 2016) and *Escherichia coli* (Al Atya et al., 2016).

Although this review mainly focuses on Gram-positive bacteriocins, it is important also to identify Gram-negative bacteriocins, which may have potential therapeutic significance. Microcins are ribosomally synthesized peptides commonly produced by Gram-negative bacteria, which are active against Gram-negative strains, and are an interesting alternative to Gram-positive bacteriocins. They have been shown to display potent antimicrobial activity *in vitro* (Patzer et al., 2003; Nolan and Walsh, 2008) and more recently also *in vivo* (Sassone-Corsi et al., 2016). It has been demonstrated that the microcin producer *E. coli* Nissle 1917 (EcN) can prevent colonisation of competing Enterobacteriaceae in the gut, while still having a minimal impact on the diversity of the gut microbiota. However, EcN microcins exhibit their mechanism of action by targeting specific siderophore receptors on other Enterobacteriaceae, which are only displayed during iron starvation, making their spectrum of activity quite narrow. Additionally to its prophylactic applications, EcN has also been demonstrated to reduce inflammation and weight loss associated with *Salmonella* infections. Another microcin produced by *E. coli* G3/10, microcin S, has been shown to inhibit other *E. coli* strains and, furthermore, can prevent the adherence of Enteropathogenic *E. coli* to intestinal epithelial cells (Zschüttig et al., 2012).

Overcoming the limitations / outlook

In previous decades, significant emphasis was placed on functional characteristics of bacteriocins, such as spectrum of activity, pH and temperature stability, which were essential for the use of bacteriocins in food applications. For their use as therapeutics, additional characteristics such as proteolytic resistance, stability and solubility of bacteriocins will also be important.

With advancements in the field of bioengineering, many intrinsic limitations have been overcome, and it has been shown using the prototypic lantibiotic nisin that bioengineering strategies can improve functional qualities such as antimicrobial activity (Field et al., 2008; Field et al., 2012b; Healy et al., 2013; Molloy et al., 2013), solubility (Rollema et al., 1995; Yuan et al., 2004) diffusion properties (Rouse et al., 2012) and effectiveness against Gram-negative bacteria (Field et al., 2012a). Indeed, similar bioengineering strategies could be applied to other bacteriocins once suitable expression systems have been developed. Although the sensitivity of bacteriocins to proteolytic cleavage was previously regarded as a desirable trait when using these peptides as food preservatives, it does represent a major concern with regard to their administration and widespread use, both orally and intravenously. Bioengineering strategies could be once again used to manipulate peptide residues, so they are no longer recognisable by host proteases and therefore are not proteolytically cleaved, thereby improving peptide functional qualities (Field et al., 2015a). Notably, the therapeutic application of the prototypic bacteriocin nisin has been in part hampered by its sensitivity to host proteases (Field et al., 2015b). Other approaches include prospecting for bacteriocins that display innate resistance to

proteases, as was achieved with pseudomycoicidin (Basi-Chipalu et al., 2015), which is naturally resistant to trypsin due to the presence of a thioether ring structure. The field of bioinformatics and the use of such programmes as BAGEL 3.0 (van Heel et al., 2013) and antiSMASH (Blin et al., 2016) could be a fundamental aspect of this prospecting, as these bacteriocin amino acid prediction tools from genome sequences may also allow researchers to identify protease-resistant peptides before investing large amounts of time and effort in characterising such bacteriocins. Finally, understanding bacteriocin pharmacodynamics and pharmacokinetics is also essential to their safe implementation as therapeutics, which has been under-investigated in comparison with other aspects of bacteriocin research. If bacteriocins are indeed to become an alternative to conventional antibiotics, a greater emphasis must be placed on research surrounding these host–drug interactions, such as was achieved with MU1140 (Ghobrial et al., 2010).

Addressing these limitations of bacteriocin research to date could provide a turning point for the flagging interest of the pharmaceutical industry and make bacteriocins an attractive therapeutic alternative to current antibiotics (Fair and Tor, 2014). Although there is considerable evidence that narrow-spectrum bacteriocins have a minimal effect on the host microbiome by comparison with current broad-spectrum antibiotics, it should also be recognised that more work in this regard is needed to strengthen the argument for the use of bacteriocins as antibiotics, along with overcoming the previously outlined limitations. Ultimately, we believe, given the safe history of use of bacteriocins in food and the large body of literature surrounding this field, that they are useful candidates

for antimicrobial therapeutics as the AMR crisis continues to worsen.

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Table 1. Bacterial infections in animal models successfully treated using purified bacteriocins.

Peptide	**Strain inhibited**	**Model**	**Purity**	**Reference**
Nisin F	*Staphylococcus aureus*	Immunosuppressed Wistar rats	Semi-pure	(De Kwaadsteniet et al., 2009)
	Staphylococcus aureus	Brushite cement in BALB/c mice	Semi-pure	(van Steden et al., 2012)
Lacticin NK34	*Staphylococcus aureus / Staphylococcus simulans*	ICR mice	Semi-pure	(Kim et al., 2010)
Nisin V	*L. monocytogenes*	BALB/c mice	Pure	(Campion et al., 2013)
Divercin V41	*L. monocytogenes*	BALB/c mice	Pure	(Rihovka et al., 2010)
Mutacin B-Ny266	*Staphylococcus aureus*	Unknown	Pure	(Mota-Meira et al., 2005)
Mersacidin	Methicillin-resistant *Staphylococcus aureus* (MRSA)	BALB/c mice	Pure	(Kruszewska et al., 2004)
Epidermicin NI01	Methicillin-resistant *Staphylococcus aureus* (MRSA)	Cotton rats	Pure	(Halliwell et al., 2017)

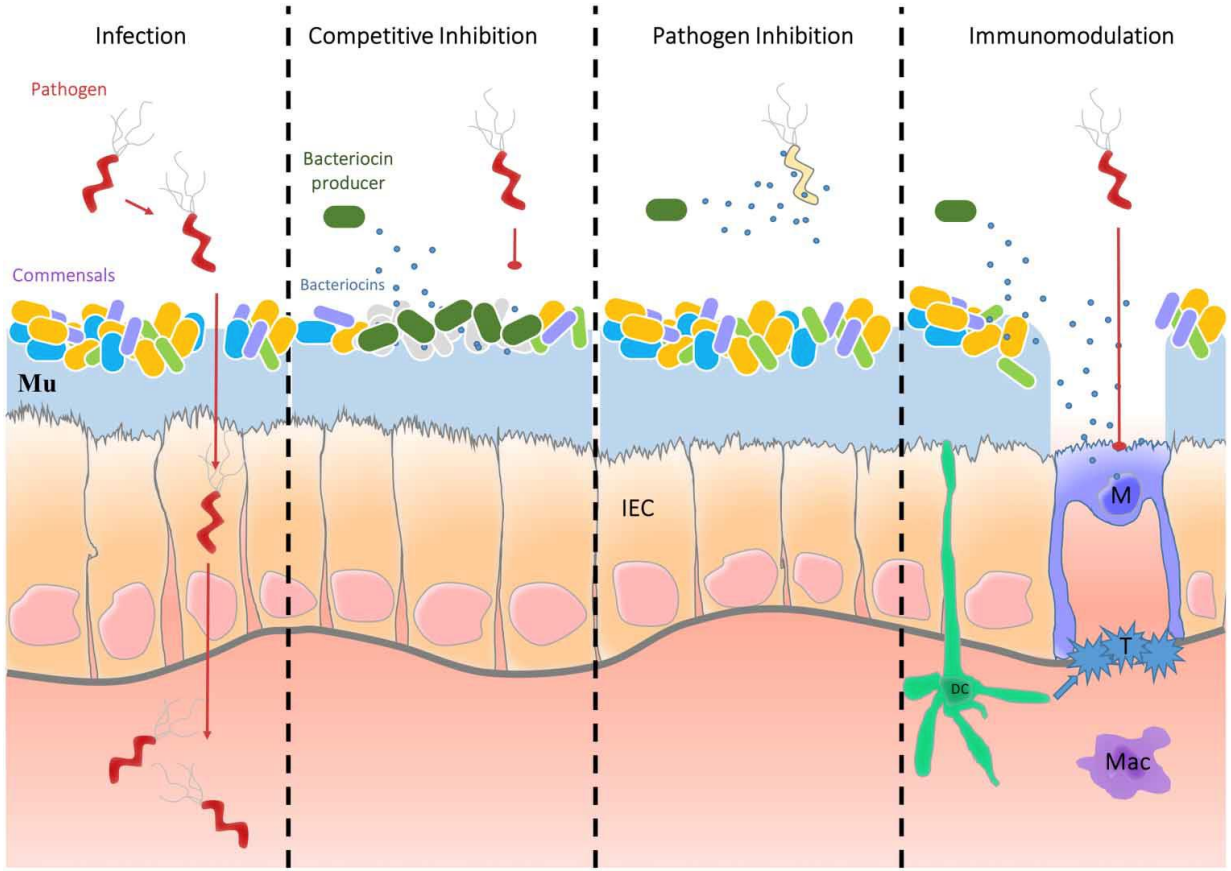

Figure 1. Bacteriocinogenic probiotics can be utilised either prophylactically or therapeutically to treat an infection. M, M cell; Mac, macrophage; Mu, mucous; T, T cell; IEC, intestinal epithelial cell; DC, dendritic cell.

Chapter II

Discovery and Partial Characterization of a Potentially Novel Antimicrobial Produced by *Geobacillus stearothermophilus* DSM 458

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Abstract

Bacteriocins are ribosomally-synthesized antimicrobial peptides produced by bacteria, which can have either broad or narrow spectrum of activity and are often active at nanomolar concentrations. We report the discovery of thermocin 458, an antimicrobial produced by *Geobacillus stearothermophilus* DSM 458 with a narrow spectrum of activity against closely related *Geobacilli*. In order to elucidate the genetic basis of the antimicrobal produced the genome of *Geobacillus stearothermophilus* DSM 458 was genome sequenced using PacBio sequencing. This resulted in a fully circular genome of 3,466,824 bp with a G+C content of 52.11% with 3361 protein coding sequences. *In silico* screening highlighted the presence of a 6.9 kDa highly hydrophobic circular bacteriocin within the genome. Thermocin 458 is heat stable and active across a broad range of pH values. The molecular mass of thermocin 458 is between 11-13 kDa as determined by native sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Digestion with proteases (proteinase K, trypsin, ficin and papain) results in decreased antimicrobial activity.

Introduction

One of the most useful defense mechanisms employed by bacteria is the ability to produce bacteriocins. Bacteriocins are small, ribsomally-synthesised peptides which may be broad or narrow spectrum in activity and are considered to be highly abundant amongst bacteria. These peptides are classified into three broad groups: Class I (post-translationally modified), Class II (unmodified) or Class III (>10 kDa) (Cotter et al., 2013). In previous decades, bacteriocin prospecting has been heavily focused on the lactic acid bacteria (LAB) because of their Generally Regarded As Safe (GRAS) status and proven safety record in food preservation. In recent years, there has been a move towards expanding screening strategies to include other bacterial genera such as *Geobacillus* (Abriouel et al., 2011). These newer initiatives have been largely driven by the need for new antibiotics to address the antimicrobial resistance crisis.

Geobacillus are facultative thermophilic bacteria which are virtually ubiquitous in the environment and are believed to play a minor role in the global carbon cycle (Zeigler, 2014). These bacteria are also biotechnologically important, as they are a source of thermostable enzymes and have highly desirable degradative properties, including the ability to break down complex sugars (e.g. lignocellulose) (Cripps et al., 2009; De Maayer et al., 2014; Studholme, 2015). Recent studies have identified a number of bacteriocins produced by this genus, (Başbülbül Özdemir and Biyik, 2012; Garg et al., 2012; Özdemir and Biyik, 2012) which have highlighted its potential as a source of novel antimicrobials. In parallel, the accelerated development of the field of bioinformatics has reduced both the time and cost required to discover

and characterize bacteriocin gene clusters through mining tools such as BAGEL 3.0 (van Heel et al., 2013) and AntiSMASH (Weber et al., 2015).

Here, we classify and characterize the potentially novel antimicrobial thermocin 458 which is produced by *Geobacillus stearothermophilus* DSM 458 previously isolated from a sugar beet factory. Furthermore, we also sequence the genome of *G. stearothermophilus* DSM 458 in an effort to identify the genetic determinants of thermocin 458.

Materials and methods

Bacterial strains and growth conditions

All strains used in this study are listed in (Table 1) All *Geobacillus* species were grown in Brain Heart Infusion (BHI) (Oxoid) at 55°C shaking at 180 RPM. *Enterococcus*, *Bacillus*, *Listeria* and *Streptococcus* were grown in BHI (Oxoid) at 37°C. *Lysinibacillus*, *Alicycobacillus*, *Paenibacillus* were grown in BHI (Oxoid) at 30°C. *Lactococcus* species were grown at 30°C in M17 (Oxoid) supplimented with 0.5% glucose.

Antimicrobial detection assays

Deferred antagonism assays were carried out by spotting 10µl of an overnight culture of the antimicrobial producer onto the surface of BHI agar plate and incubating for 18hrs at at 55°C. The resultant colonies were then subjected to UV exposure for 40 minutes and subsequently overlaid with the desired indicator in its relevant pre cooled molten agar (0.75% w/v agar). Any zones of inhibition were measured using calipers, where the producer colony diameter was subtracted from the total zone diameter. This diameter was then used to calculate the area of inhibition zone using the formula: $a = \pi r^2$.

Well diffusion assays were performed by seeding 50mls of cooled nutrient agar (1.5%) with a 0.1% v/v inoculum of an overnight culture of the relevant indicator strain and pouring into a sterile petri dish. After the agar was set, wells were bored using a Pasteur pipette (Diameter = 5.66) and 20 µl of antimicrobial containing liquid was pipetted into each well, after which the plates were incubated at the

relevant temperature for 18 hours. The zone of inhibition sizes were measured and the units of activity calculated where 1 unit = 1mm^2 as previously described.

Whole genome sequencing

The bacteriocin producer *G. stearothermophilus* DSM 458 was grown to mid log phase and the pellet harvested. 600mg of this pellet was snap frozen by placing it into ethanol at -80°C. Chromosomal DNA was isolated from this pellet by commercial sequence providers GATC Biotech Ltd. (Konstanz, Germany) and sequenced on a Pacific Bioscience PacBio RS II (GATC Biotech Ltd., Konstanz, Germany). *De novo* assembly of the genome was performed using the SMRTPortal analysis platform (version 2.3.1), using the RS_HGAP_Assembly.2 protocol.

General feature predictions

Following genome assembly, Open Reading Frame (ORF) prediction was performed using the Prodigal v2.5 prediction software (Hyatt et al., 2010). These ORFs were confirmed using BLASTX v2.2.26 (Altschul et al., 1990) alignments and annotated automatically using BLASTP v2.2.26 (Altschul et al., 1990) analysis against the non-redundant protein databases curated by the National Centre for Biotechnology Information (NCBI) (Pruitt et al., 2007). Following automatic annotation, manual curation of ORFs was completed using Artemis v16 genome browser and annotation tool (Rutherford et al., 2000). Subsequently the software tool was utilized for the combination and inspection of ORF results, adjustment of start codons where necessary and to aid in the identification of pseudogenes. Transfer RNA (tRNA) and ribosomal RNA (rRNA) genes were predicted using tRNAscan-SE v1.23 (Lowe and Eddy, 1997) and RNAmmer v1.2 (Lagesen et al., 2007) respectively. The RNA

encoded genes predicted were then manually added to the genome using Artemis v16. Phast was used to predict putative phages encoded within the genome (Zhou et al., 2011) and CRISPRfinder was used to predict clustered regularly interspaced short palindromic repeat (CRISPR)-associated repeat regions (Grissa et al., 2007).

Genome visualisation tool and bacteriocin biosynthetic gene(s) identification

The genome of *Geobacillus stearothermophilus* DSM 458 was visualised using DNA plotter (Carver et al., 2009). In order to identify putative bacteriocin operons within the genome the bacteriocin mining tool BAGEL 3.0 (van Heel et al., 2013) and antiSMASH (Weber et al., 2015) were utilized. Additionally, all the proteins encoded within the genome were aligned against the prokaryotic antimicrobial peptide database using the Protein Basic Local Alignment Search Tool (BLASTP) in order to further identify antimicrobial gene candidates.

Purification of thermocin 458

The bacteriocin producing strain *G. stearothermophilus* DSM 458 was grown overnight in BHI at 55°C. This overnight culture was then inoculated (1% inoculum) into 2L of BHI broth. Following incubation of the culture at 55°C RPM 180 for 24 hours the cells were harvested by centrifugation (6000 RPM at 4°C for 20 minutes) from the cell free supernatant (CFS). Both the CFS and cells were retained for further purification.

In order to detach any potential cell-attached antimicrobial, the cells were stirred in 70% propan-2-ol (IPA) containing 0.1% Trifluoroacetic acid (TFA) for 4 hours at 4°C and subsequently centrifuged (6000 RPM for 20 minutes). After centrifugation

the cellular debris were discarded and the volume of the aqueous / solvent mixture was reduced by 70% using rotary evaporation (Buchi) at 42°C and a pressure of 85mbar, in order to remove the propan-2-ol. This was designated solution 1.

The bacteriocin contained within the supernatant was precipitated using an ammonium sulfate precipitation method. This was completed by slowly adding ammonium sulfate to the CFS until it reached a total concentration of 90% w/v and stirred at room temperature for 4 hours. The precipitated protein was then pelleted at 7000 RPM for 30 minutes and the supernatant discarded. The precipitated pellet was then dissolved in 200mls of 150mM Tris, 150mM NaCl, pH 7.5 and retained for further desalting. This was designated solution 2.

Solution 1 and 2 were now combined and subjected to solid phase extraction (SPE) using a 10g (60ml) C-18E-SPE Giga-Tube (Phenomenex, Cheshire, UK). Initially the SPE cartridge was activated using 60mls of methanol, followed by 60mls of H_20. The aqueous solution containing the bacteriocin was then loaded onto the cartridge and washed with 30% Ethanol. The bacteriocin was eluted from the sorbent using a 60ml solution of 70% IPA and 0.1% TFA. This antimicrobial containing crude preparation was now used for further protein characterisation.

Reverse-phase high performance liquid chromatography

Liquid chromatography was performed using a Shimazdu Prominence UHPLC system (Shimazdu Biotech, Manchester, Uk) with a Proteo Widepore C18 column (250 X 4.6 mm, 5µm, 300Å, Phenomenex). The mobile phase A (water, 0.1% TFA) and B (acetonitrile 0.1%TFA) were used and a gradient of 0% to 70% of solvent B

was performed over 85 minutes. A total injection volume of 400µl and a 1.2ml/min flow rate was used. The fractions were assayed for activity and those found to be active were pooled and run on a lesser gradient of 40 – 70% solvent B. Active fractions were subsequently brought forward for mass determination and V^{max} determination. All fractions were surveyed for activity using well assays and as before 1 unit of activity was characterized at 1 mm^2 as was described by Huang et al. (2016).

Mass determination using native SDS-PAGE Gel electrophoresis

In order to estimate the molecular weight (MW) of Thermocin 458, a 4-20% TruPAGE gel (Sigma Aldrich) was used as previously described (Schagger and von Jagow, 1987). The antimicrobial containing solution was loaded onto opposite sides of the gel with a molecular marker (5-250 kDa) and after electrophoresis was completed the gel was divided in two. The first half was stained with coomassie blue to estimate the size of the antimicrobial. The second half was fixed for 30 minutes in propan-2-ol (25%) and acetic acid (10%) then washed in dH_20 for 3 hours. The fixed gel was then overlaid in a small petri dish with 15mls of BHI agar (0.75%) which had been previously seeded with a 0.1% inoculum of *Geobacillus thermoleovorans* DSM 7263. The presence of a zone was then correlated back to the visualized half of the gel in order to gain an accurate size estimation of the antimicrobial protein.

Biochemical characterization of thermocin 458

The pH stability of thermocin 458 was investigated using the cell free supernatant of an overnight culture. The pH was adjusted using 1M NaOH and HCL from pH 2 to

pH 12. The areas of the zone of inhibition were measured and expressed in terms of activity units, where 1 unit = 1mm^2.

In order to carry out temperature stability and enzyme susceptibility tests, a crude 70 % propan-2-ol/0.1% TFA thermocin 458 containing preparation which had been prepared as previously described was dispensed into 500 µl aliquots and lyophilized. The crude Thermocin 458 was then re-suspended in 500µl of Phosphate Buffered Saline (PBS) containing one of the following enzymes: proteinase K, a-chymotrypsin, papain, ficin, a-amylase and lipase. These enzymes were added at a final concentration of 1 mg/ml and incubated for 1 hour at 37°C.

To assess temperature stability, the lyophilized thermocin 458 was re-suspended in PBS (pH7) and aliquoted into 50µl aliquots in low protein binding microfuge tubes and incubated in triplicate at 60, 70, 90 or 100°C for 30 minutes or 121°C for 15 minutes.

Areas of the zone of inhibition were measured and percentage residual activity calculated. Enzymes (10mg/ml) and pH buffers without thermocin 458 were also included on the plate to serve as controls.

Results

Antimicrobial spectrum of thermocin 458

A bank of *Geobacillus* species was screened for antimicrobial activity against *L. lactis* HP, *G. stearothermophilus* DSM 7263, *S. agalactiae* ATCC 13813, *B. subtilis* 1012 and *S. aureus* SA113 (Table 2) and one isolate, *G. stearothermophilus* DSM 458 displayed antimicrobial activity. *G. stearothermophilus* DSM 458 had a narrow spectrum of activity, only inhibiting other *Geobacillus* spp (Table 3)

Whole genome sequencing and general feature prediction

In an attempt to identify the biosynthetic genes responsible for the antimicrobial activity of *G. stearothermophilus* DSM 458, whole genome sequencing and gene annotation was carried out. This yielded a fully circularised genome without any plasmids (Figure 1). The mean fold coverage was 147.88. The genome size was 3,466,824 bp with a G+C content of 52.11%. A total of 3361 protein coding sequences (CDSs) were predicted including 32 rRNA operons, 89 tRNAs, 43 pseudo genes, 4 putative phages and 5 repeat regions (Table 4).

Identification of putative bacteriocin gene clusters

Subsequently, the genome was screened using BAGEL 3.0 and antiSMASH software to identify putative antimicrobial-encoding gene clusters, both of which predicted the presence of a highly hydrophobic circular bacteriocin (Figure 2), with an amino acid sequence similar to other characterised circular bacteriocins (Figure 3). This circular bacteriocin DNA sequence was also shown to be present in a number of other *Geobacilli* genomes (Figure 4).

Three strains (*G. thermoleovorans* Y4.12MC52, *G. thermoleovorans* Y4.12MC61 and *G. thermoleovorans* C56-T3) which contained the putative bacteriocin genes were acquired to examine potential cross-immunity to the *G. stearothermophilus* DSM 458 strain and to also assess for production of the predicted circular bacteriocin encoded in their genomes. However, all three strains were sensitive to the antimicrobial produced by *G. stearothermophilus* DSM 458 and did not themselves appear to produce any inhibitory activity.

Additionally, the masses obtained using colony mass spectrometry (Figure 5) of *G. stearothermophilus* DSM 458 and HPLC purification of the inhibitory substance (11-13 kDa) using native SDS-PAGE (Figure 6) did not correspond to the predicted circular bacteriocin (6.933 kDa). The RP-HPLC profile analysis (Figure 7a) of the antimicrobial also did not resemble that of a highly hydrophobic peptide such as the circular bacteriocin circularin (Gabrielsen et al., 2014). These results suggested that the antimicrobial activity associated with *G. stearothermophilus* DSM 458 is unlikely to be the predicted putative circular bacteriocin. Furthermore, BLASTP analysis of the genome against all proteins in the prokayotic antimicrobial peptide database produced no significant matches with any other known antimicrobial peptides.

Production and partial purification of thermocin 458

In order to obtain sufficient quantities of peptide for further analysis (mass spectrometry, peptide stability, enzymatic sensitivity) *G. stearothermophilus* DSM 458 was grown in 2L of BHI broth over 24 hours. A combinatorial protein purification approach was then undertaken in order to concentrate the antimicrobial.

This method involved extracting the antimicrobial from the cells using a polar solvent (propan-2-ol) and precipitating the antimicrobial from the supernatant using ammonium sulfate precipitation. The antimicrobial displayed a retention time between 65-75 mins over a mobile phase gradient of 5-70% acetonitrile as determined through detection of the antimicrobial activity through a well diffussion assay (Figure 7b). The active fractions were subsequently pooled and run on a shallower gradient of 40-70% acetonitrile in order to further separate the proteins, however the detection of the active peptide did not correspond to a peak on the HPLC chromatogram. In order to elucidate absorbance (V^{max}) of the antimicrobial contained within this HPLC fraction a spectral scan (200-800nm) was performed, but this yielded no conclusive results as no absorbance signal was observed across this range, indicating the peptide may be present in extremely low concentrations.

Mass determination of thermocin 458

The inhibitory substance isolated from the RP-HPLC purification was subjected to ultrafiltration using a 10 kDa molecular weight cut off spin filter (MWCO). Retention of the inhibitory substance suggests that the inhibitiory substance has a mass > 10 kDa. This was further substantiated by native Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis of the antimicrobial which indicated its mass was between 11-13 kDa (Figure 6). MALDI-TOF mass spectrometry was carried out on the purified HPLC fraction and two potential masses were identified at 11362.12 and 12924.46 Da (Figure 8).

Biochemical characterisation of thermocin 458

It was observed that thermocin 458 had remarkable pH and temperature stability (Table 5). It retained full activity across a broad pH range (2-10), only becoming 11% less active at pH 12. It was also observed that the antimicrobial was temperature stable, losing only 5% of relative activity from 80-100°C and 27% at 115°C when treated for 30 and 15 minutes.

When the antimicrobial was treated with various enzymes it was shown that the antimicrobial was susceptible to a range of proteases over one hour, with papain resulting in the greatest loss of activity, suggesting that the antimicrobial contains essential peptide bonds. It was also noted that α-amylase only slightly reduced the activity of the antimicrobial, while lipase had no effect (Figure 9).

Discussion

In this study we identified an antimicrobial which we are designating thermocin 458, produced by *Geobacillus stearothermophilus* that was isolated from a sugar beet factory in Austria. This antimicrobial protein was found to be 11-13 kDa in size and is narrow spectrum, only exhibiting activity against other geobacilli. Based on these characteristics we predict this antimicrobial protein is likely a bacteriocin and belongs to the Class 3 bacteriolysins using the classification scheme set out by Cotter et al. (2013). Furthermore, by comparing our biochemical and physicochemical data with published antimicrobial peptides from the genus *Geobacillus* (Table 6) we believe that thermocin 458 is a potentially novel antimicrobial protein.

Geobacilli are an important source of thermostable enzymes, and consequently it has been suggested that they might also be a source of thermostable bacteriocins. Indeed, the thermocin 458 antimicrobial identified here is stable across a broad range of temperatures including those used by autoclaves, supporting the hypothesis of mining thermostable bacteriocins from thermophillic bacteria. Furthermore, the degradation of thermocin 458 by ficin, papain, proteinase K and trypsin confirms the proteinaceous nature of this antimicrobial compound while the slight loss of activity from α-amylase treatment could suggest the presence of a carbohydrate moiety. Although proteinaceous degradation of thermocin 458 was observed, it was not at the levels seen by Pilasombut et al., (2015), where the antimicrobial activity of *L. plantarum* KL-1 was completely reduced over the course of a three hour treatment with enzymes. Perhaps this lower sensitivity of thermocin 458 to proteinaceous enzymes could be an advantageous feature of bacteriocins from *Geobacillus* species, which may be expolited in future studies.

Although thermocin 458 displayed reproducible retention times using HPLC, as was detected by assaying fractions for antimicrobial activity, there was no detection of the bacteriocin using UV-VIS. A possible explanation for this is that the protein exhibits antimicrobial activity against sensitive *Geobacillus* even at concentrations below the level of detection for analytical HPLC.

While the availability of genomic data has led to an increase in *in silico* bacteriocin identification such as formicin (Collins et al., 2016), lichenicidin (Begley et al., 2009; Dischinger et al., 2009) and Geobacillin I and II (Garg et al., 2012) it is worth considering that neither BAGEL 3.0 (van Heel et al., 2013) nor antiSMASH (Weber et al., 2015) could successfully identify the gene(s) responsible for the production of Thermocin 458. Perhaps this could be explained by the lack of bacteriocins currently known to be produced by *Geobacillus* and a potential higher diversity compared to known bacteriocins in this genus, leading to an inability to identify the biosynthetic gene(s) responsible for its production. Ultimately until we know the sequence of the biosynthetic gene(s) responsible for the antimicrobial activity of thermocin 458 it is difficult to ascertain true novelty.

Discovering bacteriocins which are novel and functionally interesting will be a key part of the solution to the antimicrobial resistance crisis and therefore, it is important that we expand the knowledge of bacteriocin biology and prospect new sources of bacteriocin peptides such as the genus *Geobacillus* in the future.

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Table 1. Strains used in this study

Strain	Characteristics	Source/ reference *
G. stearothermophilus DSM 458	Antimicrobial producer	BGSC
G. stearothermophilus ATCC 12980	Indicator	BGSC
G. stearothermophilus DPC 6941	Indicator	DPC
G. stearothermophilus DSM 494	Indicator	BGSC
G. stearothermophilus DSM 1550	Indicator	BGSC
G. stearothermophilus NRRL B4419	Indicator	BGSC
G. thermoleovorans DSM 7263	Indicator	BGSC
G. thermoleovorans DSM 13174	indicator	BGSC
G. thermoleovorans DSM 5366	Indicator	BGSC
G. thermoleovorans Y4.12MC61	Indicator	BGSC
G. thermoleovorans Y4.12MC52	Indicator	BGSC
G. thermoleovorans C56-T3	Indicator	BGSC
G. thermoglucosidius DSM 6939	indicator	DPC
G. tobeii DSM 14590	Indicator	BGSC
G. uziensis DSM 13551	Indicator	BGSC
G. anatolyticus SB	Indicator	BGSC

G. subterraneus DSM 13552	Indicator	BGSC
G. kaue HU	Indicator	BGSC
G. uziensis X	Indicator	BGSC
G. thermodenitrificans DSM 465	Indicator	BGSC
L. lactis HP	Indicator	UCC
L. lactis KH	Indicator	UCC
L. monocytogenes L028	Indicator	This study
S. aureus RF122	Indicator	This study
B. cereus DPC 6089	Indicator	DPC
B. subtilis 1012	Indicator	BGSC
Paenibacillus polymyxa	Indicator	BGSC
E. faecium	Indicator	UCC
Alycycliobacillus acidoterrestris	Indicator	UCC

* Culture collection abbreviations

BGSC: Bacillus Genetic Stock Centre, Ohio State University, USA.

DPC: Dairy Culture Collection, Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork.

UCC: University College Cork culture collection, School of Microbiology, Western Road, Cork City, Ireland

Table 2. Bank of *Geobacilli* screened for antimicrobial activity

		Bacteria screened for antimicrobial production									
		G. thermoleovorans DSM 7263	*G. stearothermophilus* DSM 1550	*G. stearothermophilus* DSM 458	*G. thermoleovorans* DSM 13174	*G. thermoleovorans* DSM 5366	*G. thermoleovorans* Y4.MC61	*G. toebii* DSM 14590	*G. stearothermophilus* ATCC 12980	*G. stearothermophilus* DPC 6941	*G. stearothermophilus* DPC 6939
Indicator bacteria	*G. thermoleovorans* DSM 7263	-	-	+	-	-	-	-	-	-	-
	B. subtilis 1012	-	-	-	-	-	-	-	-	-	-
	S. agalactiae ATCC 13813	-	-	-	-	-	-	-	-	-	-
	L. lactis HP*	-	-	-	-	-	-	-	-	-	-
	S. aureus SA113	-	-	-	-	-	-	-	-	-	-

Table 3. Thermocin 458 has a narrow spectrum of activity against other thermophilic *Geobacilli*.

Indicator	**Zone of inhibition (mm^2)**
G. stearothermophilus ATCC 12980	186
G. stearothermophilus DPC 6941	150
G. stearothermophilus DSM 494	274
G. stearothermophilus DSM 1550	292
G. stearothermophilus NRRL B4419	263
G. thermoleovorans DSM 7263	387
G. tobeii DSM14590	420
G. uziensis DSM 13551	280
G. anatolyticus SB	178
G. subterraneus DSM 13552	257
G. kaue HU	177
G. uziensis X	328
G. thermodenitrificans DSM 465	282

Table 4. Overview of genome features of *Geobacillus stearothermophilus* DSM 458

Atributes	**Data**
Genome size (bp)	3,466,824
GC content	52.11%
rRNAs	32
tRNAs	89
Repeat regions	5
Pseudo genes	43
Phages	4
Proteins	3361
Genes	3525

Table 5. Physicochemical stability tests of thermocin 458

Treatment	
Heat treatment	**% untreated control**
Control	100
60	93.50 ± 2.25
70	91.75 ± 1.43
90	91.26 ± 2.09
100	92.38 ± 3.87
121	91.26 ± 2.09
pH	**Activity units (1 unit = 1 mm^2)**
2	80.118 ± 6.20
3	50.265 ± 7.89
4	105.68 ± 14.41
5	120.76 ± 15.31
6	120.76 ± 2.98
7	120.76 ± 2.98
8	120.76 ± 2.26
9	120.76 ± 2.26
10	120.76 ± 2.26
11	120.76 ± 5.89
12	109.35 ± 22.35

Table 6. Antimicrobials / bacteriocins produced by other *Geobacilli*

Producer	**Size**	**Spectrum of activity**	**Name**	**Refrences**
G. stearothermophilus NU-1	Unknown	Narrow	Unknown	Shafia, 1966
G. stearothermophilus NU-2	Unknown	Narrow	Unknown	Shafia, 1966
G. stearothermophilus NU-7	Unknown	Narrow	Unknown	Shafia, 1966
G. stearothermophilus NU-10	20 kDa	Narrow	Unknown	Shafia, 1966; Yule and Barridge, 1976
G. stearothermophilus NU-23W	Unknown	Narrow	Unknown	Shafia, 1966
G. stearothermophilus NU-34	Unknown	Narrow	Unknown	Shafia, 1966
G. stearothermophilus NU-37	Unknown	Narrow	Unknown	Shafia, 1966
G. stearothermophilus NU-41	Unknown	Narrow	Unknown	Shafia, 1966
G. stearothermophilus NU-44	Unknown	Narrow	Unknown	Shafia, 1966
G. stearothermophilus NCA-1373	Unknown	Narrow	Unknown	Shafia, 1966
G. stearothermophilus NCA 1492	Unknown	Narrow	Unknown	Shafia, 1966
G. stearothermophilus RS93	Unknown	Narrow	thermocin 93	Pokusaeva et al., 2009
G. toebii HBB-247	38 kDa	Broad	Unknown	Başbülbül Özdemir and Biyik, 2012

G. thermodenitrificans NG80-2	3.2 - 3.4 kDa	Broad	Geobacillin I	Garg et al., 2012
G. thermodenitrificans NG80-2	3.4 - 3.6 kDa	Broad	Geobacillin II	Garg et al., 2012
G. toebii HBB-218	5.5 kDa	Broad	Toebicin 218	Özdemir and Biyik, 2012
Geobacillus sp. ZGt-1	15-20 kDa	Broad	Unknown	Alkhalili et al., 2016

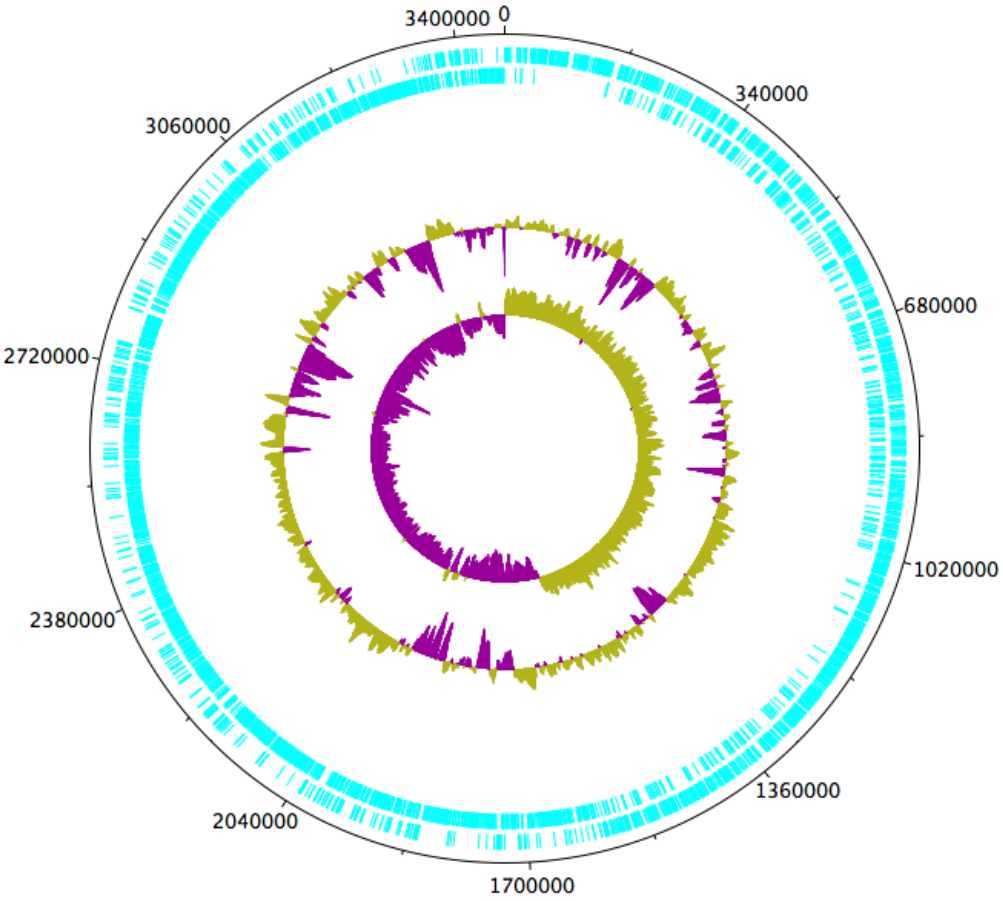

Figure 1. Genome map of of *Geobacillus stearothermophilus* DSM 458.

The tracks from the figure (starting from the outside) are: (1) forward CDS, (2) reverse CDS, (4) % GC, (4) GC skew [(GC)/(G+C)]

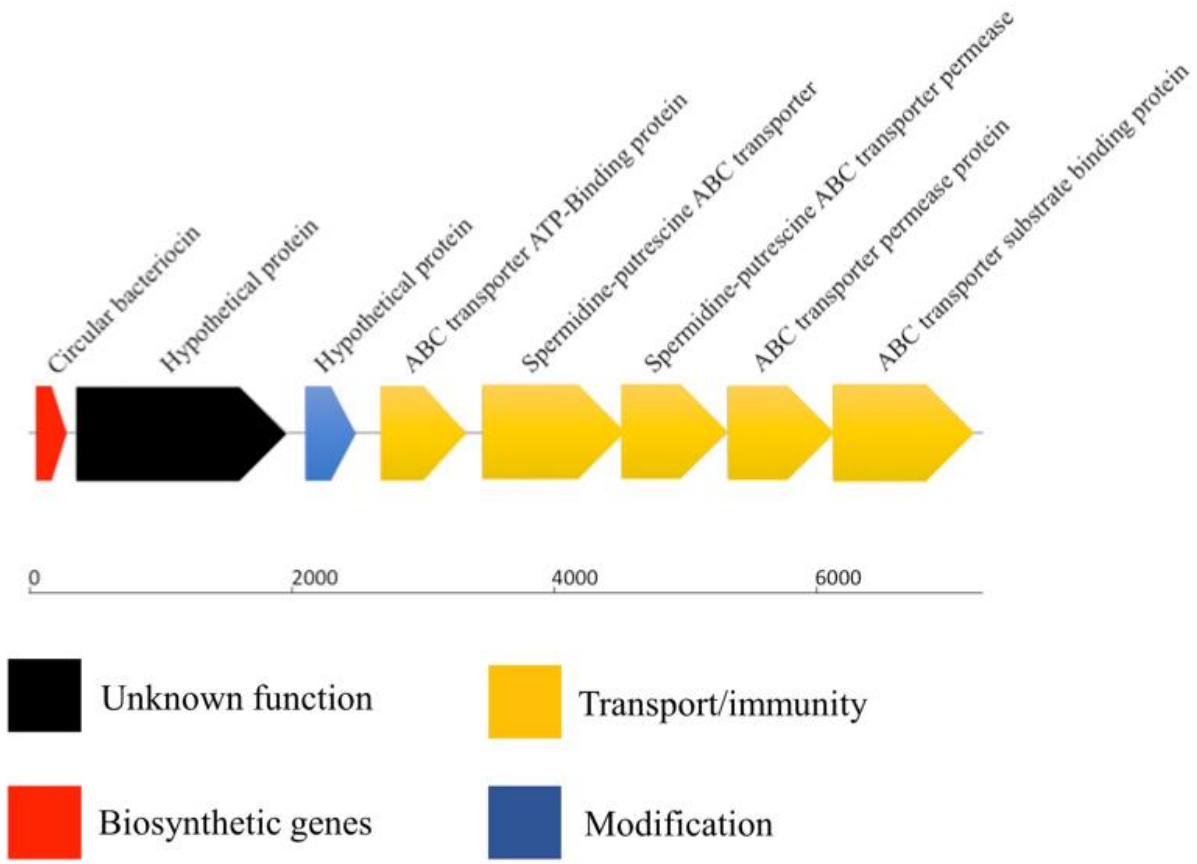

Figure 2. A putative highly hydrophobic circular bacteriocin gene cluster is contained within the genome of *G. stearothermophilus* DSM 458.

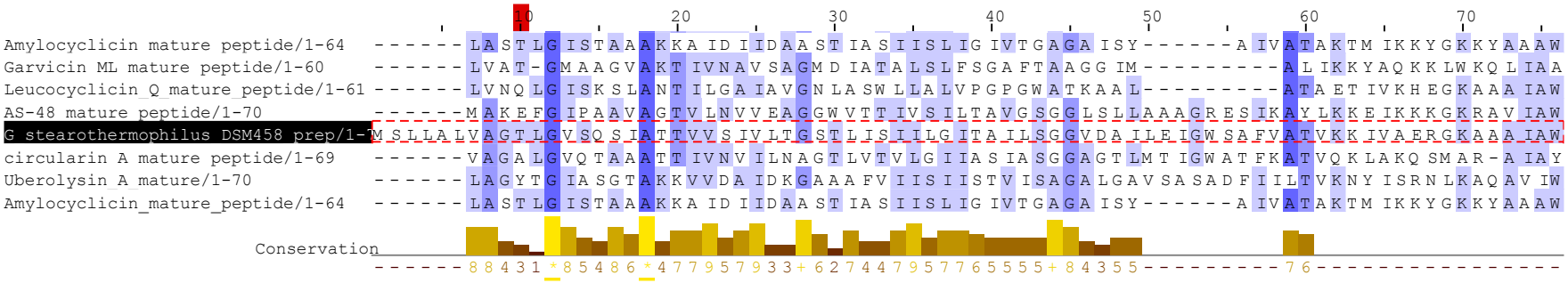

Figure 3. Alignment of predicted circular peptide with characterised circular bacteriocins

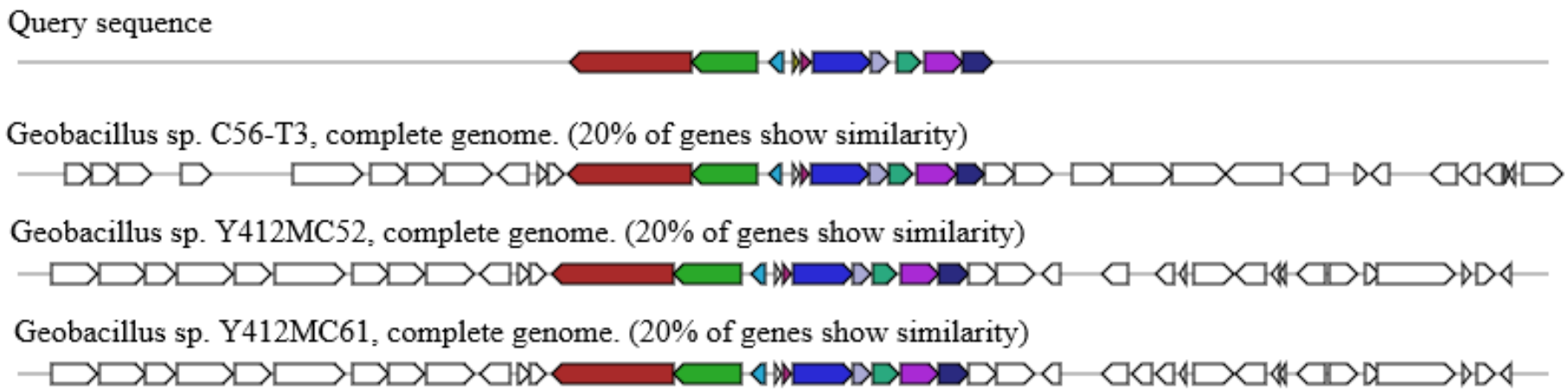

Figure 4. A putative highly hydrophobic bacteriocin is encoded in *Geobacillus* genomes

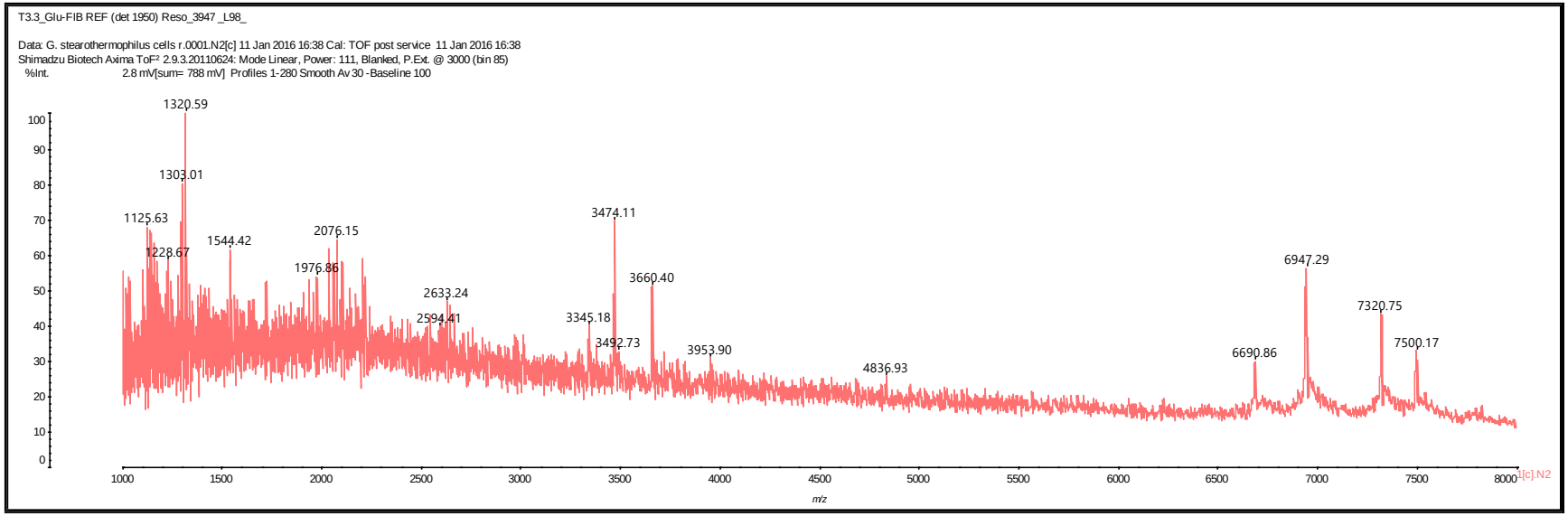

Figure 5. Colony Mass Spectrometry (CMS) of *G. stearothermophilus* DSM 458

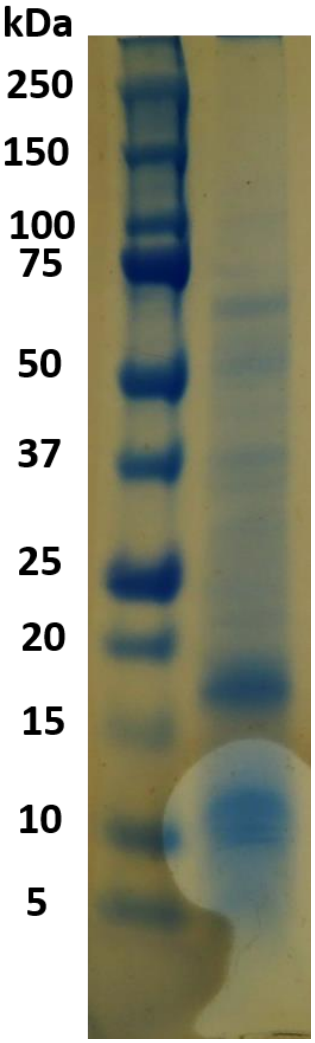

Figure 6. Thermocin 458 has an estimated molecular mass of 11-13 kDa as determined by native SDS-PAGE.

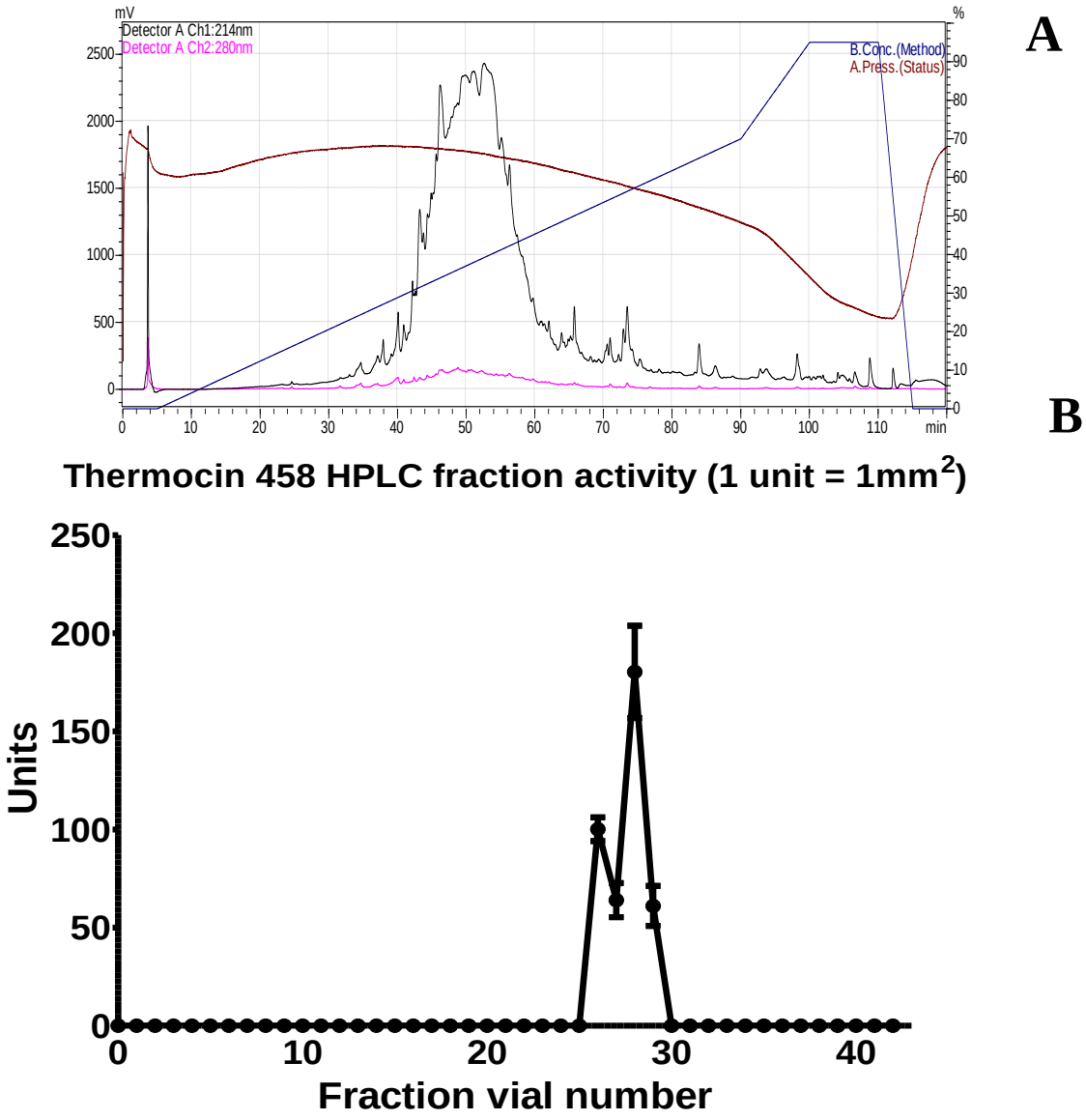

Figure 7. HPLC chromatogram of crude antimicrobial purification. Retention time of antimicrobial is 65 – 75 minutes on a gradient of 0-70% ACN / 0.1%TFA.

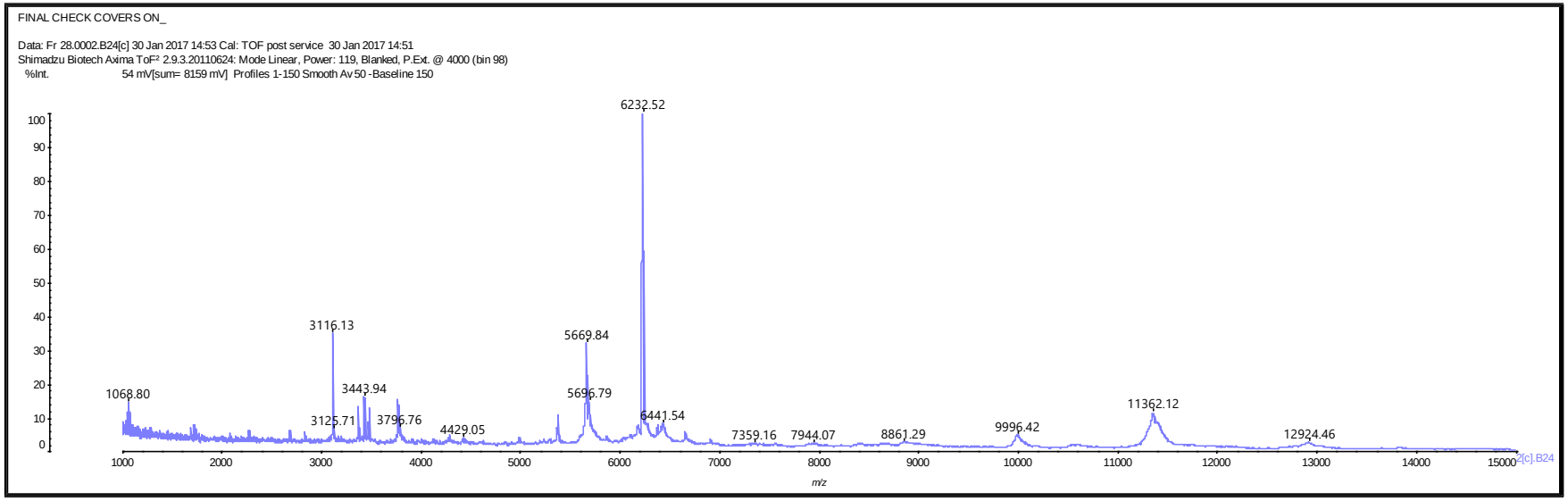

Figure 8. MALDI-TOF mass spectrometry analysis of active HPLC fractions containing putative masses of: 11362.12 and 12924.46 kDa.

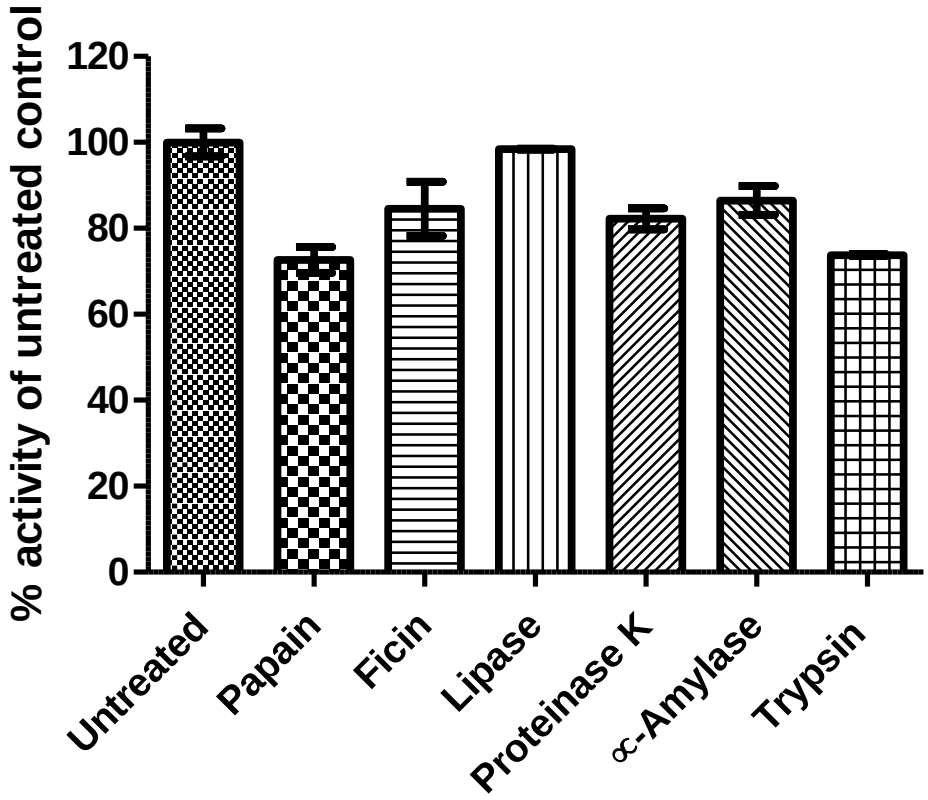

Figure 9. Thermocin 458 enzyme susceptibility assay (n=6).

Chapter III

In silico Prediction and Exploration of Potential Bacteriocin Gene Clusters within the bacterial genus *Geobacillus*

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Abstract

The thermophilic, endospore-forming genus of *Geobacillus* has historically been associated with spoilage of canned food. However, in recent years it has become the subject of much attention due its biotechnological potential in areas such as enzyme and biofuel applications. One aspect of this genus that has not been fully explored or realised is its use as a source of novel forms of the ribosomally synthesised antimicrobial peptides known as bacteriocins. To date only two bacteriocins have been fully characterised within this genus, i.e., Geobacillin I and II, with only a small number of others partially characterised. Here we bioinformatically investigate the potential of this genus as a source of novel bacteriocins through the use of the *in silico* screening software BAGEL3, which scans publically available genomes for potential bacteriocin gene clusters. In this study we examined the association of bacteriocin gene presence with niche and phylogenetic position within the genus. We also identified a number of candidates from multiple bacteriocin classes which may be promising antimicrobial candidates when investigated *in vitro* in future studies.

Introduction

The genus *Geobacillus* is composed of thermophillic, rod shaped, spore-forming, aerobic or facultative anaerobic bacteria. Their defining feature is their ability to grow at elevated temperatures of up to 80°C, with most isolates having growth temperature optima between 45 and 70°C (Nazina et al., 2001; Zeigler, 2014). Their sporulating nature makes their presence particularly challenging in food as they may survive intensive thermal processing methods and germinate when optimum conditions exist at a later period (Egan et al., 2016). In recent years this genus has attracted ever greater attention due an increased appreciation of its biotechnological potential, e.g. as sources of thermostable enzymes, as well as the biofuel and bioremediation industries (Cripps et al., 2009; Hussein et al., 2015; Kananavičiūtė and Čitavičius, 2015; Studholme, 2015). One application of *Geobacillus* which has not yet been fully explored relates to their usefulness as a source of novel and highly potent antimicrobial peptides called bacteriocins.

Bacteriocins are ribosomally synthesised, narrow or broad-spectrum, antimicrobial peptides produced by bacteria. They can be broadly classified into three classes: class I post-translationally modified, class II unmodified and class III <10 kDa in size (Arnison et al., 2013; Cotter et al., 2013). In past decades bacteriocins have been isolated primarily from lactic acid bacteria (LAB) due to their generally recognised as safe (GRAS) status which allows them to be used in food (Cotter et al., 2005). With the widespread use of *in silico* screening (Marsh et al., 2010; Azevedo et al., 2015; Walsh et al., 2015; Collins et al., 2017) and large culture based screening projects (Rea et al., 2010), bacteriocin candidates have been identified from alternative bacterial genera isolated from environmental, food or clinical samples.

However, relatively few *Geobacillus*-associated bacteriocins have been identified to date (Pokusaeva et al., 2009; Başbülbül Özdemir and Biyik, 2012; Garg et al., 2012; Alkhalili et al., 2016), with very little genetic or structural information available with respect to these peptides. Geobacillin I and II represent the only two well characterised lantibiotic (class I) bacteriocins from this genus, with a large amount of information available with regard to antimicrobial spectrum, physicochemical characteristics and genetic determinants (Garg et al., 2012).

In silico screening of bacterial genomes for novel bacteriocins has become a staple element of bacteriocin discovery and characterisation over the past decade. Its widespread use and popularity has been driven by its ability to reduce time and cost relative to culture-based bacteriocin screening studies. First generation *in silico* screening of bacterial genomes required the use of 'driver genes' to predict potential new bacteriocin genes within genomes (Begley et al., 2009; Marsh et al., 2010). However, in recent years the bacteriocin prediction software BAGEL3 (van Heel et al., 2013) has become the tool of choice for *in silico* bacteriocin discovery. BAGEL3 searches bacterial genomes in DNA FASTA format using two different approaches to discover new bacteriocins, i.e., (1) detection of bacteriocin structural genes and (2) detecting other genes commonly associated with bacteriocin production. Those bacteriocins which are identified using both approaches are compared and filtered to remove duplicate candidates. Furthermore this software can be supplemented with traditional 'driver gene' *in silico* screening or even with other programs such as antismash 3.0, which can detect other classes of antimicrobial peptides such as Non-Ribosomal PolyKetide (NRPK) antimicrobials (Weber et al., 2015).

This study set out to use BAGEL3 (van Heel et al., 2013) to perform an *in silico* screen of publically available *Geobacillus* genomes in an attempt to identify bacteriocin candidates for future *in vitro* experiments. The specific objectives were to: (1) identify potential structural peptides within *Geobacillus* genomes; (2) investigate the possibility of a relationship between genome phylogenetic position and gene presence; and (3) examine any homology between structural peptide-encoding and surrounding genes with previously characterised bacteriocin gene clusters.

Materials and Methods

Bacteriocin identification and visualisation

Using the *in silico* bacteriocin prediction tool BAGEL3 (van Heel et al., 2013), genome sequences belonging to the genus *Geobacillus* (Table 1) were aquired and analysed. Amino acid sequences of all 16 class III bacteriocins were aquired from Bactibase (Hammami et al., 2007) and aligned against the genomes as driver sequences using blastP (Altschul et al., 1990). Where necessary NisP (NCBI protein ID: AAA25200.1) and NisT (NCBI protein ID: AAA25191.1) driver sequences were used to seek and identify LanT and LanP-determinants in genome sequences. Those bacteriocin genes predicted were further visualised using Artemis genome visualisation tool (Rutherford et al., 2000). Blastn and blastP (Altschul et al., 1990) were used to determine the % identities between putative peptides / genes and those accurately curated. Structural peptides were aligned using the Multiple Sequence Alignment (MSA) tool MUSCLE (Edgar, 2004) and then visulised using Jalview (Waterhouse et al., 2009). The previously generated MUSCLE peptide alignments were then input into the MEGA 7 software package (Kumar, 2016) for phylogenetic analysis. Using a neighbour-joining method, an unrooted phylogenetic tree was generated using a Jukes–Cantor method (Dukes and Cantor, 1969) and bootstrap replication values of 1000 similarly to that by Zhang et al., (2015). In alignments where specifiec sequences contained no common sites, these were deleted. The resulting nexus tree files were exported to the interactive tree of life (itol) (Letunic and Bork, 2016) for graphical adjustment.

Phylogenetic analysis of *Geobacillus* species

Where available, 16S RNA sequences were acquired from genbank, however if no 16S sequence was available the *in silico* prediction tool RNAmmer (Lagesen et al., 2007) was used. The *B. cereus* ATCC14579 16S sequence was selected as a root for the final version of the tree. All 16S sequences were then collated and aligned as before using the MSA tool MUSCLE (Edgar, 2004). The resulting alignment output was then input into MEGA 7 (Kumar, 2016). Similaraly to Cihan (2018), a neighbour-joining tree was generated using bootstrap values based on 1000 replications and the resulting nexus tree file was then input into the itol software (Letunic and Bork, 2016) for final graphic adjustments. Where no common sites were found for specific peptides in the generation of the phylogenetic tree they were not included in the phylogenetic arrangement. The strains which had nether pre-determined or non-predictable 16S rRNA sequences were excluded from the overall study. The bacteriocin predictions by BAGEL3 were subsequently overlaid onto the phylogenetic tree using microsoft Powerpoint.

Results

Bacteriocin cluster distribution across the genus of *Geobacillus*

This study sets out to use an *in silico* approach to determine both the prevalence and diversity of bacteriocin gene clusters within the genus *Geobacillus*. Utilizing the genome sequences available in the public databases, 67 genomes (Table 1) representing 12 *Geobacillus* species, including *galactosidius, iciganius, jerrasicus, kaustophilus, liticanus, stearothermophilus, subterraneus, thermogalactosidius, thermoleovorans, thermocatenulatus, uziensis* and *vulcani* were analysed. This screen resulted in the prediction of 81 bacteriocin gene clusters, of which 2 matched the previously characterised Geobacillin I and II (Garg et al., 2012) discovered in *Geobacillus thermodenitrificans* NG80-2. The other 79 clusters represented potentially novel bacteriocin candidates belonging to class I (modified) and class II (unmodified) bacteriocin families. When characterised class III bacteriocins were used as 'driver' sequences and blasted against the entire *Geobacillus* genome database, no homologies were found. Furthermore no class III bacteriocins were predicted by BAGEL3.

In order to reveal associations between bacteriocin gene cluster presence within genomes and their phylogenetic position within the overall *Geobacillus* genus, we superimposed the BAGEL3 bacteriocin predictions onto a *Geobacillus* neighbour-joining phylogentic tree constructed from 16S rRNA sequences. Here we can see that bacteriocin clusters are both diverse and common across those genomes examined in this study (Figure 1). While lantibiotics and circular bacteriocin clusters are spread across the whole genus, Linear Azole-containing Peptides (LAPs), are associated with those strains for which a species has been designated but cluster

closely with the species *G. galactosidius* and *G. thermodenitrificans*. A higher frequency of sactibiotics can also been seen within the species *G. stearothermophilus* but these are also present in other species. Furthermore, there are a number of strains included whose genomes have not been fully sequenced and therefore it is not possible to state definitively that alternative bacteriocin clusters are absent from these genomes other than those predicted in this screen.

Similarly to Walsh et al. (2015), the homology of predicted Potential Bacteriocin Gene Clusters (PBGCs) to existing genes and the arrangement of those genes was examined. Below we group PBGCs by bacteriocin class. These arrangements will display only those genes whose function is predicted to be involved in bacteriocin bioactivity and not those genes of unknown function that exist within these clusters.

Class I Bacteriocins

Lantibiotics

29 putative lantibiotic gene clusters within 18 genomes were identified by BAGEL3 as part of this genome led bacteriocin screen (Figure 2). Lantibiotics belong to class I bacteriocins, which undergo significant post-translational modifications. These peptides are small and usually contain thioether internal bridges due to the interaction of dehydroalanine or dehydrobutyrine with intrapeptide cysteines, resulting in the formation of lanthionine or β-methyllanthionine residues. The structural gene (LanA) typically encodes a leader at the N-terminal of the prepeptide, which is transported across the cell membrane by LanT, then cleaved by LanP. The Post Translational Modification (PTM) enzyme LanB catalyses the dehydration of amino acids, while LanC catalyzes thioether formation. The two component

regulatory system genes, *lanR* and *lanK*, encode a response regulator and histidine kinase, respectively (Marsh et al., 2010; Draper et al., 2015; Field et al., 2015). While there are other PTM enzymes associated with lantibiotics they were not observed in this study so will not be described further, but they are discussed in greater detail elsewhere (McAuliffe et al., 2001).

The lantibiotics predicted in this study (Figure 2) were grouped according to their amino acid similarity. Grouping the predicted peptides in this way facilitates a comparison with characterised bacteriocins in Bactibase (Hammami et al., 2007). When aligned with the Bactibase bacteriocin peptide database the following highest homology hits was seen for each peptide group; Group 1: no hits; Group 2: 98% similarity to Geobacillin I; Group 3: no hits; Group 4: 12% similarity to LsbB; Group 5: 19% identity to nisin U; Group 6: 16% identity to nisin U; Group 7: 25% identity to nisin U; Group 8: 100% identity to Geobacillin I; Group 9: 5% identical to cinnamycin; Group 10: no hits. Furthermore, a phylogenetic analysis of those predicted peptides was carried out (Figure 3) resulting in the arrangement of five phylogroups. Phylogroups one, three, four and five were relatively homogenous showing little evolutionary distance between the group nodes. Phylogroup two however displayed a larger level of heterogenity with large evolutionary distances existing between the various nodes of the group.

The putative lantibiotics discovered consisted of 7 PBGCs (Figure 4) with some containing multiple peptide candidates per PBGC (Figure 2). These PBGCs were then typed according to their cluster structure so they could be easily compared with one another. The first cluster (lantibiotic cluster type 1) was contained within 9

genomes (*Geobacillus* sp. 1017, *G. thermocatenulatus* KTCT3921, *G. thermodenitrificans* KCTC3902, *Geobacillus* sp. PA-3, *Geobacillus* sp. Lemmy01, *G. kaustophilus* NBRC102445, *G. thermoleovorans* B23, *G. thermodenitrificans* DSM465, *G. thermocatenulatus* BGSC93A1). It consisted of genes predicted to encode a LanB, LanT, LanC, LanR and LanK consecutively and is similar to the Geobacillin I cluster with regard to its gene makeup. However, within this cluster structure, the predicted lantibiotic peptides were not completely homologous, showing differences in their amino acid composition (Figure 2). Additionally two adjacent lantibiotic peptides were predicted within this cluster type for the genomes: *G. thermocatenulatus* KTCT3921, *G. thermocatenulatus* BGSC 93A1 *G. thermoleovorans* B23 and *G. kaustophilus* NBRC102445. There were a number of exceptions to this general cluster structure: *G. thermoleovorans* CCB US3 UF5 and *G. litanicus* N3 lacked a LanK-determinant (lantibiotic cluster type 2), while *Geobacillus* sp. JS12 contained an extra LanC-encoding gene (lantibiotic cluster type 3). *G. thermoleovorans* CCB US3 UF5 encodes two peptides within this cluster type and they are located adjacent to each other. *Geobacillus* sp. 44C (Lantibiotic cluster type 4) encodes an identical peptide to *G. galactosidius* DSM18751 (lantibiotic type 5), but the PBGC of *G. galactosidius* DSM 18751 contains an additional ABC transporter after the LanC homolog. The genome for *Geobacillus* sp. G11MC16 is predicted to encode three LanA peptides. The first and second peptides are encoded within a distinct cluster from the third. These two peptides are within a cluster that also contains genes predicted to encode a PD2_2 homolog, sigma70, structural peptide, a LanM and LanT homolog (lantibiotic cluster type 6). The third putative peptide-encoding gene is not within an obvious PBGC, but is encoded 10kbs downstream of a region predicted to encode PTM enzymes SpaB-C, ABC

transporter, LanC, LanR and LanK. The peptide predicted to be encoded by *Geobacillus* sp. FW23 is within a cluster consisting of genes predicted to encode a LanB, LanT, LanC, structural peptide and response regulator (lantibiotic cluster type 7).

There were two putative LanAs encoded within the genome of *G. kaustophilus* HTA426. The gene corresponding to the first peptide was located upstream of three ABC transporter-determinants, while the gene corresponding to the second peptide was downstream of these three genes. There was a putative LanC and a further ABC transporter encoded approximately 10kbs downstream from these predicted structural peptides which appear to exist within a neighbouring gene cluster. However, no corresponding LanA-encoding gene was detected. The genome for *Geobacillus sp.* ZGt1 was predicted to encode one LanA that is situated upstream of two ABC transporter-encoding genes. However, the nearest putative LanB, ABC transporter and LanC determinants are located 10kbs upstream of these genes. Finally NisP driver sequences were aligned against all genomes containing lantibiotics, however there were no difinitive results which indicted the presence of these determinants.

Sactipeptides

Sactibiotics, like lantibiotics, are post-translationally modified and thus are a subclass of class I bacteriocins. These post-translational modifications take place in the form of intramolecular bridges of cysteine sulphur to α-carbon linkages (Mathur et al., 2015). 20 sactibiotics peptides were predicted within 17 *Geobacillus* genomes as part of this *in silico* screen (Figure 5). No conservation of amino acid residues was

observed when these peptides were aligned with known sactibiotic structural peptides. Furthermore when these predicted peptides were aligned against the Bactibase bacteriocin peptide database (Hammami et al., 2007), no strong homologies with existing sactibiotics were found. Furthermore when a phylogenetic analysis of the predicted peptides (Figure 6) was carried out three phylogroups were observed. Phylogroup one contained the Trnα peptide while phylogroup three contained all other previously characterised sactibiotic peptides. Phylogroup two however did not contain any of the previously characterised sactibiotics.

When the sactibiotic biosynthetic gene clusters were further investigated, it was seen that 8 different types of predicted sactibiotic gene clusters were encoded within the *Geobacillus* genomes (Figure 7). The putative *G. stearothermophilus* A1, *Geobacillus* sp. GS27, *Geobacillus* sp. 47-IIb, *Geobacillus* sp. Sah69, *Geobacillus* sp. 44C, G. *stearothermophilus* ATCC 12980, *G. stearothermophilus* P3 and *Geobacillus.* sp. BC02 SacA-determinants were all located upstream of a putative PTM enzyme SacCD-encoding gene (sactibiotic cluster type 1). *Geobacillus* sp. Lemmy 01 contained putative SacCD, LanK, LanR and LanD-encoding genes (sactibiotic cluster type 2). *G. jerrasicus* 107829 contained putative SacCD and LanD-determinants (cluster type 3). *Geobacillus* sp. CAMR12793 and *G. stearothermophilus* B4114 genomes encoded putative SacCD and an ABC transporter-determinant (Sactibiotic cluster type 4). *Geobacillus* sp. PA-3 contains putative SacCD, two ABC transporters and a radical SAM enzyme-determinants (sactibiotic cluster type 5). The genomes for *Geobacillus* sp. 12AMOR1 and *G. kaustophilus* et2/3 contain putative SacCD and a radical SAM enzyme-determinants (sactibiotic cluster type 6). *G. stearothermophilus* D1 and *G. stearotherophilus* A1

are predicted to encode peptides located downstream of a SacCD enzyme-determinant (cluster type 7).

The *G. stearothermophilus* 10 genome encoded a predicted structural peptide, radical SAM and two ABC transporter-determinants. While the structural peptide was encoded on the positive strand of the genome the two secondary enzymes were encoded on the negative strand and therefore are not part of the same operon but could however be part of this PBGC. A second putative sactibiotic gene cluster, predicted to be encoded within the *G. stearothermophilus* D1 genome, contains a structural peptide and SacCD enzyme-determinant, which are separated by 13 genes. The genome of *Geobacillus* sp. GS27 was predicted to encode a second sactibiotic peptide other than that predicted previously, however the SacCD-determinant driving this prediction was located on the opposite strand so is not encoded within the same operon but could still be part of the PBGC.

Linear Azole containing Peptides (LAPs)

Linear Azole containing Peptides (LAPs) are another subclass of class I bacteriocins that are distinguished by virtue of containing a variety of heterocyclic rings of thiazole and (methyl)oxazole. These are formed through an ATP-dependant cyclodehydration and further flavin mononucleotide-dependant dehydrogenation of the amino acid residues cysteine, serine and threonine. The most notable of the LAPs is streptolysin S, which is modified by the cyclodehydratase SagCD (Melby et al., 2011; Cox et al., 2015; Alvarez-Sieiro et al., 2016). Six putative LAPs were identified in six *Geobacillus* genomes (Figure 8), five of which were identified in those strains for which a species was not assigned. These peptides did not return any

strong homologies to known LAPs or other bacteriocins when aligned against the bactibase bacteriocin database (Hammami et al., 2007). When a phylogenetic analysis of the predicted LAP peptides was performed (Figure 9), three phylogroups were observed, each consisting of two nodes.

Five out of six peptides (Figure 10) are contained within a gene cluster containing a structural peptide followed by a SagD-like and SagB-like determinants (LAP cluster type 1). For *Geobacillus* sp. B4113, the only gene which is predicted to be involved in the PTM of the associated peptide is a cyclodehydration enzyme-determinant upstream of the structural peptide (LAP cluster type 2). There is a LapBotD enzyme-derminant on the opposite strand, which is close to the structural peptide, so while it is not part of the same operon it may still be part of this PBGC.

Class II Bacteriocins

Circular (a)

Circular bacteriocins belong to class IIc bacteriocins and are characterised primarily by the C to N terminal covalent linkage. They are known for their proteolytic, heat and pH resistance along with their size of 5.6 to 7.2 kDa, however to date only a handful have been characterised (Gabrielsen et al., 2014). Recently *in silico* software has been used to predict a new circular bacteriocin pumilarin (van Heel et al., 2017) and assisted in the characterisation of plantaricyclin from WGS data (Borrero et al., 2017).

Thirty-one circular peptides were predicted within 29 genomes in this screen (Figure 11). These peptides displayed a weak homology (~30-40%) to known circular

peptides when aligned against the bacteriocin database bactibase (Hammami et al., 2007). Six phylogroups were observed when a phylogenetic analysis of the peptides was performed (Figure 12). Three peptides from the strains *G. kaustophilus* Et7/4, *G. kaustophilus* Et2/3 and *G. stearothermophilus* 10 were not included in the phylogenetic tree due to the absence of common sites. While circular peptides have been predicted recently within the genomes of *Geobacillus* (van Heel et al., 2017), they have not been examined in terms of those genes which surround their structural peptide. For those circular structural peptides predicted within the genus, there are 6 general gene cluster structures (Figure 13). The genomes of *G. stearothermophilus* B4114, *G. stearothermophilus* GS27, *G. stearothermophilus* B4109, *G. stearothermophilus* 10, *G. stearothermophilus* ATCC12980, *G. stearothermophilus* A1, *G. stearothermophilus* ATCC7953, *G. stearothermophilus* P3, *Geobacillus* sp. 4113, *Geobacillus* sp. T6, *Geobacillus* sp. Y4.MC52, *G. thermocatenulatus* KTCT3921, *G. thermocatenulatus* BGSC93A1, *G. stearothermophilus* DSM458, *G. subterraneus* PSS2 and *Geobacillus* sp. Y412MC61 contain a cluster predicted to encode a structural peptide, a modification gene and two ABC transporter-determinants (Circular cluster type 1). The genomes of *Geobacillus* sp. JS12, *Geobacillus* sp. C56-T3, *Geobacillus* sp. LC300, *G. kaustophilus* Gbly and *G. thermoleovorans* CCB US3 UF5 contain a structural peptide gene, a modification gene, two ABC transporter genes and an additional 3 genes further downstream, putative LanK and Sigma5-determinants (circular cluster type 2). While it is unclear what role these gene products could play in the activity of the structural peptide, we do know that these genes are homologs of lantibiotic regulation machinery. The *Geobacillus* sp. BCO2 genome is predicted to encode a structural peptide and three ABC transporter-determinants (circular cluster type 3). *Geobacillus* sp.

CAMR12739 is predicted to encode a structural peptide, a modification protein and three ABC transporter-determinants (circular cluster type 4). *G. kaustophilus* Et7/4 encodes a structural peptide and an ABC transporter-determinants (circular cluster type 5). The genome of *G. kaustophilus* Et7/4 encodes a modification and an ABC transporter-determinant following the structural peptide (circular cluster type 6).

Circular (b)

There were an additional 5 identical circular peptides predicted (Figure 14) that had distinctly different amino acid sequences to the group a circular peptides described above and therefore were designated as a separate group. When aligned against the bacteriocin database bactibase these circular peptides displayed low homology of 17% to lacticin 481. Four of these predicted peptides were encoded within the genomes of *G. kaustophilus* HTA426, *G. thermoleovorans* N7, *G. kaustophilus* Gblys, *Geobacillus* sp. CAMR12739 and *Geobacillus* sp. LC300. These circular peptides (Figure 15) were predicted to be encoded within the previously described type 2 circular PBGC (Figure 13). These structural genes were the last genes encoded within the cluster after the putative modification, 2 ABC transporters, LanK and Sigma 5-determinants (circular (b) cluster stype 1). It is unclear if these genes have any functional role within this cluster due to a lack of any strong amino acid similarity to known bacteriocins such as to circularin (Kawai et al., 2004) and enterocin AS-48 (Burgos et al., 2014), however they could represent a family of potentially novel bacteriocins which may merit further *in vitro* testing.

The structural peptide predicted to be encoded by *Geobacillu*s sp. CAMR 12793 is located approximately 10 kbs downstream of the previously described type 4 circular

(a) cluster (Figure 13). It was accompanied by upstream putative histidine kinase and response regulator determinants (circular (b) cluster type 2) (Figure 15). As before it is unclear whether this peptide would be biologically active in this gene cluster so it may merit further *in vitro* experimentation.

Non circular class II bacteriocins

There were a number of non-circular class II bacteriocins predicted by BAGEL3 (Figure 16), which are heterogenous with regard to both their predicted amino acid composition and those genes predicted to be involved in their bioactivity (Figure 17). When a phylogenetic analysis was performed, two phylogroups were observed for these predicted peptides (Figure 18). The predicted peptide encoded within the genome of *Geobacillus* sp. Y4MC1 structural peptide is 54% identical to Lacticin Z, however it is located on the opposite strand to its predicted ABC transporters so it is unclear whether they may have a role in its production (class II cluster type 3). The *G. stearothermophilus* D1 predicted cluster contained a circularisation enzyme and two ABC transporters, meanwhile the predicted structural peptide could be potentially novel as it displayed no homology to any known bacteriocins (class II cluster type 2). The *Geobacillus* sp. Lemmy 01 putative peptide did not display any homology to known bacteriocins and its prediction as a class II peptide was most likely based on the presence of a circularisation gene-determinant located 16 genes downstream of the structural peptide (class II cluster type 4). *G. stearothermophilus* 10 is predicted to produce a class II unmodified peptide (class II cluster type 1), which is encoded before the previously described circular (a) cluster type 1 (Figure 13). It is unclear if either or both peptides are bioactive. *G. litanicus* N3 is predicted to encode a bacteriocin which is two genes upstream of a circularisation gene-

determinant, however it has no further transport or modification genes associated with it (class II cluster type 5). *G. vulcani* PSS1 encodes a class II peptide with no homology to existing bacteriocins and is situated on the opposite strand to four ABC transporter and modification gene-determinants (class II cluster type 6).

Discussion:

Bacteriocin prospecting has typically been a long and expensive process, based on trial and error in order to isolate bacteriocin producing bacteria and then optimise their growth conditions for bacteriocin production and protein purification. Further characterisation of these bacteriocins then typically required the use of trained personnel to carry out High Performance Liquid Chromatography (HPLC), mass spectrometry and other steps. Since the advent of *in silico* screening this process of bacteriocin discovery has been significantly reduced in terms of time and cost. Indeed this technology allows the bioinformatician to characterise to a high level putative bacteriocin candidates in terms of their amino acid content, physicochemical characteristics and surrounding genes which may be related to its function. Interestingly, it is these elements which had previously been the most laborious and expensive elements of bacteriocin discovery. This ability to identify candidates *in silico* ultimately removes a large portion of this trial and error process as so much is known about the bacteriocin once it is produced *in vitro*.

This *in silico* screen resulted in the identification of seven lantibiotic, seven sactibiotic, two LAPs, eight circular and six class II PBGCs which are potentially novel. The putative bacteriocins identified through this *in silico* screening approach will require further investigation through *in vitro* experimentation. However, it was possible to study the genes surrounding the structural peptide to more accurately predict that the bacteriocin cluster was indeed likely to be functional. Notably, in some cases those genes predicted by BAGEL3 were situated within annotated genes and could be determined to be pseudogenes. This study serves to therefore validate and critically assess BAGEL3 as a tool for bacteriocin discovery which could be

advantageous for future improvements. When we consider the report that 30-99% of bacteria produce at least one bacteriocin (Riley and Wertz, 2002), it does seem likely that this may also be the case for *Geobacillus*, though a more complete picture will not become apparent until *in vitro* experiments are carried out to validate the findings of this study. Within the genomes examined here, only 23 of 67 were completely sequenced genomes. Where a genome only contains a partially sequenced bacteriocin cluster BAGEL3 will likely return a bacteriocin hit due to its dual detection method, distinguishing both structural peptides and associated bacteriocin genes. In order to fully explore the potential of *Geobacillus* as a reservoir of bacteriocin discovery, the generation of complete assembled genome sequences would be advantageous. A more conclusive picture of its potential will be revealed when the magnitude of genome sequences reaches that of *Lactobacillus*, which was examined recently *in silico* for its bacteriocinogenic potential (Collins et al., 2017). It could be expected that over the next number of years the amount of completely sequenced *Geobacillus* genomes will increase due to the wealth of data generated by way of the widespread use of metagenomic sequencing technologies and the ease / lower cost of WGS which is enabled by third generation sequencing technologies, such as PacBio (Rhoads and Au, 2015) and or Oxford Nanopore (Lu et al., 2016) instruments, that allow for *de novo* genome assembly. With this expected increase in genome sequence data, associations between niche and bacteriocin presence could be investigated in the future.

In the case of lantibiotic peptide predictions, LanT-determinants were not however identified always by BAGEL3 and in most cases LanT-determinant identification was made possible through the alignment of putative ABC transporter-determinants

and NisT driver sequences, highlighting the importance of using a hybrid approach of BAGEL and driver sequence homology searching to peptide prediction. Furthermore, a LanK-determinant was absent from a number of lantibiotic gene clusters yet was found in circular PBGCs predicted in the same genomes. It is unclear what role (if any) these LanK-determinants play in these lantibiotic PBGCs. Another interesting observation which merits further investigation was the absence of LanP-determinants from the *Geobacillus* genomes as was seen in the study of the geobacillin I and II biosynthetic genes (Garg et al., 2012). This could be due to effects of incomplete genome sequencing or perhaps the absence of LanP-homologs for peptide leader cleavage as seen in geobacillin I and II. Issues surrounding absent bacteriocin gene-determinants have however been overcome in various studies through the use of heterologous expression systems and such technology will be important for future validation of the various *in-silico* screening studies that have taken place to date (Mesa-Pereira et al., 2017; Piper et al., 2011; van Heel et al., 2016).

A common method of bacteriocin molecular mass size determination involves the use of Native Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS-PAGE), where the protein preparation is loaded onto an SDS gel and subjected to electrophoresis. It is then washed and overlaid with agar containing a sensitive indicator bacteria. A zone of inhibition surrounding a protein band provides an estimation of its molecular mass when compared to a molecular-weight size marker or ladder. While we have seen this method used to estimate the molecular mass of a bacteriocin produced by *Geobacillus* sp. ZGt-1 of 15-20 kDa, no such class III bacteriocin was predicted within this genome in our *in silico* screen (Alkhalili et al.,

2016). This may indicate the presence of a potentially highly novel class III bacteriocin within *Geobacillus* sp. ZGt-1 given the lack of homology to any known class III peptide, the presence of an uncommon gene cluster not identified in this study or the presence of another type of peptide antimicrobial other than a bacteriocin. Toebicin 218 is produced by *G. stearothermophilus* DSM22 with a molecular mass of 5.5 kDa (Özdemir and Biyik, 2012) and it is interesting to note that no bacteriocin was detected within this genome in the current study. Pokusaeva et al. (2009) used SDS-PAGE to estimate the size of bacteriocins produced by various *G. stearothermophilus* at 6.8, 5.6, 7.1 and 7.2 kDa. However, the genomes of these strains have not been sequenced and therefore the identity of potential bacteriocin candidates cannot be determined through bioinformatics. This is also the case for *G. toebii* HBB-247, that has been shown to produce a bacteriocin with an estimated mass of 38 kDa (Başbülbül Özdemir and Biyik, 2012). There are a number of other bacteriocins of undetermined mass which have been characterized within *Geobacillus* prior to modern sequencing or mass spectrometry methods (Shafia, 1966; Yule and Barridge, 1976; Sharp et al., 1979; Fikes et al., 1983). Indeed, it is notable that there is a significant lack of mass spectrometry data for all *Geobacillus*-associated bacteriocins other than the lantibiotics Geobacillin I and II discovered within *G. thermodenitrificans* NG80-2.

In chapter II of this thesis a circualar bacteriocin (Figure 2) was predicted within the genome *Geobacillus stearothermophilus* DSM 458, however, it was determined that this was not the basis of this strains antimicrobial activity. Additionally *G. thermoleovorans* Y4.12MC52, *G. thermoleovorans* Y4.MC61 and *G. thermoleovorans* C56-T3 were also shown to predict this circular bacteriocin, but

also did not display any antimicrobial activity when tested *in vitro*, highlighting the limitations of *in silico* sequencing. This limitation could be overcome using a heterologous expression system similarly that used by Collins et al., (2018), where the circular bacteriocins may be expressed and characterised fully.

While *Geobacillus* appears to represent a potential reservoir for novel bacteriocin discovery, its route to commercial application in food or medicine remains unclear. The nature of *Geobacillus* when in the form of a thermally resistant spore makes it difficult to remove once introduced into an processing environment (Egan et al., 2016). Furthermore, the associated high temperature growth requirements would translate to high processing and energy costs. Typically its direct addition to food, albeit a GRAS bacterial genus, is not applicable due to its history as a bacterial spoilage agent. Despite this, *Geobacillus* do already have applications in the biotechnology industry in a number of ways (such as biofuel and chemical production), so perhaps it is within this niche where bacteriocins produced by *Geobacillus* could be of commercial relevance. Additionally, these bacteria could serve as a platform for research into protein thermostability and as a source of not only heat stable bacteriocins but also post-translational modification enzymes. Finally, with the oncoming antimicrobial resistance (AMR) crisis, humankind is looking outside of the traditional antimicrobial candidate reservoirs and increased investment in other classes of antimicrobials such as defensins (Oppedijk et al., 2015) are visibly apparent. Given the abundance of potentially novel bacteriocins identified by this study, *Geobacillus* spp. could yet develop their full potential as a source of new peptide structures with enhanced functionality.

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Table 1. List of *Geobacillus* genomes examined in this *in silico* screen. Also included is their accession numbers, location and type of bacteriocin predicted by BAGEL3

Number	Species	Strain ID	Accession no:	Source	Assembly level	Country	Sample type	Bacteriocin encoded?	Reference
1	*G. galactosidius*	DSM18751	GCA_002217735.1	Compost	Contig	Italy	Environmental	Lantibiotic; LAPs	
2	*G. icigianus*	G1w1	GCA_000750005.1	Hydrothermal samples	Contig	Russia	Environmental	No	(Bryanskaya et al., 2014)
3	*G. kaustophilus*	Et2/3	GCA_000948165.1	Geyser	Contig	Chile	Environmental	Circular; Sactipeptide	
4	*G. kaustophilus*	Et7/4	GCA_000948285.1	Geyser	Contig	Chile	Environmental	Circular	
5	*G. kaustophilus*	HTA426	GCA_000009785.1	Deep sea sediment	Complete	Marina trench	Environmental	Lantibiotic; circular	(Takami et al., 2004a; Takami et al., 2004b)
6	*G. litanicus*	N-3	GCA_002243605.1	High temp oilfield	Complete	Litunia	Environmental	Lantibiotic; Circular	
7	*Geobacillus* sp.	Y4.1MC1	GCA_000166075.1	Hot Spring	Complete	USA	Environmental	LAPs; Class II	(Brumm et al., 2015a)
8	*Geobacillus* sp.	FJ8	GCA_000445995.2	Compost	Complete	Japan	Environmental	No	(Shintani et al., 2014)
9	*Geobacillus* sp.	44B	GCA_002077755.1	Deep subsurface	Contig	USA	Environmental	Sactibiotic; LAPs	(Singh et al., 2017)

10	*Geobacillus* sp.	44C	GCA_002077865.1	Deep subsurface	Scaffold	USA	Environmental	Lantibiotic; Circular; LAPs	(Singh et al., 2017)
11	*Geobacillus* sp.	WCH70	GCA_000023385.1	Compost	Complete	USA	Environmental	Class II; LAPs	(Brumm et al., 2016)
12	*Geobacillus* sp.	46C-IIa	GCA_002077765.1	Deep subsurface	Scaffold	USA	Environmental	No	(Singh et al., 2017)
13	*Geobacillus* sp.	47C-IIb	GCA_002077775.1	Deep subsurface	Scaffold	USA	Environmental	Sactibitoic	(Singh et al., 2017)
14	*Geobacillus* sp.	PA-3	GCA_001412125.1	Soil	Contig	Litunia	Environmental	Lantibitoic; Sactibitoic	(Petkauskaite et al., 2017)
15	*Geobacillus* sp.	12AMOR1	GCA_001028085.1	Deep sea hydrothermal vent	Complete	Unknown	Environmental	Sactibiotic	(Wissuwa et al., 2016)
16	*Geobacillus* sp.	LEMMY01	GCA_002042905.1	Soil	Contig	Brazil	Environmental	Lantibitoic; Sactibiotic; Circular	(de Souza et al., 2017)
17	*Geobacillus* sp.	1017	GCA_001908025.1	Oil water	Contig	China	Environmental	Lantibiotic	(Kadnikov et al., 2016)

18	*Geobacillus* sp.	GHH01	GCA_000336445.1	Soil sample	Complete	Germany	Environmental	No	(Wiegand et al., 2013)
19	*Geobacillus* sp.	Y4.12MC61	GCA_000024705.1	Hot spring	Complete	USA	Environmental	Circular	(Brumm et al., 2015c)
20	*Geobacillus* sp.	Y4.12MC52	GCA_000174795.2	Hot spring	Complete	USA	Environmental	Circular	(Brumm et al., 2015b; Brumm et al., 2015c)
21	*Geobacillus* sp.	Sah69	GCA_001414205.1	Soil	Contig	Algeria	Environmental	Sactibitoic	(Bezuidt et al., 2015)
22	*Geobacillus* sp.	JS12	GCA_001592395.1	Compost	Complete	South Korea	Environmental	Lantibiotic; Sactibiotic	(Jeon et al., 2016)
23	*Geobacillus* sp.	T6	GCA_001025095.1	Hot water spring	Contig	Argentina	Environmental	Circular	(Ortiz et al., 2015)
24	*Geobacillus* sp.	BC02	GCA_001294475.1	Bore well isolate	Contig	Australia	Environmental	Circular; Sactibiotic	
25	*Geobacillus* sp.	WSUCF1	GCA_000422025.1	Soil	Contig	USA	Environmental	No	(Bhalla et al., 2013)
26	*Geobacillus* sp.	FJAT-46040	GCA_002335725.1	Hot spring	Scaffold	China	Environmental	No	
27	*Geobacillus* sp.	ZGt-1	GCA_001026865.1	Hot spring	Scaffold	Jordan	Environmental	Lantibiotic	(Alkhalili et al., 2015)

28	*Geobacillus* sp.	A8	GCA_000447395.1	Deep mine	Contig	South africa	Environmental	No	
29	*Geobacillus* sp.	CAMR5420	GCA_000691465.1	Unknown	Contig	Unknown	Environmental	No	(De Maayer et al., 2014)
30	*G. stearothermophilus*	10	GCA_001274575.1	Hot spring	Complete	USA	Environmental	Sactibiotic; Circular	
31	*G. stearothermophilus*	22	GCA_000743495.1	Hot spring	Contig	Russia	Environmental	No	(Rozanov et al., 2014)
32	*G. stearothermophilus*	53	GCA_000749985.1	Hot Spring	Contig	Russia	Environmental	No	(Rozanov et al., 2014)
33	*G. stearothermophilus*	C1BS50MT1	GCA_001620045.1	Water sediment	Contig	Australia	Environmental	Circular	
34	*G. subterraneus*	KCTC3922	GCA_001618685.1	Subsurface Oil field	Complete	China	Environmental	No	(Lee et al., 2017)
35	*G. subterraneus*	K	GCA_001632595.1	Oilfield	Contig	Russia	Environmental	No	(Poltaraus et al., 2016)
36	*G. thermocatenulatus*	KCTC3921	GCA_002243665.1	Gas well isolate	Complete	USSR	Environmental	Lantibiotic; Circular	
37	*G. thermocatenulatus*	BGSC93A1	GCA_002217655.1	Oilfield	Contig	Russia	Environmental	Lantibiotic; Circular	
38	*G. thermocatenulatus*	SURF-48B	GCA_002077815.1	Deep subsurface	Scaffold	USA	Environmental	No	(Singh et al., 2017)
39	*G. thermodenitrificans*	NG80-2	GCA_000015745.1	Deep subsurface	Complete	China	Environmental	Geobacillin I; Geobacillin II	(Feng et al., 2007)
40	*G. thermodenitrificans*	T12	GCA_002119625.1	Compost	Complete	Netherlands	Environmental	No	(Daas et al., 2018)

41	*G. thermoleovorans*	CCB US3 UF5	GCA_000236605.1	Hot spring	Complete	Malaysia	Environmental	Lantibiotic; Circular	(Muhd Sakaff et al., 2012)
42	*G. thermoleovorans*	FJAT-2391	GCA_001719205.1	Soil	Complete	China	Environmental	No	
43	*G. thermoleovorans*	KCTC3570	GCA_001610955.1	Soil	Complete	USA	Environmental	No	
44	*G. thermoleovorans*	N7	GCA_001707765.1	Hot spring	Contig	India	Environmental	Circular	
45	*G. thermoleovorans*	B23	GCA_000474195.1	Deep oil reserve	Contig	Japan	Environmental	Lantibiotic	(Boonmak et al., 2013)
46	*G. uzenesis*	BGSC92A1	GCA_002217665.1	Oilfield	Contig	Russia	Environmental	No	
47	*Geobacillus* sp.	B4113	GCA_001587475.1	Mushroom soup	Scaffold	Netherlands	Food	LAPs; Circular	(Berendsen et al., 2016)
48	*G. kaustophilus*	NBRC102445	GCA_000739955.1	Pasteurized milk	Contig	Unknown	Food	Lantibiotic	
49	*G. stearothermophilus*	A1	GCA_001183895.1	Milk powder facility	Scaffold	New Zealand	Food	Sactibiotic; Circular	(Burgess et al., 2015)
50	*G. stearothermophilus*	B4114	GCA_001587395.1	Buttermilk power	Scaffold	Neatherlands	Food	Sactibiotic; Circular	(Berendsen et al., 2016)
51	*G. stearothermophilus*	D1	GCA_001183885.1	Milk powder facility	Scaffold	New Zealand	Food	Sactibiotic; Circular	(Burgess et al., 2015)
52	*G. stearothermophilus*	P3	GCA_001183915.1	Milk powder facility	Scaffold	New Zealand	Food	Sactibiotic; Circular	(Burgess et al., 2015)

53	*G. stearothermophilus*	DSM 458	GCA_002300135.1	Sugar beet juice	Complete	Austria	Food	Circular	(Egan et al., 2017)
54	*G. stearothermophilus*	GS27	GCA_001651555.1	Casein pipeline	Scaffold	Netherlands	Food	Sactibiotic; Circular	
55	*G. stearothermophilus*	ATCC 12980	GCA_001277805.1	Spoilled canned food	Scaffold	USA	Food	Sactibiotic; Circular	
56	*G thermodenitrificans*	DSM 465	GCA_000496575.1	Sugar beet juice	Contig	Austria	Food	Lantibiotic	(Yao et al., 2013)
57	*G. thermodenitrificans*	KCTC3902	GCA_002072065.1	Sugar Beet juice	Complete	Austria	Food	Lantibiotic	(Lee et al., 2017)
58	*G. jurassicus*	NBRC107829	GCA_001544315.1	Unknown	Contig	Unknown	Unknown	Sactibiotic	
59	*G. kaustophilus*	GBlys	GCA_000415905.1	Unknown	Contig	Unknown	Unknown	Circular	(Doi et al., 2013)
60	*Geobacillus* sp.	G11MC16	GCA_000173035.1	Unknown	Contig	unknown	unknown	Lantibiotic	(Brumm et al., 2015c)
61	*Geobacillus* sp.	LC300	GCA_001191625.1	Bioreactor	Complete	USA	Unknown	Circular	(Cordova et al., 2015)
62	*Geobacillus* sp.	C56-T3	GCA_000092445.1	Unknown	Complete	Unknown	Unknown	Circular	(Brumm et al., 2015c)

63	*Geobacillus* sp.	CAMR12739	GCA_000691445.1	Unknown	Contig	Iceland	unknown	Sactibitoic; Circular	(De Maayer et al., 2014)
64	*Geobacillus* sp.	FW23	GCA_000617945.1	Oil well	Contig	India	unknown	Lantibiotic	(Pore et al., 2014)
65	*G. stearothermophilus*	ATCC7953	GCA_000705495.1	Unknown	Contig	Unknown	unknown	Circular	
66	*G. subterraneus*	PSS2	GCA_000744755.1	Unknown	Contig	Unknown	unknown	Lantibiotic; Circular	
67	*G. vulcani*	PSS1	GCA_000733845.1	Human Microbiome isolate	Contig	Japan	Human	Circular	

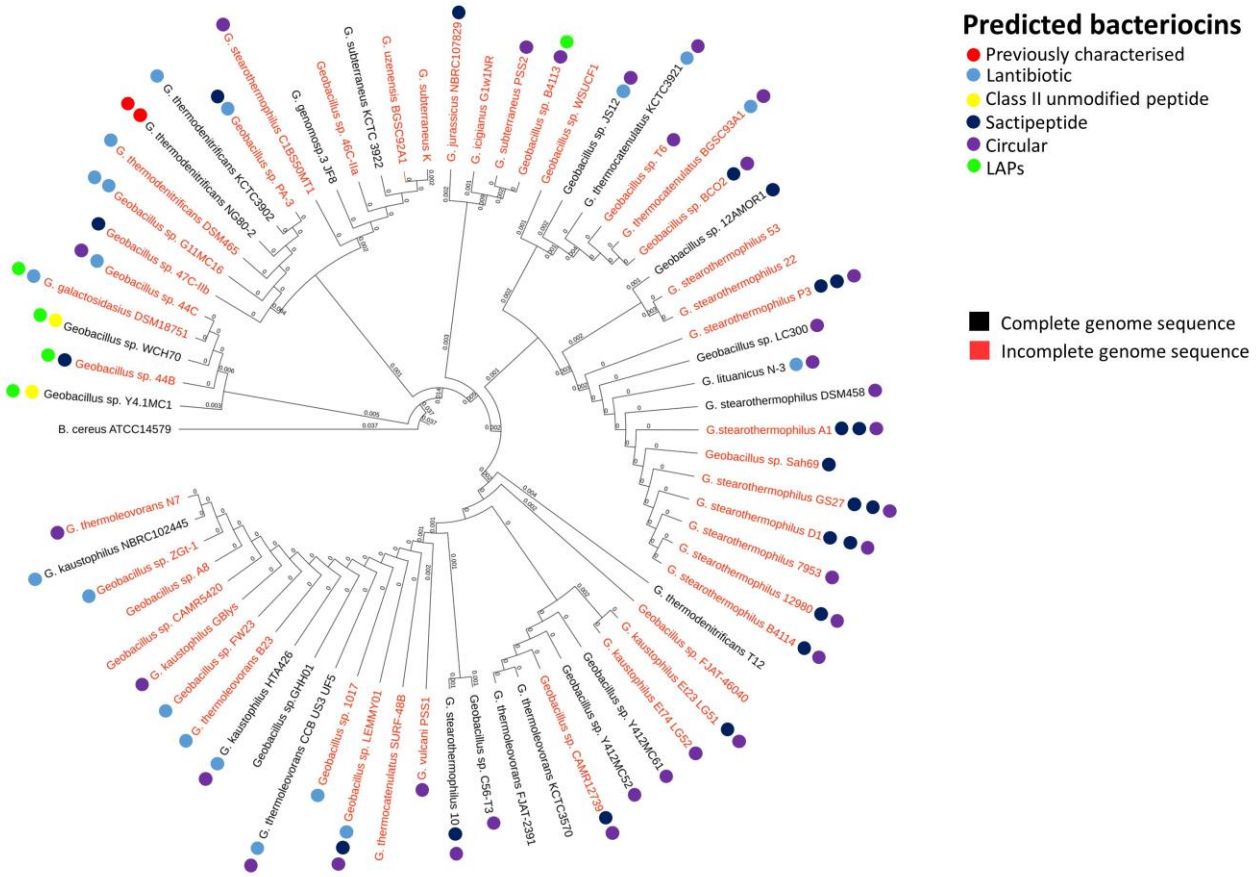

Figure 1. Phylogenetic arrangement of *Geobacillus* genomes investigated in this study. The BAGEL3 peptide predictions are overlaid in order to examine associations between bacteriocin gene presence and position within the *Geobacillus* phylogenetic arrangement

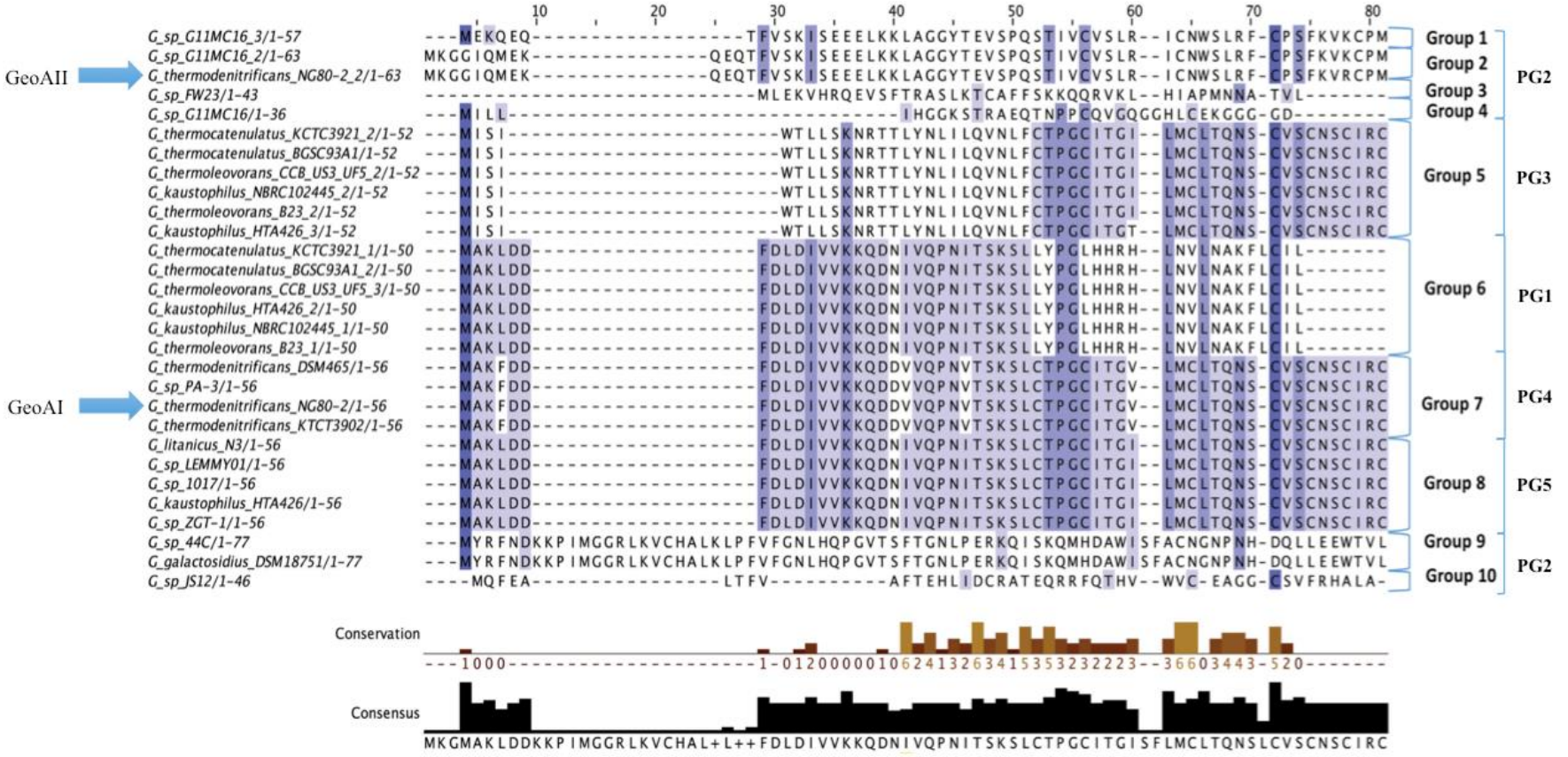

Figure 2. Multiple Sequence Alignment (MSA) of those lantibiotic peptides predicted. In some genomes multiple peptides were predicted within a single bacteriocin cluster and were therefore included as part of this alignment**.** PG: Phylogenetic group; GeoAI: Geobacillin I; GeoAII: Geobacillin II.

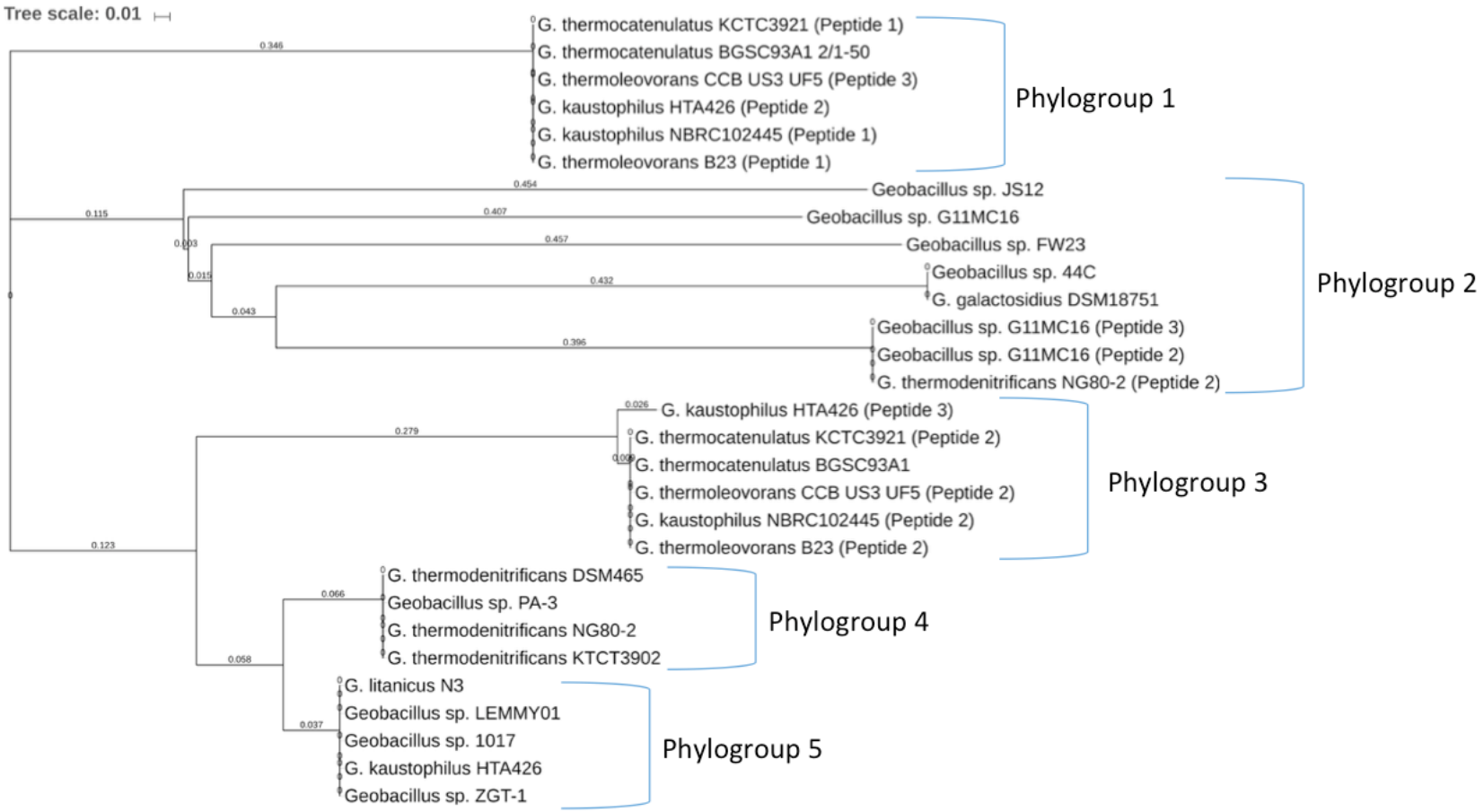

Figure 3. Phylogenetic arrangement of predicted lantibiotics

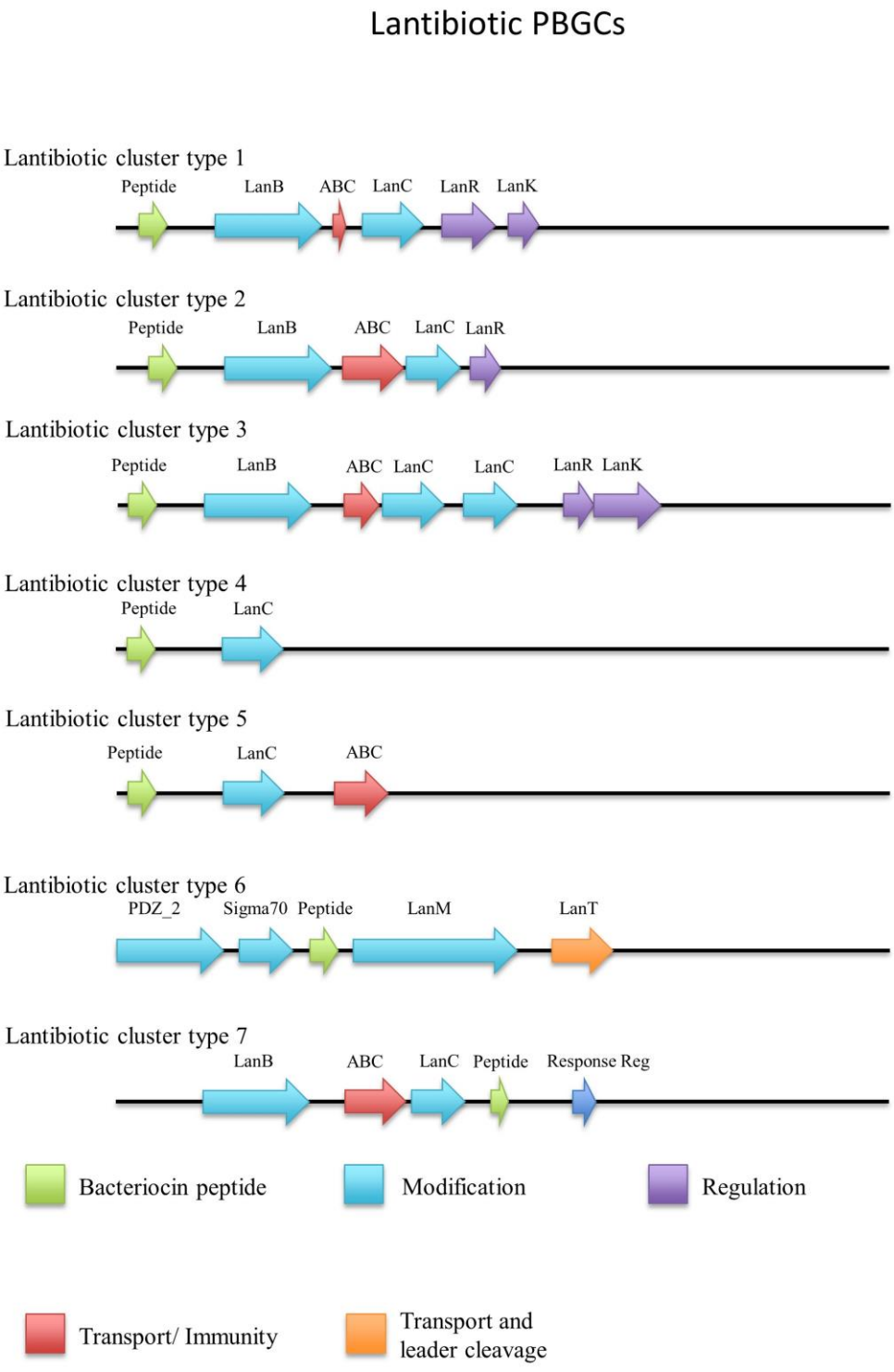

Figure 4. Lantibiotic cluster types predicted by BAGEL3

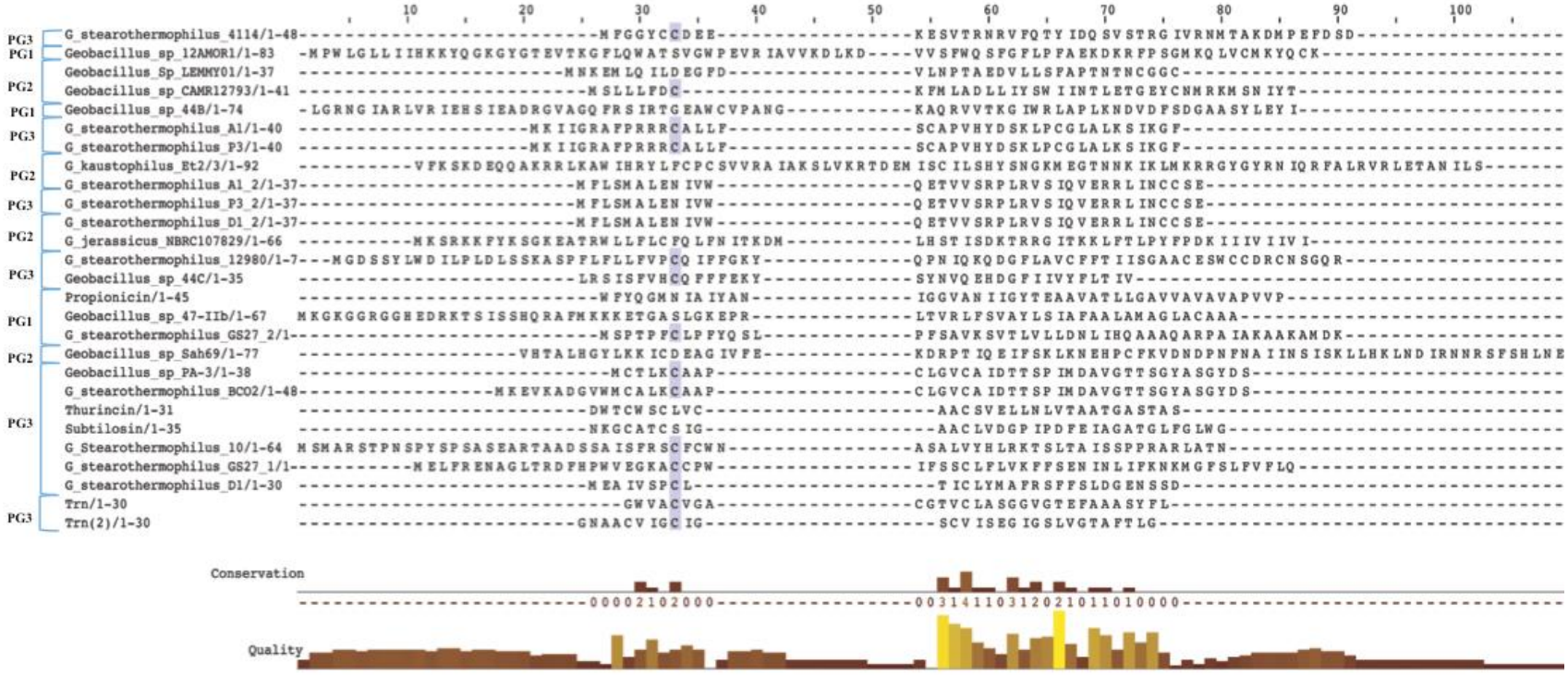

Figure 5. MSA alignment of Sactibiotic peptides predicted by BAGEL3 with characterised sactibiotic peptides. PG: phylogroup.

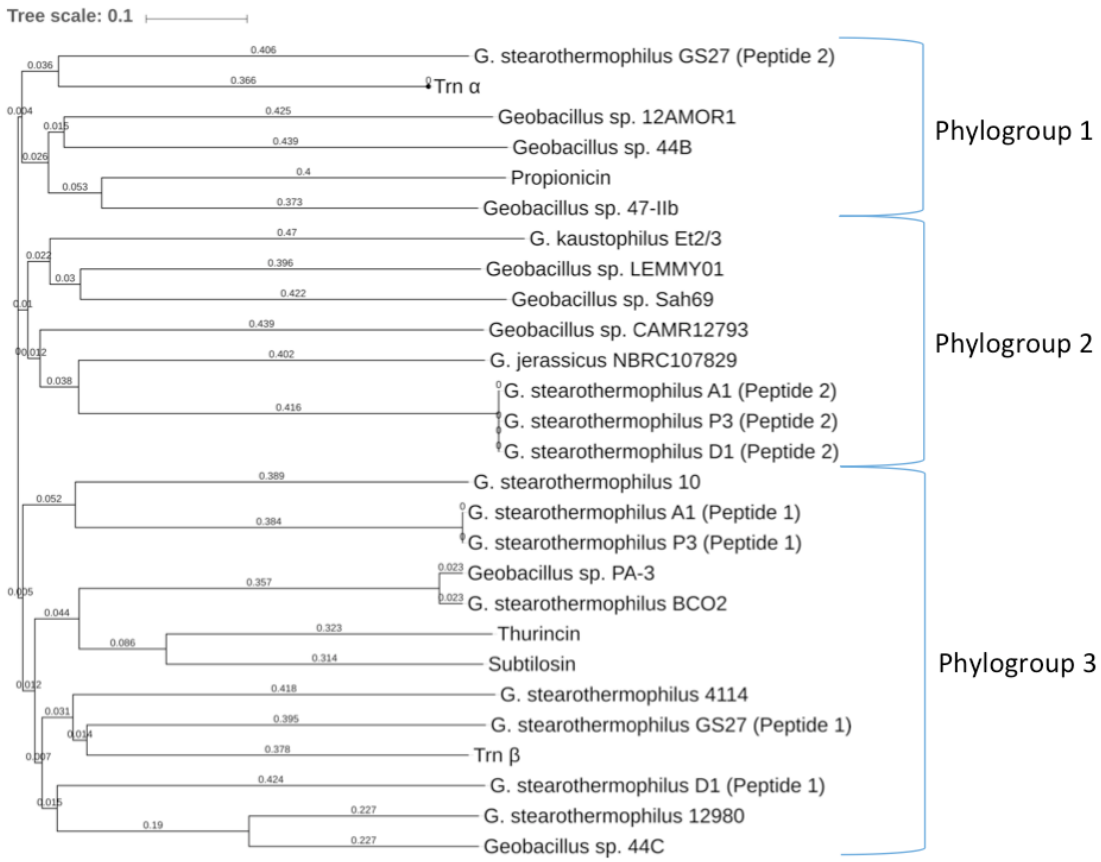

Figure 6. Phylogenetic arrangement of predicted sactibiotics

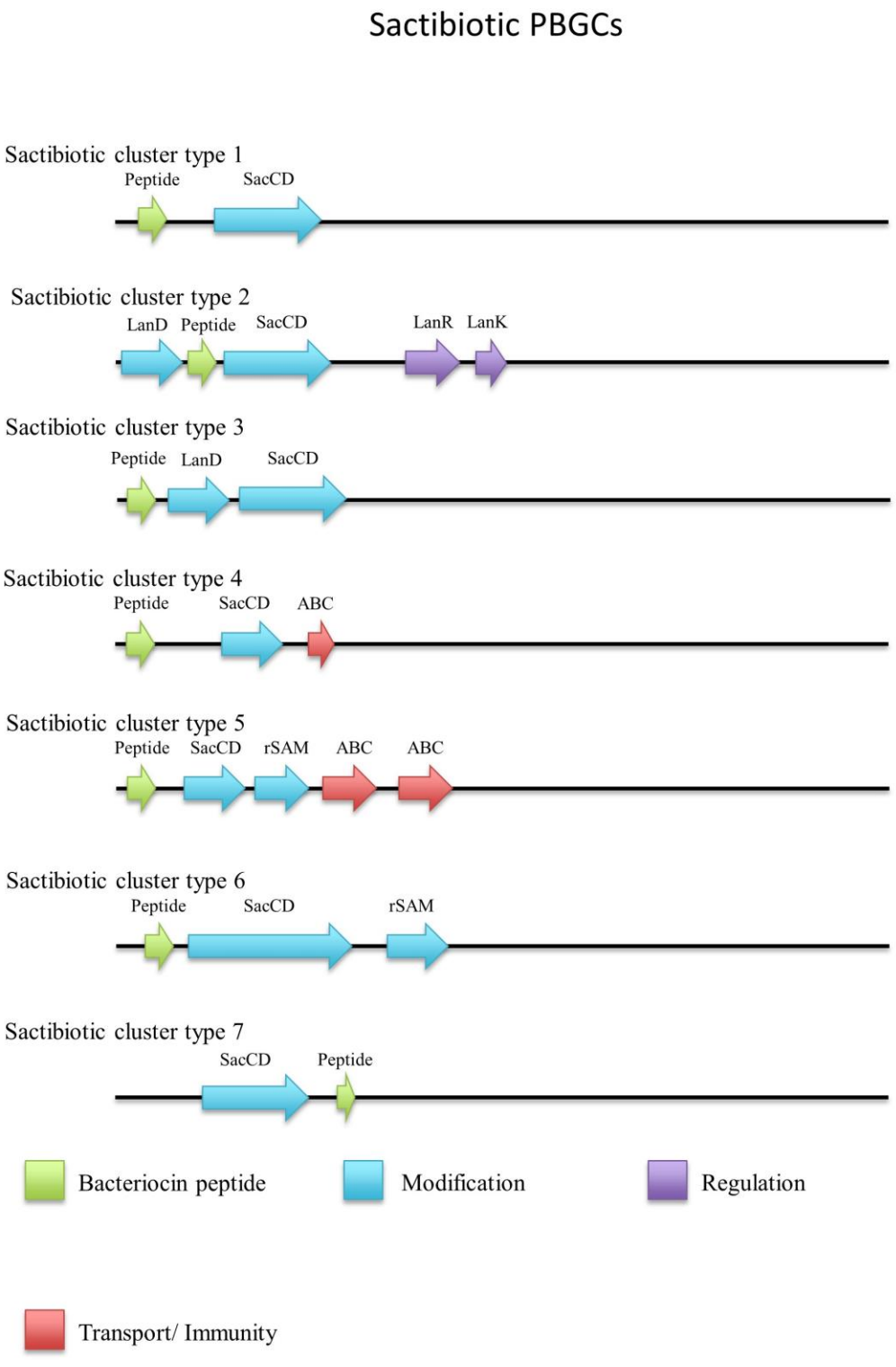

Figure 7. Sactibiotic cluster types predicted by BAGEL3

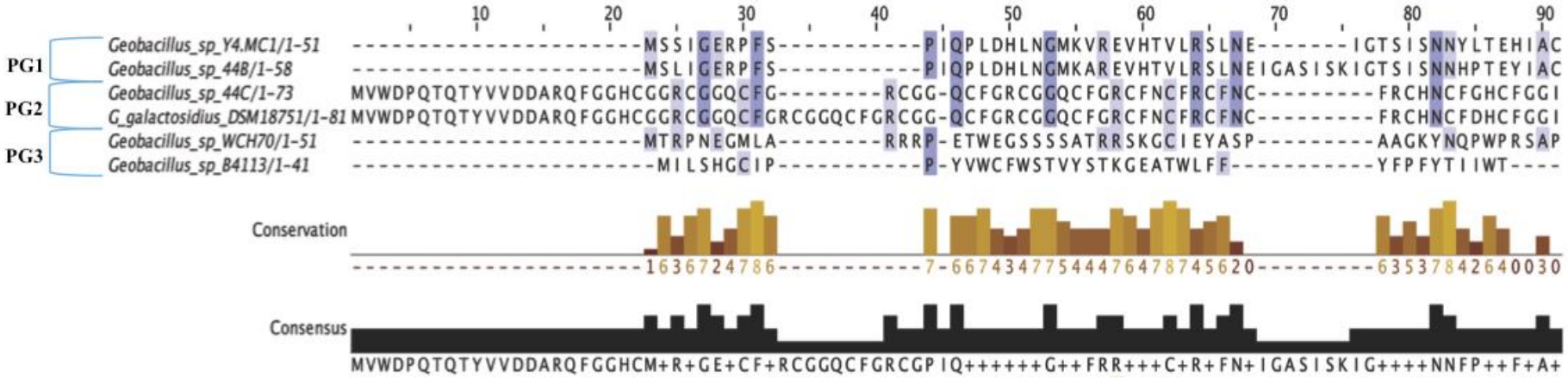

Figure 8. Linear Azole containing peptides predicted by BAGEL3. PG: phylogroup.

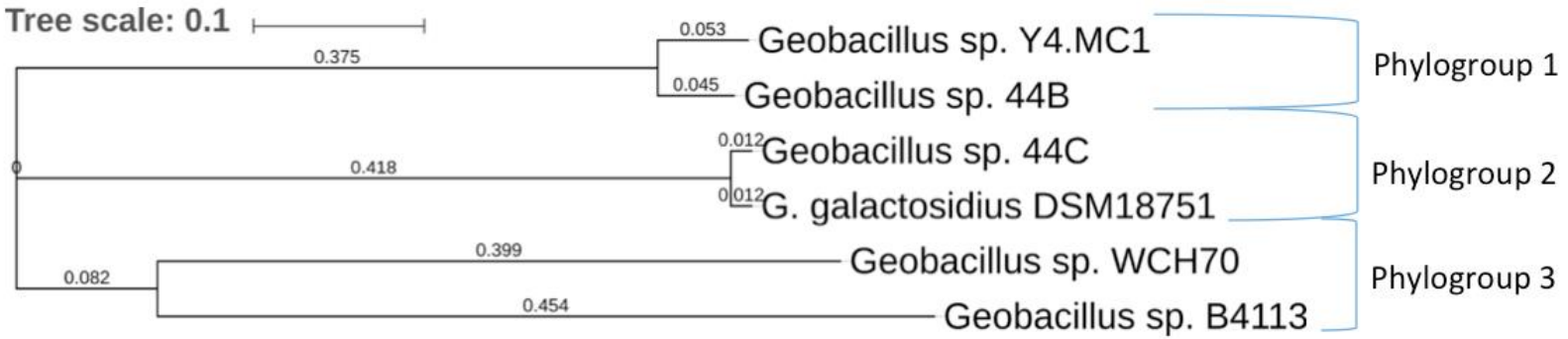

Figure 9. Phylogenetic arrangement of predicted LAPs

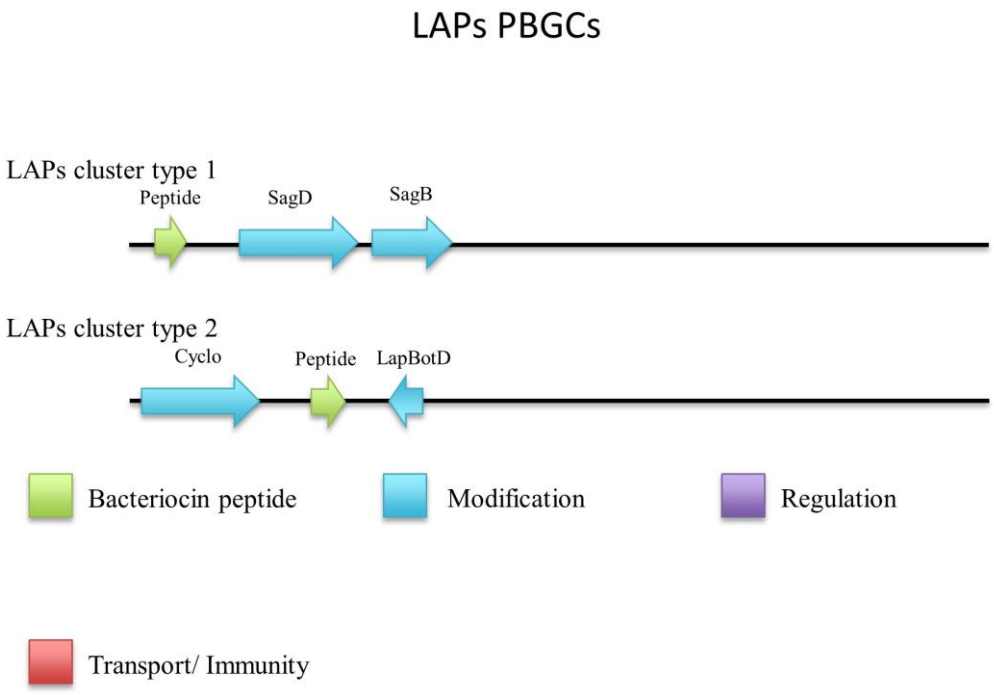

Figure 10. Linear Azole containing peptides (LAPs) cluster types predicted by BAGEL3

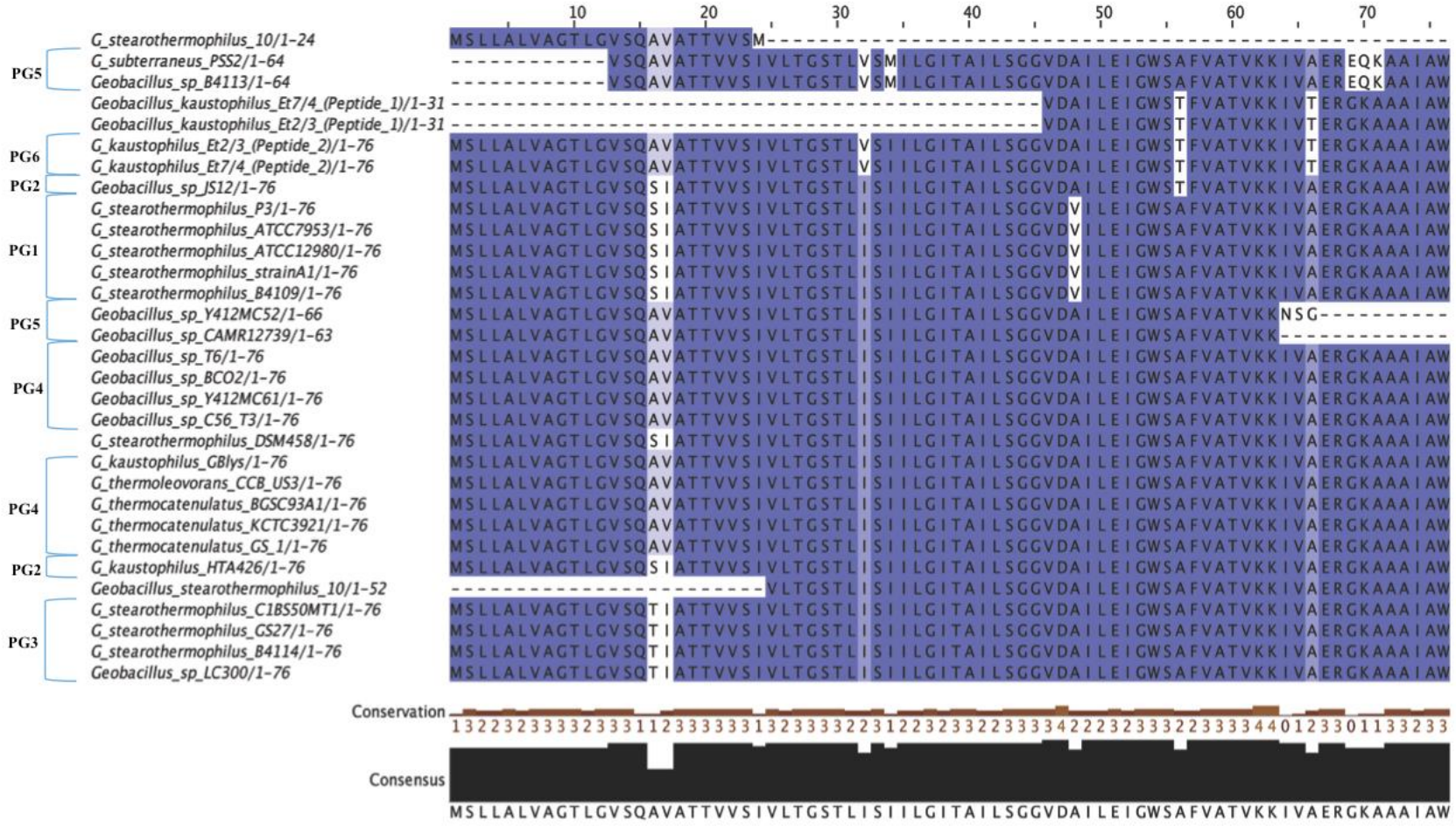

Figure 11. MSA of circular peptides predicted by BAGEL3. PG: phylogroup.

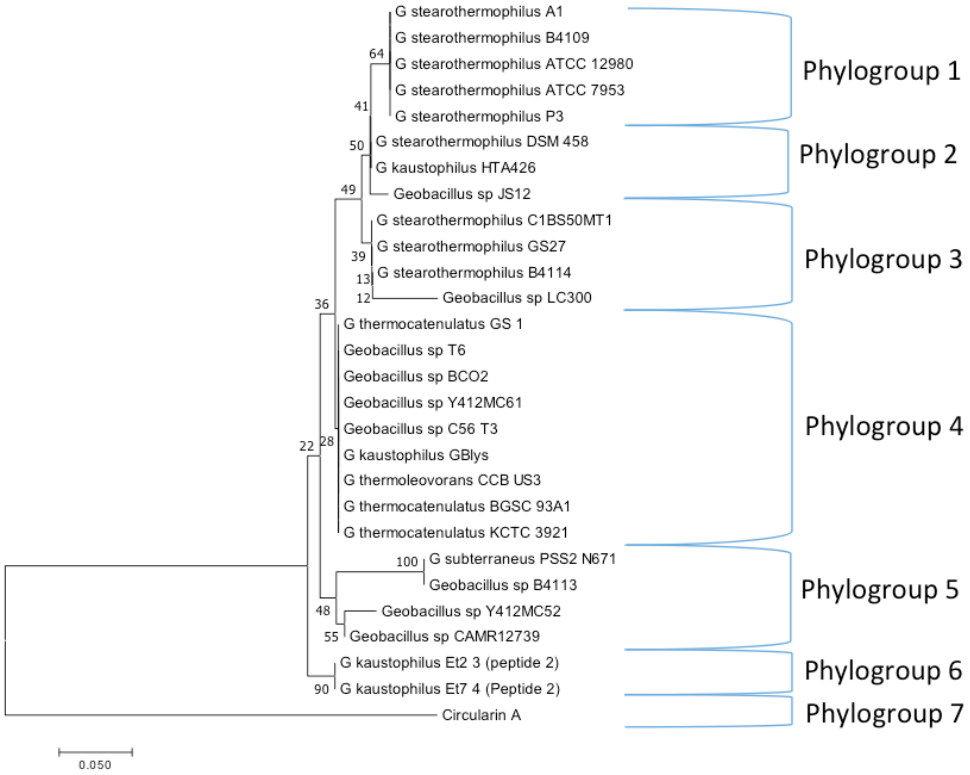

Figure 12. Phylogenetic arrangment of predicted circular bacteriocins

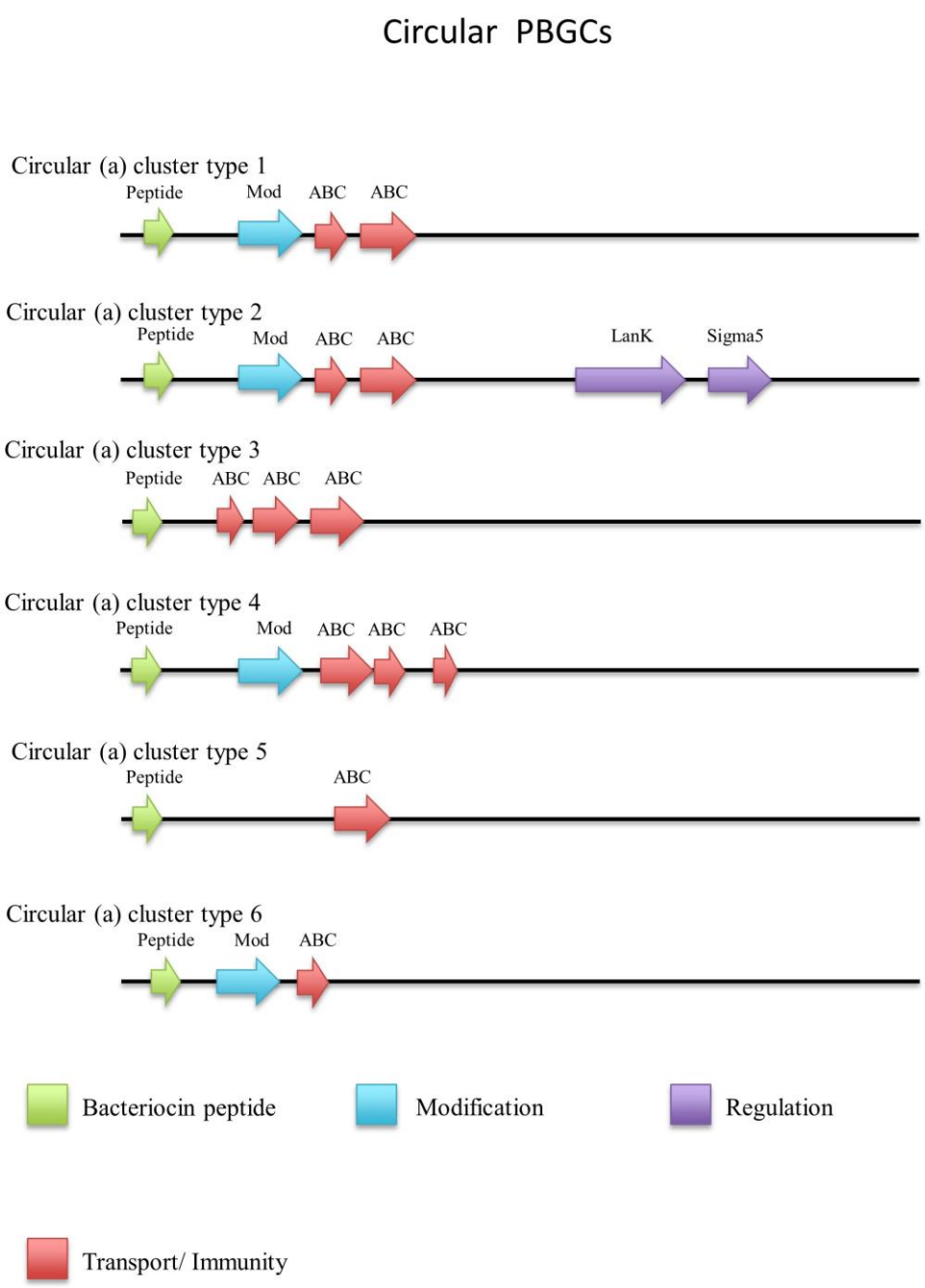

Figure 13. Circular (a) cluster types predicted by BAGEL3

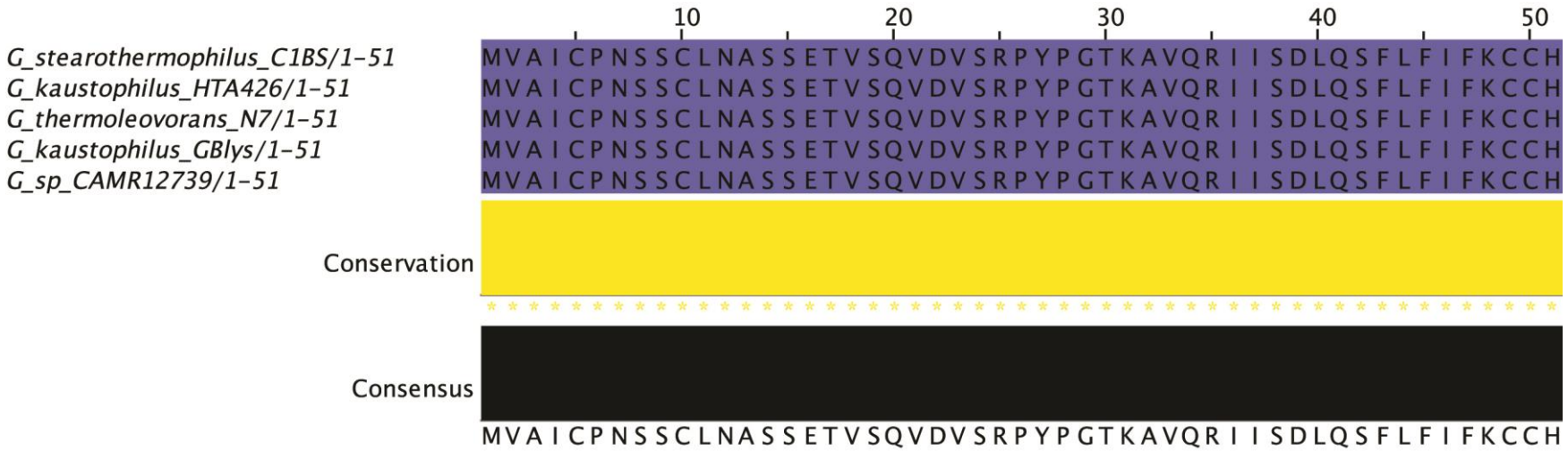

Figure 14. Circular peptides (b) predicted by BAGEL

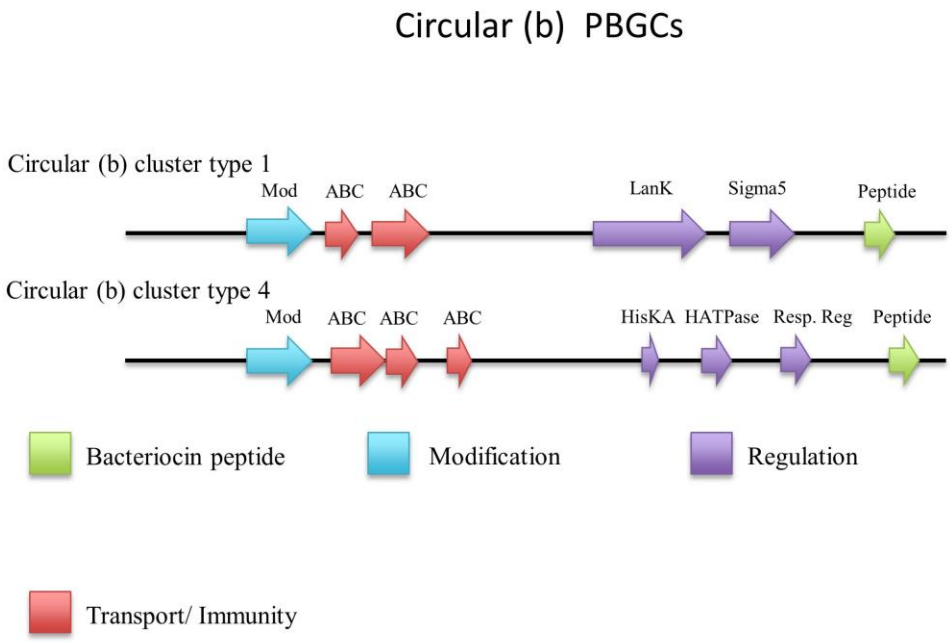

Figure 15. Circular (b) cluster types predicted by BAGEL

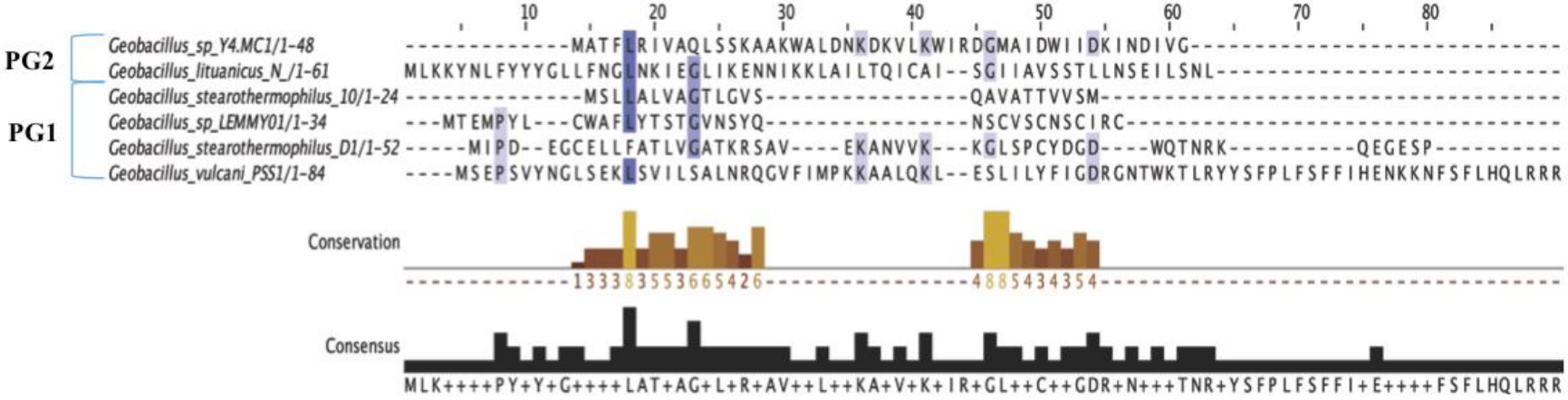

Figure 16. MSA of Class II bacteriocins predicted by BAGEL3**.** PG: phylogroup.

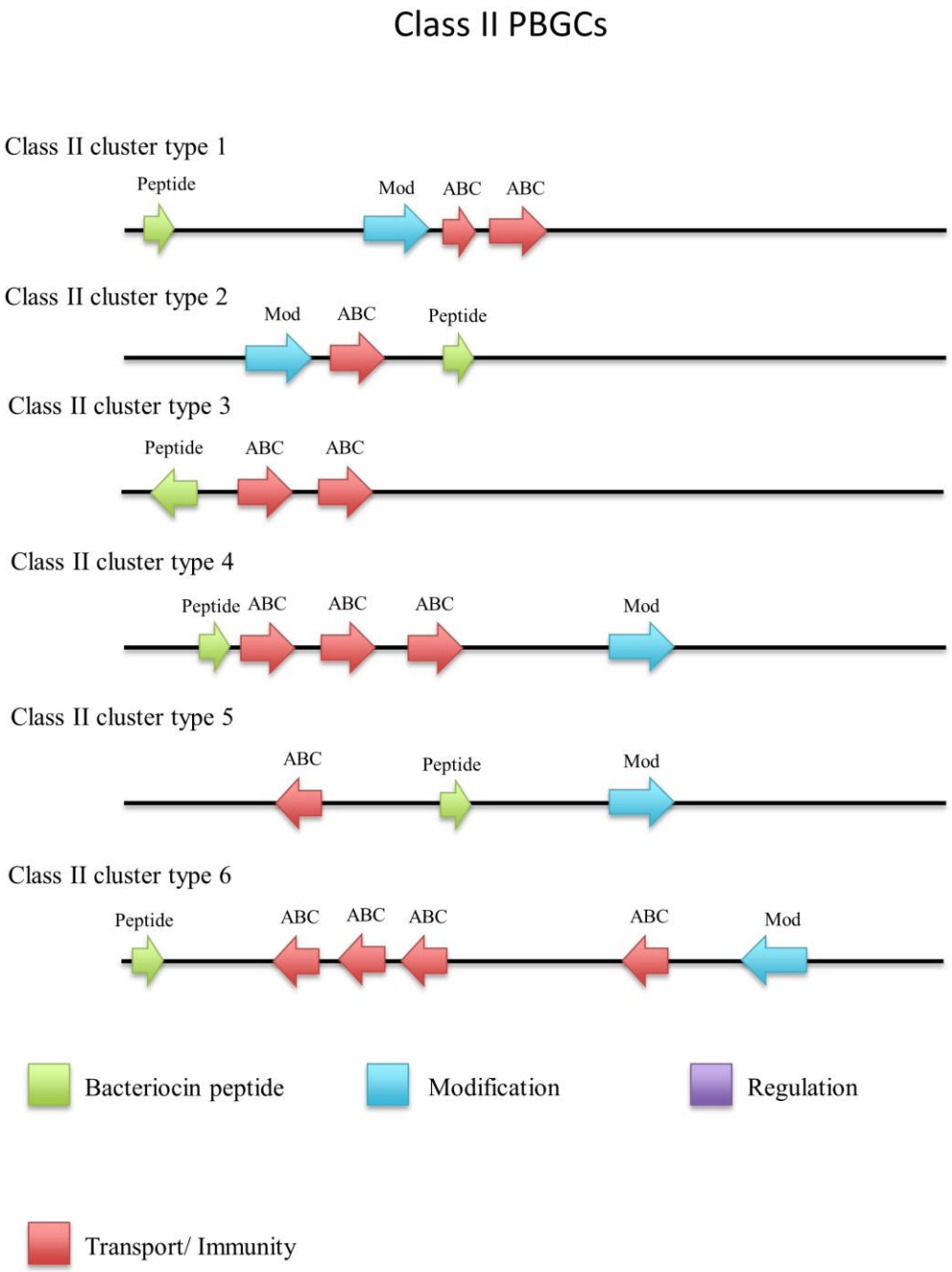

Figure 17. Class II cluster types predicted by BAGEL3

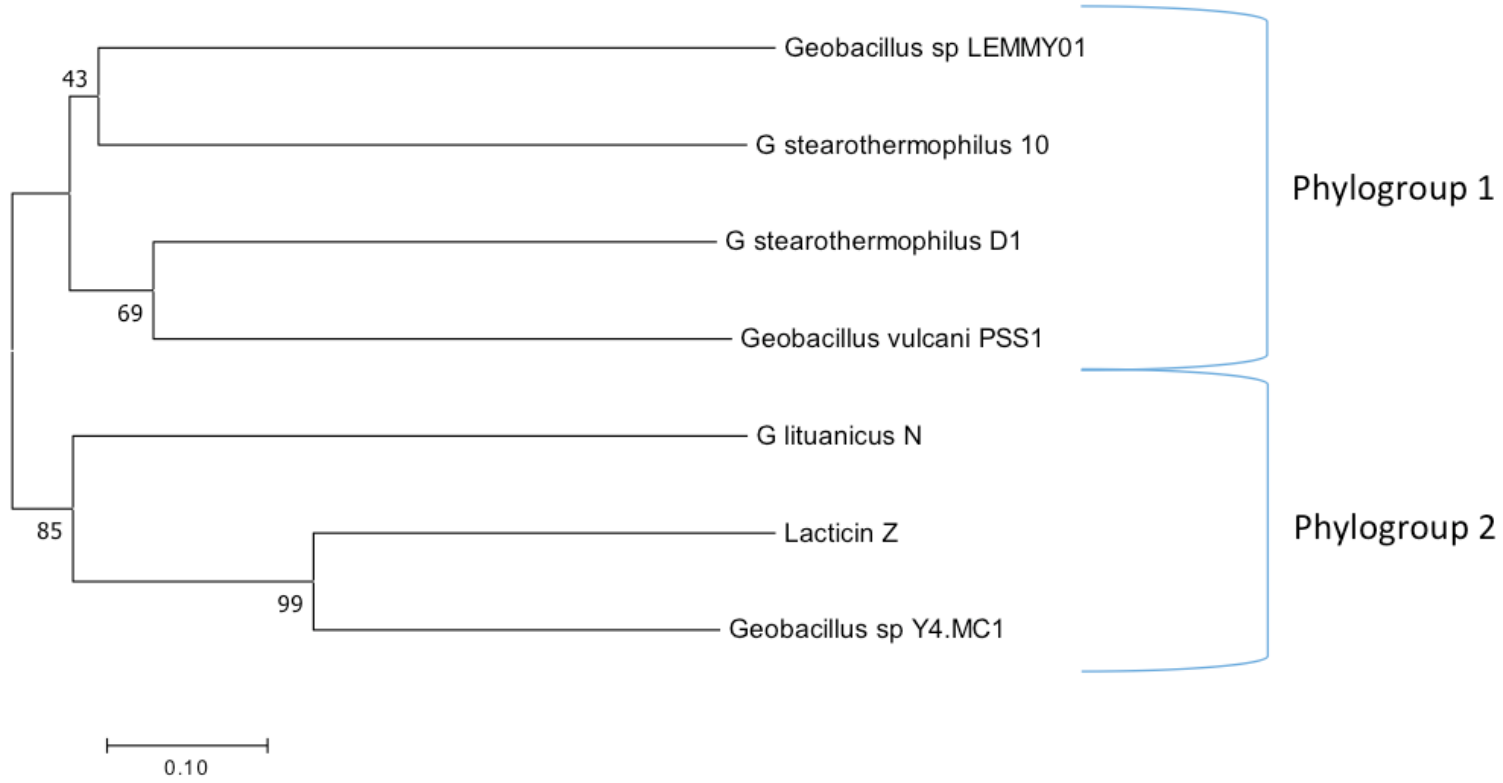

Figure 18. Phylogenetic arrangement of predicted class II bacteriocins.

Chapter IV

Generation and Development of Nisin Variant Producing Starter Cultures and Analysis of their Functional Qualities

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Abstract:

Starter culture innovation, research and development is extremely important for ensuring the future growth and development of the cheese industry. Enhanced food safety can be achieved though the production by starter cultures of a group of antimicrobial peptides called bacteriocins. In this study we use a food-grade mutagenesis approach to alter the amino acid structure of the bacteriocin nisin in *L. lactis* NZ9700 to produce the enhanced nisin S29A variant. Furthermore, crucial starter characteristics were conferred to the previously creatined nisin M21V and K22T variant producing strain by conjugating the lactose utilization plasmid into these strains. Their potential as starter cultures was assessed across a number of functional assays and their anti-*Listerial* capacity was assessed during acidification and as part of the cottage cheese production process. It was shown that all starter cultures created using this process could acidify milk effectively, however, only M21V and S29A variant producing starters displayed anti-*Listerial* traits equivalent to the wild-type peptide-producing starter. The K22T variant producing starter displayed reduced anti-*Listerial* activity which was comparable to the non-peptide producing starter culture. Overall this proof of concept work reveals a novel approach to starter culture improvement and also a potential means of enhancing food safety.

Introduction

The discovery and development of new starter cultures that display enhanced technological characteristics is important to meet the modern demands of both consumers and industry. Characteristics such as the inhibition of undesirable microbial growth, bacteriophage resistance, enhanced proteolysis, flavour development and exopolysaccharide formation are perhaps the phenotypic traits of highest priority (Fox et al., 2000). Indeed recently technological advancements and innovations have resulted in the development of lower fat and salt cheese which is gaining popularity among health conscious consumers (Johnson, 2016). Importantly, many of the aforementioned desirable starter culture traits are encoded on mobile genetic elements such as plasmids and when transmission occurs via conjugation, the resultant recipient strain continues to retain its food-grade status.

One such phenotypic trait that has played a significant role in cheese manufacture is the production of small, ribosomally synthesised antimicrobial peptides called bacteriocins (Cotter et al., 2005). These peptides can be encoded within the bacterial genome (such as gassericin A) (Kawai et al., 1998), on plasmids (such as lacticin 3147) (Dougherty et al., 1998) or sometimes on both as is the case with nisin (Rauch and De Vos, 1992). Bacteriocin production is widespread and diverse amongst the lactic acid bacteria (LAB) group and they are commonly produced *in situ* during the cheese making process by a wide variety of starter cultures from a number of genera (Guinane et al., 2005). The use of the bacteriocin nisin has proved popular in a variety of foods due its broad spectrum of activity and potent antimicrobial activity. Nisin is a 3.4 kDa peptide belonging to the class of bacteriocins that are know as lantibiotics due to the presence of post translationally modified amino acid residues

(Arnison et al., 2013). Over the last decade a number of studies (Field et al., 2008; Field et al., 2010; Rouse et al., 2012; Healy et al., 2013; Molloy et al., 2013) have sought to improve the functional qualities of nisin through the use of bioengineering. This has coincided with the discovery of a number of natural nisin variants in nature (F, H, Q, O, P, U, U2, Z) (Mulders et al., 1991; Zendo et al., 2003;Wirawan et al., 2006; de Kwaadsteniet et al., 2008; Zhang et al., 2012; O'Connor et al., 2015; Hatziioanou et al., 2017). Bioengineering has also allowed the production of a numbe of these natural variants in an isogenic background (Mierau and Kleerebezem, 2005; Piper et al., 2011).

Precise molecular biology techniques such as double cross over mutagenesis have made it possible to make changes to chromosomally encoded bacteriocin genes without the presence of leftover exogenous DNA or antibiotic resistance markers (Cotter et al., 2003). Indeed there have been a number of chromosomal nisin variants generated via double cross over mutagenesis such as M21V, K22S and K22T (Field et al., 2008; Field et al., 2010). As a result of the food-grade approaches utilised, the European Food Safety Agency (EFSA) / Environmental Protection Agency (EPA) have ruled that a number of nisin variants made in this way are not regarded as genetically modified microorganisms (Appendix 1).

Previously it has been shown that nisin M21V and S29A display enhanced activity against *L. monocytogenes* (Field et al., 2010; Field et al., 2012). While the enhanced activity of M21V against *L. monocytogenes* has been validated in foods (i.e. frankfurter meat and chocolate milk models) (Field et al., 2010; Field et al., 2015), corresponding studies have not been performed, to date with nisin S29A.

Furthermore, while the use of nisin has been examined in cottage cheese models through direct addition (Ferriera and Lund., 1996) or through the *in situ* production by nisin producing starter cultures (Dal Bello et al., 2012), the efficacy of nisin variants to control *L. monocytogenes* in cheese has yet to be tested. The use of nisin variants with enhanced phenotypes in this way has the potential to be of societal and commercial value as, despite the low incidence of listerosis in the EU, it remains a serious concern due to the various pathologies and mortalities which result from infection by *L. monocytogenes* (Oliver et al., 2005).

In this study, we utilize double crossover mutagenesis to alter the nisin structural gene and to conjugate the important industrial plasmid pLP712 (a 55.39 kb plasmid encoding genes for lactose catabolism and a serine proteinase involved in casein degradation (Wegmann et al., 2012) into these nisin variant producing strains in order to evaluate their potential as cheese starter cultures.

Materials and Methods

Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Table 1. *L. lactis* was grown at 30 °C on M17 agar and broth (Oxoid) supplemented with 0.5% glucose. *L. lactis* strains containing the plasmid pLP712 were grown on M17 supplemented with 0.5% lactose (Sigma-Aldrich). Broth culture of *Listeria monocytogenes* was carried out in Brain Heart Infusion (BHI) (Oxoid), and meanwhile Listeria was selectively recovered onto agar plates using Listeria Selective Agar (Oxoid) at 37 °C.

Creation of electrocompetent *L. lactis*

An overnight culture of the desired *L. lactis* strain a 1% v/v dilution was performed in fresh sterile GM17. Once the OD_{600nm} had achieved between 0.4 – 0.6, the culture was diluted serially to the 10^{-4} and 10µl of each dilution was added to 100 ml volumes of SGM17 (14.9g M17, 14g glycine, 27.4g sucrose and 2g glucose in 400 mls of H_2O). Following overnight incubation, cultures that had attained an OD_{600} of 0.2 – 0.4 were centrifuged at 4000 RPM for 10 mins after which the cell pellets were resuspended in 100mls of ice cold glycerol-sucrose (85.5g sucrose, 50mls glycerol in 500mls H_2O). This mixture was then centrifuged again as before and the supernatant removed. The cell pellet was once again resuspended in 1ml of the glycerol-sucrose solution and aliquoted into 50 µl aliquots and frozen at -80 °C.

Construction of food grade nisin-derivative producing strains

Using a combination of Quickchange site directed mutagenesis (stratagene) and double cross-over mutagenesis with pORI280 ($LacZ^+$, $RepA^-$) as previously described (Cotter et al., 2003; Field et al., 2008; Field et al., 2010), mutagenesis of the *nis*A gene could be achieved using *E. coli* EC101 ($RepA^+$) as a host. In order to introduce the desired mutations, the plasmid pDF06 (encompassing ~300bp either side of the *nis* A gene cloned into pORI280) was amplified with the quickchange system using primers (S29Afor/S29Arev) (Table 2). The resulting PCR products were then transformed into *E. coli* EC101. Potential candidates were identified using 'check' primers which were specific for the desired mutation in each case. Those potential candidates were then re-amplified using pORI280For and pORI280Rev and sequenced to confirm the successful mutagenesis and that no undesired alterations to the pORI208 plasmid had taken place. The pORI280nisXXX plasmids were then electroporated into *L. lactis* NZ9800 pVE6007 and transformants were grown on GM17 containing Xgal (80 µg/ml), chloramphenicol (5µg/ml), and erythromycin (2.5µg/ml). Single crossover recombination was achieved by passaging/curing the temperature sensitive pVE6007 plasmid at 37°C in GM17 broth containing Erythromycin over a period of 4 days followed by isolation of colonies on GM17 agar containing Erythromycin at the same temperature. The loss of pVE6007 was confirmed by the inability of colonies to grow on GM17 agar containing chloramphenicol. To achieve the second recombination event a number of potential candidates were brought forward for passaging in GM17 without antibiotic selection. After a period of 7 days the subcultures were plated daily on GM17 containing Xgal in order to identify variants which had lost the LacZ phenotype. Candidates were then analysed using deferred antagonism assays to observe enhance zones of

inhibition against a suitable indicator organism, which differentiated the successful mutant candidates from those which had reverted back to a non-producer phenotype. These Bac^+ candidates were further analysed using mass spectrometry in order to confirm the presence of the desired variant peptide mass. Finally in order to verify the loss of the pORI280 plasmid, the successful variants were checked for their sensitivity to erythromycin.

Conjugation of pLP712 into nisin producing strains

Conjugations were carried out between the donor strain *L. lactis* MG1614:pLP712 and the relevant recipient strain at a donor-to-recipient ratio of 1:1, 1:2 and 1:2.5 using a solid mating procedure adapted from Coakley et al., (1997). Briefly the donor and recipient strains were grown overnight in the appropriate media and after 18 hours of growth were sub-cultured (2%) and grown for 4 hours at 30°C. Three 1ml samples of the donor and recipient were pelleted and washed in maximum recovery diluent (MRD) twice and re suspended in 50µl of GM17. At this point the donor and recipient were combined at the desired ratios, spotted onto GM17 agar and incubated at 30°C for 18 hours. These spots were then harvested and diluted using MRD and plated onto agar that was selective for recipients and trans-conjugants. The selective agar contained Xgal (80 µg/ml) and was composed on 1:1 ratio of double strength (ds) LM17 and the cell free supernatant (CFS) of an overnight nisin producing culture that had been passed through a 0.45µm filter. Those colonies which appeared blue were then analysed for the presence of pLP712 by their acidifying capacity compared to the non-pLP712 containing nisin producing strain. Furthermore mass spectrometry and deferred antagonism assays were used to confirm the presence of the desired variant peptide production.

Strain validation

Acidification potential

Starter cultures which had been previously grown overnight in 10% RSM were inoculated into sterile 10% RSM (1% v/v inoculation). Following inoculation, the pH was monitored over a period of 24 hours in order ascertain the acidification profile of the starters. The rate of acidification was calculated as the mean rate of change in pH over the first 8 hours of fementation (dpH/dt) and was expressed as change in pH per hour. This dataset was then statistically analysed using a one-way ANOVA to determine if any of the acidification rates were statistically different ($P< 0.05$). The software package Graphpad Prism 8 was used to conduct this analysis.

Colony mass spectrometry

Colony mass spectrometry was performed as previously described by (Field et al., 2008). Briefly, bacteria were collected using sterile plastic loops and mixed with 50 µl of 70% isopropanol (IPA) then adjusted to pH 2 with HCl. The bacterial IPA suspension was vortexed and centrifuged at 14,000 r.p.m. for 2 minutes. The supernatant was retained for analysis. Mass spectrometry in all cases was performed with an Axima CFR plus matrix-assisted laser desorption/ionisation time-of-flight (MALDI TOF) mass spectrometer (Shimadzu Biotech, Manchester, UK.). A 0.5 µl aliquot of matrix solution (alpha-cyano-4-hydroxy cinnamic acid (CHCA), 10mg ml^{-1} in 50% acetonitrile-0.1% (v/v) trifluoroacetic acid) was placed onto the target. This matrix solution was left for 1-2 minutes and then removed. The residual solution was then air dried and the sample solution that was previously prepared was positioned onto the pre-coated sample spot. Matrix solution (0.5 ml) was added to the sample

and allowed to air dry. The sample was subsequently analysed in positive-ion reflectron mode.

Minimum inhibitory concentration assays

Minimum inhibitory concentrations assays for chloramphenicol and erythromycin were determined for the nisin variant producing cultures. Briefly,stock solutions containing 4X the desired starting concentrations of antibiotic (100 and 20 μg/ml of chloramphenicol and erythromycin respectively) were generated. 100μl of GM17 was added to each well of a 96 well plate. To the first well 100μl of the antibiotic stock solution was added and diluted two fold across the remaining wells of the plate.

A 2% inoculum of each strain to be tested was grown to 0.5 OD_{600nm} and diluted to give a final cell number of ~10^5 CFU/ml. 100μl of this inoculum was then added to each well containing the various concentrations of antibiotic. The MIC was determined visually as the minimum concentration at which the specific antibiotic inhibited growth.

Deferred antagonism for confirmation of antimicrobial activity

The nisin producing strains were grown in LM17 overnight for 18hrs. 10uL of this culture was spotted onto the surface of LM17 and incubated for an additional 18hrs. The surface of the LM17 plate was UV treated for 40 mins to eliminate the presence of bacteriocin producing bacteria. The plate was then overlaid with *Listeria monocytogenes* 33413 and *L. lactis* HP in an appropriate media containing (0.75%)

agar and incubated for 18 hours at 37ºC and zone sizes measured accordingly. All zone sizes were expressed as total area of inhibition (mm2).

Effect of nisin producing starter cultures on contaminated milk with *L. monocytogenes* during acidification

10 mls of sterile RSM (10% w/v) was inoculated the $1X10^5$ CFU/ml of indicator microorganism (*L. monocytogenes* 33413). The contaminated milk was then inoculated with 1% w/v of the desired starter culture overnight culture and dispensed into 6 1ml Eppendorf tubes (each Eppendorf tube representing one sampling time point) and incubated at 30°C. An Eppendorf tube was then removed at each time point (0, 2, 4, 6, 8, and 24 hours) and the indicator enumerated using *Listeria* selective agar for detection of *L. monocytogenes*. This was carried out in triplicate.

Cottage cheese manufacture

Sterile 10% RSM was inoculated with the cheese starter cultures (*L. lactis* NZ9800, NZ9700, NZ9700::M21V, NZ9700::K22T and NZ9700::S29A) and grown for 18 hours at 30 °C. 40mls commercial low fat milk which had previously been dispensed into sterile 50ml centrifuge tubes was then inoculated (1% v/v) with the overnight starter cultures and incubated for 30 mins at 32°C, after which time 60µl of rennet was added (from a 1:100 dilution of stock rennet; Maxiren, DSM, Netherlands). The centrifuge tube(s) were incubated at 21°C for 18 hours after which the coagulum was cut in vertical strokes using a 1µl inoculating loop. The curds were then cooked by placing the tubes into a water bath and increasing the temperature over a 90 minute period to 52°C. The whey was then removed via centrifugation (5000 RPM for 60 seconds) and the curds washed at 20 minute intervals using sterile deionised water at

20, 10 and 4 °C. Once all liquid was once more removed from the curds they were allowed to stand overnight at 4°C. After overnight resting a cream dressing (composed of 54% v/v single cream, 42% v/v non-fat milk and 4% w/v NaCl) was added at a ratio of 1 part cream to 3 parts curd.

Analysis of cottage cheese

Listeria contamination of cottage cheese

This dressing was spiked with *L. monocytogenes* 33413 from a fresh overnight culture bringing the final concentration of *L. monocytogenes* in the cheese to $1X10^5$CFU/g. The cheese was then incubated in triplicate at 4°C, 8 °C and also at room temperature. The cheese was sampled on days 0, 1, 2, 3 and 7, and the *Listeria* enumerated on Listeria selective agar (Oxoid).

Evaluation of pH during storage at 4°C

In order to evaluate the changes in pH of the cottage cheese at refrigeration temperature, 1g of cheese was resuspended in 10mls of deionised H_2O. This mixture was vortexed for 10-20 seconds and the pH of the resultant slurry measured using a digital pH meter (Mettler toledo).

Results

Strain construction and validation

This study set out to create a panel of novel *Lactococcus lactis* cheese starter cultures capable of producing the nisin variant peptides: M21V, K22T and S29A. This production of variant peptides was achieved through the process of double crossover recombination, where the *nis*A gene on the *L. lactis* NZ9800 chromosome was altered to encode the desired variant peptide. Importantly *L. lactis* NZ9800 encodes a non-functional *nis*A gene (due to a 4 base pair deletion and introduction of a stop codon (Miereau and Kleerebezem., 2005) and thus nisin production is restored following successful gene replacement via double crossover recombination. Furthermore this process does not require the new strain to be regarded as a genetically modified microorganism (GMM) as the resultant nisin variant producing *L. lactis* does not contain any residual antibiotic resistance genes or other plasmid remnants following the mutagenesis process. Furthermore, this non-GMM status was confirmed by the EPA/EFSA in 2014 (Appendix 1.). *L. lactis* NZ9800::M21V and *L. lactis* NZ9800::K22T had been previously created (Field et al., 2008; Field et al., 2010), and *L. lactis* NZ9800::S29A was created in this study using the double crossover mutagenesis process.

Confirmation of successful replacement of the wild type *nis*A gene in *L. lactis* NZ9800 to nisS29A was achieved through the combined use of colony mass spectrometry and deferred antagonism (Fig 1). More specifically, an antimicrobial producing phenotype was restored to the newly generated strain, this activity was greater than that associated with *L. lactis* NZ9700 (nisin A producers) and the mass

of the antimicrobial produced, 3336.65 was consistent with that of a S29A variant (and contrasts with the wild type mass of 3352.36).

The sensitivity of the recombinant *L. lactis* NZ9800::S29A to chloramphenicol and erythromycin was indicative of the loss of the pORI280 and pVE6007 plasmids used to carry out the double crossover mutagenesis strategy. This sensitivity is evident through the use of agar plates containing these antibiotics, however it was further confirmation involved the use of more accurate minimum inhibitory assays (Table 3). This loss antibiotic resistance genes involved in the double crossover recombination process is critical to retain the non-GMM status of these starter cultures.

While the nisin variant producing *L. lactis* derived from *L. lactis* NZ9800 can grow in a dairy environment, they do not always have the potential to be used as cheese starter cultures due to their inability to rapidly acidify milk. Here these phenotypic characteristics were bestowed on these strains through the conjugation of the important lactose utilization and proteinase plasmid pLP712. Nisin variant-producing trans conjugates were created through the use of the donor strain *L. lactis* MG1614:pLP712. A selective media was employed which utilized the sensitivity of donor *L. lactis* MG1614 cells to wild type nisin and detection of lactose utilizationon the basis of the ability to degrade Xgal to produce blue colonies by transconjugates.

Acidifying ability of starter cultures

Successful conjugation of the pLP712 plasmid into the stable variant peptide producers resulted in the ability of the transconjugates to rapidly acidify milk. It was

observed that the nisin wild type and variant-producing *L. lactis* transconjugates similarly acidified 10% RSM (Table 4). Initally, it was observed that the transconjugates had similar acidification profile to that of the pLP712 donor strain (*L. lactis* MG1614). When the rate of acidification (Table 4) was calculated over the first eight hours however, it was observed that the pLP712 transconjugates showed slightly reduced acidification rates when compared to the *L. lactis* MG1614 plasmid donor (0.198 dpH/h). Indeed these rate(s) of acidification were 0.190, 0.185, 0.160, 0.189, and 0.186 (dpH/h) for the wild type, non nisin-producer, M21V, K22T and S29A transconjugates respectively. The commercially relevant starter cultures: *L. lactis* HP, *L. lactis* 303 and *L. lactis* AM2 had acidification rates of 0.079, 0.168 and 0.173 (dpH/h) respectively. When the data was statistically compared using a one-way ANOVA and it was determined that the differences in rate of acidification were not statistically different ($P<0.05$) for any of the starter cultures.

Antimicrobial potential of starter cultures during acidification of milk

While the nisin producing starters created in this study have the ability to rapidly acidify milk, their ability to control potential food pathogens in milk is also of great interest. Here, the starter cultures were inoculated into milk containing high levels of *L. monocytogenes* 33413 in a contaminated bulk milk tank model. It was shown that those starters producing the nisin variant peptides S29A, M21V and wild-type nisin completely eradicated *L. monocytogenes* 33413 after an acidification period of 24 hours (Figure 2). The K22T producing starter did not have the same effect with approximately $5X10^5$ CFU/ml remaining after 24 hours, which was comparable in activity to the non-producer which contained $1X10^6$ CFU/ml of *L. monocytogenes* 33413.

Cottage cheese model

Cottage cheese was selected as a model to investigate if the newly generated starters could be used to control *Listeria* in cheese. In order to facilitate the screening of the 5 starter cultures in this study, a cottage cheese protocol described by Dal Bello et al. (2012) was adapted to allow for the creation of a mini-cottage cheese model (Figure 3). This model proved very valuable as a high throughput cheese model, and allowed all 5 starter cultures to be examined: *L. lactis* NZ9800, *L. lactis* NZ9700, *L. lactis* NZ9800::M21V, *L. lactis* NZ9800::S29A and *L. lactis* NZ9800::K22T. The cottage cheese dressing was spiked to give a final concentration of $1X10^5$ CFU/g *L. monocytogenes* 33413 and the survival of the pathogen was tested over 7 days at 3 temperatures: 4, 8 and 21 °C. Although at every temperature sampled the non-nisin producing starter culture contained the highest levels of *Listeria*, there were no notable differences between the various nisin variant producing starter cultures and the wild type control (Figure 4).

The pH values of the cottage cheese made with nisin variant producing starter cultures was also analysed over a fourteen day period and again no differences were observed between the various starter cultures. Furthermore, it was noted that the mini cottage cheeses had an initial pH of approximately 4.8, however this pH value dropped to 4.15 after 7 days and increased to 4.25 after 14 days (Table 5).

Discussion

This study sought in principle to combine both modern and classical microbiological techniques in order to create and develop a novel cheese starter culture system. Here the application of double crossover recombination and plasmid conjugation were employed to alter the amino acid structure of the nisin peptide and also to confer phenotypic starter culture characteristics respectively. Mass spectrometry and deferred antagonism assays were used to confirm that the accurate chromosomal mutagenesis of the gene corresponding to nisin S29A had occurred. Furthermore, MIC assays with chloramphenicol and erythromycin also confirmed that the *L. lactis* strains now producing the nisin variants had lost all plasmid-related DNA (pORI280 and pVE6007) utilized in the recombination process, thereby confirming their food-grade status (Cotter et al., 2003). Bacteriocin production by starter cultures originating from chromosomally-encoded genes is more advantageous over plasmid DNA due to the instability of plasmids without specific selection (i.e. lactose). Chromosomally encoded bacteriocin genes are however more difficult to manipulate in comparison to plasmid DNA, requiring greater resources in both time and technical expertise. However, recent advancements have demonstrated the ability to quickly and efficiently generate targeted point mutations in the chromosome without the need for antibiotics, though in some instances the technology may result in the loss of the food-grade status of the recombinant strains (van Pijkeren and Britton., 2012). Furthermore, advancements in whole genome sequencing (Rhoads and Au, 2015; Lu et al., 2016; Raine et al., 2018) and CRISPR genome editing technology (Sander and Joung, 2014) in the coming years may perhaps facilitate the routine mutagenesis of genes within the chromosome rather than using recombinant technology that was common-place over the last number of decades, and that

employed in this study. Furthermore, the precise mechanism of CRISPR-Cas gene mutagenesis should theoretically allow for the development of further food-grade bacteriocin variants based on the previously outlined criteria.

Conjugation of the plasmid pLP712 facilitated the acquisition of acidification and proteolytic abilities. While previously it has been shown that the presence of the nisin transposon has a metabolic load on acidification rates (Mills et al., 2017), here this metabolic load was observed with *L. lactis* NZ9700 pLP712 and nisin variant transconjugates acidifying RSM at a lower rate that the donor strain. Meanwhile the presence of pLP712 did in fact facilitate acidification by the wild-type, S29A and K22T transconjugates at a rate that could be considered of commercial interest when compared to those industrial starter cultures *L. lactis* 303 and *L. lactis* AM2 (Sheehan et al., 2006; Cavanagh et al., 2015). Interestingly there were differences in the rate of acidifcation between the four transconjugate strains for which the difference cannot be explained, but could be due in part to genetic / structural instability previously described for the pLP712 plasmid, resulting in the loss of casein utilization and / or lactose utilization (Wegmann et al., 2012).

Potentially the most interesting finding in this study was that wild type and the M21V and S29A nisin producing variants can eradicate *L. monocytogenes* 33413 during the acidification stage of the cheese making process. While the variants did not demonstrate enhanced activity corresponding to that as observed in *in vitro* studies with purified peptides (Field et al., 2008; 2010; 2012), they did perform equally as well as the wild type, which could be due in part to the effects of cheese matrix. Another study by Lianou and Samelis (2014) demonstrated that the removal

of the autochthonous antagonistic microbiota in raw milk through heat thermisation facilitated the growth of *Listeria*. When the thermised milk was re-supplemented with a nisin A producer, *Listeria* numbers remained at initial inoculum levels and were not totally eradicated, as observed in this study. Although K22T has been previously reported to have identical specific activity against *L. monocytogenes* as wild-type nisin, we see here that its activty is greatly diminished and is equivalent to that of the non-nisin producing starter (Field et al. 2010). Furthermore, changes to position 22 of nisin have previously been reported to increase its activity in complex matrices (Rouse et al., 2012), however this is not the case here. It could be reasoned that this could be due to non-specific binding of K22T to a component of the whey-curd matrix, conferred by the specific alteration to the peptide amino acid sequence.

In this study a mini-cottage cheese model containing ~5g curd was developed using modified cottage cheese protocol set out by Dal Bello et al. (2012). It has been shown previously that the presence of 2000IU g^{-1} nisin in cottage cheese increases *L. monocytogenes* inactivation by 1000 fold (Ferriera and Lund., 1996), however in this study we did not observe such a reduction when the cottage cheese was produced using nisin variant or wild-type nisin producing starter cultures. One reason for this difference may be related to the capacity of the strains to produce sufficient levels of nisin, which is calculated to be approximately 10 mg/L (data not shown). In the study by Field et al., (2010), the two most nisin A resistant *L. monocytogenes* strains had nisin A minimum inhibitory concentrations of 12.57 mg/L. Thus, an issue of insufficient bacteriocin production *in situ* may be a factor in the inability of these nisin producers to completely eradicate *L. monocytogenes* 33413 in Cottage cheese. Another explanation could be that the various washing steps could have diluted nisin

peptide from the curd. Additionally, the chemical composition and treatment of foods as well as the initial level of *L. monocytogenes* contamination are all of crucial importance (Bhatti et al., (2004). Overall, our results are in agreement with those of (Dal Bello et al., 2012) where a decrease in *L. monocytogenes* was observed for the nisin A producing starter culture compared to the bacteriocin negative starter culture. It should also be noted that the nisin variants produced by the starter cultures display enhanced activity against many other bacteria when assessed *in vitro* as purified peptides (Field et al., 2010). Thus, future work will also focus on the ability of the starter cultures to target bacteria that are relevant/undesirable in cheese such as non-starter lactic acid bacteria (NSLAB) and *Staphylococcus aureus*, and so perhaps could find applications in controlling other bacteria in cheese and milk.

While these starter cultures do not provide a major bio protective effect to cottage cheese during long-term storage, they do show promise in performing essential starter culture functions and also at lowering and eradicating *L. monocytogenes* during the milk acidification process. Perhaps these cultures could find applications in non-pasteurised cheeses where *L. monocytogenes* control is vital to their safe widespread use. These starters may also be of use in pasteurised cheeses where they can provide a second defence as part of the hurdle concept post pasteurisation. The impact of the starter cultures generated in this study on the cheese microbiota (Yeluri Jonnala et al., 2018) is also worth investigating, as we have seen certain amino acid changes in nisin confer specific differences in activity towards a variety bacterial species (Field et al., 2010; Field et al., 2012; Molloy et al., 2013). Finally this study nicely demonstrates the successful application of mutagenesis to starter culture

technology using a food-grade approach for future starter development to enhance functional qualities.

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Table 1. Strains used in this study

Strains/plasmids	Characteristics	Reference/Source
Strains		
L. lactis NZ9800	*L. lactis* NZ9700Δ*nisA*	Kuipers et al., (1993)
L. lactis NZ9800 pVE6007	*L. lactis* NZ9700Δ*nisA*, $RepA^+$	Kuipers et al., (1993)
L. lactis NZ9800 pLP712	Lac^+ Pro^+ *L. lactis* NZ9800	UCC culture collection
L. lactis NZ9700	Wild type nisin A producer	
L. lactis NZ9700 pLP712	Lac^+ Pro^+ *L. lactis* NZ9700	This study
L. lactis NZ9700::M21V	Stable nisin M21V producer	Field et al., (2008)
L. lactis NZ9700::M21V pLP712	Lac^+ Pro^+ stable nisin M21V producer	UCC culture collection
L. lactis NZ9700::K22T	Stable nisin K22T producer	Field et al., (2008)
L. lactis NZ9700::K22T pLP712	Lac^+ Pro^+ stable nisin K22T producer	This study
L. lactis NZ9700::N20P	Stable nisin N20P producer	UCC culture collection
L. lactis NZ9700::N20P pLP712	Lac^+ Pro^+ stable nisin N20P producer	This study
L. lactis NZ9700::S29A	Stable nisin S29A producer	This study
L. lactis NZ9700::S29A pLP712	Lac^+ Pro^+ stable nisin S29A producer	This study
L. lactis MG1614 pLP712	Lactococcal donor of Lac^+ Pro^+ phenotypes via	O'Sullivan et al. (1998)
E. coli EC101	*E. coli* host for pORI280	(Law et al., 1995)
L. lactis 303	Representative cheese starter	UCC culture collection
L. lactis AM2	Representative cheese starter	UCC culture collection
L. lactis HP	Representative cheese starter/ nisin sensitive	UCC culture collection
Indicator bacteria		
L. monocytogenes 33413	Indicator	UCC culture collection
L. lactis HP	Indicator	UCC culture collection
Plasmids		
pORI280	$RepA^-$, $LacZ^+$, Ery^R	(Leenhouts et al., 1996)
pDF06	pORI280-*nis*A	(Field et al., 2010)
pDF11	pORI280-*nis*S29A	This study
pVE6007	$RepA^+$ Cm^R, Temperature sensitive	(Maguin et al., 1992)

Table 2. Oligonucleotides used

Oligonucleotides	**Sequence**
S29AFor	5’ TGT CAT TGT GCT ATT CAC GTA AGC AAA TAA 3’
S29ARev	5’TACGTGAATAGCACAATGACAAGTTGCTGTTTTCATGTT 3’
S29ACheck	5’ GCA ACT TGT CAT TGT GC 3’
pORI280For	5’ CTCGTTCATTATAACCCTC 3’
pORI280Rev	5’ CGCTTCCTTTCCCCCCAT 3’

Table 3. MICs of stable variants against chloramphenicol and erythromycin

Strain	Characteristics	Cm (µg/ml)	Ery (µg/ml)
L. lactis NZ9700	Wild type nisin A producer. Progeny of the conjugation between nisin producer strain NIZO B8 with MG1614 (Rif^R $Strp^R$ derivative of MG1363). Carries nisin–sucrose transposon Tn5276.	1.56	0.156
L. lactis NZ9800	Derivative of NZ9700. Has a 4-bp deletion in *nisA* gene, leading to inactivation of the nisin operon except for the *nisRK* genes that are transcribed by a constitutive promoter.	1.56	0.156
L. lactis NZ9800 pVE6007	*L. lactis* NZ9800 harboring pVE6007 plasmid [$RepA^+$, Cm^r, temperature-sensitive].	12.5	0.156
L. lactis NZ9800 pORI280	*L. lactis* NZ9800 harbouring pORI280 plasmid [$RepA^-$; ori^+; *lacZ*; Ery^r; integration vector].	1.56	2.5
L. lactis NZ9800::M21V	Stable derivative producing M21V variant	1.56	0.156
L. lactis NZ9800::K22S	Stable derivative producing K22S variant	1.56	0.156
L. lactis NZ9800::S29A	Stable derivative producing S29A variant	1.56	0.156
L. lactis NZ9800::K22A	Stable derivative producing K22A variant	1.56	0.156
L. lactis NZ9800::K22T	Stable derivative producing K22T variant	1.56	0.156
L. lactis NZ9800::N20P	Stable derivative producing N20P variant	1.56	0.156

Table 4. Acidifying ability of cheese starter cultures

	pH						Rate of acidification
Starter culture	**0h**	**2h**	**4h**	**6h**	**8h**	**24h**	**(d)pH/h^{-1}**
L. lactis MG1614 (no nisin biosynthetic machinery)	6.52±0.05	6.44±0.02	6.18±0.06	5.64±0.03	4.94±0.16	4.21±0.09	0.198
L. lactis NZ9700 (MG1614 with nisin operon)	6.52±0.05	6.39±0.06	6.25±0.02	5.73±0.05	5.00±0.12	4.20±0.05	0.190
L. lactis NZ9800 (4bp deletion in *nisA* gene)	6.52±0.05	6.45±0.04	6.29±0.06	5.72±0.11	5.04±0.22	4.29±0.03	0.185
L. lactis NZ9700::M21V:pLP712	6.52±0.05	6.39±0.04	6.24±0.15	5.95±0.09	5.24±0.09	4.29±0.03	0.160
L. lactis NZ9700::K22T:pLP712	6.52±0.05	6.42±0.01	6.23±0.02	5.68±0.02	5.03±0.09	4.24±0.02	0.189
L. lactis NZ9700::S29A:pLP712	6.52±0.05	6.34±0.04	6.18±0.07	5.71±0.04	5.03±0.11	4.25±0.03	0.186
L. lactis 303	6.52±0.05	6.45±0.02	6.30±0.03	6.01±0.03	5.18±0.13	4.15±0.03	0.168
L. lactis AM2	6.52±0.05	6.45±0.03	6.18±0.03	5.85±0.03	5.14±0.07	4.35±0.02	0.173
L. lactis HP	6.52±0.05	6.42±0.08	6.34±0.05	6.21±0.11	5.89±0.09	4.51±0.10	0.079

Table 5. pH values of cottage cheese stored over 14 days

Day	**NZ9800**	**NZ9700**	**N20P**	**M21V**	**K22T**	**S29A**
0	4.85±0.00	4.62±0.08	4.8±0.04	4.77±0.09	4.72±0.01	4.83±0.05
7	4.15±0.08	4.25±0.06	4.26±0.05	4.25±0.04	4.14±0.14	4.12±0.05
14	4.26±0.09	4.38±0.08	4.22±0.13	4.30±0.10	4.20±0.05	4.28±0.09

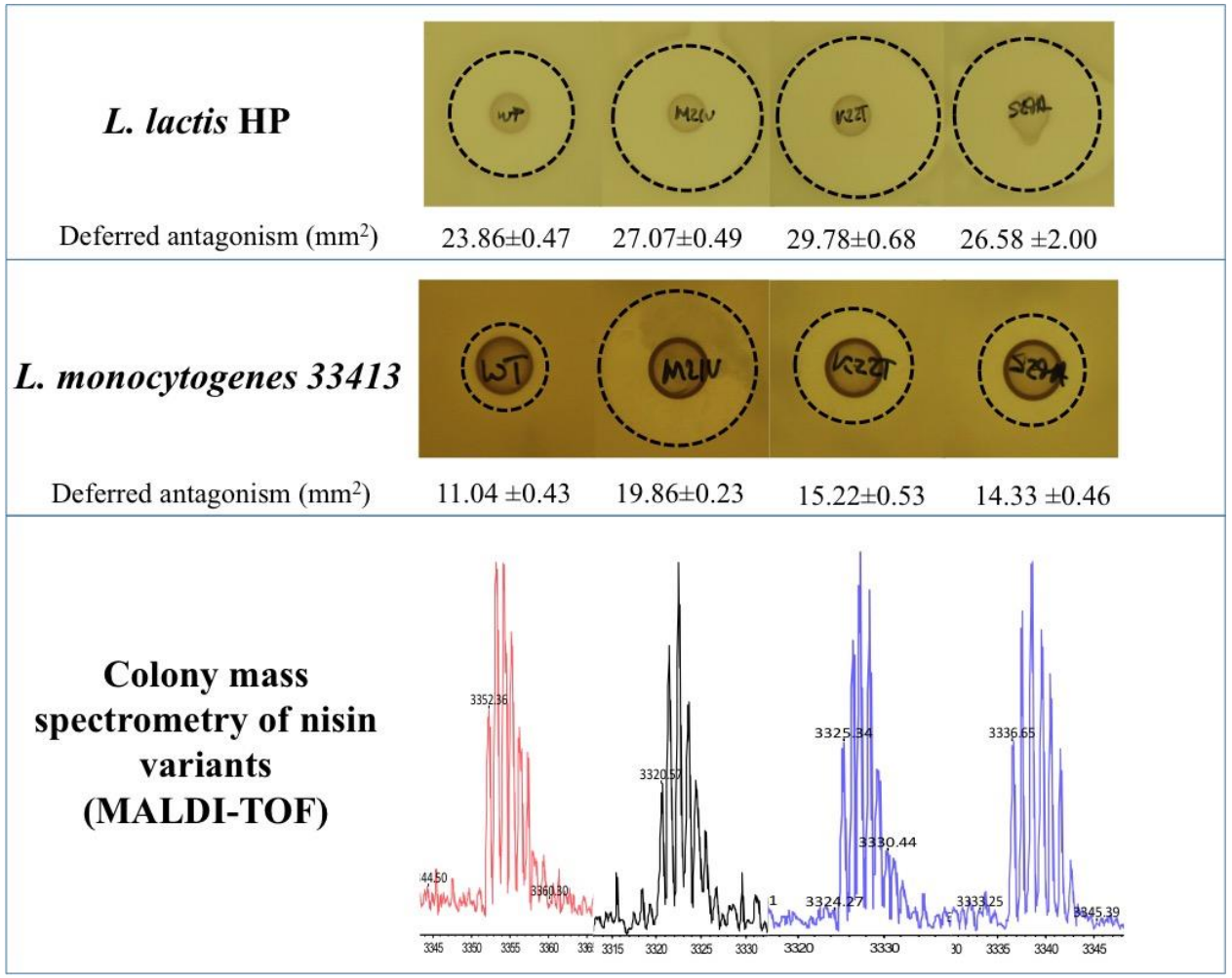


Figure 1. Deferred antagonism assay and mass spectrometry highlight successful double crossover recombination.

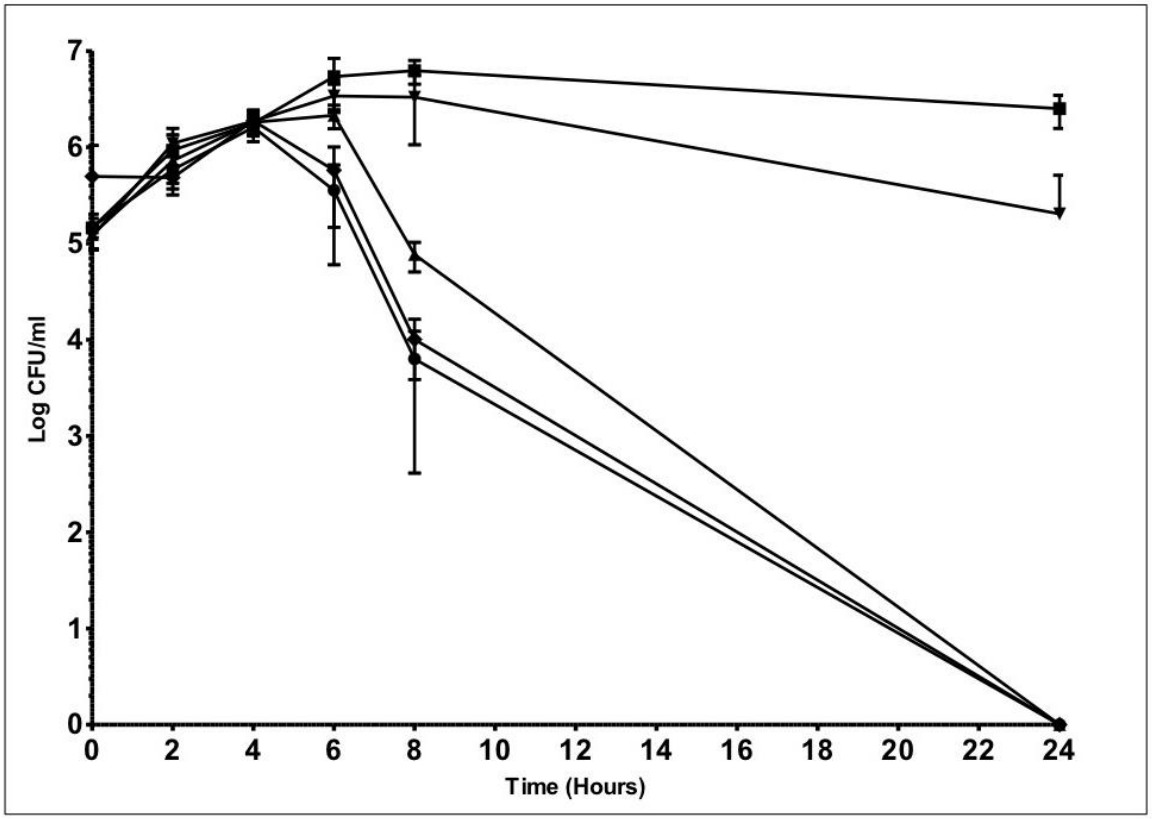


Figure 2. Fate of *L. monocytogenes* 33413 during acidification by nisin producing starter cultures

The following shapes denote the starter culture characteristics used: Circles WT nisin producer; Square, non-producer; diamond, nisin S29A; Triangle, nisin M21V; upside-down triangle nisin K22T.

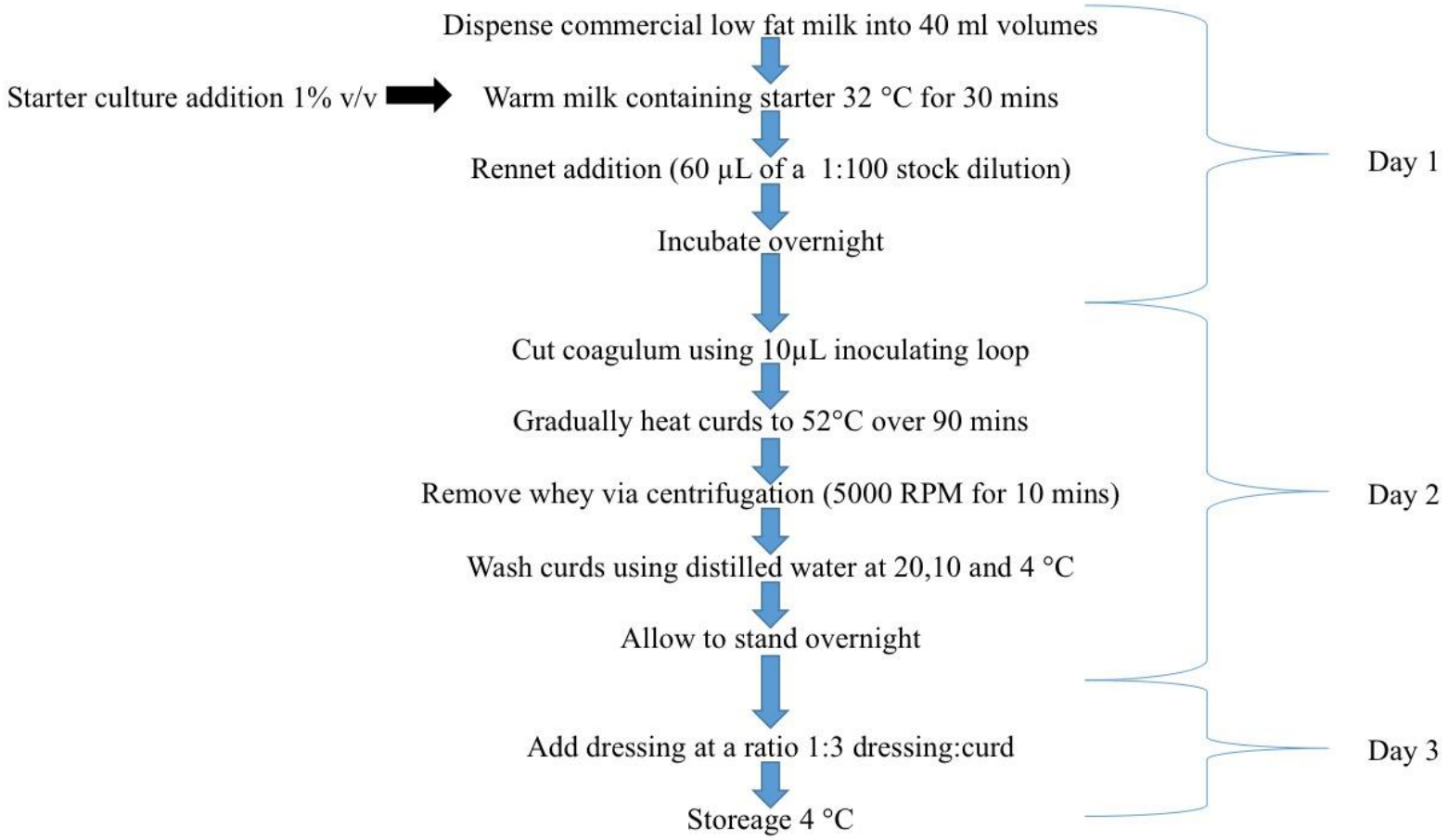


Figure 3. Flow chart of the mini cottage cheese making process

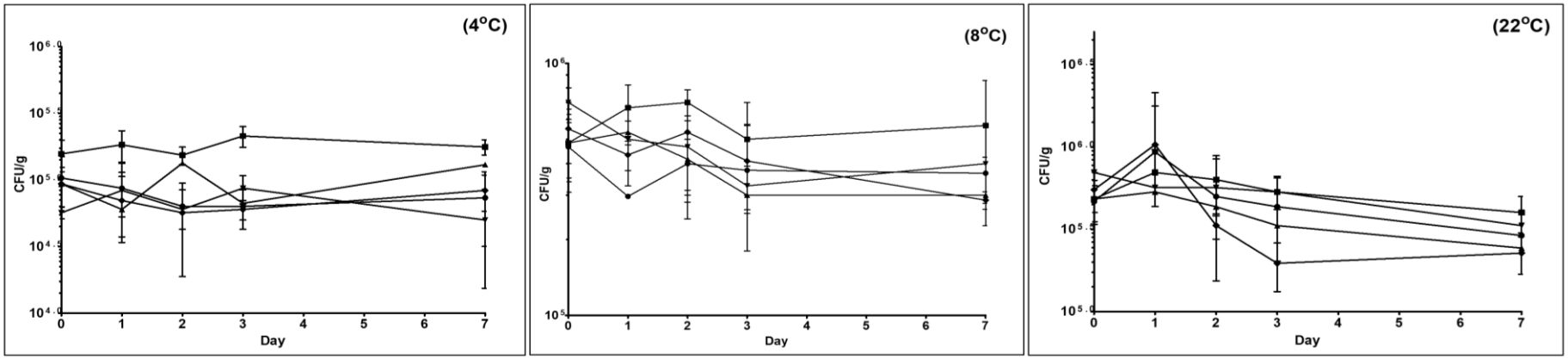


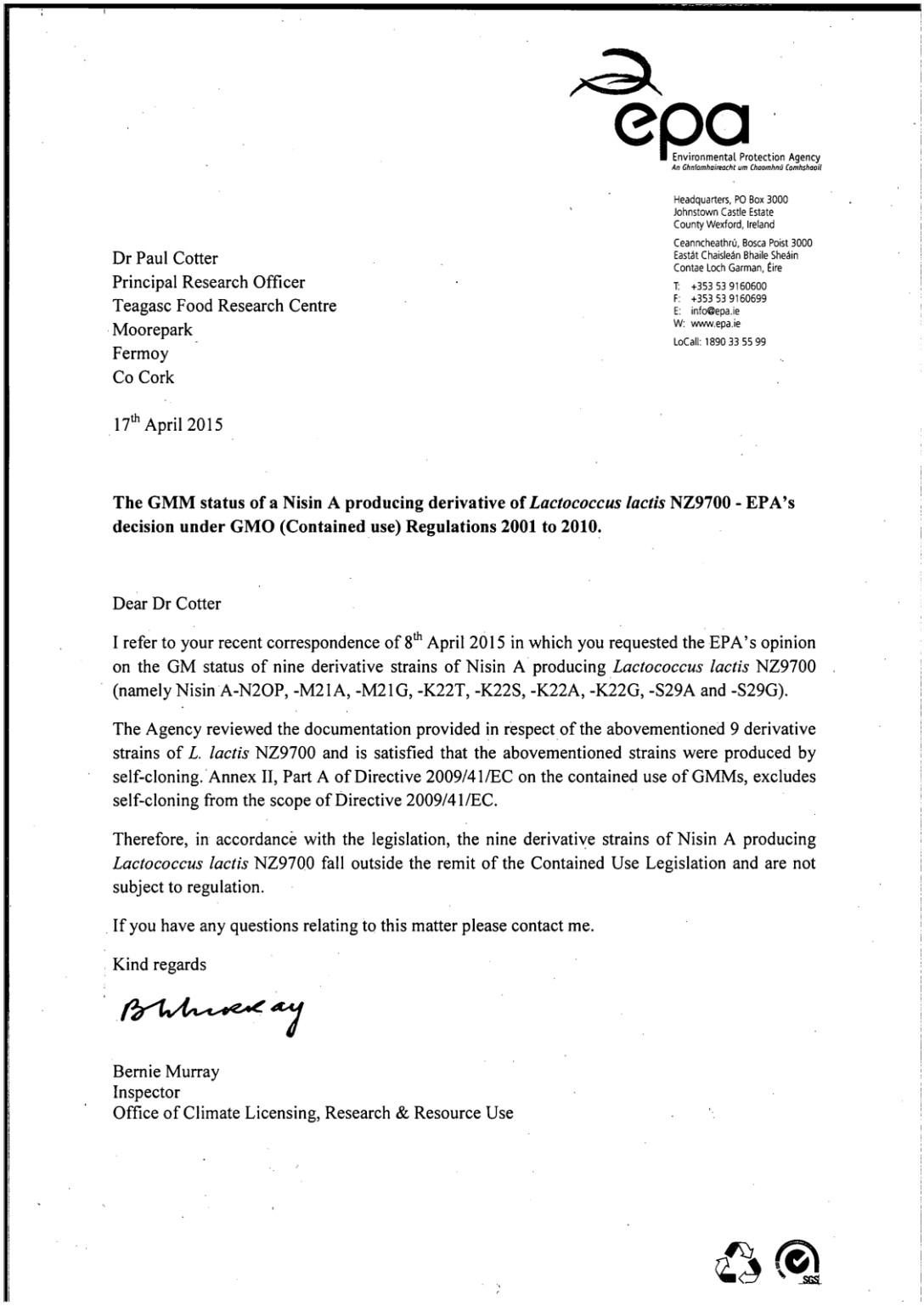
Figure 4. Fate of *Listeria monocytogenes* 33413 in cottage cheese manufactured with nisin (and nisin variant) producing starter cultures.

The growth/decline of *L. monocytogenes* 33413 in cottage cheese was monitored over a 7 day period at three different temperatures (4, 8 and 22). The following shapes denote the starter culture characteristics used: Circles WT nisin producer; Square, non-producer; diamond, nisin S29A; Triangle, nisin M21V; upside-down triangle nisin K22T.

Thesis conclusion

This thesis contributes to the overall knowledge and literature in three key ways: (1) discovery of numerous bacteriocin candidates for future characterisation and application in food and biomedicine, (2) futher exploration / validation of *in silico* bacteriocin mining methodology, and (3) further application of bacteriocin mutagenesis in food models. The knowledge advances and challenges described herein will ultimately drive further investigation in the field of bacteriocin research and microbiology in general.

Appendix



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17th April 2015

The GMM status of a Nisin A producing derivative of *Lactococcus lactis* NZ9700 - EPA's decision under GMO (Contained use) Regulations 2001 to 2010.

Dear Dr Cotter

I refer to your recent correspondence of 8th April 2015 in which you requested the EPA's opinion on the GM status of nine derivative strains of Nisin A producing *Lactococcus lactis* NZ9700 (namely Nisin A-N2OP, -M21A, -M21G, -K22T, -K22S, -K22A, -K22G, -S29A and -S29G).

The Agency reviewed the documentation provided in respect of the abovementioned 9 derivative strains of *L. lactis* NZ9700 and is satisfied that the abovementioned strains were produced by self-cloning. Annex II, Part A of Directive 2009/41/EC on the contained use of GMMs, excludes self-cloning from the scope of Directive 2009/41/EC.

Therefore, in accordance with the legislation, the nine derivative strains of Nisin A producing *Lactococcus lactis* NZ9700 fall outside the remit of the Contained Use Legislation and are not subject to regulation.

If you have any questions relating to this matter please contact me.

Kind regards

Bernie Murray
Inspector
Office of Climate Licensing, Research & Resource Use

Appendix 1. EPA decision on non-genetically modified microbe (GMM) status of the stable nisin producing variants.

Appendix 2. CommBeBiz 2018-2019 magazine. I submitted the cover photo for the CommBeBiz bioeconomy photo competition and was selected as the winner.

The photo was captioned as follows: The human body can be a source of bacteriocin producing bacteria capable of inhibiting antibiotic resistant superbugs. Bacteriocins are a group of antimicrobial peptides or proteins, produced by bacteria that can kill

other bacteria. Bacteriocins have been safely used in food for over half a century and are found in a variety of probiotic dairy products. Here, at the APC Microbiome Institute, we are actively mining the human gut for new bacteriocins that can meet the unprecedented challenge that antimicrobial resistance (AMR) presents. The Image shows a bacteriocin (nisin) producing colonies of Lactococcus lactis inhibiting vancomycin resistant enterococci (VRE), an important nosocomial (hospital acquired) superbug.

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