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An investigation into novel bacteriocin producers isolated for shelf life extension of dairy products

Thesis presented for the degree of

Doctor of Philosophy

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.

Contributions:

Chapter 1 - Part 2. Ms. Daragh Hill co-authored the review, contributing the sections 'Prebiotics', 'Health-focused research', and 'Regulation of fermented dairy products'.

Chapter 2. Volatiles analysis were performed by Ms. Emer Garvey, Mr. David Mannion, and Dr. Kieran Kilcawly.

Chapter 4. Mass spectrometry was performed by Ms. Paula O'Connor.

Chapter 5. Mass spectrometry, peptide purification and alkylation were performed by Ms. Paula O'Connor.

Chapter 6. Mass spectrometry and peptide synthesis were performed by Ms. Paula O'Connor.

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List of Publications

Book Chapters

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Reviews

Hill, D.*, **Sugrue, I**.*, Arendt, E., Hill, C., Stanton, C. and Ross, R.P., 2017. Recent advances in microbial fermentation for dairy and health. *F1000Research*, *6*. DOI: 10.12688/f1000research.10896.1

Research Papers

Sugrue, I., O'Connor, P.M., Hill, C., Stanton, C. and Ross, R.P., 2020. *Actinomyces* produce defensin-like bacteriocins (actifensins) with a highly degenerate structure and broad antimicrobial activity. Journal of bacteriology, 202(4). DOI: 10.1128/JB.00529-19.

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Abbreviations and Acronyms

AU	Activity units
BA	Biogenic amine
BGC	Bacteriocin gene cluster
CAS	Chemical abstract service
CFS	Cell-free supernatant
CSαβ	Cysteine-stabilised α-helix β-sheet
DM	Dry matter
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
FOS	Fructo-oligosaccharide
FOSHU	Food of specified health use
GABA	Gamma-aminobutyric acid
GC-MS	Gas chromatography-mass spectrometry
GRAS	Generally recognised as safe
GRS	Perennial ryegrass
HPLC	High-performance liquid chromatography
HS-SPME	Head space-solid phase micro extraction
IDF	International Dairy Federation
ISAPP	International Scientific Association for Probiotics and Prebiotics
ICK	Inhibitor cysteine knot
IU	Individual unit

HTST	High-temperature short-time
LAB	Lactic acid bacteria
LRI	Linear retention indices
MALDI-TOF	Matrix assisted laser deionised time-of-flight
MF	Micro-filtration
MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
MS	Mass spectrometry
MW-SPPS	Microwave-assisted solid phase peptide synthesis
NCBI	National Center for Biotechnology Information
ORF	Open reading frame
PF	Pre-fermentation
PMR	Partial mixed ration
PSI-BLAST	Position-specific iterative-BLAST
QPS	Qualified presumption of safety
RT	Retention time
SBSEC	*Streptococcus bovis/Streptococcus equinus* complex
STEC	Shigatoxin-producing *Escherichia coli*
TBC	Total bacterial count
TMR	Total mixed ration
UHT	Ultra-high temperature
UP	Ultra-pasteurisation

Abstract

This thesis investigates extension of the shelf life of dairy products and outlines the discovery and characterization of novel antimicrobials for application in food and health. The results demonstrate factors which affect the quality of yoghurt and extend the shelf life of pasteurised milk, and detail the identification of antimicrobial producing organisms and their characterization.

Chapter 1 first describes food-borne pathogens associated with raw milk and the risks associated with its consumption followed by an overview of microbial by-products of dairy fermentation. Metabolites such as biogenic amines, bioactive peptides, and antimicrobial peptides known as bacteriocins which affect the product and the consumer are described.

Chapter 2 outlines the production and techno-functional characterization of yoghurt produced using milk derived from different diets, perennial ryegrass (GRS) and total mixed ration (TMR). Using compositional, textural, microbial, and volatile analyses GRS yoghurts were found to contain significantly more protein, less lactose, altered colour and improved late shelf life texture characteristics. TMR yoghurts had increased quantities of volatile compounds associated with natural yoghurt.

Chapter 3 describes the application of the well-studied bacteriocin and preservative, nisin A, to extend the shelf life of pasteurised milk. Using a range of concentrations in commercially produced pasteurised milk, nisin A impacted spoilage organisms at 1 - 10 μg ml^{-1} and completely prevented growth at 1 mg ml^{-1} as far as 49 days in cold storage. This study determines the efficacy of using a bacteriocin for shelf life extension of pasteurised milk.

Chapter 4 details the identification of novel bacteriocin producing lactic acid bacteria using traditional screening methods. Of 823 isolates mainly from raw milk sources, seven strains were identified as putative bacteriocin producers. Using whole genome sequencing and predictive analysis four high quality genomes were generated, each of which contained gene clusters for bacteriocin production. Two closely related but separate *Streptococcus* sp. strains were found to encode a novel nisin variant, nisin I, detectable by mass spectrometry. A *Lactococcus lactis* isolate was found to encode a novel two component lantibiotic. One *Streptococcus uberis*

isolate contained four operons for bacteriocin production, one of which encoded a novel two component lantibiotic.

During the screen for bacteriocin producing isolates described in chapter 4, a strain of *Actinomyces ruminicola* (a species and genus without previous recorded bacteriocin production) was identified. Using mass spectrometry, N-terminal amino acid sequencing and comparative genomics, chapter 5 outlines the purification of a novel antimicrobial peptide, actifensin, which could then be identified within the sequenced genome. Using the gene encoding the structural peptide, 161 *Actinomyces* genomes were searched, finding 47 homologous genes displaying a remarkable level of sequence diversity. Actifensin peptides were found to bear similarity to conserved ubiquitous eukaryotic antimicrobial peptides, defensins. This study highlights conserved antimicrobial structures across kingdoms and describes a novel group of bacteriocins.

In Chapter 6 an investigation was carried out into another *Actinomyces* sp. and other genera harbouring actifensin-like gene clusters. Synthesised actifensin was found to be less active than natural actifensin and lacked highly conserved disulphide bonds. *Actinomyces oris* CCUG 34286 containing seven *afnA* copies did not exhibit characteristic actifensin activity. As defensin-like structures are conserved throughout nature, other actifensin-like operons were sought using gene neighbouring analysis of hypothetical proteins within the actifensin operon. Gene clusters were detected across the phylum Actinobacteria bearing similarity to the actifensin operon, but traditional culturing and mass spectrometry did not detect actifensin homolog production. This study identified a range of encoded actifensin-like peptides outside the genus *Actinomyces* to be investigated for future applications.

Overall, the results of this thesis detail quality properties of dairy products and expand upon the current knowledge of antimicrobial peptides.

Chapter 1.

Part 1 - Foodborne pathogens and zoonotic diseases associated with raw milk

Abstract

Milk consumption is estimated at billions of litres worldwide, the majority of which is consumed as pasteurised cow's milk. In recent years there has been an increase in the consumption of raw milk due to perceived health benefits compared with heat-treated milk, including increased nutritional value and the potential presence of probiotic bacteria. However, the consumption of raw milk and its derived products poses a significant health risk associated with ingestion of foodborne pathogens and consequent zoonotic illnesses. Contamination of raw milk generally occurs from environmental sources, or from sick animals. Good farming practices and effective post-processing are generally sufficient for the eradication of milk-borne pathogens. Annually, a number of human illnesses are associated with consumption of raw milk worldwide, and the severity of these varies from mild symptoms to life threatening infections. In recent times, there have been calls for legislation preventing the sale of un-pasteurised milk in many developed countries.

Introduction

Milk and milk product consumption has long been associated with good health, but it can also pose a potential health risk when ingested in the raw state, particularly if it has been improperly processed, or when product manufacturing conditions are not of sufficiently high standard. Raw milk is defined by the European Food Safety Authority (EFSA) as 'milk produced by farmed animals which has not been heat treated to more than 40 °C nor had any equivalent treatment' (1). Human and zoonotic animal pathogens and their toxins may be present in raw milk and raw milk products which can lead to many illnesses, the severity of which depends on the pathogen(s) present, the infectious dose and the health of the individual consuming the product. The US Centres for Disease Control and Prevention estimate a number of 48 million foodborne illnesses in the United States every year, of which 128,839 lead to hospitalizations and as many as 3,037 deaths (2). While the percentage of the US population who consume unpasteurised milk and cheese is relatively small (3.2% and 1.6%, respectively), they are >800 times more likely to become ill and 45 times more likely to require hospitalisation (3). Consumption of raw milk increases the risk of foodborne illness due to the potential presence of pathogenic microbes. According to EFSA the major risk organisms present in raw milk include *Campylobacter* spp., *Salmonella* spp., shigatoxin producing *Escherichia coli* (STEC), *Bacillus cereus*, *Brucella abortus*, *Brucella melitensis*, *Listeria monocytogenes*, *Mycobacterium bovis*, *Staphylococcus aureus*, *Yersinia enterocollitica*, *Yersinia pseudotuberculosis*, *Corynebacterium* spp., and *Streptococcus suis* subsp. z*ooepidemicus* (1). The parasites, *Toxoplasma gondii* and *Cryptosporidium parvum*, and the virus, tick-borne encephalitis virus, are also considered a microbiological hazard of raw milk, though this chapter will focus on the more common bacterial pathogens found in cow's milk. The potential of raw milk to cause illness after contamination depends largely on the storage conditions of the milk which is usually designed to prevent overgrowth of harmful organisms, though for certain low infectious dose pathogens such as STEC the potential is entirely dependent on the degree of product contamination. Most milk collected, thermally processed, and packaged under high quality conditions poses little risk to the consumer, though problems with ineffective heat treatment,

high initial microbial load or poor packaging conditions can lead to contaminated milk. Products of raw milk also pose risk to consumers, with unpasteurised cheeses and other soft-style cheeses being a potential vector for foodborne pathogens which can survive or grow at refrigeration temperatures. Great care must therefore be taken when choosing to ingest raw milk over its processed counterpart as there are many risk factors which could lead to illness.

Campylobacter spp.

The genus *Campylobacter* is composed of Gram-negative, non-spore forming spiral rods which colonise the intestinal tract of many animal species, can be shed in faeces intermittently, and therefore are commonly found in the farm environment (4). *Campylobacter jejuni* and *Campylobacter coli* are the most important species regarding health, with *C. jejuni* acting as the predominant pathogen. The infectious dose of *C. jejuni* is estimated at between 500 and 800 individual cells (5). Symptoms of campylobacteriosis are like that of other lower GI tract bacterial infections and include abdominal discomfort, cramps, fever, diarrhoea, and bloody stools. Severe cases can lead to the development of Guillain Barré Syndrome, an autoimmune disorder of the peripheral nervous system (6). *Campylobacter* are a leading cause of foodborne illnesses worldwide, though the number of reported infections are decreasing globally (7). They are an environmental contaminant of milk, and their presence is generally due to contamination from faeces, though their direct excretion into milk has also been described (8, 9). Pasteurisation is effective at eliminating *Campylobacter* spp. from milk, though care must be taken that effective pasteurisation is performed as poor processing and post-processing environments can lead to contamination of milk or milk products (10).

Escherichia coli

E. coli is a Gram-negative, facultative anaerobe and a normal commensal of the human gut, which is often used as an indicator of faecal contamination and poor hygiene practices. Some strains of *E. coli* have acquired virulence factors enabling pathogenesis in the human gut leading to illness. Shiga toxin-producing *Escherichia*

coli (STEC), also known as verotoxigenic *E. coli*, are human enteric pathogens, the most well-known of which is *E. coli* serotype O157:H7, which causes diarrhoea, haemorrhagic colitis, and haemolytic uremic syndrome. Haemolytic uremic syndrome can potentially lead to loss of kidney function, and in extreme cases can be fatal (11). *E. coli* O157:H7 is a major hazard due to its extreme virulence, with an infectious dose as low as 5-50 cells (12). STEC growth has been recorded in milk at temperatures below 15 °C, due to mechanisms not present in non-pathogenic *E. coli* (13). Ruminants are a significant reservoir for STEC, and frequently shed them in faeces. Defecation of the cows during milking is considered a critical event for potential transmission of STEC to raw milk, therefore good milking and subsequently good hygiene practices must be maintained (14). STEC have shown to be susceptible to heat treatment at 72 °C for 15 s, and therefore pasteurisation is sufficient to eliminate them from milk (15).

Yersinia enterocolitica

Yersinia enterocolitica are a heterogenous group of Gram-negative facultatively anaerobic pathogens associated with raw milk, raw or undercooked pork, untreated water, and faeces (16). The species is classified into six biovars, five of which are pathogenic to humans, represented by more than 30 serotypes (17, 18). *Y. enterocolitica* is the most common aetiological agent of yersiniosis followed by *Yersinia pseudotuberculosis.* Yersiniosis is an illness with a range of symptoms from acute gastroenteritis, to terminal ileitis, mesenteric lymphadenitis, and in severe cases septicaemia (19). *Y. enterocolitica* are ubiquitous in the environment, and have been shown to grow at low temperatures, propagating even at refrigeration temperatures (20). Pasteurisation of milk at 72 °C for 15 s has been shown to be effective for inactivation of *Y. enterocolitica* in milk (15). However, as *Y. enterocolitica* can grow at low temperatures, it remains a hazard if pasteurisation is not performed effectively. Yersiniosis has previously been associated with outbreaks following consumption of pasteurised milk, due to deficiencies in the heat treatment facility (21).

Staphylococcus aureus

Staphylococcus aureus is an important opportunistic pathogen which causes mastitis, inflammation of the mammary gland in the udder of dairy cows, leading to major economic losses worldwide. While most strains are commensals of human and other animals' skin, many of the Gram-positive facultative anaerobic cocci have been implicated as potential hazards in raw milk. Many *S. aureus* strains can produce a host of extracellular protein toxins and virulence factors which contribute to their pathogenicity, such as heat stable enterotoxins which remain stable during and after pasteurisation (22). Of the multiple toxins produced by *S. aureus* ingestion of food contaminated with staphylococcal enterotoxin(s) is the cause of staphylococcal food poisoning (23). The illness is characterized by acute gastroenteritis, with vomiting and diarrhoea within two to six hours of consumption (24). Other virulence factors and toxins associated with *S. aureus* are relevant to systemic infections rather than foodborne infections. Antimicrobial resistance is a significant problem with strains of *S. aureus*, with some strains having developed high level of resistance to β-lactam antibiotics through acquisition of resistance genes which are now well established in farm populations (25). Methicillin resistant *S. aureus* (MRSA), the increasingly common nosocomial acquired pathogen, have been found in milk samples since the 1970s (26), and pose a serious risk to consumers (27). Overuse of antibiotics in the dairy and agriculture industries has contributed to the prevalence of antimicrobial and multidrug resistant (MDR) strains among herds, and in milk, with MDR strains frequently playing a role in mastitis (27-29). Significantly higher levels of antibiotic resistance have been found in milk from lactating Holsteins with clinical mastitis than without mastitis (30). A recent study investigating the effect of pasteurisation as a means for inactivating staphylococcal enterotoxins found that heat treatment of 40 milk samples at 72 °C, 85 °C, and 92 °C all had samples containing toxin post treatment (87.5%, 52.5%, 45% of samples respectively) (31).

Sporeformers: *Bacillus* and *Clostridium* spp.

Gram-positive bacteria of the phylum Firmicutes which can form spores when placed under environmental stress are a major problem in the food industry and the

dairy sector in particular (32). A spore can form in a bacterial cell which experiences harsh conditions for growth and survival such as high osmotic pressure, nutrient deficient environments or large temperature differentials (33). Spores can overcome these conditions, surviving pH changes, radiation, heat, cold and chemical damage until conditions become favourable and allow for germination (34). Spores are commonly found in soil, silage, animal faeces and on udders with poor hygiene, all of which are common in the milking environment which can lead to contamination of bulk tank milk (35-37). Food pathogens of note which form spores in milk are those of the genera *Bacillus* and *Clostridium* which are aerobic and anaerobic, respectively. Many species of these genera are psychrotophic thermophilic bacteria, which can not only survive but also grow at refrigeration temperatures. They pose a very real threat of contaminating milk and multiplying in the refrigerated bulk milk tank (38).

Bacillus cereus, while not on EFSA's list of harmful pathogen risk factors associated with raw milk (1), is considered a major hazard due to the ability of some strains to cause illness in humans through the production of toxins. Such strains can release emetic and/or diarrhoea causing toxins while growing in milk prior to heat treatment and when growing in the small intestine after consumption of contaminated milk (39). In 2010, 3.8% of all milk samples which were tested in the EU indicated as positive for *Bacillus* toxin (40). A recent study investigating the effects of storage temperature and duration on the microbial quality of bulk tank milk in Ireland isolated what was denoted as presumptive *B. cereus* in 8 to 12% of all bulk milks samples, with no significant difference between altered storage conditions, though inadequate sample size may have been a factor (41). They are also a notable spoilage hazard, given their ability to produce lipolytic enzymes which can act at temperatures close to those of pasteurisation and thermization (42). Indeed, the thermoduric sporeformers are a major hazard associated not only with raw milk, but also with pasteurised milk products, fermented products and powders, and great care must be taken to prevent their contamination and ensure the absence of any toxins. Some investigated methods for the inactivation of *Bacillus* spores in milk include high pressure homogenisation, mild pressure and heat treatment, the

combined effects of high temperature and the food preservative and bacteriocin, nisin (43-46).

Clostridium spp. are a major problem in the dairy industry, many of which are toxigenic, neurotoxigenic, or spoilage bacteria (32). Contamination of bulk tank milk can occur during and after milking from the farm environment, including feeds, faeces, soil and animal bedding (47). *Clostridium* sp. have been found in raw milk, and pasteurisation is insufficient to eradicate this bacterium due to the spores formed (47, 48). Spores pose huge problems for manufacturing standards as they are difficult to eradicate. Illnesses from *Clostridium* spp. are due to ingestion of toxins produced by the genus or germination of ingested spores in the milk or milk product once inside the GI tract. *Clostridium perfringens*, and *Clostridium botulinum* are the species of most concern in milk, as both are frequently isolated from the farm environment and are capable of toxin production, most notably enterotoxin and botulinum toxin, the potent neurotoxin (32). Good farm practices must be ensured to avoid contamination with clostridia as some toxins produced in milk are heat stable, and are not inactivated by heat treatment (49). Similarly to *Bacillus* spp*.,* high pressure heat and treatment with nisin have been investigated as methods for elimination of *Clostridium* spores in milk (50).

Listeria monocytogenes

Listeria monocytogenes is a non-spore forming Gram-positive facultative anaerobe which causes listeriosis, a condition of particular concern for pregnant women, the immunocompromised and the elderly (51). *Listeria* spp. are ubiquitous in the environment and contamination of products is common from poor manufacturing conditions such as open water tanks or poor water heating systems (52). The species *L. monocytogenes* is genetically heterogenous and has been categorised into high/low virulence clonal complexes by multilocus sequence typing (53). Recent investigation of such complexes has found that clones from the dairy and mammalian gut niches are virulent in humans but are poorly adapted to food production environment (54). Risk of *Listeria*-related illness is high from consumption of raw milk and milk products such as unpasteurised cheeses held for extended

periods of time at low temperatures at which they grow (55, 56). *L. monocytogenes* has a long history of pathogenesis in milk and dairy products and is arguably one of the most worrying foodborne pathogens associated with the dairy industry (57). Several outbreaks have occurred globally in recent years connected with raw milk and raw milk products, however, illnesses due to *L. monocytogenes* are more often associated with consumption of pasteurised cheeses (3, 58).

Other Zoonoses and Toxins

Other zoonotic bacterial species of note found in milk are *Mycobacterium bovis* and *Coxiella burnettii. M. bovis* causes bovine tuberculosis (TB) in animals, a chronic disease which is now rare in the developed world. The pathogen can spread to humans through consumption of raw milk which is contaminated from infected cows, causing zoonotic TB which presents as identical to TB caused by the well-known human pathogen *Mycobacterium tuberculosis* (59). *M bovis* is rarely found in milk outside the developing world except in outbreaks associated with the consumption of non-pasteurised milk (60). Contaminated milk when treated by standard high-temperature short-time pasteurisation has been demonstrated to eliminate *M. bovis* (60).

C*. burnetii* is the causative agent of Query fever, a ubiquitous zoonosis which can infect many animal species including humans, cattle, sheep, and goats. Infection by *C. burnetii* has largely been investigated in sheep and goats, where infections usually remain asymptomatic until pregnancy where the bacterium can increase incidence of miscarriage (61). Bacterial shedding has been described in raw milk among dairy cattle, though consumption of raw milk is considered an inefficient route of transmission (62).

Brucella spp. are similar to *Y. enterocolitica* as they not only survive but may grow at refrigeration temperature, both in raw milk (63), and after contamination of pasteurised milk (64). *Brucella* spp. are Gram-negative aerobes which are known to cause brucellosis, a zoonotic infection common among cattle populations. Practically all human cases of brucellosis are due to close contact with an infected animal or

through ingestion of unpasteurised dairy products contaminated with the organism (65). It is an infection associated with poor hygiene practices particularly in developing regions of the world as major eradication programs have taken place in much of Northern Europe, North America and Australia (66). *Brucella abortus* and *Brucella melitensis* are the two main species which infect cattle and pose risk associated with raw milk consumption (67). Manifestations of brucellosis in humans present as high, undulating fever, but chronic brucellosis can lead to organ damage, arthritis, hepatitis, encephalomyelitis, and endocarditis (68). Bovine brucellosis is associated with miscarriage, reduced fertility and milk yields (69).

Mycotoxins are compounds produced in mould-contaminated foods and can be harmful to humans if ingested at high enough concentrations. Aflatoxins and ochratoxins are two such mycotoxins which can be found in raw milk. Mycotoxins are secondary metabolites from species within the *Aspergillus* and *Penicillium* genera (70). These toxins are not associated with an infection in a dairy animal but are associated with the dairy animal ingesting mould contaminated foodstuff causing toxins to enter the milk. Both aflatoxins and ochratoxins are regarded as carcinogenic, and classified as class 1 known human carcinogens and class 2b possible human carcinogens, respectively (71, 72). Exposure to high levels of these mycotoxins can lead to disease and possibly death. Elevated quantities of aflatoxin can cause hepatic necrosis while ochratoxin is linked to nephropathy (73, 74). Aflatoxin is a relatively heat stable compound and pasteurization is not sufficient to completely destroy the toxin in milk but it significantly reduces its level (75).

Epidemiological concerns

The outbreak-related disease burden associated with consumption of unpasteurised cow's milk and cheese is estimated at 761 illnesses and 22 hospitalisations annually in the US, of which 95% are salmonellosis and campylobacteriosis (3). Between 2007 and 2012, there were 27 reported epidemics associated with consuming raw milk in Europe, 24 of which were bacterial in nature, largely *Campylobacter* spp. Of 24 outbreaks, 21 were likely due to contamination by *C. jejuni*, two due to *Salmonella enterica* ser. Typhimurium and one from STEC (1).

Four of the 27 outbreaks were due to consumption of raw goat's milk and the remaining 23 were due to raw cow's milk consumption. Over the same time period in the US, there were a total of 81 reported outbreaks throughout 26 states associated with raw milk consumption, which lead to 979 illnesses and 73 individuals hospitalized. This was a 4-fold increase in outbreaks associated with the consumption of unpasteurised milk over a period of 6 years with the number of outbreaks caused by *Campylobacter* spp. nearly doubling in that time (76). Seventy-eight of the 81 outbreaks were linked with a single infectious agent, the most common of which were *Campylobacter* spp., causing 81% (62) of outbreaks. Unlike in Europe, STEC was the next most common with 17% (13) of outbreaks, followed by *Salmonella enterica* ser. Typhimurium present in 3% (2) of outbreaks, and *Coxiella burnettii* was the causative agent in one outbreak (76). Between 2007 and 2009, outbreaks caused by raw milk consumption accounted for 2% of outbreaks related to food in the US, and this increased to 5% between 2010 and 2012 (76), most likely due to recent relaxation of laws banning the sale of unpasteurised milk in certain states (77). In 2012 alone, there was an outbreak of *Campylobacter* infections in multiple states of the USA which was traced back to a single dairy farm in Pennsylvania with a permit to sell unpasteurised milk, and which was carrying out the recommended testing for microbial contaminants of milk. The outbreak resulted in 148 individuals falling ill, 10 of whom had to be hospitalised (78). With the number of outbreaks associated with raw milk consumption having increased in the US in recent years, there have been calls for the sale and distribution of unpasteurised milk to be legislated against, and continued public education with regard to the dangers of consuming unpasteurised milk (76).

Raw milk products such as unpasteurised cheeses were responsible for 38 outbreaks between 1998 and 2011 in the US, the pathogens responsible were *Salmonella* (34%), *Campylobacter* (26%), *Brucella* (13%), and STEC (11%) in order of most common single organism cause to least common, and soft cheeses were implicated in 26 of these outbreaks (79). Soft cheeses have higher moisture content enabling bacterial growth and are commonly manufactured using raw milk. Thus, soft cheeses have been commonly linked with pathogens such as *E. coli, S. aureus,*

Salmonella, and *Listeria* (80-83). The Food and Drug Administration in the US requires that unpasteurized cheese be aged for 60 days to improve microbiological quality. The ageing process ensures sufficient time for acid producing starter cultures to act, thereby limiting the growth and survival of potential pathogens. Though this has been the standard for over 60 years, its effectiveness is still a matter of debate as studies have shown both its efficacy and its limitations (84-86). The pathogenic potential of raw milk remains a very real risk, even in areas where raw milk may be legally sold. A recent study of 902 raw drinking milk samples for retail sale in the United Kingdom found almost half to contain indicators of poor hygiene, and 1% were deemed "unsatisfactory and potentially injurious to health" because of the presence of STEC, *Campylobacter*, *L. monocytogenes* or coagulase-positive staphylococci (87). Those who are immunocompromised, pregnant, elderly, and very young are discouraged from ingesting raw milk as they are high risk subjects for complications which arise from infections from milk-associated pathogens.

Conclusion

Controversy remains on the potential benefits of raw milk consumption versus heat treated milk. The arguments for raw milk consumption are increased nutritional content, prevention of lactose intolerance and consumption of 'good' bacteria, but these have largely been debunked (88). Raw milk can pose a serious risk to consumers, due to the presence of pathogens and toxins of pathogenic organisms, and due to overuse of antibiotics the increasing prevalence of antibiotic and multidrug resistance. Standard HTST pasteurisation is an effective means of reducing most harmful microbial organisms in milk, ensuring safety for consumption, though not all microbes, such as sporeformers, are susceptible. Similarly, some toxins may not be heat labile and remain post-processing if conditions allow for their production prior to heat treatment. Good farming practices are essential to reduce the risk of contamination from the environment, along with proper cold chain storage. Current legislation allows for the sale of raw milk in certain states in the US, and Europe, under strict regulation and monitoring, and it is recommended that raw milk is not ingested by those at the extremes of life, infants and the elderly, or by the

immunocompromised as they are more susceptible to infections by potential contaminants. The potential risk foodborne pathogens and zoonotic elements in raw milk consumption seriously outweighs any argued benefit as raw milk consumption will not protect against pathogenic bacteria or toxins present.

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Part 2 – Recent advances in microbial fermentation for dairy and health

Abstract

Microbial fermentation has been used historically for preservation of foods, the health benefits of which have since come to light. Early dairy fermentations depended on the spontaneous activity of the indigenous microbiota of the milk. Modern fermentations rely on defined starter cultures with desirable characteristics to ensure consistency and commercial viability. The selection of defined starters depends on specific phenotypes that benefit the product by guaranteeing shelf life and ensuring safety, texture, and flavour. Lactic acid bacteria can produce a number of bioactive metabolites during fermentation, such as bacteriocins, biogenic amines, exopolysaccharides, and proteolytically released peptides, among others. Prebiotics are added to food fermentations to improve the performance of probiotics. It has also been found that prebiotics fermented in the gut can have benefits that go beyond helping probiotic growth. Studies are now looking at how the fermentation of prebiotics such as fructo-oligosaccharides can help in the prevention of osteoporosis, obesity, and colorectal cancer. The potential to prevent or even treat disease through the fermentation of a food is a medically and commercially attractive goal and is showing increasing promise. However, the stringent regulation of probiotics is beginning to detrimentally affect the field and limit their application.

Introduction

The fermentation of food by microbes has been employed for millennia as a process to ensure extended shelf life, and improve the functionality, texture, and flavour of food products. The first evidence of dairy fermentation exists from approximately 7,000 years ago, where early Europeans are thought to have produced cheese (1, 2). Methods have evolved from spontaneous fermentation by the indigenous microbial population to pre-selection of starter cultures with known attributes. Lactic acid bacteria (LAB) are the major bacteria used in food fermentations worldwide. LAB consist of a myriad of genera including but not limited to *Lactobacillus, Lactococcus, Streptococcus, Leuconostoc, Pediococcus* and *Enterococcus,* some of which have recently been reclassified (3). Though the LAB are a diverse group of bacteria including pathogenic and opportunistic pathogenic genera not limited to *Enterococcus* and *Streptococcus*, many species enjoy historical "generally regarded as safe" (GRAS) and "qualified presumption of safety" (QPS) status by the Food and Drug Administration (FDA) and European Food Safety Authority (EFSA), respectively (4). LAB fermentation has long been recognised to confer beneficial effects on human health through the modulation of the intestinal microbiota. These either directly or indirectly affect the host microbiota, which in turn can lead to an effect on health. The use of these bacteria in fermentations to produce functional foods has greatly increased in recent years. Consumption of fermented foods has been associated with a range of health benefits from disease prevention to enhancing the bioregulation of behavioural issues such as stress and anxiety (5-7).

While the consumption of traditional fermented foods in cultures around the world is believed to have beneficial effects, not all of these foods have been subjected to appropriate trials in which these beliefs could be credited or discredited. The benefits of fermented dairy products are being researched extensively in parts of the world, but other traditional fermentations are also beginning to be studied in more detail (8). These traditionally fermented foods use uncharacterized starter cultures that could possess novel properties or be useful in other fermentations,

some of which will be discussed in more detail later (8-10). The potential application of microbial fermentation is enormous both in health and in biotechnology and will continue to be an important area of research and production in coming decades (11). Yeasts and moulds are also prominent fermenting organisms in alcoholic and certain cheese fermentations. The focus of this review is to look at advances in the past three years in the field of microbial fermentation with a focus on food and added health benefits of fermentation including extraneous commercial and legislative factors impacting the field (Fig. 1).

Fermentation starter cultures and by-products of fermentation

Starter cultures, which carry out the fermentation process, are used to ensure consistency in commercial products by using known species with desirable traits, such as a high rate of acidification through the production of lactic acid, and/or the secretion of secondary metabolites into the fermentate matrix (Fig. 2). Novel starter cultures are continually in demand for the development of new commercial products along with greater characterization of those currently in use to ensure safe and functional products. There are many positive and negative factors that impact the selection of starter cultures in dairy fermentations such as a history of safe use; acidification rate during fermentation; exopolysaccharide production (12); proteolytic activity, particularly during cheese production, and the generation of bioactive metabolites and peptides (13, 14).

Bacteriocins

Bacteriocins are small ribosomally synthesised antimicrobial peptides against which the producer species is immune and which act against other bacteria in a bactericidal or bacteriostatic manner (15). Great care must be taken with regards to bacteriocin production in starter cultures, as they may target other fermenting cultures or adjuncts, however, their ability to inhibit potential spoilage bacteria and pathogens can be of great use (16)**.** The identification of bacteriocinogenic strains has mostly relied on agar diffusion based assays (17). Increasing interest in

bacteriocins as alternatives to antibiotics and chemical food preservatives has led to new methods for identifying bacteriocin producers. *In silico* screening using programs such as BAGEL (18) and antiSMASH (19) enables the discovery of new bacteriocin operons where whole genome data is available. Such methods avoid any potential problems with unsusceptible indicator strains (20) and can allow for faster initial screening. Collins *et. al* (2017) identified 11 bacteriocins, five of which were novel using an *in silico* screen of 213 lactobacilli genomes (21, 22). Another *in silico* study which mined human gut microbiome sequence data found 74 bacteriocin gene clusters from 382 fully sequenced genomes (23). *In silico* screens such as these rely on previously described peptides or the identification of bacteriocin accessory genes, and such it is unlikely in that initial agar diffusion-based assays will be completely replaced with *in silico* screening. However, they may represent an opportunity to search for new bacteriocins in complex microbiotas such as that of a traditional fermented product.

Biogenic amines

Biogenic amines (BAs) are biologically active, low molecular weight organic compounds produced mainly through the decarboxylation of certain amino acids, which can accumulate during fermentation. Traditionally, the presence of biogenic amines in food products is associated with undesirable microbial activity, indicating food spoilage or defective manufacture (24). Dairy products, in particular cheese, can accumulate high levels of BA, mainly histamine and tyramine, which are known to be toxic (25-27), but as of yet legal limits have only been set for histamine in fish products (28). The accumulation of more than one kind of BA in products is of particular concern due to their synergistic toxicity at dietary concentrations, which has recently been demonstrated with intestinal cells *in vitro* (29). BAs are detected in dairy products by chromatographic detection of the BA compounds, or through detection of BA-producer organisms using PCR based methods, which correlate with HPLC results (30). In a recent study, levels of tyramine in a model cheese were reduced by 85% through the use of a bacteriophage to limit the population of BA-producing bacteria (31). Pre-selection of starter cultures lacking BA genes in the

future may be necessary to avoid unwanted build-up of BA compounds and continued avoidance of contamination, which is known to occur during post-ripening processing (32, 33).

Bioactive peptides

Bioactive peptides are encrypted in larger proteins, and when released after proteolysis have been associated with health promotion through a number of mechanisms such as inhibition of angiotensin 1-converting enzyme (ACE) activity, antithrombotic activity, antihypertensive activity, antioxidant activity, immunomodulation, apoptosis modulation, and by opioid and anti-opioid activities (13, 34). LAB possess a myriad of proteases and peptidases that can release encrypted peptides during fermentation, or following the ingestion of fermented products containing LAB in the intestinal lumen (35). In recent years, potential anticarcinogenic peptides have been found encrypted in bovine milk casein and whey proteins, including the previously known cationic antimicrobial lactoferricin (36). The known cancer-preventative peptide lunasin has been found to be proteolytically released during sourdough fermentation by LAB (37). Further research has subsequently revealed increased protease resistance during *in vitro* gastrointestinal transit in the presence of naturally occurring protease inhibitors to allow lunasin to reach the large intestine (38). Another recent study has found that the administration of milk fermented by a probiotic *Lacticaseibacillus casei* strain modulated the immune response against a breast cancer tumour in a mouse model, with delayed or blocked tumour development in the fermented milk fed group as compared with unfermented milk as a control (39). The mechanism of action has not yet been elucidated and studies of a similar nature have not yet passed the animal trial preclinical stages of investigation.

Recently-characterized traditional fermented milk products

There is increasing interest in novel LAB strains isolated from previously uncharacterized fermented milk products relative to well characterised commercially

utilised isolates. Products such as matsoni, a fermented milk product of Armenian origin, and kule naoto, the traditional fermented milk product of the Maasai in Kenya, are having their previously undescribed microbiotas characterized using sequencing based analysis (40, 41). Indeed, such products may be of great value, for example shubat, a probiotic fermented camel milk of Kazakh origin, has recently been found to demonstrate positive hypoglycaemic activity in type 2 diabetic rats (42), and the indigenous Indian fermented beverage Raabadi has been investigated as a source of probiotic hypocholesterolaemic lactobacilli (43). However, a study of mursik, a milk product from Kenya that is traditionally fermented in a gourd, has been suggested as a possible aetiological factor for oesophageal cancer, because of high levels of ethanol and acetaldehyde present post fermentation (44). This is in stark contrast to the health claims made for most commercial fermented dairy products that indicate the benefits of using safe, known starter cultures.

Probiotics, prebiotics, and synbiotics

The International Scientific Association for Probiotics and Prebiotics (ISAPP) define 'probiotics' as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (45). Probiotic species may be applied in food fermentation as starter or adjunct cultures, but not all fermenting cultures are considered probiotics. In 2008 prebiotics were defined by ISAPP as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health'' (46), a definition that was updated to "a substrate that is selectively utilized by host microorganisms conferring a health benefit" recently (47). Prebiotics are fermented by the gastrointestinal microbiota and contribute to healthy modulation of the gut (48). The ingestion of specific prebiotics has been shown to increase antibacterial capabilities of a probiotic strain (49). Synbiotics are a relatively new area that involve a combination of probiotic and prebiotic in one product; the prebiotic is intended to improve the survival/growth/performance of the probiotic or other beneficial bacteria in the colon, which in turn has beneficial health effects on the host (50).

Currently, research is being conducted on the role of the gut microbiota in the development of cancer, with a focus on colorectal cancer (51). While this research is in its early stage, there is evidence for the use of probiotics, prebiotics and synbiotics in the treatment or prevention of this disease. There is potential for these to act as anticarcinogens or antimutagenic agents through diet-based interventions. More detailed analysis could lead to huge strides in the prevention of cancer, but as of yet the field is open to new research (52).

Health-focused research

Research into the use of fermented foods as a potential approach to fight disease is growing, but it must be appreciated that many of these functional foods are intended to prevent disease onset, or alleviate symptoms, and not necessarily act as a curative agent (53). This increases the burden of proof on the researcher to prove that the fermentation of the prebiotic was indeed the reason the host remained healthy. Modulation of the gut microbiota is the focus of many studies relating microbial fermentation to measurable health benefits. One emerging area of study using microbial fermentation is in osteoporosis. Osteoporosis is common in postmenopausal women and the elderly, and presents itself as weakened bones prone to breaks or fractures due to poor calcium absorption (6). The consumption of a prebiotic fructo-oligosaccharide (FOS) has the potential to be a preventative method for osteoporosis (54). The prebiotic is fermented in the gut, causing a drop in the pH of the lumen to such an extent that previously insoluble calcium phosphate will dissolve. This plays a beneficial role in bone mineral density (55). The fermentation of FOS releases short-chain fatty acids and lactic acid, which cause the drop in pH. This dissolved calcium results in an increase in passive diffusion; thus, it could help to treat or even potentially prevent the onset of osteoporosis.

Obesity is a global issue and has generated much interest in whether and how our gut bacteria could be a contributing factor in the development of this complex syndrome. On-going studies into the gut microbiota are aimed at identifying whether a specific bacterium or bacterial group could be contributing to obesity (56). While this is an emerging area of research, there are exciting developments on how to

potentially fight this syndrome through the modulation of the gut microbiota (57). The so called “obese microbiota profile” can be characterized as a decreased Bacteroidetes/Firmicutes ratio in individuals (56). One study looked at the administration of prebiotics such as FOS as a potential method of reducing the likelihood of obesity by increasing the levels of “lean microbiota” through fermentation of the prebiotic in the gut. This in turn led to a decrease in the permeability of the intestine with improved tight junction integrity. While this is in early stages of research it does present a potentially new method by which obesity could be treated through microbial fermentation within the gut (58). This area of research has great potential medically and commercially (59).

Bacteriocins, as discussed previously, are antimicrobial peptides that target and kill other bacteria, which could potentially be utilised as an anti-obesity tool. If specific strains which are contributing to obesity are identified then modulation of the gut microbiota, through introduction of specific bacteriocins or bacteriocin producers has the potential to reduce the risk of obesity. Likewise, specific bacteriophage (obligate bacterial parasite viruses) could be used to target such strains. Bacteriophage, and phage components such as endolysins, can be applied to control the growth of undesirable microorganisms in fermented foods, and are also of interest as attractive alternatives to current antibiotics, particularly due to their highly discriminatory nature (60). Phage are abundant in the environment and have no harmful effect when consumed (61). Phage may be more suitable for inhibition of many Gram negative organisms which are not inhibited by bacteriocins due to the protection of the outer membrane. Their applications face some disadvantages such as the negative consumer perception associated with the terminology “viruses” or “viral components” in foods, and the requirement for a cocktail of different phage to inhibit multiple different spoilage organisms.

Current evidence supports the notion that microbes in the gut could be a contributing factor to mental disorders through the brain gut axis (62). Psychobiotics are an emerging area of study on the role of the microbiota in brain health. A psychobiotic is a bacterium that, when ingested in adequate amounts, can have a positive mental health benefit (63). The permeability of the intestinal barrier can be

compromised by the westernised diet of processed foods and carbonated beverages. The bacteria present in our gut are capable of producing neurotransmitters through the metabolism of indigestible fibres, these include dopamine, noradrenalin, gamma-aminobutyric acid (GABA), and acetylcholine (64). The consumption of probiotics in fermented foods could have a positive influence on maintaining the intestinal barrier and preventing chronic inflammation. Dietary interventions in adolescents of more fermented foods containing beneficial brain bacteria could help prevent the onset of depression and anxiety among other mental health issues which are becoming more prevalent.

National recommendations

Fermented foods have been consumed worldwide for thousands of years before any direct health benefits were truly understood. While the demand from consumers for functional foods is growing the national recommendations are not following suit. Now that the mechanisms by which these fermentations can beneficially affect human health are beginning to be elucidated, food guidelines around the world are slowly beginning to recommend their consumption. This inclusion is not universal, despite historical use and clinical trials proving the benefits of these fermented products in the diet. Given the strong tradition of fermented foods in Asia, it is somewhat surprising that they are not specifically included in food guidelines, however, the Chinese Nutrition Society suggests the consumption of yogurt for those with lactose intolerance.

There is a high incidence of lactose intolerance in Asian countries, and there are clinically proven studies that show the inclusion of fermented dairy foods can help to alleviate the symptoms of intolerance (65). Japanese authorities list fermented foods in the Food of Specified Health Use (FOSHU) category, and in India the guidelines specifically encourage the consumption of fermented foods. The Indian guide highlights specifically that pregnant women should consider including more fermented foods in their diet owing to the increased bioavailability of iron that is associated with these foods.

Regulation of fermented dairy products

The regulation surrounding microbial fermentations in the food industry is beginning to have a detrimental effect on the industry as a whole. For example, there is currently no legal definition for the term “probiotics”. Until scientific, legal, and industrial teams are all working together under one solid definition, the term “probiotics” will begin to lose its meaning. Along with this, the general community are losing confidence in the benefits of fermented dairy products that are supplemented with probiotic/prebiotics. Since December 2012, in Europe, labelling of a probiotic was banned along with the use of health claims in any product without receiving approval from EFSA, which has yet to approve any probiotic health claim. This is despite the numerous clinical trials proving the benefits of probiotic yogurts in health. This change has led to consumer confusion as to whether the claims were ever true. It is essential that labelling of fermented food products with clinically proven health benefits is permitted to allow industry to begin to profit from funding these trials, or they will begin to invest in marketing strategies rather than the much-needed research (46). The International Dairy Federation (IDF) represents the dairy sector at relevant CODEX meetings regarding the international standards for dairy products (66). The IDF are currently involved in investigating product labelling with regard to nutritional information and health claims and how these affect the consumer’s choice of different products (67). These studies will hopefully lead to a change in labelling laws to allow for clinically proven health claims to be present on fermented dairy products.

Conclusion

Microbial fermentation holds the key to some extremely complex interactions between bacterial species and the food matrix they are fermenting. The studies highlighted in this review show the potential of utilising these microbial fermentations in a more knowledge-based fashion than that of the past. Regarding microbial fermentation in food, this represents an area with potential well beyond the extension of shelf life. The work in these areas is continuing and, with the help of better regulation, could lead to exciting new discoveries on managing disease

symptoms through food. Though fermented products have long been associated with health promotion, the lack of regulation has been a confounding factor in consumer attitudes. Indeed, other legislation must be put in place in the near future for harmful levels of BAs in fermented dairy products, given that at present no upper limit for potentially toxic levels of histamine and tyramine are available. The search for probiotics is on-going using both genetic and traditional screening methods (68, 69). Probiotics have a bright future in the area of supplemented fermented foods for health promotion.

There have been numerous advances in fermented products, the microbes which produce them, and fermentable polysaccharides in recent years. With public opinion shifting towards healthier lifestyles and viewing chemical preservatives in a negative light, fermented products show great commercial promise. New starter cultures are being identified using more sophisticated methods to ensure their effectiveness and viability. *In silico*-based methods and research in the health-promoting activities of LAB in fermentates are on the rise, along with the characterization of traditional products that have been associated with good health.

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Figures and Tables

Figure 1**.** Schematic representing the relationships between fermenting microbes, fermented dairy products, and the consumer.

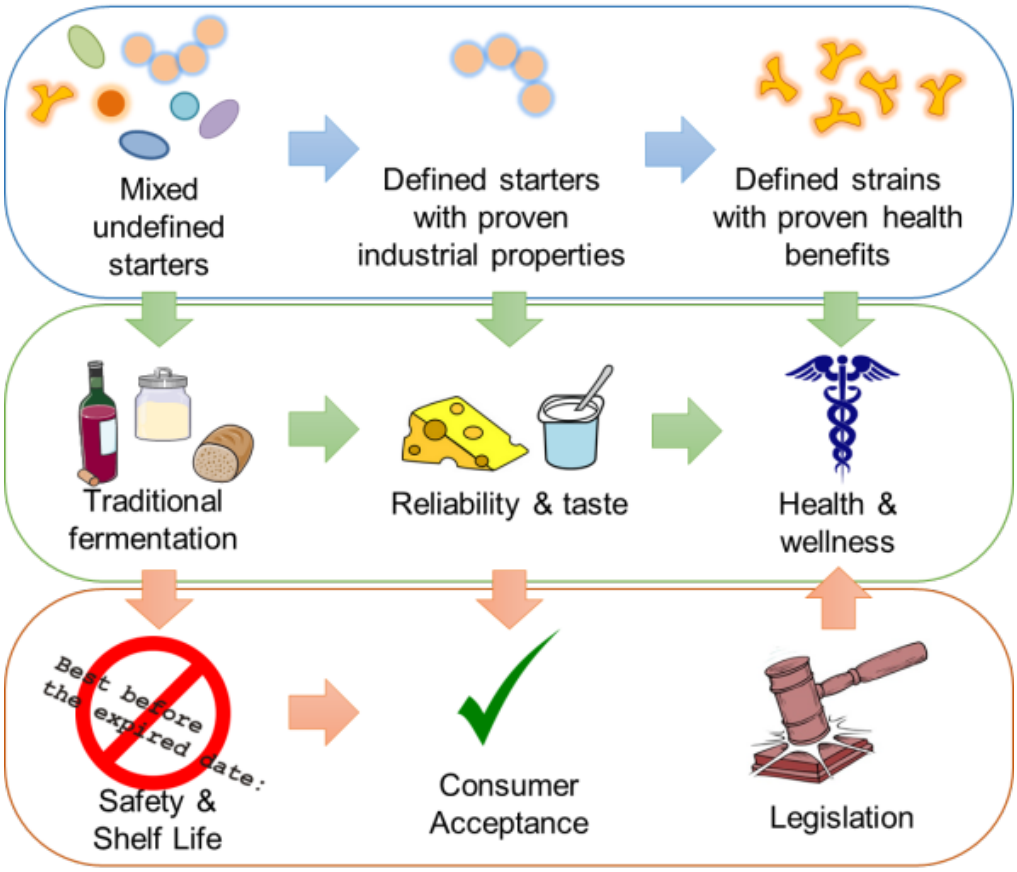
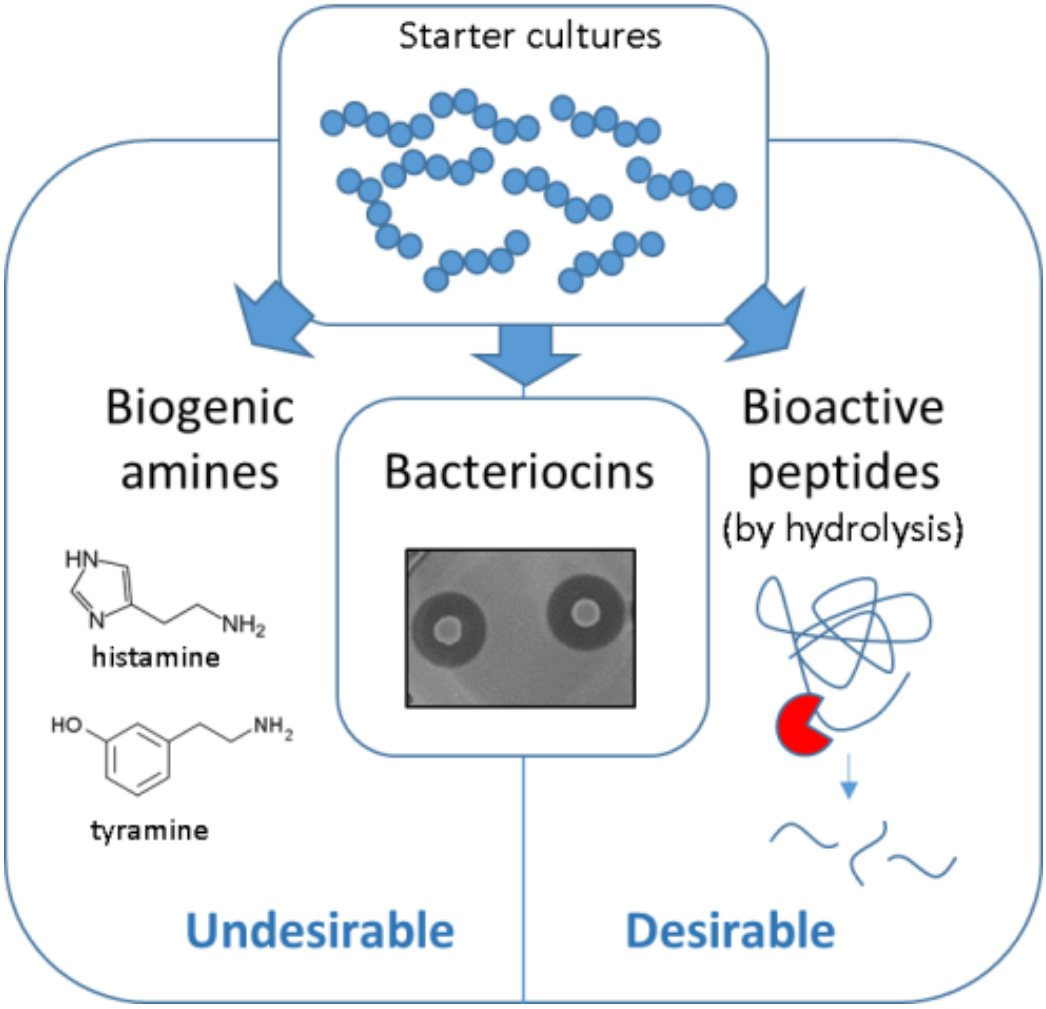


Figure 2. Desirable and undesirable bioactive metabolites produced during fermentation which can impact choice of starter cultures. Fermentation starters can produce a number of desirable and undesirable bioactive metabolites. Biogenic amines (left) are an undesirable product in most fermentations due to their toxicity. Bioactive peptides (right) produced through enzymatic release are desirable by-products due to positive biological activity. Bacteriocins (centre) are desirable as a known probiotic trait, but potentially undesirable in a starter culture due to possible impact on other fermenting cultures.



Chapter 2. Shelf life properties of artisanal style yoghurt produced with milk from grass-fed versus indoor-housed Irish cows

Abstract

Yoghurt quality is directly affected by the quality of the milk used in production which in turn can be affected by cow's diet. Using milks derived from a perennial ryegrass (*Lolium perene* L.) pasture and total mixed ration diets, full fat artisanal-style yoghurts were produced, referred to as perennial ryegrass (GRS) yoghurt and total mixed ration (TMR) yoghurt, respectively, and the techno-functional and volatile properties were investigated over 28 days of cold storage. Pasture feeding increased the protein content of milk by 0.032% ($p = 0.002$) but led to a significantly lower lactose content ($p = 0.019$) compared with total mixed ration diets. *S. thermophilus* growth during yoghurt fermentation was affected by the bovine diet, increasing by 0.64 $\log_{10}$ CFU ml^{-1} in TMR yoghurt ($p = 0.015$). GRS derived yoghurt was significantly greener and yellower than TMR yoghurt at every timepoint throughout shelf life. Texture analysis showed an increase in firmness ($p = 0.04$) and consistency ($p = 0.045$) in GRS yoghurts at day 28 of storage. Volatile profiles assessed by head space-solid phase micro extraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS) found a higher concentration of characteristic yoghurt flavour and aroma compounds such as acetaldehyde, acetic acid, 2,3-butanedione, and 2-butanone in TMR yoghurts and increased off-flavour aldehyde compounds during the early shelf life of GRS yoghurts. These data illustrate a potentially negative effect of GRS derived milk on yoghurt flavour counterbalanced against an improvement in late-shelf life texture and health.

Introduction

Yoghurt is consumed globally for its highly nutritious and desirable sensory properties. Yoghurt is produced through the addition of starter cultures, generally a combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*, to milk followed by incubation at a temperature ranging from 30 to 42 °C until a pH of 4.6 or below is achieved. During fermentation, the starter cultures act symbiotically to digest lactose, producing lactic acid which causes gel formation giving yoghurt its characteristic texture and contributing towards flavour. Yoghurt has a long history of production and comes in many forms such as natural, sweetened, fruit, fortified, strained, probiotic, whole-fat, skimmed fat, lactose free, and more recently 'alternative milk' yoghurts (1-3). The consistency, flavour and aroma of natural yoghurt vary by region depending on the quality of milk, production process, and the use of specific starter cultures (4, 5). Volatile flavour and aroma compounds such as those produced during fermentation or present from the source milks can alter the sensory properties of yoghurt, impacting consumer acceptance. Yoghurt is most commonly produced using bovine milk though others such as ovine and caprine milks are also used, that develop distinct texture, taste, and aroma profiles (6, 7).

Temperate countries such as Ireland and New Zealand primarily maintain dairy herds on pasture-based diets as opposed to total mixed ration (TMR) diets which are common in other parts of Europe and worldwide (8). Irish pasture consists mainly of perennial ryegrass (*Lolium perene* L.) and clover (*Trifolium repens* L.) though this can vary according to country and climate (9). There are many benefits to a pasture based-diet for cows including improved animal welfare, a lower environmental impact with better sustainability and improved consumer perception (10-13). It has been established that the diet of the producing animal can affect the composition of bovine milk and increase the concentration of compounds beneficial to human health (14-20). Recent research has shown that cows fed perennial ryegrass pasture (GRS) based diets compared to total mixed ration (TMR) diets produce milk with improved protein and mineral content containing increased levels

of unsaturated fatty acids (UFAs) (21, 22). The differences in milk composition have been shown to further impact products developed such as butter, Cheddar cheese, milk powders, and mozzarella cheese (23-25). Pasture derived products have different sensory attributes and improved nutritional aspects compared to those derived from TMR diets.

No studies to date have investigated the effect of GRS versus TMR feeding systems on the techno-functional and volatile properties of yoghurts throughout an entire shelf life. Akbaridoust *et al.* (2015) investigated the effect of a partial mixed ration diet (TMR incorporated into grazing diet) (PMR) on bovine milk and yoghurt composition, organic acids and flavour compounds (26). They identified differences in the relative abundances of flavour compounds between diets, notably in important aroma compounds such as acetaldehyde and ketone compounds and determined that PMR improves the overall aroma of yoghurt (26).

The objective of this study was to investigate the effect of the two widely used feeding systems, outdoor GRS and indoor TMR on the techno-functional characteristics and volatile properties of set yoghurt throughout 28 days of shelf life.

Materials and Methods

Experimental design, sample collection and yoghurt Manufacture

The experimental design for this study was the same as that previously described by O'Callaghan et. al (2016) (21), without the inclusion of milk from cows maintained on perennial ryegrass/white clover pasture. Briefly, thirty-six spring-calving Friesian cows were allocated to 2 groups (n = 18) at the Teagasc Animal and Grassland Research and Innovation Centre (Moorepark, Fermoy, Co. Cork, Ireland). Groups were randomized based on milk yield, milk solids yield, calving date (mean calving date: February 19, 2015), and lactation number. Group 1 was housed indoors and fed a TMR diet; group 2 was maintained outdoors on perennial ryegrass-only pasture (GRS). For further information on the chemical and nutritional values of each of the diets, see O'Callaghan et al. (2016). Briefly, the TMR diet consisted of 7.15 kg grass silage, 7.15 kg maize silage, and 8.3 kg of concentrates, on dry matter (DM) basis. TMR was available *ad-libitum* at 0830 h daily from electronically controlled individual feed bins (Mealmaster; Griffith Elder and Company Ltd., Suffolk, UK). GRS fed cows consumed ~18 kg of DM/d as estimated by pre-grazing herbage mass and daily post-grazing sward heights, as previously described (27). Milking took place at 0730 and 1530 h daily. To obtain a representative sample of milk, the cows in each of the feeding systems were milked separately into designated 5,000-L refrigerated tanks. The evening milk was stored at 4 °C overnight, to which the morning milk was then added. Tanks were maintained at 4 °C and agitated before sample collection. Milk was collected from both groups for yoghurt manufacture on 3 separate occasions over a 3-wk period in August 2017. Milk samples were homogenized using 2-stage homogenization at 5,000 to 150,000 kPa [GEA Niro Soavi S.p.A. Type: NS2006H]. The milk was then pasteurized using a Microthermics unit (UHT/HTST Electric Model 25HV Hybrid, Liquid Technologies, Wexford, Ireland) heated to 72 °C and held for 15 s, then cooled to 42 °C. Each milk sample was transferred at 42 °C to the sterile product outlet and aseptically poured into sterile 5-l glass bottles. Additional milk was aliquoted into sterile 100 ml pots (Sarstedt, Germany) and frozen at -20 °C for volatiles analysis. Five litre volumes of pasteurised milk were inoculated

with 0.2 g l^{-1} of CH-1 (Chr-Hansen, Denmark). After mixing to ensure thorough dispersion of the culture into the milk the five-litre volume was poured aseptically into sterile 100 ml pots (Sarstedt, Germany) and incubated at 42 °C until a pH of 4.6 was achieved. Pots were transferred to tubs of ice water until cool and stored at 4 °C for 28 days for analysis. Triplicate batches of yoghurt were produced from the two feeding systems and each of the yoghurts within each batch was manufactured on the same day.

Compositional analysis

Prior to heat treatment and homogenisation milks were analysed for fat, protein, lactose, and total solids by infrared absorption spectroscopy using a FT6000 Milkoscan (Foss Ireland Ltd., Dublin, Ireland.) Total protein and solids were measured at day seven of shelf life. Protein nitrogen content was assessed by Kjeldahl method (28) and values were multiplied by 6.38 to obtain total protein values. Total solids content of yoghurt was measured by recording the weight lost from samples (initial weight of 10 ± 0.5 g) after drying in an oven at 102 °C for at least 15 h.

Shelf life analyses

Shelf life analyses were carried out on day 1, 7, 14, 21, and 28 of storage unless stated otherwise. Viable organisms were enumerated from 1 ml aliquots milk and yoghurt at specific timepoints. Serial dilutions were performed in maximum recovery diluent (MRD; Oxoid c/o Fannin Healthcare, Dublin, Ireland) and plated onto the appropriate selective medium for each strain. *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* were enumerated on de Man, Rogosa and Sharpe (MRS; Difco laboratories, Detroit, MI USA) adjusted to pH 5.4 with glacial acetic acid (Sigma Aldrich, Wicklow, Ireland) and M17 (Merck, Darmstadt, Germany) supplemented with 1% lactose (Oxoid c/o Fannin Healthcare, Dublin, Ireland). pH was determined throughout shelf life, pre- and post-fermentation using a bench-top pH meter (model MP220, Mettler-Toledo, Greifensee, Switzerland).

Colour was measured prior to pasteurisation, post-pasteurisation and throughout shelf life using the CIE L*a*b* method. Three millilitres of milk or stirred

yoghurt aliquots were pipetted into a plastic cuvette (Sarstedt) and measured for L* (lightness-darkness), a* (green-red), and b* (yellow/blue) using a Minolta Chroma-Meter CR-400 (Mason Technology Ltd.).

Syneresis was assessed by the centrifugation method as outlined previously (29) and expressed as:

$$\text{Syneresis (\%)} = \frac{\text{Weight of separated whey (g)}}{\text{Initial weight of yoghurt sample (g)}} \times 100$$

Titratable acidity of yoghurt was measured by adding one drop 1% phenolphthalein (Sigma Aldrich, Wicklow, Ireland) to 10 g yoghurt and titrating with 0.1 M NaOH until a light rose pink colour persisted. Titratable acidity was expressed as % of lactic acid, calculated as below:

$$\text{\% Lactic acid} = \frac{\text{0.1 M NaOH (ml)}}{10}$$

Single pots, taken from 4 °C storage, were measured for firmness, consistency, cohesiveness, and index of viscosity using a 35-mm flat-disk backward extrusion rig on a Texture Expert Exceed system (Stable Microsystems, Godalming, UK). A 2-kg weight mounted on a 5-kg load cell was used to calibrate probe force. Trigger force was set at 2 g. The probe penetrated to a depth of 25 mm and returned to the starting point. All test probe speeds were set at 1 mm/s.

Volatiles analysis

At each timepoint post-fermentation 100 ml pots of yoghurt were frozen at -20 °C for collective volatiles analysis. Analysis of volatiles was carried out by HS-SPME and GC-MS of thawed yoghurt samples. 4 g of sample was added to a 20 ml screw capped SPME vial and equilibrated to 40 °C for 10 mins with pulsed agitation of 5 sec at 500 rpm. Samples were analysed in triplicate using a Shimadzu AOC-5000 autosampler (Shimadzu UK Ltd., Milton Keynes, UK). A single 50/30 µm Carboxen™/divinylbenzene/ polydimethylsiloxane (DVB/CAR/PDMS) fibre was used for the analysis. The SPME fibre was exposed to the headspace above the samples for 20 min at a depth of 1 cm at 40 °C. The fibre was retracted and injected into the

GC inlet and desorbed for 2 min at 250 °C. Injections were made on a Shimadzu 2010 Plus GC with an Agilent DB-624 UI (60m x 0.32mm x 1.8µm) column using a split/splitless injector with a 1/10 split. A merlin microseal was used as the septum. The temperature of the column oven was set at 40 °C, held for 5 min, increased at 5 °C/min to 230 °C then increased at 15 °C/min to 260 °C, yielding at total GC run time of 50 min. The carrier gas was helium held at a constant flow of 1.2ml/min. The detector was a Shimadzu TQ8030 mass spectrometer detector, ran in single quad mode. The ion source temperature was 220 °C and the interface temperature was set at 260 °C. The MS mode was electronic ionization (70v) with the mass range scanned between 35 and 250 amu. Compounds were identified using mass spectra comparisons to the NIST 2014 mass spectral library, a commercial flavour and fragrance library (FFNSC 2, Shimadzu Corporation, Japan) and an in-house library created using authentic compounds with target and qualifier ions and linear retention indices for each compound using Kovats index. Retention indices were matched against peer reviewed publications where possible to confirm compound identification. Spectral deconvolution was also performed to confirm identification of compounds using AMDIS. Batch processing of samples was carried out using MetaMS (30). MetaMS is an open-source pipeline for GC-MS-based untargeted metabolomics. An auto-tune of the GC-MS was carried out prior to the analysis to ensure optimal GC-MS performance. A set of external standards was run at the start and end of the sample set and abundances were compared to known amounts to ensure that both the SPME extraction and MS detection was performing within specification.

Statistical analysis

Statistical analyses were performed using SPSS v24.0.0 (IBM Statistics Inc., Armonk, NY). Data sets were examined for normality using the Shapiro-Wilk test, and equal variances using Levene's test. An independent T-test or Mann-Whitney U test was used to compare between diets at each timepoint. Characteristics over time were compared at each timepoint by independent T-test. Characteristics between timepoints were compared using delta values by independent T-test. P-values ≤ 0.05 were considered statistically significant.

Results and Discussion

Milk and yoghurt composition

Milk composition was determined prior to pasteurisation and yoghurt production. Solids and fat content were not significantly affected by diet, though greater variation was observed in the fat content of TMR milk (Fig. 1a and b). GRS milk contained less lactose (0.11 ± 0.02%, mean difference ± SD) and more protein (0.32 ± 0.05%) than TMR derived milk (Fig. 1c and d). True protein and total solids of yoghurt at seven days post fermentation were consistent with the raw milk data (Fig. 1e and f). GRS yoghurts contained more protein (0.21 ± 0.06%) (Fig. 1f) and there was no significant difference in solids content (Fig. 1e).

Diet is known to impact the composition and quality of bovine milk (14-20, 31). Some differences were found between this study and milks from the same diets across mid- to late-lactation (LL) periods generated in previous reports. Diet did not influence the total fat or total solids content of the milks (Fig. 1a and b). Previous research has shown increased fat and total solids content in GRS derived milks and despite fat being one of the components most affected by bovine diet (21, 32). This difference may be attributed to the large variation (Fig. 1b) in fat content of the TMR milks which in turn increased the variation in the total solids content, reducing statistical significance. Lactation period can affect the composition of cow's milk, and milk from TMR fed cows has been previously found to have reduced fat content and higher lactose than GRS in late lactation (33). This study was performed over a three-week period spanning early to late August in the transition period between mid- and late-lactation which may account for some of the variation in fat content of the TMR milk. The increased lactose and decreased protein content of TMR milk (Fig. 1c and d) are consistent with previous studies investigating the effects of the same diets (21, 22). Solids and true protein content of yoghurts measured were consistent with the milk data (Fig. 1e and f).

Protein and fat content are key factors which influence the texture of yoghurt as total solids significantly affect its physical properties (34-36). Higher fat and

protein content result in increased thickness/viscosity and mouthfeel and improved sensory properties (37).

Shelf life analyses

Mean colony counts of *Lactobacillus delbrueckii* ssp. *bulgaricus* (hereafter referred to as *L. bulgaricus*) and *Streptococcus thermophilus* were not significantly different in the GRS and TMR milks prior to fermentation (PF) or after fermentation (day one) (Fig. 2a). The growth of *S. thermophilus* during fermentation ($\Delta\log_{10}$ CFU ml^{-1} between PF and day 1), however, was significantly higher in TMR yoghurts (2.35 ± 0.08 log increase) than GRS (1.71 ± 0.26 log increase) ($p = 0.015$). By the end of fermentation TMR yoghurts reached 7.31 ± 0.16 $\log_{10}$ CFU ml^{-1} and 6.30 ± 0.88 $\log_{10}$ CFU ml^{-1} for *L. bulgaricus* and *S. thermophilus* respectively, and GRS yoghurts reached 7.60 ± 0.33 $\log_{10}$ CFU ml^{-1} and 5.54 ± 0.43 $\log_{10}$ CFU ml^{-1}, respectively (Fig. 2a). Commercial starter cultures are typically included at an inoculum concentration of 6 – 7 $\log_{10}$ CFU ml^{-1} at the start of fermentation in order to impact the flavour and chemical profiles of the product. Though the starter culture was included at the manufacturer recommended concentration (0.2 g l^{-}1), colony counts of *S. thermophilus* were >100 fold below 6 – 7 $\log_{10}$ CFU ml^{-1} at the start of fermentation, and failed to achieve 7 $\log_{10}$ CFU ml^{-1} during fermentation. *L. bulgaricus* viability was stable throughout shelf life. *S. thermophilus* counts were higher on average in TMR yoghurts on day seven and day 21 by 0.82 log and 1.04 log respectively (Fig. 2a). No significant difference was observed at any other time points. Growth of starter culture species *L. bulgaricus* and *S. thermophilus* has been subject to extensive research and their relationship during fermentation is well established (38-41). Many studies report a stimulating effect on the growth of *S. thermophilus* strains by the presence of free amino acids such as histidine, valine, methionine, and glutamic acid (42-44). However previous studies of milks from GRS and TMR diets in Cheddar cheese production found no significant difference in the relative abundance of free amino acids during ripening (24). Initially starter cultures grow exponentially using free sugars, peptides and amino acids that are available in the milk, subsequently amino acids become limiting and cultures enter a non-exponential growth phase during which proteolytic enzymes are expressed. The moderately lower lactose

content of the GRS milk may explain some of the more growth of *S. thermophilus* during fermentation, though other compositional factors such as protein content and volatile profiles may have had an impact. Given the large amount of variation and the lack of significant difference in *S. thermophilus* counts between diets on days 1, 14, and 28, this observed difference could be due to a confounding factor such as the reduction in starter culture as described above. Diet did not impact the pH of yoghurts during shelf life (Fig. 2b).

Colour analysis was performed on raw milk and pasteurised milk prior to fermentation, and on the yoghurts throughout shelf life (Fig. 3). No difference was found in L* (lightness) scores or a* (green/red) scores of the raw and pasteurised milks between diets (Fig. 3a and b). Raw GRS milk had a significantly higher b* score (blue/yellow) than TMR milk ($p = 0.036$) (Fig. 3c). Yoghurt L* scores were not significantly altered by diet during cold storage (Fig. 3a). GRS yoghurt had a lower a* score and higher b* score than TMR at every time point post-fermentation (Fig. 3b and c). The feeding system is known to affect the colour of dairy products with pasture diets producing butter which is yellower (higher b* values) and redder (higher a* values) than butter produced with TMR milk (23). A number of studies have found the increased b* value of butters to correlate with an increase in the quantity of trans-β-carotene, a beneficial carotenoid precursor to vitamin A which is low in TMR concentrates (23, 45, 46). GRS yoghurt also had a lower a* score, indicating a greener colour than TMR. The greener colour may result from the high levels of riboflavin in whey which is present in greater quantities in pasture derived milks (21, 47).

Extent of syneresis (expressed as % of whey separated from yoghurt over initial weight of yoghurt) and titratable acidity were not significantly affected by diet or shelf life (Fig. 4). Syneresis in yoghurt is directly related to the fat and protein content of the initial milk but can also be affected by other factors such as heat treatment of the milk and exopolysaccharide producing starter cultures (38, 48, 49). Though the protein content of the GRS yoghurt was higher, the mean total solids content was similar between diets, and as such the overall extent of syneresis was not affected by milk type. TMR and GRS yoghurts reached a concentration of 0.97 ±

0.05 % and 0.95 ± 0.07 % lactic acid, respectively, higher than the minimum of 0.6 % according to CODEX standards for yoghurt (50).

In order to determine the effect of diet on the development of texture during storage, yoghurts were characterized using a flat-disk backward extrusion method. Mean texture measurement profiles and corresponding texture characteristic data are shown in Fig. 5. Firmness is described as the force necessary to penetrate the gel structure of the yoghurt, and cohesiveness is the amount of deformation the gel matrix can withstand before rupturing (51, 52). GRS derived yoghurts had higher mean firmness at every time point, reaching significance at the final timepoint after 28 days of storage (453.2 ± 63.7 g and 279.0 ± 77.7 g for GRS and TMR, respectively) (Fig. 5b). Cohesiveness was not significantly affected by diet at any timepoint (Fig. 5d). Consistency has been described as the property by which the yoghurt gel resists a change in shape (53). Consistency values were significantly higher in GRS (9373.9 ± 1414.6 g sec^{-1}) than TMR (5662.2 ± 1731.3 g sec^{-1}) yoghurts at day 28 (Fig. 5c). A previous study comparing the effects of cow's diet on the production of skim milk powder and their yoghurt gel structures found that the GRS diet improved firmness, cohesiveness and consistency when compared with TMR (25). As this study did not standardise the base milk for protein and fat content as would be common in commercially produced yoghurt, the same result was not evident on day one post-fermentation.

Volatiles profile

Using head space solid phase micro extraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS), we identified 36 volatile compounds present in the heat-treated milks and yoghurts (Table 1 and 2). Four of 39 compounds (propanal-2-methyl, 3-methyl-2-butenal, 2,5-dimethyl furan, 3-hexanone) were found only in the yoghurts. Twenty compounds differed significantly between milk types for at least one timepoint (Table 1.) Of the 20 compounds affected by diet, 16 were increased in TMR derived yoghurt (Table 2.). The distinct flavour of yoghurt arises from the combination of aroma compounds of the milk source, by specific compounds generated by bacterial metabolism during fermentation and via

degradation of milk constituents during heat processing (54, 55). Over 90 flavour and aroma compounds have been identified in yoghurt, a number of which are essential contributors to the characteristic sensory profile (5, 54). Lactic acid, acetic acid, ethanol, acetaldehyde, acetoin, dimethyl sulphide 2,3-butanedione (diacetyl), 2,3-pentanedione, and 2-butanone are some of the key contributors to flavour and aroma (5, 54, 56-59).

Of these important flavour contributors, acetaldehyde, acetic acid, dimethyl sulphide, 2,3-butanedione, 2,3-pentanedione, and 2-butanone were increased in TMR derived yoghurts at various timepoints during storage (Table 1 and 2.). Acetaldehyde was significantly higher in TMR yoghurts at every timepoint following fermentation. The carbonyl compound acetaldehyde has been described as the compound which drives the most typical yoghurt flavour (54, 60). It is produced by both *L. bulgaricus* and *S. thermophilus* during fermentation (55, 61). 2,3-butanedione (diacetyl) is also a by-product of starter culture fermentation and is associated with a creamy, buttery, sour milk flavour and contributes to the delicate, full-bodied flavour of yoghurt (54, 55). 2,3-butanedione was increased on day one of storage in TMR yoghurts but was found to be similar between both yoghurt types at each subsequent timepoint, as was 2,3-pentanedione. Acetic acid was significantly increased in TMR yoghurts on day 15 and 28 of storage. Acetic acid produced by starter cultures in lactose fermentation is associated with a vinegary, pungent and acidic odour and flavour and high concentrations can be unpalatable to consumers (54, 55, 60, 62). Dimethyl sulphide was significantly higher in TMR yoghurts on day one and day 14 of storage. Sulphur compounds are produced through free amino acid catabolism of the starter cultures and though they can have positive effects on cheese sensory profiles, their presence in yoghurt can contribute towards off flavours (63, 64). The ketone compound 2-butanone was increased nearly tenfold in TMR yoghurts at every timepoint measured, the biggest relative difference in a single compound between diets (Table 2). This difference was already apparent in the base milks (Table 1). 2-Butanone has been described as a compound of minor importance to the flavour of milk, but it elicits ‘yoghurt’ odour and contributes to the flavour of yoghurt (65, 66). Ketones, certain acids and alcohols are produced by fat hydrolysis

during yoghurt fermentation (59). Akbaridoust et al. (2015) investigated the effect of partial mixed ration and pasture based diets on organic acids and volatile organic flavour compounds in yoghurt (26). They attributed the generation of different relative volatile compound concentration in yoghurts mostly to the increased fat content of the partial mixed ration milk (26). As fat content in this study was not significantly altered by diet, the differences in some of these compounds may be due differences in the growth of *S. thermophilus* during fermentation. Dan *et al* (2017) previously described an increase in aldehydes and ketones in fermented milk products with a higher ratio of *S. thermophilus* to *L. bulgaricus* (67).

Yoghurts produced from GRS milk were found to have higher quantities of heptanal, nonanal, butanoic acid and cyclohexane, mainly during the early stages of shelf life (storage days 1 – 14) (Table. 1). Butanoic acid (otherwise known as butyric acid) was significantly increased on day 21 of storage (Table 2). Butanoic acid is a primary source of energy for colonocytes and has been associated with gut health (68). It is produced in the mammary gland and secreted directly into milk and can also be a product of microbial fermentation by *L. bulgaricus* and *S. thermophilus* (32, 55). There was no evidence that diet affected the levels of butanoic acid between the milks, as has been observed previously (21, 45). Previous studies which have identified butanoic acid in yoghurt have not found an increase in short chain fatty acids during storage and we are unable to explain the significant increase after 21 days (69). The concentration of volatile aldehydes (C_5 – C_9) in yoghurt has been suggested as a marker for degradation of quality within storage (70). Heptanal and nonanal C_7 and C_7 aldehydes respectively, originate from the oxidation of unsaturated fatty acids (71). It has been established that GRS derived milk contains an increased amount of healthier unsaturated and poly-unsaturated fatty acids relative to saturated fatty acids which improves health related attributes such as the thrombogenicity index of the milk (21). Future studies will benefit from the use of sensory panels to evaluate if the altered volatile profiles affect the organoleptic quality of the yoghurts and impact on consumer acceptability. TMR profiles are more likely to possess desirable 'natural yoghurt' sensory characteristics. However, GRS yoghurt could present a healthier alternative base for addition of fruit and flavours.

Recent Irish consumer trends indicate a preference for plain and natural yoghurt flavours which can then be sweetened through the addition of fruit and berries, and as such the difference between diets if detectable could impact consumer choice (72).

Conclusion

Yoghurts produced from grass derived milk contained more protein and had a more intense colour than their indoor TMR diet counterpart. They also had improved texture characteristics, firmness, and consistency in late-shelf life. However, TMR derived yoghurts showed higher concentrations of flavour and aroma compounds known to drive characteristic natural yoghurt flavour, likely due to a higher lactose content and improved starter strain growth during fermentation. GRS yoghurt had higher concentrations of compounds unpalatable to consumers as off flavours in early shelf life. Further studies will be required to determine if the organoleptic properties associated with the different volatile profiles affect consumer acceptability.

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Figures and Tables

Figure 1. Composition of milks used in the production of yoghurt, and yoghurt at 7 days post-fermentation. (a) Total solids, (b) total fat, (c) lactose, (d) and protein content of raw milks used in yoghurt production. Total solids (e), and protein (f) of yoghurts produced.

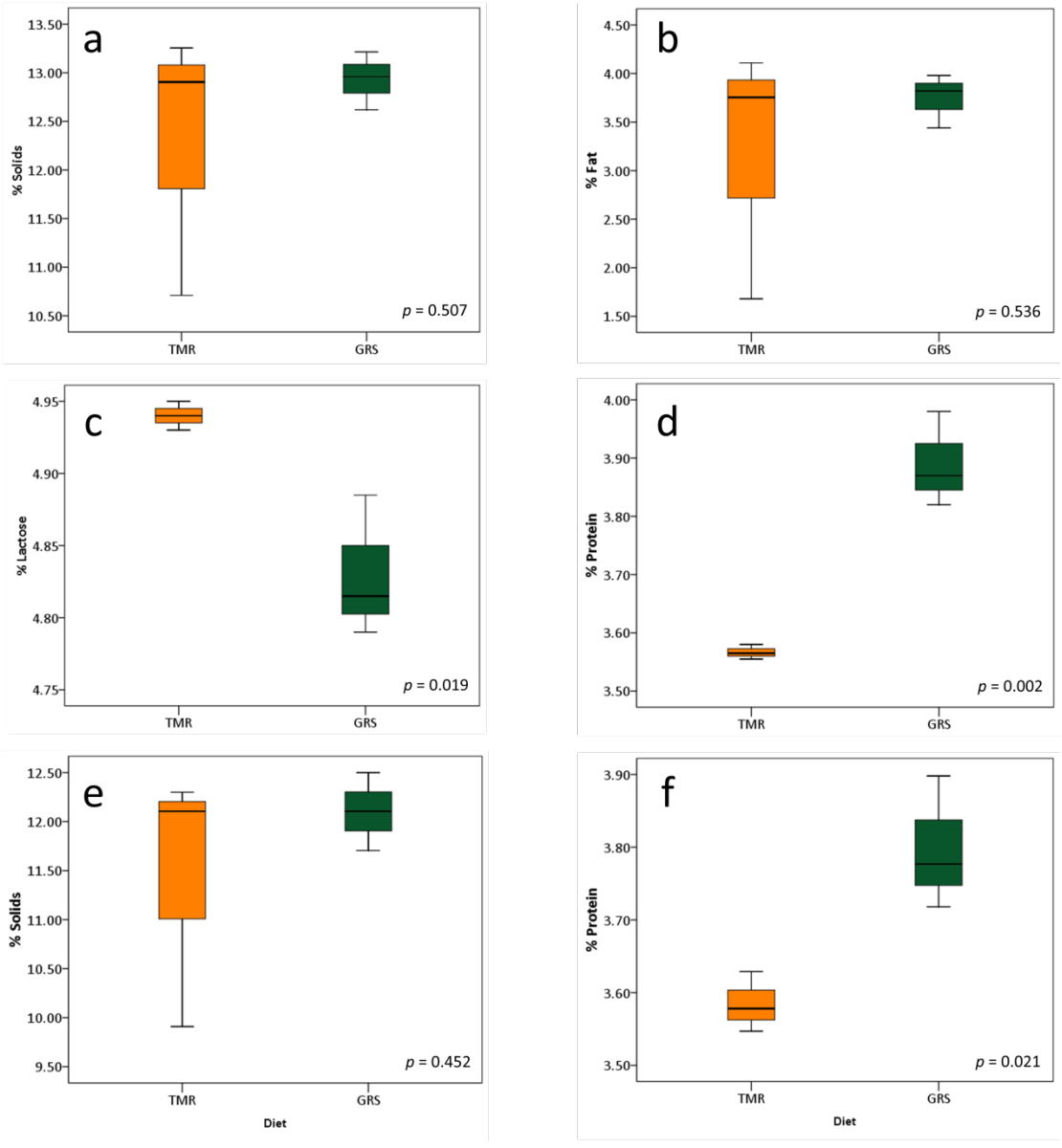


Figure 2. Stability of starter culture (a) and pH (b) during shelf life (PF: pre-fermentation).

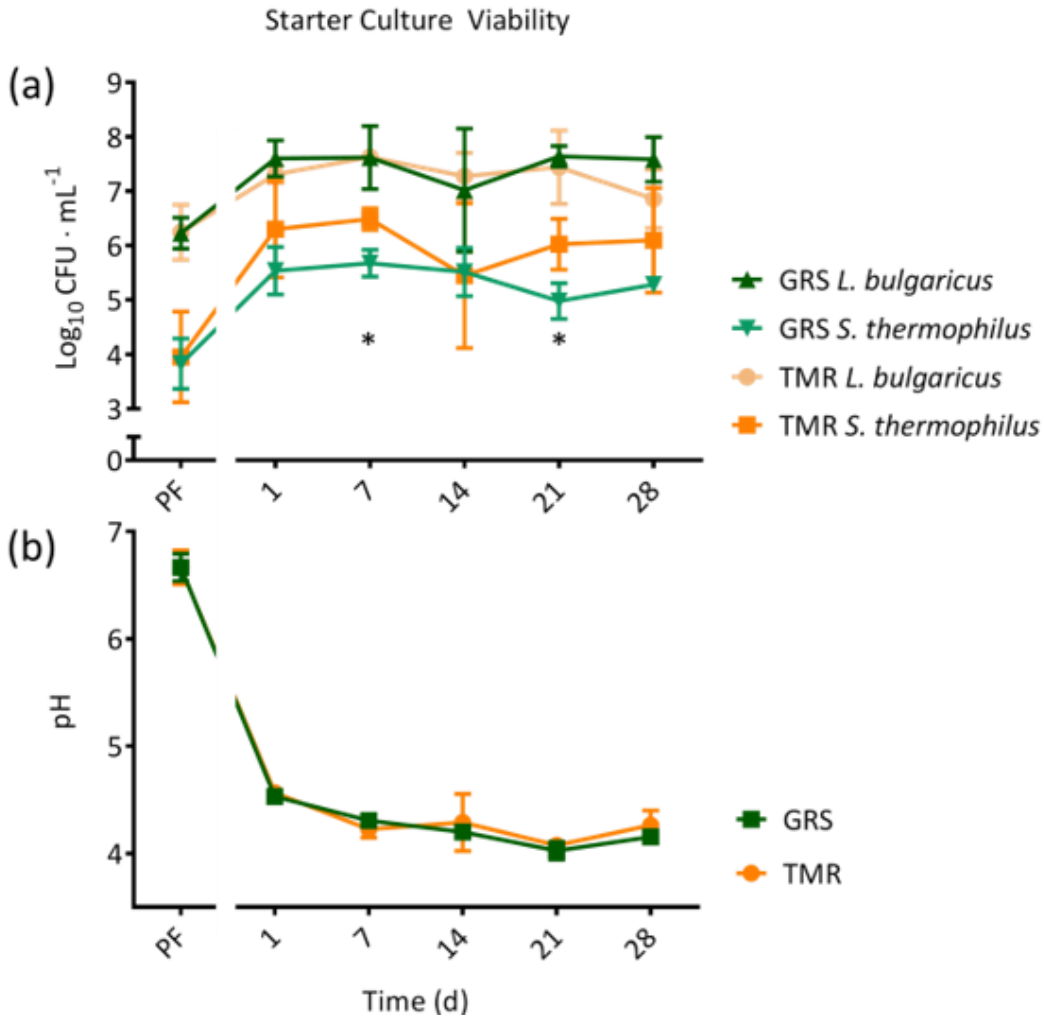


Figure 3. Colour characteristics of raw milk (R), pasteurised milk (P), and yoghurt over 28 days of storage. L* (lightness), a* (green/red), b* (blue/yellow). (* = p ≤ 0.05.)

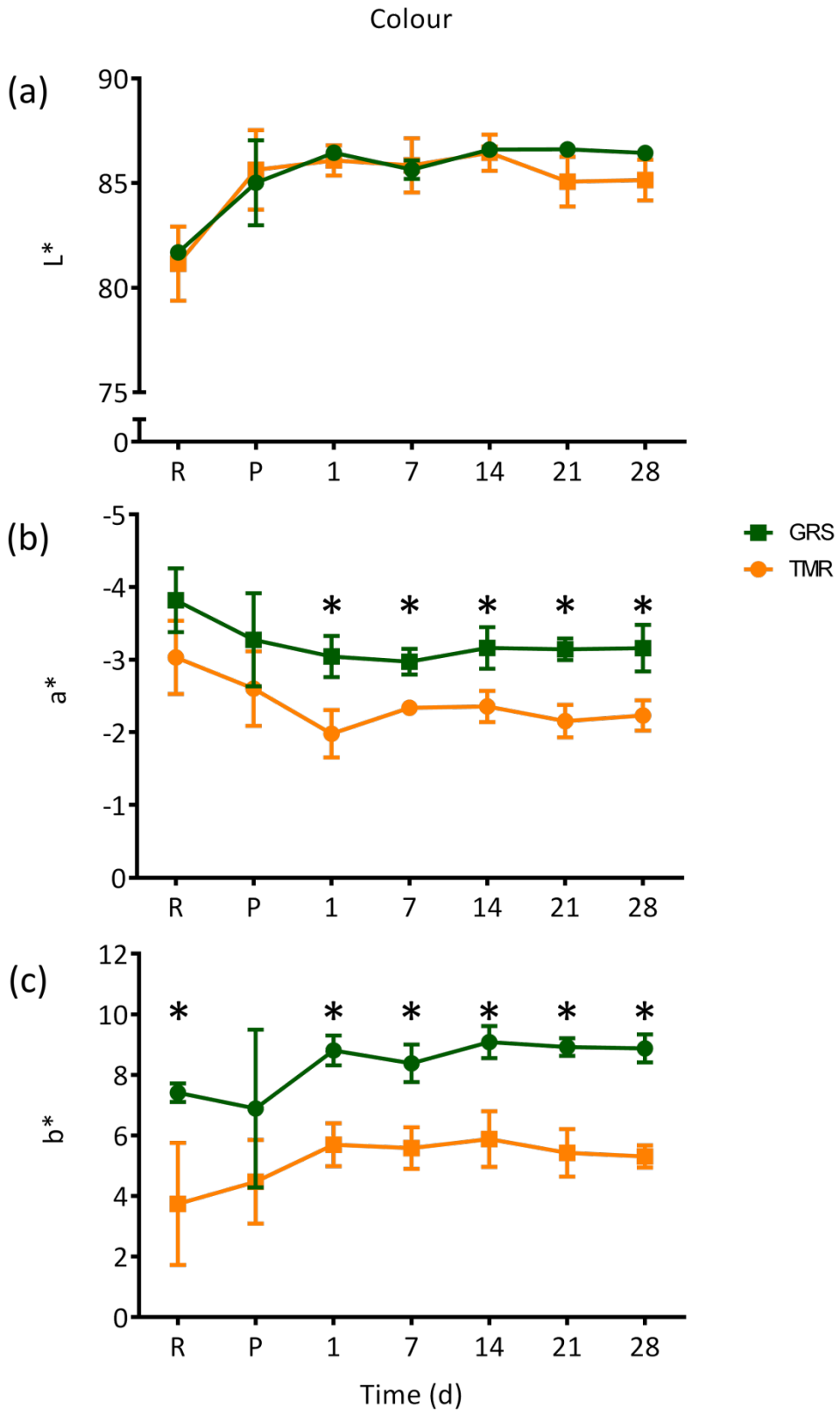


Figure 4. Quality characteristics of yoghurts during shelf life. (a) Extent of syneresis and (b) percentage lactic acid.

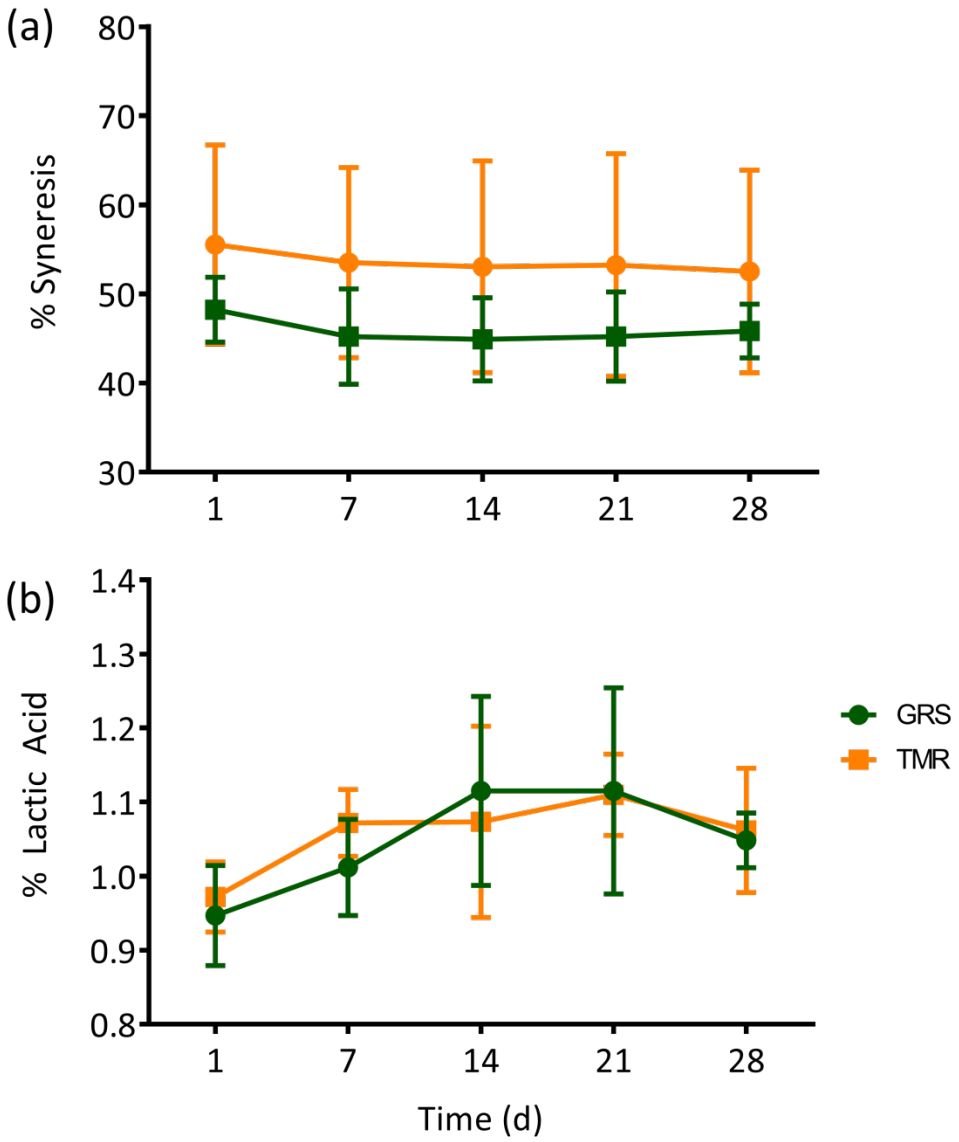


Figure 5. Texture characteristics of yoghurts throughout 28 days of storage. (a) Mean texture profiles of yoghurts produced from GRS and TMR yoghurts during shelf life. Corresponding (b) firmness, (c) consistency, and (d) cohesiveness values of yoghurts at each timepoint during shelf life. (* = $p \leq 0.05$.)

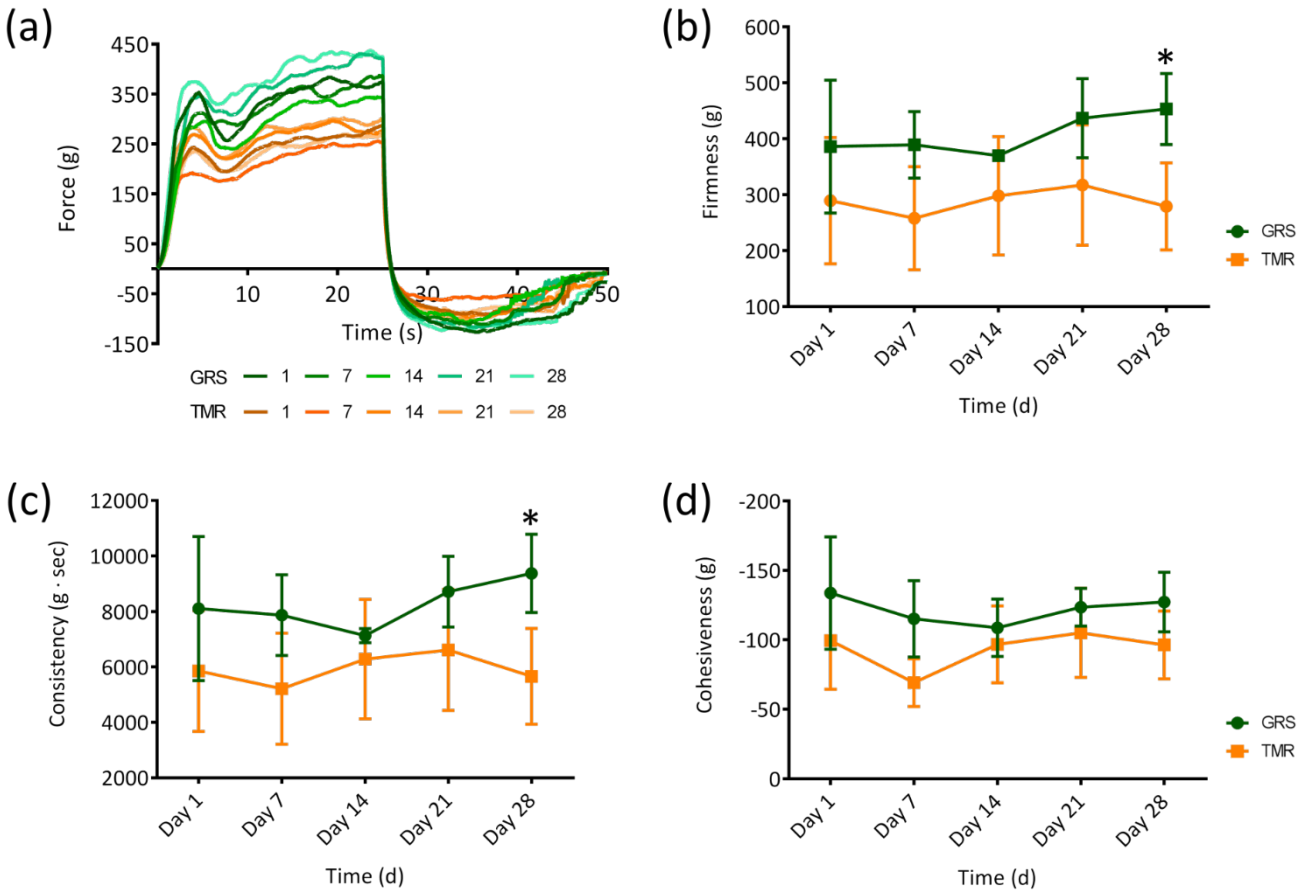


Table 1. Mean volatile compounds peak areas from pasteurised milk pre-fermentation and yoghurts during shelf life, prepared from perennial ryegrass (GRS) and TMR feeding systems.

Compound	PF		*p*	1 d		*p*	7 d		*p*	14 d		*p*	21 d		*p*	28 d		*p*
	GRS	TMR		GRS	TMR		GRS	TMR		GRS	TMR		GRS	TMR		GRS	TMR	
Acids																		
Acetic acid	3.17E+05	6.51E+05	0.294	3.18E+06	3.98E+06	0.302	4.89E+06	6.16E+06	0.374	4.05E+06	7.84E+06	**0.041**	5.84E+06	8.35E+06	0.071	5.23E+06	7.42E+06	**0.033**
Butanoic acid	5.14E+04	4.69E+04	0.927	9.64E+05	2.24E+05	0.177	1.48E+06	2.94E+05	0.116	1.35E+06	3.66E+05	0.198	1.62E+06	3.54E+05	**0.042**	1.57E+06	3.42E+05	0.071
Hexanoic acid	6.27E+04	5.78E+04	0.840	1.01E+06	2.15E+05	0.156	1.38E+06	2.36E+05	0.119	1.34E+06	2.75E+05	0.143	1.48E+06	2.86E+05	0.053	1.46E+06	2.78E+05	0.071
Octanoic acid	1.08E+04	8.57E+03	0.515	1.61E+05	3.85E+04	0.193	1.44E+05	3.44E+04	0.166	1.92E+05	3.82E+04	0.152	1.66E+05	4.86E+04	0.184	1.69E+05	3.47E+04	0.131
Alcohols																		
Ethanol	2.93E+04	1.10E+05	0.244	8.23E+03	4.58E+04	0.16	1.27E+04	4.95E+04	0.152	1.95E+04	4.72E+04	0.247	2.14E+04	5.41E+04	0.265	7.05E+03	4.72E+04	0.179
2-Butanol	0.00E+00	8.65E+04	**0.001**	0.00E+00	4.89E+04	**0.001**	0.00E+00	4.76E+04	**0.004**	0.00E+00	4.19E+04	**0.004**	0.00E+00	4.79E+04	**<0.001**	0.00E+00	4.34E+04	**0.001**
1-Hexanol	1.71E+04	5.06E+04	0.334	1.89E+05	2.95E+05	**0.045**	1.65E+05	2.87E+05	0.07	1.68E+05	2.82E+05	0.117	1.62E+05	3.08E+05	**0.05**	1.71E+05	3.04E+05	**0.031**
2-Ethylhexanol	5.01E+04	9.05E+04	0.136	1.67E+05	2.61E+05	0.146	1.34E+05	2.51E+05	0.153	1.46E+05	2.64E+05	0.194	1.43E+05	2.84E+05	0.148	1.47E+05	2.81E+05	0.115
Aldehydes																		
Acetaldehyde	4.53E+04	9.94E+04	0.269	2.35E+06	4.08E+06	**0.002**	2.27E+06	4.07E+06	**0.007**	2.05E+06	3.56E+06	**0.012**	1.81E+06	3.98E+06	**0.004**	1.95E+06	3.29E+06	**0.003**
Propanal, 2-methyl-	–	–	–	5.05E+04	3.31E+04	0.282	5.06E+04	3.05E+04	0.255	4.05E+04	3.08E+04	0.459	4.53E+04	3.09E+04	0.326	4.47E+04	2.93E+04	0.284
Hexanal	2.81E+05	7.20E+05	0.453	2.29E+05	3.63E+05	0.084	2.44E+05	2.33E+05	0.84	1.16E+05	1.89E+05	**0.02**	1.42E+05	2.10E+05	0.252	1.71E+05	3.07E+05	0.291
2-Butenal, 3-methyl-	–	–	–	5.99E+04	7.12E+04	0.78	7.80E+04	1.18E+05	0.481	9.93E+04	1.14E+05	0.814	7.62E+04	1.51E+05	0.169	7.45E+04	1.02E+05	0.507
Heptanal	9.04E+04	1.88E+05	0.524	7.05E+04	4.04E+04	**0.002**	7.41E+04	3.47E+04	**0.005**	4.43E+04	3.13E+04	0.06	6.53E+04	3.53E+04	0.062	7.13E+04	4.43E+04	0.274
Benzaldehyde	3.14E+04	9.49E+04	0.082	9.33E+04	1.10E+05	0.774	7.73E+04	7.62E+04	0.954	5.54E+04	7.02E+04	0.192	5.10E+04	7.33E+04	**0.048**	6.87E+04	6.25E+04	0.748
Nonanal	1.77E+05	3.71E+05	0.424	1.03E+05	6.00E+04	**0.019**	6.57E+04	4.96E+04	0.089	5.75E+04	3.65E+04	**0.043**	6.60E+04	4.40E+04	0.315	6.35E+04	4.40E+04	0.218
Alkenes																		
1-Pentene, 2-methyl-	1.78E+06	2.55E+05	0.176	1.73E+05	1.09E+05	0.232	1.32E+05	3.05E+05	0.202	8.80E+04	1.88E+05	0.155	7.01E+04	2.09E+05	0.09	1.09E+05	1.64E+05	0.542
Benzenes																		
Toluene	6.68E+05	1.38E+05	0.181	8.50E+05	3.78E+05	0.355	9.15E+05	4.11E+05	0.315	9.00E+05	4.38E+05	0.369	8.41E+05	4.40E+05	0.352	9.33E+05	4.31E+05	0.352
o-Xylene*	1.83E+04	1.91E+04	0.838	4.03E+04	5.58E+04	0.285	4.34E+04	6.54E+04	0.322	4.09E+04	6.06E+04	0.31	3.77E+04	6.53E+04	0.219	4.43E+04	6.42E+04	0.379

Continued.

Compound	PF		*p*	1 d		*p*	7 d		*p*	14 d		*p*	21 d		*p*	28 d		*p*
	GRS	TMR		GRS	TMR		GRS	TMR		GRS	TMR		GRS	TMR		GRS	TMR	
Benzene, 1,3-bis(1,1-dimethylethyl)-	1.62E+05	2.18E+05	0.487	7.52E+05	9.29E+05	0.237	6.45E+05	8.36E+05	0.36	5.96E+05	8.70E+05	0.201	3.98E+05	9.90E+05	0.053	5.02E+05	7.64E+05	0.22
Ethers																		
Ethyl ether	1.18E+06	8.78E+05	0.784	1.59E+05	1.17E+05	0.552	1.09E+05	8.05E+05	0.391	1.55E+05	1.58E+05	0.983	1.95E+05	6.64E+04	0.164	6.59E+04	1.84E+05	0.242
Furans																		
2,5 Dimethyl furan	-	-	-	0.00E+00	2.56E+04	**0.035**	0.00E+00	5.41E+04	**0.004**	0.00E+00	5.75E+04	**0.003**	0.00E+00	8.05E+04	**0.001**	0.00E+00	6.05E+04	**<0.001**
Hydroxy ketones																		
Acetoin	0.00E+00	2.22E+05	0.374	1.47E+07	1.98E+07	0.083	1.48E+07	2.12E+07	0.095	1.33E+07	2.07E+07	0.095	1.36E+07	2.16E+07	0.059	1.39E+07	1.87E+07	0.101
2-Hydroxy-3-pentanone	1.67E+04	3.69E+04	0.556	5.71E+05	1.07E+06	**0.032**	5.80E+05	1.08E+06	**0.018**	5.60E+05	1.12E+06	**0.005**	4.98E+05	1.36E+06	**0.002**	5.25E+05	1.10E+06	**0.009**
Ketones																		
Acetone	6.77E+06	1.34E+07	0.186	5.87E+06	1.02E+07	0.143	6.27E+06	9.46E+06	0.204	6.19E+06	1.03E+07	0.144	6.21E+06	1.07E+07	0.146	6.50E+06	9.96E+06	0.206
2,3-Butanedione	0.00E+00	2.14E+05	0.184	1.25E+07	1.71E+07	**0.007**	1.78E+07	2.24E+07	0.213	1.81E+07	2.57E+07	0.133	1.97E+07	2.28E+07	0.212	2.15E+07	2.49E+07	0.52
2-Butanone	2.49E+05	4.61E+06	**0.003**	3.25E+05	3.20E+06	**0.001**	3.24E+05	2.95E+06	**0.005**	2.92E+05	3.07E+06	**0.002**	2.87E+05	3.09E+06	**0.001**	3.00E+05	2.85E+06	**0.001**
2-Pentanone	5.78E+05	5.25E+05	0.881	6.87E+05	1.10E+06	**0.018**	8.94E+05	1.30E+06	**0.004**	9.15E+05	1.61E+06	**0.002**	1.00E+06	1.91E+06	**0.001**	1.09E+06	1.66E+06	**0.018**
2,3-Pentanedione	0.00E+00	2.33E+04	0.374	7.47E+06	1.26E+07	**0.002**	1.27E+07	1.80E+07	0.223	1.30E+07	2.25E+07	0.183	1.48E+07	2.04E+07	0.1	1.52E+07	2.32E+07	0.219
3-Hexanone	-	-	-	9.65E+04	1.74E+05	**0.001**	1.74E+05	2.60E+05	0.235	1.75E+05	3.16E+05	0.146	2.02E+05	2.97E+05	0.091	2.03E+05	3.25E+05	0.205
2-Heptanone	1.04E+06	1.27E+06	0.672	1.33E+06	2.31E+06	**0.001**	1.49E+06	2.80E+06	**0.011**	1.57E+06	2.79E+06	**0.006**	1.48E+06	3.02E+06	**0.006**	1.48E+06	2.81E+06	**0.001**
Undecan-2-one	7.99E+03	1.92E+04	0.345	3.24E+04	5.10E+04	**0.035**	2.60E+04	5.72E+04	**0.035**	3.14E+04	4.77E+04	0.078	2.36E+04	5.16E+04	**0.01**	2.38E+04	4.45E+04	**0.005**
Sulphurs																		
Methanethiol	3.48E+04	1.58E+04	0.290	4.68E+04	5.12E+04	0.531	5.58E+04	4.64E+04	0.129	4.51E+04	4.85E+04	0.44	4.54E+04	4.38E+04	0.783	4.03E+04	3.87E+04	0.615
Dimethyl sulphide	1.99E+04	6.85E+04	0.092	1.99E+04	2.64E+04	**0.04**	1.93E+04	2.22E+04	0.429	1.53E+04	2.36E+04	**0.001**	1.56E+04	2.05E+04	0.121	1.38E+04	1.86E+04	0.093
Carbon disulphide	1.64E+06	1.33E+06	0.707	1.57E+06	1.64E+06	0.822	1.76E+06	1.43E+06	0.419	1.41E+06	1.48E+06	0.819	1.49E+06	1.10E+06	0.402	1.36E+06	1.11E+06	0.568
Dihydro-2-methyl-3(2H)-thiophenone	-	-	-	1.12E+06	3.45E+05	0.108	1.04E+06	3.59E+05	0.142	1.10E+06	2.70E+05	0.117	9.08E+05	4.17E+05	0.284	9.33E+05	2.89E+05	0.134
Other																		
Cyclohexane	5.87E+04	1.19E+04	**0.004**	8.55E+04	6.14E+04	**0.05**	9.39E+04	8.15E+04	0.49	8.54E+04	7.59E+04	0.309	7.54E+04	7.87E+04	0.772	8.51E+04	7.79E+04	0.662

*or isomer of this compound, PF = pre-fermentation

Table 2. Volatile compounds and their odour descriptors detected in pasteurised milk and yoghurts.

Compound	CAS	RT	LRI	Ref LRI	Descriptor[1]	Compound increased	Timepoint(s) affected (d)	Mean fold increase from diet[2]
Acids								
Acetic acid	64-19-7	15.465	697	690	Vinegar, pungent, acidic	TMR	14, 28	1.68
Butanoic acid	107-92-6	22.87	865	864	Sharp, cheesy, rancid, sweaty, sour, putrid	GRS	21	4.58
Hexanoic acid	142-62-1	30.075	1053	1052	Pungent, rancid, flowery	-	-	-
Octanoic acid	124-07-2	36.455	1246	1244	Wax, soap, goat, musty, rancid, fruity	-	-	-
Alcohols								
Ethanol	64-17-5	7.88	515	506	Mild, ether	-	-	-
2-Butanol	78-92-2	13.075	645	648	Wine	TMR	1, 7, 14, 21, 28	*
1-Hexanol	111-27-3	25.105	920	916	Resin, flower, green	TMR	1, 21, 28	1.74
2-Ethylhexanol	104-76-7	30.95	1077	1077	Rose, green	-	-	-
Aldehydes								
Acetaldehyde	75-07-0	5.865	460	452	Ethereal, fresh, green, pungent	TMR	1, 7, 14, 21, 28	1.83
Propanal, 2-methyl-	78-84-2	11.095	602	592	Pungent, malt, green	-	-	-
Hexanal	66-25-1	21.88	842	839	Green, cut-grass	TMR	14	1.63
2-Butenal, 3-methyl-	107-86-8	21.89	842	N/A	Metallic, aldehydic, herbaceous	-	-	-
Heptanal	111-71-7	26.1	946	943	Green, sweet	GRS	1, 7	1.94
Benzaldehyde	100-52-7	29.41	1034	1032	Almond, burnt sugar	TMR	21	1.44
Nonanal	124-19-6	33.52	1153	1150	Sweet, floral, citrus, grass-like	GRS	1, 14	1.65
Alkenes								
1-Pentene, 2-methyl-	763-29-1	10.89	597	N/A	-	-	-	-
Benzenes								
Toluene	108-88-3	19.945	797	794	Paint	-	-	-
o-Xylene*	95-47-6	25.595	933	N/A	Geranium	-	-	-
Benzene, 1,3-bis(1,1-dimethylethyl)-	1014-60-4	37.835	1291	1286	Plastic	-	-	-
Ethers								
Ethyl ether	60-29-7	7.975	518	515	Ethereal	-	-	-
Furans								
2,5 Dimethyl furan	625-86-5	16.685	724	N/A	Chemical, ethereal, meaty, gravy, roast beef, bacon	TMR	1, 7, 14, 21, 28	*
Hydroxy ketones								

Compound	CAS	RT	LRI	Ref LRI	Descriptor[1]	Compound increased	Timepoint(s) affected (d)	Mean fold increase from diet[2]
Acetoin	513-86-0	19.405	785	778	Buttery	-	-	-
2-Hydroxy-3-pentanone	5704-20-1	23.335	876	N/A	Truffle, earth, nut	TMR	1, 7, 14, 21, 28	2.12
Ketones								
Acetone	67-64-1	8.635	535	533	Sweet, fruity	-	-	-
2,3-Butanedione	431-03-8	12.605	635	631	Buttery, creamy, vanilla	TMR	1	1.37
2-Butanone	78-93-3	12.85	640	639	Varnish-like, sweet, fruity	TMR	1, 7, 14, 21, 28	9.94
2-Pentanone	107-87-9	16.995	731	730	Fruity, acetone	TMR	1, 7, 14, 21, 28	1.65
2,3-Pentanedione	600-14-6	17.34	738	736	Butter, vanilla, mild	TMR	1	1.69
3-Hexanone	589-38-8	22.255	851	N/A	Sweet, fruity	TMR	1	1.80
2-Heptanone	110-43-0	25.775	937	936	Fruity, spicy	TMR	1, 7, 14, 21, 28	1.87
Undecan-2-one	112-12-9	39.545	1350	1353	Floral, rose-like, herbaceous	TMR	1, 7, 21, 28	1.96
Sulphurs								
Methanethiol	74-93-1	6.36	474	462	Cooked cabbage, boiled potato, sulphurous	-	-	-
Dimethyl sulphide	75-18-3	9.04	546	538	Intense, lactone-like, sulphurous, cabbage	TMR	1, 14	1.42
Carbon disulphide	75-15-0	9.345	555	546	-	-		-
Dihydro-2-methyl-3(2H)-thiophenone	13679-85-1	30.435	1063	N/A	Cabbage, onion, must	-	-	-
Other								
Cyclohexane	110-82-7	14.305	672	666	-	GRS	1	1.39

CAS: Chemical CAS (Chemical Abstract Service), RT: Retention time, LRI: Linear Retention Indices as determined as by Van Den Dool & Kratz, 1963

[1]Odor descriptors sourced from Cheng et al. (2011), Chen et al. (2017), and where unavailable from http://flavornet.org and http://thegoodscentscompany.com

[2]Mean of every significant timepoint post fermentation, *****Compound only detected in one diet

Chapter 3. Nisin as a preservative for pasteurised milk

Abstract

The bacteriocin nisin inhibits a wide range of Gram-positive bacteria including spore forming clostridia and bacilli. This study investigated the effect of nisin addition on the quality of pasteurized whole milk. The nisin used in these experiments was either purified nisin A or a commercially available Nisaplin® which contains 2.5% (w/w) of the antimicrobial peptide. Nisaplin® significantly reduced the total bacterial count (TBC) in whole milk during storage at a concentration (50 mg ml^{-1}) over a 13 d period at 4 °C. In addition, pure nisin A at high concentrations (1, 10 mg ml^{-1}) was also found to eliminate detectable levels of microbes present in pasteurised milk and inhibition was maintained during 49 d of storage at 4 °C. Nisin A included at 1 µg ml^{-1} and 10 µg ml^{-1} reduced the TBC from 3.46 log CFU ml^{-1} to 2.82 log CFU ml^{-1} and to below 2.48 log CFU ml^{-1} (the limit of detection) after addition, respectively. An effect was also observed for these concentrations after seven days of cold storage at 4 °C, but not at subsequent timepoints up to 49 d, or in milk stored at 21 °C for the same period. These data demonstrate that nisin is effective at preventing bacterial spoilage of pasteurized milk, but only at relatively high concentrations.

Introduction

The dairy industry has developed numerous methods to extend the shelf life of commercial fluid milk beyond the standard 12-14 days of high-temperature short-time (HTST) treated milk. The benefits of an improved shelf life milk include of increased distribution distances and times, longer shelf life for the consumer prior to spoilage, and enhanced food safety. Several methods are currently used to produce extended shelf life milk and improve the quality of milk for cheese production such as ultra-pasteurisation (UP), ultra-high temperature (UHT) pasteurisation and microfiltration (MF) (1-3). UP and UHT treatments utilize a temperature of 138 °C/135 °C for at least 2 seconds, followed by different packaging techniques which produces fluid milk that can last for several weeks at refrigeration temperature and up to six months at room temperature (1, 2). However, the extended shelf life can be offset by a decline in perceived milk quality due to considerable chemical changes caused by Maillard reactions from heating and storage which lead to browning and a cooked or caramelised flavour (4-6). Newer low-temperature and cold treatment technologies continue to be investigated to combat the negative effects of high heat treatment, such as low-temperature short-time variable-pressure, thermo-sonication, and MF paired with pulsed electric fields. These methods have seen limited uptake by the dairy industry and some may not be as effective as standard HTST pasteurisation (7-10).

HTST pasteurisation involves heating of milk to 72 °C for a minimum of 15 seconds followed by rapid cooling, thus ensuring reduction of spoilage microbes (11). Subsequent spoilage of pasteurised milk is mainly caused by the growth of Gram-positive psychrotolerant endospore-forming bacteria, typically *Bacillus* spp. and *Paenibacillus* spp., which survive the pasteurisation process (12-14). Gram-negative species such as *Pseudomonas* spp. also cause milk spoilage, but generally are post-pasteurisation contaminants which gain access due to poor post-processing conditions (15). Microbial growth is widely acknowledged as the main limiting factor in the shelf life of fluid milk, with 5 – 6 $\log_{10}$ CFU ml^{-1} marking the end of shelf life (16). Spoilage organisms produce lipolytic and proteolytic enzymes which cause the

deterioration of milk sensory properties and, in rare cases, can also the produce toxins (12, 17).

Bacteriocins are small ribosomally produced antimicrobial peptides that are of continued interest for applications in food and health, particularly for the inhibition of spore-forming bacteria (18-20). The antimicrobial effect of bacteriocins can be improved when used with other antimicrobial treatments, such as in combination with other bacteriocins, other antimicrobial compounds (e.g. organic acids, essential oils), and in combination with physical treatments (e.g. high pressure) (21-23).

Nisin is a 34 amino acid lantibiotic produced by *Lactococcus lactis* subsp. *lactis* and was first identified in 1928 (24). It is the most well-studied bacteriocin and has been designated generally recognised as safe (GRAS) by the FDA in 1988 and approved by EFSA as a natural food preservative under the E number E234 (25). Nisin is widely available as a partially purified powdered food preservative under the product name Nisaplin® (Danisco). Nisaplin® is produced by fermentation of a sugar-based medium including yeast extract by *L. lactis* subsp. *lactis* followed by microfiltration and ultrafiltration to remove cells and concentrate the product. The resulting nisin concentrate is subjected to salt precipitation, centrifugation, spray drying, and balancing with sodium chloride which produces a powder with a defined potency of 1,000 international units (IU) nisin A/mg (2.5% nisin A wt/wt) (26).

Nisin is known to be effective for controlling growth of spoilage organisms and human pathogens in various foods (27, 28) The peptide is heat stable, non-toxic and is broken down by pancreatin in the small intestine (29). Nisin inhibits a broad range of Gram-positive bacteria, including spore-formers such as *Bacillus* spp., making it of particular interest for application in pasteurised dairy products (30). The use of nisin in dairy products is not without limitations. The peptide is known to be more effective at acidic pH, and can interact with, and can bind to the membranes of fat globules and anionic casein and so activity may be reduced by higher fat concentrations (31, 32). There are also concerns over the use of nisin in terms of the development of resistance (33). Despite these concerns, it has proven to be effective

at inhibiting pathogenic *Listeria monocytogenes* in cottage cheese, extending the shelf life of milk based pudding, and has previously been tested in reduced-heat UHT treated milk, as well as in combination with lysozyme in whole milk for shelf life extension (34-37).

This study formed part of the European funded Joint Programming Initiative – A Healthy Diet for a Healthy Life (JPI-HDHL) LONGLIFE project which aimed to develop food products with improved functionality and shelf life. As part of this project we examined the application of nisin in commercial pasteurised whole milk and determined a concentration at which spoilage organism growth is reduced up to 14 days of storage at refrigeration temperatures and beyond producing an HTST pasteurised extended shelf life milk.

Materials and Methods

All milk shelf life experiments were performed in biological and technical triplicate at laboratory scale in 2 ml screw cap tubes, unless stated otherwise. Experiments were performed over three-day intervals, with commercial milk (Avonmore, 3.5% fat) purchased in a different location each morning.

Application of Nisaplin® in milk

A 50 mg ml^{-1} solution of Nisaplin® (2.5% nisin from *L*. *lactis* subsp. *lactis* balanced with Sodium chloride and denatured milk solids, Sigma-Aldrich, Wicklow, Ireland) in pasteurised milk was prepared and vortexed until homogenous. The solution was then aliquoted into two volumes for technical replicates. Samples were stored at 4 ° for 13 d. At 0, 7, and 13 d, 100 µl was aseptically removed, serially diluted, and plated on plate count skim milk agar (MPCA) (Merck). Plates were incubated at 30 °C aerobically and enumerated after 72h.

Application of pure nisin in milk

Two sets of experiments were performed using different concentrations of pure nisin A from *L*. *lactis* subsp. *lactis* (Handary, Brussels, Belgium), high concentration and low concentration of pure nisin. For the high concentration experiments a 100 mg ml^{-1} stock solution of nisin in pasteurised milk was prepared and vortexed until homogenous. The stock solution was then used to prepare 10 mg ml^{-1} and 1 mg ml^{-1} solutions. For the low concentration replicates a 10 mg ml^{-1} stock solution of nisin in milk was prepared and vortexed until homogenous. The stock was then serially diluted in milk to achieve the final desired concentrations of 10 µg ml^{-1}, 1 µg ml^{-1}, and 0.1 µg ml^{-1}. High concentration samples were stored at 4 °C for 49 d. Low concentration samples were stored at 4 °C and 21 °C for 49 d. At 0, 7, 14, and 49 d, 100ul was aseptically removed, serially diluted, and plated on plate count skim milk agar (MPCA) (Merck). Plates were incubated at 30 °C aerobically and enumerated after 72h.

Statistical analyses

Statistical analyses were performed using SPSS v24.0.0 (IBM Statistics Inc., Armonk, NY). Statistical difference between groups was assessed using one-way analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) for post-hoc comparisons. P-values ≤ 0.05 were considered statistically significant.

Results and Discussion

A high concentration of Nisaplin® inhibits bacterial growth in milk

The total bacterial count (TBC) (log CFU ml^{-1}) of the base milk (commercial pasteurised, 3.5 % fat) was below the limit of detection (2.48 log CFU ml^{-1}) on day 0 (Fig. 1). TBC in the base milk increased to 4.35 ± 0.70 log CFU ml^{-1} and 6.24 ± 0.8 log CFU ml^{-1} after storage for 7 d and 13 d at 4° C, respectively. The addition of Nisaplin® powder to milk at 50 mg ml^{-1} inhibited TBC number to below the limit of detection at the same timepoints ($p < 0.0005$). Reduction of TBC with the addition of 50 mg ml^{-1} of Nisaplin® is unsurprising. Nisaplin® consists of 1000 IU nisin A/mg of Nisaplin®, where 1 IU corresponds to 0.025 µg of pure nisin, resulting in a final concentration of 1.25 mg ml^{-1} pure nisin A in the milk (25). The powder is prepared with denatured milk solids and sodium chloride at a concentration ranging from minimum 50% (wt/wt) to a maximum 75% (wt/wt) (26, 38). As such, the addition of 50 mg ml^{-1} Nisaplin® includes the addition of 25 - 37.5 mg ml^{-1} sodium chloride to milk . The addition of sodium chloride to milk has previously been found to inhibit bacterial growth at similar concentrations and, therefore, the inhibitory effect cannot be attributed solely to the presence of nisin (39). Consumer attitudes have shifted in recent years towards food products with healthier attributes such as reduced fat, sugar, and salt, and though Nisaplin® is considered a clean label ingredient with a more consumer friendly image than synthetic preservatives, the addition of salt could negatively impact on consumer attitudes towards a product containing high quantities of Nisaplin® (40, 41).

Effect of pure nisin A on bacterial growth in milk

To eliminate any potential salt effect, pure nisin A was tested at 1 mg ml^{-1}, a final concentration similar to the amount of active nisin included with Nisaplin®. As nisin activity is known to be improved by the presence of sodium chloride, a higher concentration of 10 mg ml^{-1} was also tested (42, 43). The effects of both concentrations in whole milk were similar to the effect of Nisaplin® at 50 mg ml^{-1} (Fig. 2). TBC of the base milk was 1.73 ± 1.50 log CFU ml^{-1} on day 0 after addition, which

was not significantly affected by 1 mg ml^{-1} and 10 mg ml^{-1} ($p = 0.079$). TBC in the milk without nisin increased to 3.03 ± 0.76 log CFU ml^{-1}, 4.92 ± 2.08 log CFU ml^{-1}, and 4.87 ± 1.45 log CFU ml^{-1} by days 7, 14 and 49, respectively (Fig. 2). Nisin at 1 mg ml^{-1} and 10 mg ml^{-1} significantly reduced TBC after 7, 14 , and 49 days of storage at 4 °C ($p < 0.0005$, $p = 0.004$, and $p = 0.001$), respectively, maintaining a level below the limit of detection (Fig. 2).

The addition of 1 µg ml^{-1} and 10 µg ml^{-1} pure nisin reduced the TBC in the base milk on day 0 from 3.46 ± 0.23 log CFU ml^{-1} to 2.82 ± 0.02 log CFU ml^{-1} ($p = 0.013$) and to below the limit of detection ($p < 0.0005$), respectively (Fig. 3a). The addition of nisin at a concentration of 0.1 µg ml^{-1} did not affect TBC at the same timepoint ($p = 0.987$) (Fig. 3a). After seven days of cold storage TBC in the base milk rose to 3.76 ± 0.20 log CFU ml^{-1}, whereas milk containing 1 µg ml^{-1} and 10 µg ml^{-1} nisin reached 1.18 ± 2.04 log CFU ml^{-1} ($p = 0.033$), 0.75 ± 1.30 log CFU ml^{-1} ($p = 0.017$), respectively (Fig. 3a). TBC in milk containing 0.1 µg ml^{-1} nisin was not significantly different from the base milk after seven days ($p = 0.691$). After 14 and 49 days of cold storage, the difference between groups was no longer observed ($p = 0.616$, and $p = 0.518$, respectively) (Fig. 3a). The lack of significant difference at timepoints after day seven can be attributed to high levels of variation in TBC between milk samples, which is reflective of the range in quality of milk on day of purchase. The reduction in TBC in milk containing 1 µg ml^{-1}, and 10 µg ml^{-1} nisin stored at 4 °C after 7 days was not observed in milk stored at 21 °C ($p = 0.615$). After 14 days at 21 °C the TBC of the 10 µg ml^{-1} group was significantly higher than the base milk and lower nisin concentration groups ($p = 0.05$). This effect was not apparent after 49 days of storage at 21 °C ($p = 0.686$) (Fig. 3b).

Schaffner *et. al* (2003) determined that a reduction of initial microbial contamination by 0.5 log CFU ml^{-1} significantly lengthens shelf life of HTST milk (44). However, the reduction we observed in TBC of 0.69 log CFU ml^{-1} by 1 µg ml^{-1} nisin and of more than 0.98 log CFU ml^{-1} by 10 µg ml^{-1} nisin on day 0 did not significantly affect milk TBC numbers after 14 days. Saad *et. al* (2019) investigated the effect of Nisaplin® at a single concentration, 0.5 mg ml^{-1} (12.5 µg nisin per ml), on refrigerated whole milk with and without lysozyme over the course of 18 days, exposing the milk

to air at every timepoint (37). In the same study, TBC in milk without preservatives increased after opening the vessels whereas the TBC decreased over 12 days in milk containing nisin and nisin with lysozyme, indicating that the presence of nisin may also inhibit spoilage organisms introduced after opening (37). We found that nisin at a similar concentration was less effective than the quantity reported by Saad *et. al* (2019) which may be due to the slightly lower concentration used in this study (10 µg ml^{-1} vs. 12.5 µg ml^{-1}), and/or the absence of sodium chloride included with Nisaplin®.

The differential response to nisin between replicates could potentially be attributed to the variable presence of nisin insensitive spoilage organisms, such as Gram-negative *Pseudomonas* spp. It is worth noting that because nisin has a limited range of inhibition, the microbiota of the milk would be shifted from Gram-positive spoilage organisms towards Gram-negatives, potentially eliminating competitors and even accelerate spoilage. Other research has investigated the effectiveness of nisin in combination with other compounds or treatments which sensitise Gram-negative species in milk. High pressure treatment in combination with nisin has previously been found to be effective at eliminating *Pseudomonas fluorescens* cultured in milk, and Nisin in combination with reuterin (β-hydroxypropionaldehyde) also inhibits Gram-negative organisms (45, 46). In recent years, several studies have investigated the application of nisin in combination with other compounds such as phenolics and bacteriophage in pasteurised whole milk against the Gram-positive pathogenic species *Staphylococcus aureus* and *L. monocytogenes* (recently reviewed by Ibarra-Sánchez *et. al* (2020)) (47-55). We note an absence of studies investigating nisin in combination with other compounds against main spoilage species such as *Bacillus* spp., *Paenibacillus* spp. during refrigerated shelf life, which may be explored in the future.

The price of milk for direct consumption varies around the world. In Ireland, 536.7 million litres of milk for human consumption was sold in 2018, of which 61.1% was pasteurised whole milk, and the average manufacturing price of whole milk in 2019 was €0.34 per litre (56). In Australia, where UHT milk accounted for 17.6% of milk purchased for consumption in 2014, the sales price of branded whole milk averaged at AU$1.81 (€1.12) per litre compared to AU$1.60 (€0.99) per litre for UHT

milk (57). In the same period Italian whole milk was priced at €1.37 per litre, compared to €1.20 per litre of UHT milk (58). The longer shelf life of UHT milk enables its reduced cost, as it can be stored and distributed without refrigeration. A concentration of 400 μg ml^{-1} of Nisaplin® in milk would be required to achieve the same concentration of pure nisin in milk (10 μg ml^{-1}) at which an effect was found on TBC after 7 days of cold storage. The price of Nisaplin® from advertised sources ranges from $32 to $100/kg (€27.70 – €86.52/kg)(59). Thus, the addition of 400 μg ml^{-1} would increase the manufacturing cost of milk production by €0.011 to €0.035 per litre.

This study did not assess sensory characteristics of milks containing Nisaplin® or nisin, but previous research has found no difference in flavour, odour, or appearance between pasteurised milk and milk containing 0.5 mg ml^{-1} Nisaplin® (12.5 μg nisin per ml) (37). Therefore, it is unlikely that pure nisin at the lower concentrations utilised in this study (1 and 10 μg ml^{-1}) would have an effect on sensory characteristics, though this has yet to be confirmed. EFSA defines 'milk' as "the normal mammary secretion obtained from one or more milkings without either addition thereto or extraction therefrom", and 'whole milk' as heat-treated milk containing a fat concentration of at least 3.5% (60). Milk containing a preservative such as nisin would require additional labelling e.g. extended shelf life, or long-life milk, which consumers may perceive as more processed.

This study demonstrates the effectiveness of the bacteriocin nisin at inhibiting bacterial spoilage organism growth in pasteurised whole milk. The data are relevant to future studies and applications to produce extended shelf life milks.

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Figures and Tables

Figure 1. Impact of Nisaplin® on bacterial growth in milk during shelf life. Dotted line indicates limit of detection (* $p < 0.05$).

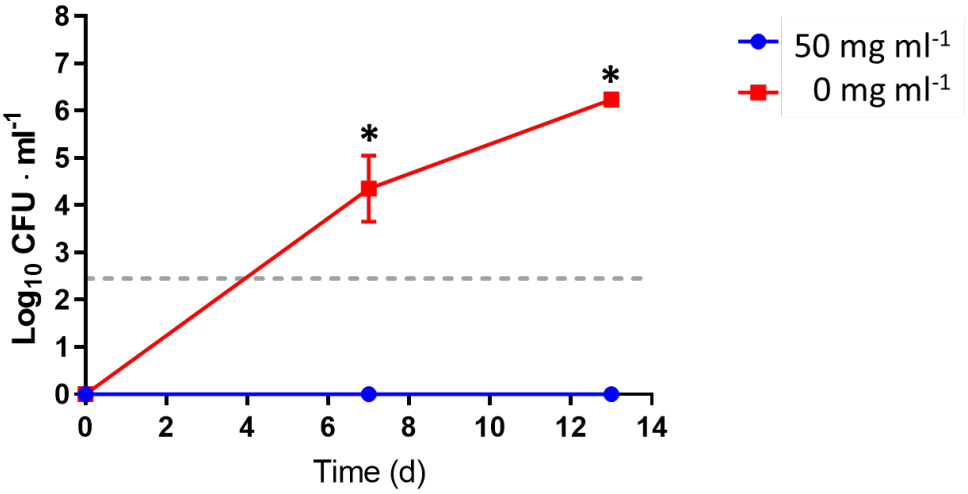


Figure 2. Effect of nisin A on growth of spoilage bacteria in milk during shelf life and beyond. Dotted line indicates limit of detection. Legend: concentration of nisin A. (* $p < 0.05$).

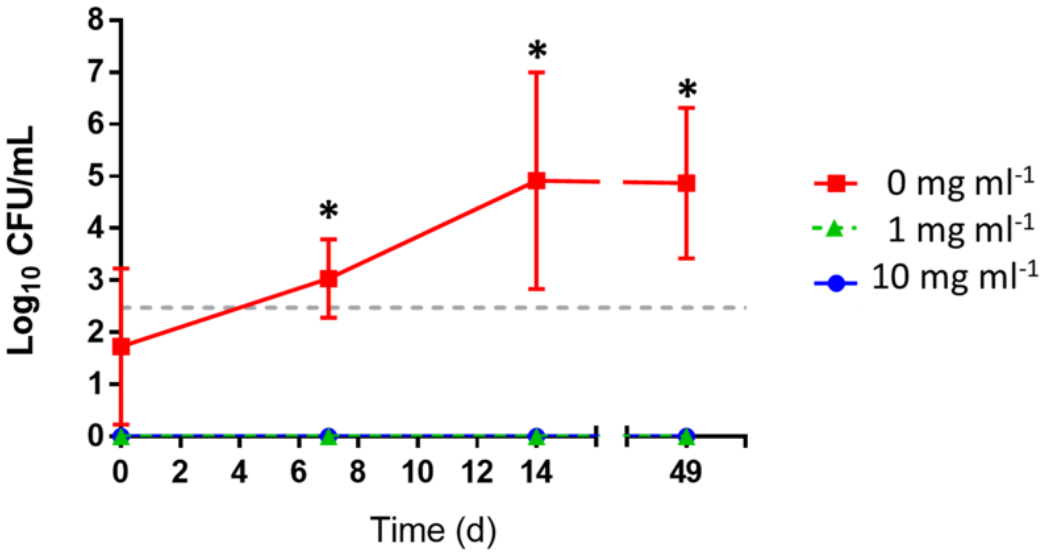
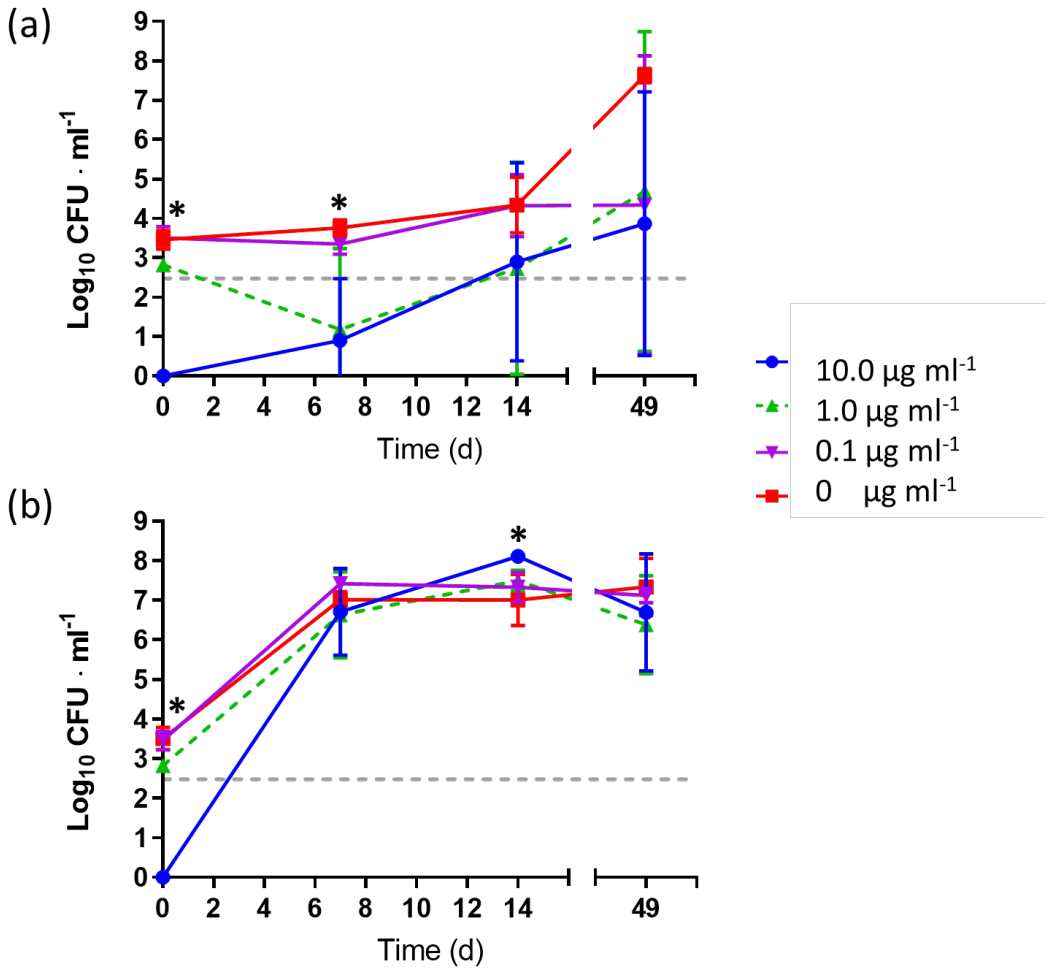


Figure 3. Effect of lower concentrations of nisin A on growth of spoilage bacteria in milk during shelf life and beyond at (a) 4 °C and (b) 21 °C. Dotted line indicates limit of detection. Legend: concentration of nisin A. (* $p < 0.05$).



Chapter 4. Diverse bacteriocins from lactic acid bacteria of raw milk

Abstract

The aim of this study was to recover and characterise bacteriocin producing lactic acid bacteria (LAB), mainly from ruminant milk samples. From 104 bovine, ovine, and caprine milk samples, 50 isolates exhibited potent antimicrobial activity in agar overlay assays, seven of which exhibited antimicrobial activity against *Lactobacillus delbrueckii* ssp. *bulgaricus* in the cell free supernatant. Following whole genome sequencing, four high quality genomes were constructed for three *Streptococcus* spp. and one *Lactococcus lactis,* all isolated from ovine milk. *In silico* analysis revealed the presence of seven putative bacteriocin gene clusters in the four strains, including three lantibiotics (one class 1 and two class 2). Four gene clusters were identified within a single *Streptococcus uberis* genome, encoding for uberolysin, nisin U, a two-component gene cluster similar to the lantibiotics C55 and lacticin 3147, and a gene cluster containing two class II bacteriocin core peptides. Two closely related *Streptococcus* species that were isolated from different samples contained near identical gene clusters for a novel nisin variant, nisin I. This variant shares 75% identity with nisin A with ten differences, two deletions (Ser29 and Ile30) and eight substitutions (Ile4Lys, Gly18Thr, Asn20Pro, Met21Ile, His27Gly, Val32Phe, Ser33Gly, Lys34Asn). The nisin I structural peptide is highly similar to nisin U (93.6% identity) but the biosynthetic operon has an alternative gene arrangement. The seventh gene cluster was a class I unmodified bacteriocin detected within the genome of *Lactococcus lactis* that is similar to carnolysin and an enterococcal cytolysin. These data confirm that raw milk LAB are capable of producing novel peptides that could be utilised in food fermentation and human health.

Introduction

Raw milk is a highly nutritious and complex environment which contains a diverse microbiota (1). Lactic acid bacteria (LAB) present in raw milk produce many compounds which may be beneficial or detrimental to human health and have potential commercial applications. In food products many LAB can produce a range of compounds *in situ* during fermentation or which can be isolated and added in post-fermentation to improve flavour, texture, nutritional quality and extend shelf life. Many LAB have been investigated for their health promoting properties and some conform to the definition of probiotic (2). LAB also produce many antimicrobial metabolites such as organic acids, hydrogen peroxide, reuterin, and ethanol as well as other antimicrobial compounds such as reutericyclin, and bacteriocins (3, 4). Bacteriocins are small (<10 kDa) ribosomally synthesised antimicrobial peptides which can have a broad or narrow spectrum of inhibition (4). They have the potential to be used as natural food preservatives and are potential alternatives or adjuncts to currently used antibiotics given the global crisis of antimicrobial resistance (5). Raw milk is a good source of diverse bacteriocin producing LAB, and strains have been isolated from the milk of cows, goats, sheep, camels, mares and donkeys (6-13). Recently, human milk has also been identified as a potent source of novel and diverse bacteriocins (14).

Bacteriocins are grouped into two broad categories according to their structure; post-translationally modified (class 1) and unmodified or cyclic (class II), though alternative classification schemes exist (4, 15). Lantibiotics are class 1 bacteriocins which contain β-thioether crosslinked bis-amino acids lanthionine (Lan) and methyllanthionine (MeLan) (16). Lantibiotics are further divided based on the enzymes involved in their post-translational modifications. Class II bacteriocins are not enzymatically post-translationally modified and are also divided into structural subgroups IIa (pediocin-like), IIb (two peptide), IIc (cyclic peptides), and IId (non-pediocin-like linear single peptides) (5).

Nisin is a type I lantibiotic produced by *Lactococcus lactis* ssp. *lactis.* First identified in 1928, nisin is the most studied bacteriocin, has been extensively applied

in food products and is FDA and EFSA approved as a natural food additive (E234) (17, 18). A number of natural variants have been identified since the discovery of nisin A, including nisin Z, F, and Q which are produced by members of the genus *Lactococcus* (19-21), as well as nisin U, U2, H and P from *Streptococcus* spp. (22-24), nisin O and O4 from *Blautia obeum* A2-162 (25), and more recently, nisin J from *Staphylococcus capitis* APC 2923 (26). Nisin U and U2 are natural variants of nisin A produced by *Streptococcus uberis,* and were the first to be characterized from a species other than *L. lactis* (22). These 31 amino acid peptides contain one conservative substitution between each other (Ile1Val) and 11 differences from nisin A, three deletions (Ser29, Ile30 and Lys34) and eight substitutions (Ile4Lys, Ala15Ile, Gly18Thr, Asn20Pro, Met21Leu, His27Gly, Val32Phe, and Ser33Gly) (22). Nisin variants can be subjected to genetic modification and certain amino acid substitutions can result in altered activity and stability (27).

Type I lantibiotics use separate enzymes for dehydration (LanB) and cyclisation (LanC) of the core peptide, while type II lantibiotics are characterized by a single enzyme (LanM) which performs both functions (28). Type II lantibiotics and other class I bacteriocins have exhibited useful properties for applications in human and animal health, such as the narrow spectrum sactibiotic thuricin CD for the treatment of *Clostridioides difficile* (formerly *Clostridium difficile*) infection (29), lacticin 3147 for the elimination of *Staphylococcus aureus* in a murine model of human infection and elimination of mastitis in cows (30, 31).

In this study, we screened 112 samples, mainly raw milk (104 samples) from cows, sheep, and goats, for bacteriocin producing LAB. We identified four bacteriocin producing strains from 823 isolates with initial antimicrobial activity, three *Streptococcus* spp. and one *Lactococcus lactis* containing seven diverse bacteriocin gene clusters. A single *Streptococcus uberis* strain harboured four different operons, encoding two class I bacteriocins (nisin U and novel two peptide lantibiotic) and two class II bacteriocins (uberolysin and novel bovicin 255/enterocin like peptides). Two closely related *Streptococcus* spp. isolated from sheep milk from different farms were found to possess near identical gene clusters to produce a new nisin variant that we

designate nisin I, and a novel two component lantibiotic homologous to carnolysin was detected in the genome of the *Lactococcus lactis* strain.

Materials and Methods

Strain isolation and initial bacteriocin screen

One hundred twelve samples, consisting mainly of raw milks of different origin (Table 1) were streaked or spread plated following serial dilution in maximum recovery diluent (MRD; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) on several media (Table 7) for the isolation of putative LAB. Briefly, *Streptococcus thermophilus* selective agar was incubated aerobically at 42 °C; M17 (Merck, Darmstadt, Germany) was supplemented with 10% (wt/vol) lactose incubated at 30 °C aerobically; de Man, Rogosa, and Sharpe (MRS; Difco Laboratories, Detroit, MI) agar was supplemented with 30 mg litre^{-1} vancomycin hydrochloride and incubated at 37 °C; MRS was adjusted to pH 5.4 incubated at 42 °C anaerobically; *Lactobacillus* selective agar (LBS, Difco) was incubated at 30 °C anaerobically; and TOS (transgalactosylated oligosaccharide) agar (Sigma-Aldrich, Wicklow, Ireland) supplemented with 50 mg litre^{-1} lithium mupirocin was incubated at 37 °C anaerobically.

Following incubation (Table 7), three to five distinct appearing colonies were re-streaked for purity on the appropriate culture medium without selective agents, inoculated into broth and stocked at -20 °C. Isolates were subject to an initial bacteriocin production screen by overlaying pure streaks with 10 ml 'sloppy' MRS agar (7.5 g litre^{-1} agar) tempered to 50 °C and seeded with an overnight culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901 (0.25% [vol/vol]). Isolation plates were also subject to overlay after colonies were picked. Colonies which were found to produce zones of inhibition with defined edges indicative of bacteriocin production (Fig. 1) were cut from under the overlay with a sterile scalpel blade, re-streaked for purity and stocked.

Determining bacteriocin production

Strains which produced zones of inhibition with defined edges were re-screened following broth culture and spotting (5 µl) onto square 120 mm x 120 mm agar plates (Sarstedt, Germany). Overlays were performed as described above using

40 ml tempered sloppy agar. Strains with positive results were subject to well diffusion assay using pH neutralised cell free supernatant (CFS) of 24-72 h grown cultures. For well diffusion assays, 40 ml of sloppy MRS agar seeded with *L. bulgaricus* LMG 6901 as described above was poured and allowed to set in 120 mm x 120 mm petri dishes, in which 6-mm-wide wells were then bored using a sterilised glass Pasteur pipette. Fifty microlitres of cell-free supernatant was added to each well, and plates were incubated at 37 °C overnight. Zones of inhibition were indicative of antimicrobial activity. To determine if the antimicrobial activity was of proteinaceous nature, CFS were subject to protease treatment. One hundred microlitre aliquots of pH neutralised CFS were subjected to treatment with 20 mg ml^{-1} proteinase K (Sigma-Aldrich) at 37 °C for 3 h, followed by a 10 min incubation at 100 °C to denature the enzymes. Fifty-microlitre aliquots were assayed on *L. delbrueckii* subsp. *bulgaricus* LMG 6901 indicator plates.

Strain identification and whole genome sequencing

To identify putative bacteriocin producers, DNA was extracted using a GenElute bacterial genomic DNA kit (Sigma) and the 16s rRNA gene was subject to sanger sequencing (Genewiz, UK) following PCR amplification using B27F and U1492R universal primers. Whole genome sequencing was performed using a Nextera XT kit (Illumina) for library preparation. DNA was quantified using a Qubit 2.0 fluorometer. Shotgun sequencing was carried out using an Illumina MiSeq platform with paired-end 2 x 300-bp reads by the Teagasc Sequencing Centre, Teagasc Food Research Centre, Moorepark, Fermoy, Ireland. Assembly was performed using tools available on the public server at https://usegalaxy.org (32). Assembly was performed de novo using SPADES (version 3.0.0) and annotated with RAST (version 2.0) (33, 34). Contigs were aligned to reference genomes acquired from the NCBI GenBank database using Mauve (version 20150226, build 10) (35). For speciation of *Streptococcus* sp. with inconclusive 16s gene sequencing, the GroEL nucleotide sequence was sourced from the whole genome sequence and analysed using nBLAST. Where GroEL sequence comparison was unsuccessful average nucleotide identity was calculated using OrthoANI (version 0.93.1) and closely related species genomes (36). Constructed genomes were also subject to analysis using PlasmidFinder2.0 and

PHASTER for the *in silico* detection and typing of plasmid DNA and prophage sequences, respectively (37, 38).

Bacteriocin operon comparisons

Annotated genomes were analysed for predicted bacteriocin and secondary metabolite production clusters using BAGEL4 and antiSMASH and any further annotation was carried out using Artemis genome browser (version 16.0.0) (39-41). Multiple sequence alignments and percentage identities were generated using MUSCLE (42) and visualized with Jalview (43).

Colony mass spectrometry

Following extended storage at -80°C in glycerol (20% vol/vol) stocks, strains were streaked and cultured under appropriate conditions. Actively inhibitive bacterial colonies were subjected to colony mass spectrometry. Colonies were mixed with 50 µl of 70% isopropanol (IPA) containing 0.1% trifluoroacetic acid (TFA). The bacterial IPA suspension was vortexed and centrifuged at 20,817 x g. for 1 min. Supernatant was retained for analysis. Mass spectrometry in all cases was performed with an Axima TOF^2 matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometer (Axima TOF^2 MALDI-TOF mass spectrometer; Shimadzu Biotech, Manchester, UK). A 0.5 µl aliquot of matrix solution (alpha-cyano-4-hydroxy cinnamic acid), 10mg ml^{-1} in 50% acetonitrile-0.1% (v/v) trifluoroacetic acid) was placed onto the target. This matrix solution was left for 30 seconds and then removed. The residual solution was then air dried and the previously prepared supernatant was positioned onto the pre-coated sample spot. Positive-ion linear mode was used to detect peptide masses.

Results

Isolation of bacteriocin producers and draft genome analysis

More than 10,000 colonies were screened for bacteriocin production from a biobank of putative LAB isolated from a range of mainly unpasteurised milks (Table 1). Of 823 potential antimicrobial isolates, 386 (46.9%) displayed some level of inhibition against the acid tolerant indicator strain *Lactobacillus delbrueckii* ssp. *bulgaricus* LMG 6901 (Fig. 1). Four hundred and thirty-seven isolates (53.1%) had no effect on growth of the indicator in the overlay. Three hundred thirty-five (40.7%) of the isolates produced zones of inhibition with a diffuse boundary characteristic of acid production (Fig. 1). Fifty-one isolates (6.2%) exhibited zones of inhibition with sharp defined edges characteristic of bacteriocin activity and were subject to a well diffusion assay. The 51 isolates originated from sheep's milk (54.9%), goat's milk (35.3%), cow's milk (5.9%) and sheep faeces (2.0%). Using neutralised cell-free supernatant (CFS), eleven strains (1.3%) produced zones of inhibition in well diffusion assays. Seven of these were consistently active and could be disrupted by treatment with proteinase K, indicating a soluble proteinaceous antimicrobial compound (Fig. 2). Six of the seven strains were isolated from unpasteurised sheep's milk from New Zealand, and one isolate was sourced from Irish sheep faeces (Table 2). Using 16s rRNA gene sequencing the six isolates from sheep's milk were identified as three *Streptococcus* sp., two *Enterococcus* sp., and one *Lactococcus lactis*, and the isolate from sheep faeces was identified as *Actinomyces ruminicola* (Table 2).

The putative bacteriocin producing LAB were subjected to whole genome shotgun sequencing and the four highest quality genome sequences (LL-383, LL-387, LL-427, and LL-514) were taken forward for analysis (Table 3). As *Streptococcus* spp. LL-387 and LL-514 could not be speciated by 16s gene alone, their GroEL genes were analysed using BLAST. GroEL from *Streptococcus* sp. LL-514 was found to be 99.45% identical to *Streptococcus equinus* CNU-23 and was designated as *Streptococcus equinus.* The GroEL nucleotide sequence of *Streptococcus* sp. LL-387 was found to be 99.39% identical to both *Streptococcus equinus* NCTC8140 and *Streptococcus gallolyticus* ssp. *gallolyticus* NCTC8140. Subsequent average nucleotide identity

calculation using representative genomes, *Streptococcus equinus* AG46 and *Streptococcus gallolyticus* ssp. *gallolyticus* DSM 16831 (RefSeq accessions: NZ_JNLO00000000.1 and NZ_CP018822.1, respectively) found LL-387 was 98.35% identical to *S. equinus* and 82.35% identical to *S. gallolyticus* ssp. *gallolyticus.*

The four draft genomes were subsequently analysed using BAGEL4 and antiSMASH to identify bacteriocin encoding gene clusters. Operons capable of potentially encoding bacteriocin production were identified within all four genomes (Fig. 3, Table 4). *S. uberis* LL-383 contained four distinct bacteriocin gene clusters (Fig. 3). The predicted clusters corresponded to uberolysin (Cluster 1), a class II bacteriocin cluster (Cluster 2), nisin U (Cluster 3) and a two component lantibiotic (Cluster 4) (Fig. 3, Table 5). Nisin U-like operons were also detected in the genomes of *S. equinus* LL-387 (Cluster 5) and *S. equinus* LL-514 (Cluster 6) (Fig. 3, Table 5). Cluster 6 was truncated by a contig boundary. An operon for a two component lantibiotic similar to carnolysin was detected within *L. lactis* LL-427 (Cluster 7) (Fig. 3). Further genes were detected within the genome of *L. lactis* LL-427 related to sactipeptide production, but the cluster lacked core peptide genes (data not shown).

The draft genomes were also subject to *in silico* analyses for prophage and plasmid sequences. An intact prophage sequence spanning 31.4 kbp was detected in the genome of *L. lactis* LL-427, as well as potential regions (13.7 kbp and 27.7 kbp) and seven incomplete sequences (data not shown). *S. uberis* LL-383 and *S. equinus* LL-387 were predicted to contain a potential 40.6 kbp prophage and two incomplete regions, respectively. No prophage sequences were detected in *S. equinus* LL-514 (Table 4). The plasmid replication gene *repA* was detected within contig 13 of the draft genome of *L. lactis* LL-427, indicating potential plasmid DNA (Table 4). Genome alignment of *L. lactis* LL-427 sequences to the reference genome *L. lactis* ssp. *lactis* A12 revealed that contigs 13, 19, 21, 22, 23, 24, 25, and 27 failed to align to the reference genome (Fig. 4). Nucleotide BLAST analyses found that contigs 13, 19, 23, and 25 had homology to *Lactococcus* spp. plasmids (Table 6). Contigs 21, 24 and 27 were homologous to other *Lactococcus* sp. genomes, and contig 22 was homologous to a *Lactococcus* phage genome (Table 6).

Four bacteriocin production operons in *S. uberis* LL-383

S. uberis LL-383 contains four clusters potentially capable of bacteriocin production. Cluster 1 contains six open reading frames bearing >99% amino acid identity to proteins encoded by the uberolysin operon in *Streptococcus uberis* str. 42 (Fig. 5). The LL-383 4,401 bp operon is 99.75% identical to the *ublABCD* from *S. uberis* str. 42, with 11 single nucleotide polymorphisms (SNP) (data not shown). Clusters 2, 3, and 4 were detected on one large contig and were separated by 655.1 kbp and 49.5 kbp, respectively. A 40.6 kbp prophage is encoded within the 49.5 kbp region between clusters 3 and 4 (data not shown). Cluster 2 spans 7.2kbp and harbours two open reading frames (ORFs) encoding transporters, an ORF encoding a predicted ComE response regulator, two structural genes and four predicted immunity genes (Fig. 3). *orf5* and *orf8* encode peptides which share some similarity to class II bacteriocins (Fig. 6). The product of *orf5* is 62.0% and 37.1% identical to the lactococcin-like class IId bacteriocins bovicin-255 from *Streptococcus* sp. LRC0255 and garviaecin Q from *Lactococcus garvieae* BCC 43578, respectively (Fig. 6a). The product of *orf8* shares 31.7% and 30.0% amino acid identity with the class IIa pediocin-like bacteriocins enterocin A and mundticin KS, respectively (Fig. 6b). We note the absence of a complete 'pediocin box' motif (YGXGVXC) within the product of *orf8* which is shared by class IIa bacteriocins, though the residues GIYC at positions +6 to +9 of the mature peptide (following the GG cleavage signal) may constitute the latter part of the motif.

Cluster 3 spans 13.5 kbp containing 12 ORFs for nisin U production (Fig. 7a). Nucleotide blast analysis of the operon determined it is 98.45% identical to the *Streptococcus uberis* str. 42 nisin U gene locus (data not shown). Each encoded peptide is nearly identical to the corresponding *nsu* gene with a mean identity of 98.3% for all genes. The structural peptide contains one amino acid substitution, Asn3Ser, in the leader sequence (Fig. 7b), corresponding to a single SNP from 5'-AAC-3' to 5'-AGC-3'. The mature peptide sequence shares 100% identity with mature NsuA, and as such is predicted to undergo the same posttranslational modification and have the same 3,029 Da mass as NsuA (Fig. 7b).

The fourth bacteriocin gene cluster identified within *S. uberis* LL-383 was a 12.8 kbp operon similar to the two component lantibiotics staphylococcin C55 from *Staphylococcus aureus* C55 and lacticin 3147 from *Lactococcus lactis* DPC3147 (Fig. 8a). The predicted mature A1 peptide is 30 residues in length and is closely related to C55A1 and LtnA1, sharing 90% and 86.7 % amino acid identity, respectively (Fig. 8b). Three residues are substituted with respect to C55A1 (Ser2Thr, Ala21Leu, and Ser27Asn) and four residues are different from LtnA1 (Ser2Thr, Asn15Lys, Ala17Asn, and Ala27Asn) (Fig. 8b). The resulting peptide has a predicted unmodified mass of 3,355.0 Da, and a predicted mass of 3,424 Da following seven dehydrations. The A2 peptide is more closely related to LtnA2 than C55A2, sharing 62.1% and 46.9% amino acid identity, respectively (Fig. 8c). The peptide is 39 amino acids, with an unmodified molecular mass of 3,798.3 Da and 3,600 Da following 11 dehydrations. It also contains a seven residue insert not present in C55A2, consisting of Thr-Pro-Try-Thr-Pro-Ala-Ile, and an additional three residues not present in LtnA2, Gly-Lys-Gly (Fig. 8c). Both predicted A1 and A2 peptides have shorter leader sequences than their c55 and lacticin counterparts (Fig. 8b and c).

The overall operon organisation of Cluster 4 is similar but not identical to C55. Predicted dehydrogenase and transport genes are immediately downstream from encoded core biosynthesis proteins (Fig. 8a). *orf1* is predicted to encode a response regulator protein but has low homology (<30% identity) to *orf38* in the same position in the C55 operon which encodes a regulator (Fig. 8a). The operon contains conserved lanthipeptide synthetase family (LanM) genes (*orf457*) corresponding to *ltnM1M2* and *sacM1M2* from the lacticin 3147 and c55 production operons, respectively. *orf4* is 2,109 bp in length, compared to 2,943 bp and 2,898 bp of *ltnM1* and *sacM1*, respectively. The 750bp *orf5* follows *orf4* 62 bp downstream and has encodes a protein predicted to have LanM functionality. The protein sequences encoded by *orf4* and *orf5* mainly align to the N-terminus and C-terminus of LanM1 type proteins, respectively (Fig. 9), and their combined length (951 residues) approximates that of the SacM1 and LtnM1 proteins.

Nisin I, a nisin variant encoded within *Streptococcus* spp. LL-387 and LL-514

The genomes of *S. equinus* LL-387 and *S. equinus* LL-514 contain highly similar biosynthetic gene clusters (Clusters 5 and 6) which are homologous to nisin U (Fig. 7a). Cluster 6 is truncated within a *nisB/nsuB* gene homolog by the contig boundary and genes corresponding to *nisTCI/nsuTCI* were not detected (Fig. 7a). The 6,052 bp region corresponding to *nsuPRKAFEG* is 99.8% identical between strains, containing 14 SNPs. The gene cluster organisation does not match any previously described nisin variant operon (Fig. 10a). Clusters 5 and 6's organisation resemble the nisin U operon, with genes corresponding to *nisBTCI* translocated downstream of *nisPRKFEG* homologs. The position of the structural gene between *nisFEG* and *nisPRK*-like genes is unlike nisin A or U (Fig. 10a). Both clusters encode a structural peptide that we designate nisin I, that shares 76.4% and 75% amino acid identity with the nisin A prepropeptide and leaderless peptide, respectively (Fig. 7b). Nisin I is 32 amino acids in length and has ten differences from nisin A: two deletions, Ser29 and Ile30, and eight substitutions, Ile4Lys, Gly18Thr, Asn20Pro, Met21Ile, His27Gly, Val32Phe, Ser33Gly, and Lys34Asn. The peptide is highly similar to nisin U, sharing 93.6% identity, two amino acid substitutions, Ile15Ala, Leu21Ile, and one additional C-terminal Asn residue (Fig. 10b). The unmodified mass is 3,245.9 Da, and 3,101 Da following eight predicted dehydrations. Colony mass spectrometry analysis detected a mass of 3,101.6 Da corresponding to the mature peptide produced by *S. equinus* LL-387 (Fig. 7c). An additional peak of 3,119.8 Da was detected which may correspond to nisin I dehydrated seven times. *Streptococcus equinus* LL-514 did not exhibit the same mass spectrometry profile, lacking a peak at 3,101 Da matching nisin I, though a small peak was found at 3,139 Da which may correspond to nisin I following six dehydrations (Fig. 7c).

Identification of a carnolysin homologue in *Lactococcus lactis* LL-427

L. lactis LL-427 contains a 9 kbp predicted gene cluster (Cluster 7) potentially encoding the production of a two peptide lantibiotic homologous to carnolysin from *Carnobacterium maltaromaticum* and cytolysin from *Enterococcus faecalis* (Fig. 11a).

The overall genetic architecture is similar to the carnolysin production operon except for the position of *orf3*, which corresponds to *crnA2. orf3* precedes another structural gene*,* and there is an additional coding sequence of unknown function (*orf5*) immediately downstream from the encoded core peptides (Fig. 11a).The immature peptides encoded by *orf4* and *orf3* from *L. lactis* LL-427 share 57.4% and 61.0% amino acid identity to CrnA1 and CrnA2*,* and 49.1% and 52.7% identity to $CylL_L$ and $CylL_{S,}$ respectively (Fig. 11a). The products of *orf2* and *orf6* are homologous to core biosynthesis genes CrnM and CrnJ, sharing 47.5% and 50.4 % identity, respectively. Similarly, the proteins encoded by *orf7* and *orf9* are homologous to the transport and regulatory genes CrnT and CrnY with 52.8 and 32.8% identity, respectively. Orf8 is more closely related to CylA (a protease) than CrnP, sharing 44.4% amino acid identity (Fig. 11a). Multiple sequence alignments show a conserved leader sequence prior to the cleavage sites of CylB/CrnT, including a VQGS sequence at positions -4 to -1 which is identical between Orf3/4 and CrnA1/2 (Fig 11.b). Both *Lactococcus* structural peptides also contain a GDVQVE motif at positions -6 to -1 which is nearly identical to enterococcal $CylL_L$ and $CylL_S$ (Fig. 11b).

Discussion

In this study, novel bacteriocin producing LAB were isolated and characterized, mainly from the raw milk of sheep, cows, and goats. From a total of 823 potential antimicrobial isolates isolated from 112 samples, almost half (46.9%) exhibited a level of inhibition against the Gram-positive indicator species *L. bulgaricus* LMG6901 (Fig. 1). Only a small percentage of total isolates (6.2%) displayed the distinct sharp boundary of inhibition in agar overlay characteristic of bacteriocin activity, and a smaller percentage (1.3%) were active in a well diffusion assay. This relatively low number may be due to the use of a single indicator strain (*L. delbrueckii* ssp. *bulgaricus*) as some bacteriocins can have a narrow range of inhibition, targeting a small group or only closely related species (44). Other studies have found a similar range of bacteriocin production incidence among isolates from dairy sources, varying from 0.2% of colonies, to between 2% and 5% of isolates screened (45-47).

The main sample type was unpasteurised milk, constituting 92.9% of the samples screened (Table 1). Of 104 milk samples 74 were ovine, 21 were caprine and 9 were bovine. Sheep milk was the most prevalent sample type which is likely to be the reason it was the source for most antimicrobial producing isolates. Isolates from goat milk exhibiting antimicrobial activity were overrepresented in the initial screening phase, with 18/51 isolates (35.3%) when goat milk samples comprised only 21/112 samples (18.8%), however many isolates were inactive or lost activity in well diffusion assay. Bacteriocin expression is a highly regulated process and removing strains from a complex environment and growing them in pure culture could result in loss of bacteriocin production (48, 49). Future studies may revisit these isolates and determine their bacteriocin production capability using molecular methods.

We identified seven isolates with potent activity in agar overlay (Fig. 2), six of which were isolated from sheep milk and were identified as *Enterococcus* spp., *Streptococcus* spp. and a *Lactococcus lactis* (Table 2). The seventh was isolated from sheep faeces and is described in detail elsewhere (50). Recently, *Staphylococcus, Lactobacillus, Corynebacterium, Streptococcus, Escherichia* and *Shigella* were identified as the core bacterial genera present in milk of healthy Assaf dairy ewes in

Spain (51). Previous research determined that *Staphylococcus aureus*, *Salmonella* sp. *Escherichia coli*, *Clostridium perfringens* and *Bacillus* sp. are a large component of the sheep milk microbiota (52) and that sheep milk is a rich a source of anti-listerial *Enterococcus* spp. and bacteriocinogenic staphylococci (47, 53). As *Enterococcus* spp. and *Streptococcus* spp. are established to be producers of diverse bacteriocin types and are known to be members of the sheep milk microbiota, it is unsurprising that we identified several in our study (54). Bacteriocin producing *Lactococcus* spp. have also been isolated from sheep milk and from unpasteurised sheep cheese, including producers of nisin and the type II lantibiotic lacticin 481 (7, 55). Bacteriocin gene cluster prediction programs BAGEL4 and antiSMASH identified seven gene clusters within four draft genomes (Fig. 3). *In silico* gene cluster prediction is now an increasingly common method for the identification of novel bacteriocins, particularly among large genomic/pangenomic datasets (56-58). Combining a functional screen and *in silico* approach has previously been successful for the identification of novel bacteriocins of human and cow milk isolates (14, 59). This study employed several selective media to isolate a range of LAB (Table 7). However, it is worth noting that lactobacillus selective (LBS) agar does not support the growth of all heterofermentative lactobacilli, and thus the chosen media may not have selected the full complement of lactobacilli and other LAB present in the source samples.

Four gene clusters were present in the genome of a single isolate, *S. uberis* LL-383 (Fig. 3). *S. uberis* is one of the most commonly found bacterial species associated with clinical and subclinical bovine mastitis, a leading cause of milk loss worldwide (60). A number of bacteriocins are known to be produced by *S. uberis* including the type I lantibiotic nisin U, and the class IIc bacteriocin uberolysin, both of which we identified within *S. uberis* LL-383 (Figs. 5 and 7) (22, 61). In addition to these, two other gene clusters were predicted, one containing two encoded peptides homologous to class IIa and IId bacteriocins, and the second encoding a two component lantibiotic (Figs. 6 and 8). Cluster 2 contains two genes encoding core peptides homologous to different class II bacteriocins (Fig. 6). Orf5 shares >60% identity with the class IId bacteriocin bovicin-255 produced by *Streptococcus* sp.

LRC0255 (62), and Orf8 is similar to enterococcal pediocin-like peptides mundticin KS and enterocin A, albeit with low identity (63, 64).

The production of multiple bacteriocins by a single strain has been described previously, including an *Enterococcus faecium* isolate from raw bovine milk which was shown to produce three antimicrobials and a *Streptococcus salivarius* isolate with four megaplasmid encoded lantibiotic gene clusters (12, 65, 66). As no plasmid sequences were detected within the genome of *S. uberis* LL-383 (Table 4), and three of the four gene clusters are predicted within a single large contig which aligns to the reference genome *S. uberis* 0140J (Fig. 4), it is likely that the bacteriocins are chromosomally encoded.

Bacteriocin expression has a fitness cost, but also provides a competitive advantage to the producing strain against sensitive strains (67). As such the repertoire of bacteriocins encoded within the genome of *S. uberis* LL-383 may provide a distinct advantage against a broad range of competing bacteria. The bacteriocins are diverse in sequence, structure, and probable mode of action. Nisin is known to act by a dual mechanism of lipid II binding and pore formation (68). The inhibitory mechanism of uberolysin is unknown but related circular bacteriocins enterocin AS-48 and carnocyclin A have been found to interact directly with and permeabilise cell membranes (69-71). The peptide encoded by *orf5* of cluster 2 shares homology with class IId bacteriocins garviaecin Q and lactococcins, and that encoded by *orf8* is similar to class IIa enterocins, all of which target the man-PTS system (72, 73). The two component lantibiotics lacticin 3147 and C55 that are similar to cluster 4 also bind to lipid II and cause pore formation in a mechanism distinct to nisin (74). Harbouring several gene clusters for bacteriocins with diverse modes of action could help the producing strain compete against a broad range of bacteria, particularly against strains which may have acquired resistance to single bacteriocins such as nisin (75). Future work should characterise the competitive ability of *S. uberis* LL-383 and determine if possession of multiple bacteriocin gene clusters impacts on its pathogenesis in mastitis.

Streptococcus spp. LL-387 and LL-514 were predicted to carry genes encoding a novel nisin variant homologous to nisin U from *S. uberis* str. 42 (22). MALDI-TOF MS could detect the 3,101 Da mass from colonies of *S. equinus* LL-387, but not from *S. equinus* LL-514. Given the truncated nature of the *nsi* operon by a contig boundary in the *S. equinus* LL-514 draft genome, we cannot be certain if genes for transport biosynthesis and immunity (*nsiTCI*) are present (Fig. 7a). However, a peak of 3,139 Da was observed from *S. equinus* LL-514 which could represent immature nisin I following six dehydrations. Purification of the active compound from cells/supernatant will be required to confirm nisin I as the source of antimicrobial activity.

Nisin I is the 11th natural nisin variant described to date following nisin Z (19), U/U2(22), F (20), Q (21), H (23), O123 and O4 (25), P (24, 76), and J (26), and the fifth of streptococcal origin. Though the gene cluster was truncated by a contig boundary in *S. equinus* LL-514, the 6,052 bp region corresponding to *nsiPRKAFEG* was 99.8% identical between *S. equinus* LL-514 and *S. equinus* LL-387, and the predicted mature peptides are identical. Therefore, the same novel variant was found in two separate isolates of sheep milk from different farms. Both strains are of the taxonomically complicated *Streptococcus bovis/Streptococcus equinus* complex (SBSEC) (77). Bacteriocin production has been described within the SBSEC previously including the lantibiotic bovicin HJ50 (78). Nisin P production has recently been described by multiple strains including *Streptococcus agalactiae* DPC7040 and *Streptococcus gallolyticus* subsp. *pasteurianus* AB39 which are also of the SBSEC (24, 76, 78). Gene clusters for nisin I production have also been detected within publicly available genomes of the SBSEC (Hill *et al.*, personal communication).

Nisin I is the second natural variant to be described of 32 residues following the description of the distantly related nisin O4 produced by the human gut bacterium, *Blautia obeum* (25). Though closely related to nisin U, nisin I contains an Ile15Ala substitution which is conservative, as nisin A, Z, F, and J all have Ala at this position (19, 20, 26) (Fig. 10b). Another substitution from nisin U is present within the hinge region of the peptide (+20 to +22) of Leu21Ile, which is also present in nisin P (24). The substitution within the hinge region may affect activity, as the hinge

region has been established as a target for bioengineering to improve activity (79). Though the other substitutions are conservative, the additional C-terminus Asn residue at position 32 is unique among nisin variants, with nisin A, Z, F, Q, H, and J having Val, nisinO123 all having Gly while O4 has Glu at position +32 (19-26) (Fig. 10b). The peptide is one amino acid longer than its closest relative nisin U (22). Extending the C-terminus has been found to improve the permeation of cell membranes by the peptide and increase activity against Gram-negative bacteria (80). However, it remains to be determined if the additional Asn residue impacts on activity relative to nisin U, particularly as Asn is a polar amino acid. The nisin I operon includes all the genes involved with typical nisin production including transport, modification, and immunity, however, the gene order differs from that of all other nisin operons, with the structural peptide immediately upstream from the *lanFEG* transport and immunity genes (Fig. 10a). In the nisin U operon, transposase sequences flank the operon and are directly upstream from *nsuA*, to which the reorganisation of the operon relative to nisin A is attributed (22).

The final bacteriocin gene cluster identified was within the genome of *L. lactis* LL-427. The operon shares similarity with the two component lantibiotic carnolysin from *Carnobacterium maltaromaticum* (81) and cytolysin of *Enterococcus faecalis* (82) (Fig. 11). The operon structure and identity of most predicted genes are similar to the carnolysin operon, however, one open reading frame is more closely related to the cytolysin gene which encodes an extracellular serine protease, *cylA* (Fig. 11a). In *Enterococcus*, CylA is responsible for the final second proteolytic cleavage and activation of the cytolysin peptides, removing the identical N-terminal 6 amino acid sequence GDVQAE from $CylL_L$' and $CylL_S$', generating the final active forms $CylL_L$" and $CylL_S$" (83). Natural carnolysin peptides have been detected in the singly cleaved inactive state (CrnA1' and CrnA2') and active forms of the peptide have been produced by heterologous expression and treatment with an endoproteinase (81, 84). As with the other two peptide lantibiotics, both peptides were required for maximum antimicrobial activity (84). Peptides encoded by *orf3* and *orf4* share the $CylL_L$ and $CylL_S$ GDVQAE motif, and as such may be subject to an extracellular cleavage step to produce their mature active forms. Cytolysin was first experimentally

characterized as a haemolytic toxin (85) and is now known to have a role in outcomes of infection models (86). However, the active form of carnolysin is not haemolytic and inhibits a broad spectrum of Gram-positive strains, including *Enterococcus faecium* (84). *L. lactis* LL-427 is faintly α-haemolytic on horse blood agar (data not shown) which is distinct from the true (β-) haemolysis produced by the enterococcal cytolysin (87). Future work should characterise potential haemolytic or cytolytic activity of the strain and peptides and assess their applicability for food fermentation.

In conclusion, this study described the identification of diverse bacteriocin gene clusters within LAB isolated from unpasteurised sheep milk. This study also generated a biobank of raw milk isolates which could be screened for other phenotypes such as exopolysaccharide production and bile salt hydrolase activity which may be of interest for application in food fermentation or as probiotic candidates. This study illustrates some of the broad range of streptococcal bacteriocins and is the first to describe the novel nisin variant nisin I. Nisin I is the 11th natural variant to be described and contains two conservative amino acid substitutions from nisin U, and a C-terminus Asn residue unique among natural variants. Nisin has a long history of use in food preservation and variants may have useful properties such as increased stability, protease resistance, or improved activity over related compounds. Future characterization of nisin I and the other compounds identified in this study may lead to applications in shelf life extension or the development of novel antimicrobials to combat the global crisis of antimicrobial resistance.

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Figures and Tables

Figure 1. Workflow diagram of strain isolation and screen for bacteriocin producers.

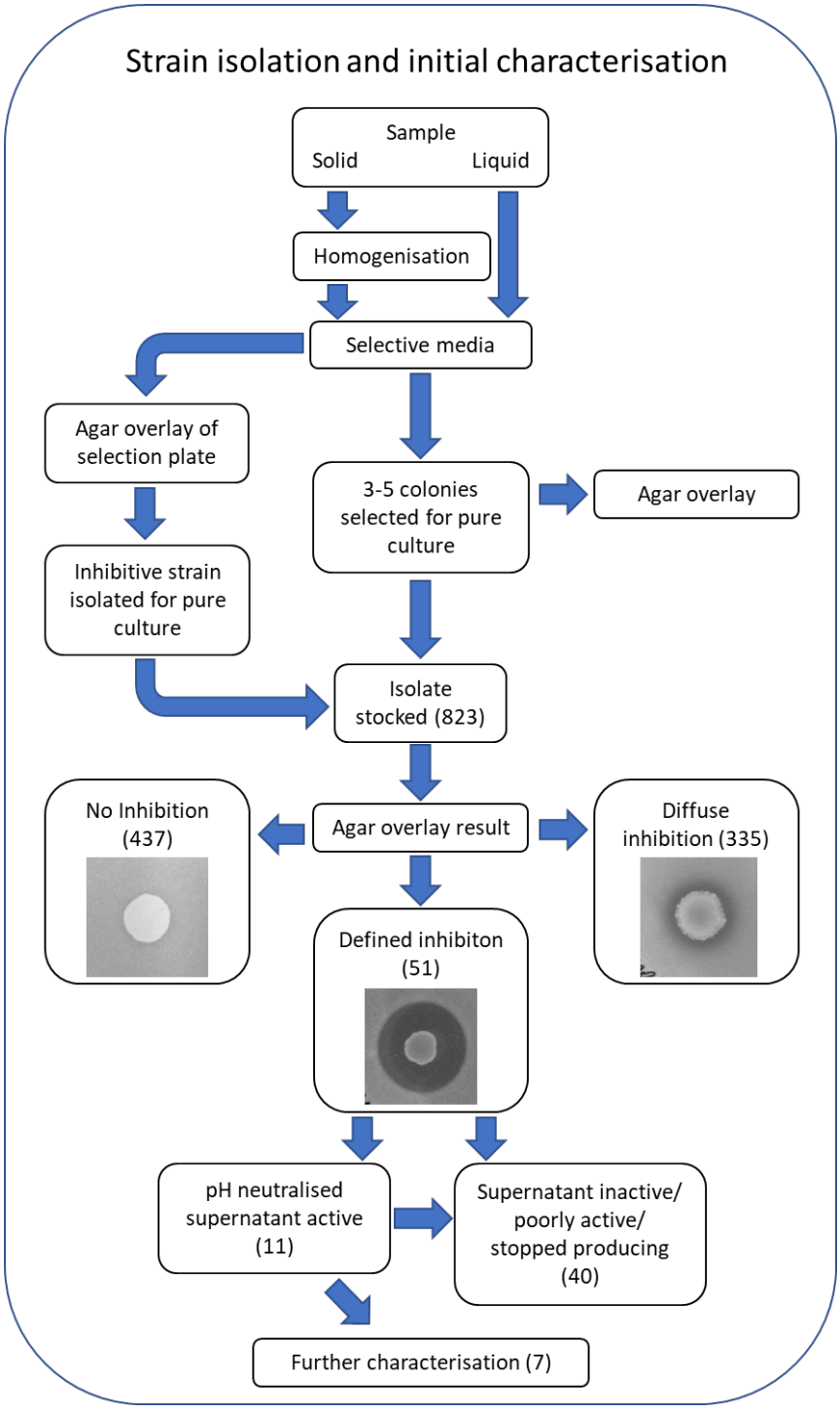


Figure 2. Antimicrobial activity of strains identified from bacteriocin screen. Inhibition is observed as a zone of clearing in the indicator strain (*L. delbrueckii* ssp. *bulgaricus*) in agar overlay and well diffusion. When treated with protease activity is disrupted, indicating proteinaceous activity.

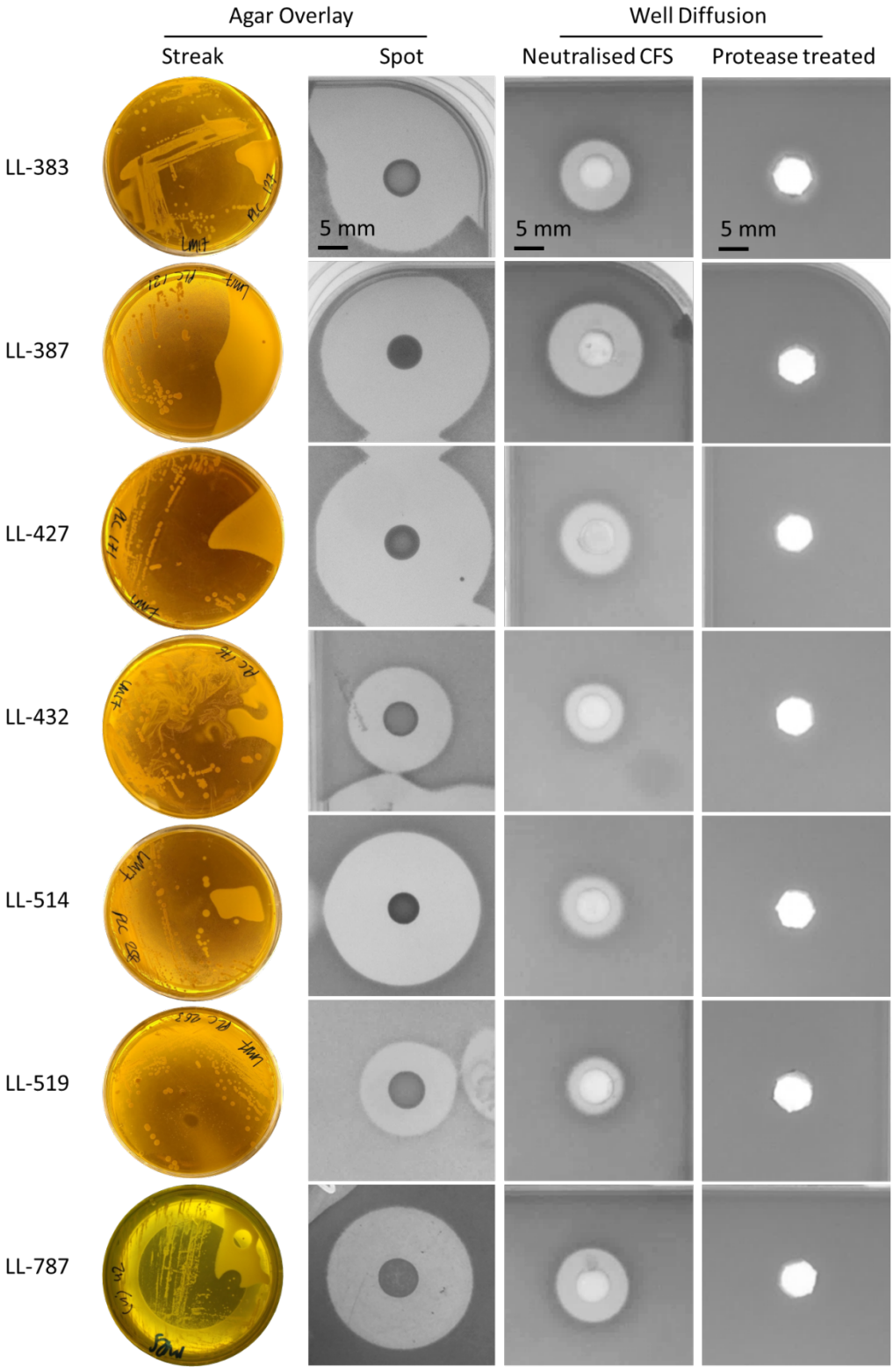


Figure 3. Diagrams of predicted bacteriocin gene clusters in *S. uberis* LL-383, *S. equinus* LL-387, *S. equinus* LL-514 and *L. lactis* LL-427.

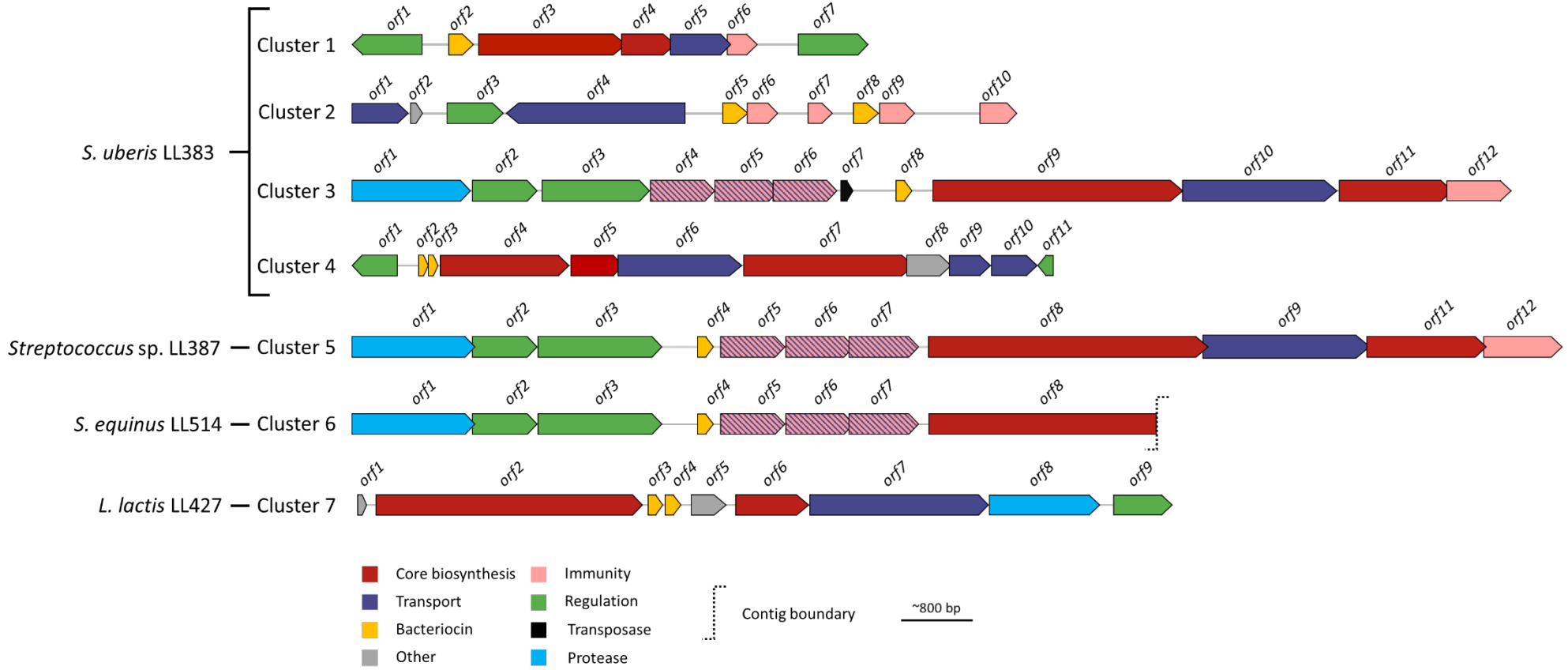


Figure 4. Mauve alignments of bacteriocin producer draft genomes to reference genomes. Contig boundaries indicated by vertical red lines.

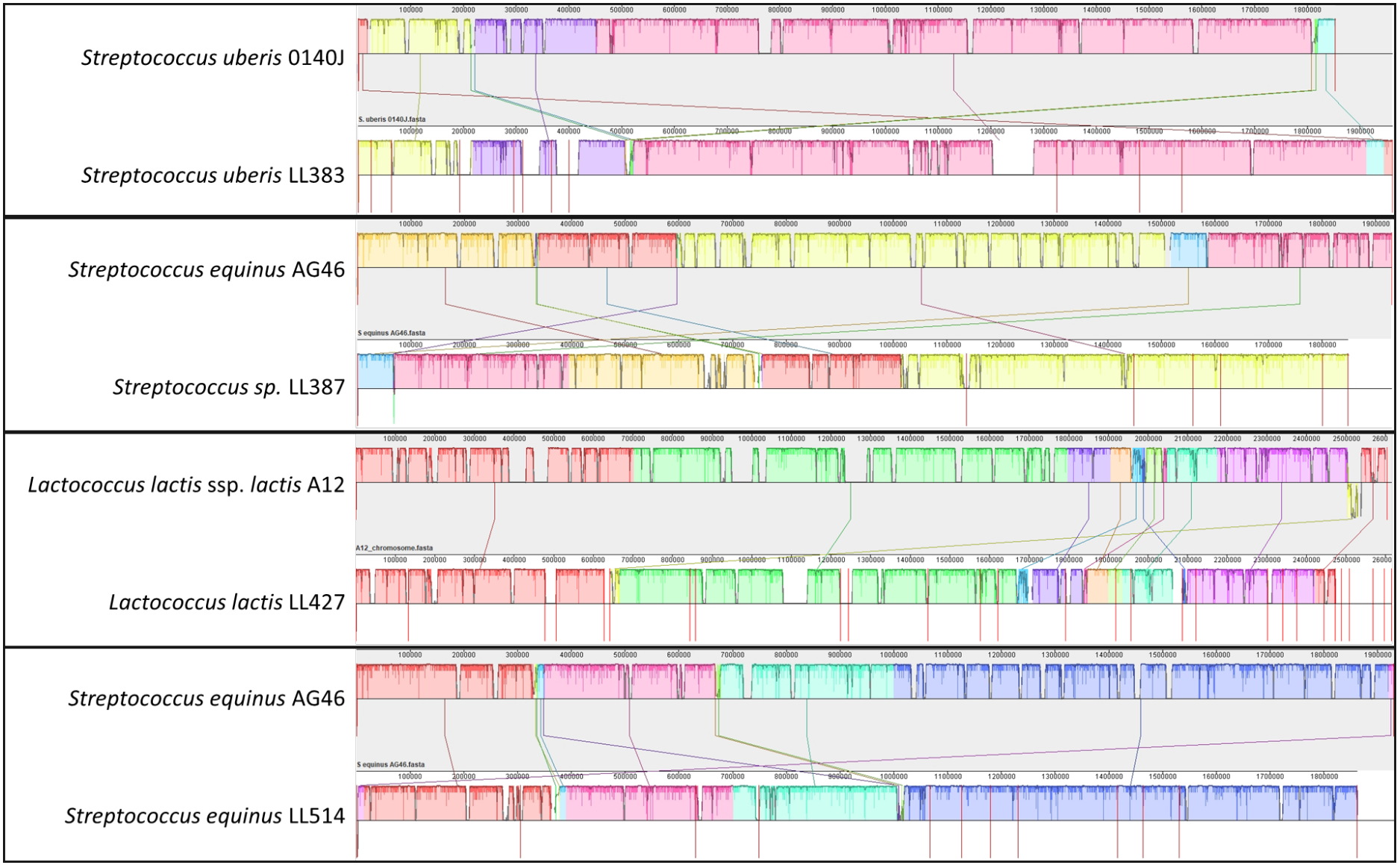


Figure 5. Gene cluster comparison of 4.4kb uberolysin operon (*Streptococcus uberis* str. 42) and Cluster 1 (*S. uberis* LL-383). Amino acid percentage identity between operons is shown above the corresponding CDS.

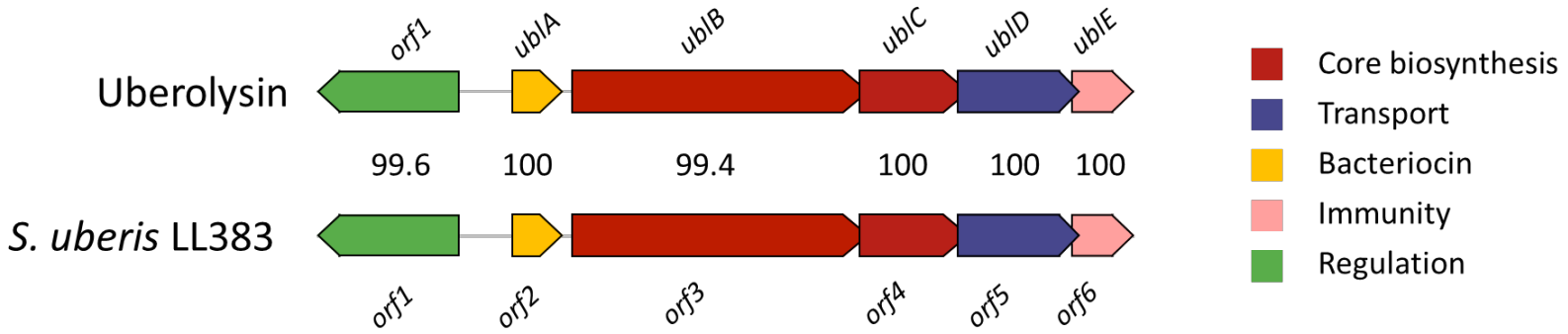


Figure 6. Multiple sequence alignments of (a) ORF5 and (b) ORF8 from Cluster 2 (*S. uberis* LL-383) to similar known bacteriocins. Conserved residues coloured with Clustalx scheme.

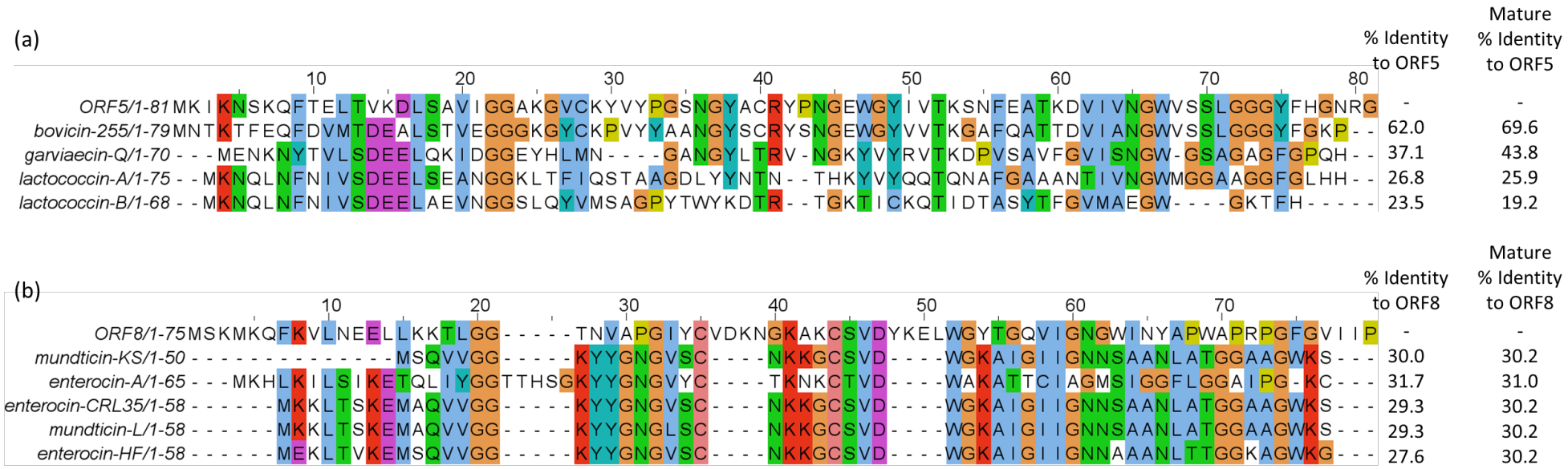


Figure 7. (a) Operon comparison of nisin A (*Lactococcus lactis* subsp. *lactis*), nisin U (*Streptococcus uberis*), Cluster 3 (*S. uberis* LL-383), Cluster 5 (*Streptococcus equinus* LL-387), and Cluster 6 (*Streptococcus equinus* LL-514). Amino acid percentage identity to nisin A genes shown above the corresponding CDS. *Percent identity of truncated sequence to nisB. (b) Sequence alignment of nisA, nisU and predicted peptides. Conserved residues coloured with Clustalx scheme. Italicized mass = predicted mass. (c) MALDI-TOF mass spectra of colonies. Peaks corresponding to predicted bacteriocin genes are indicated with arrows.

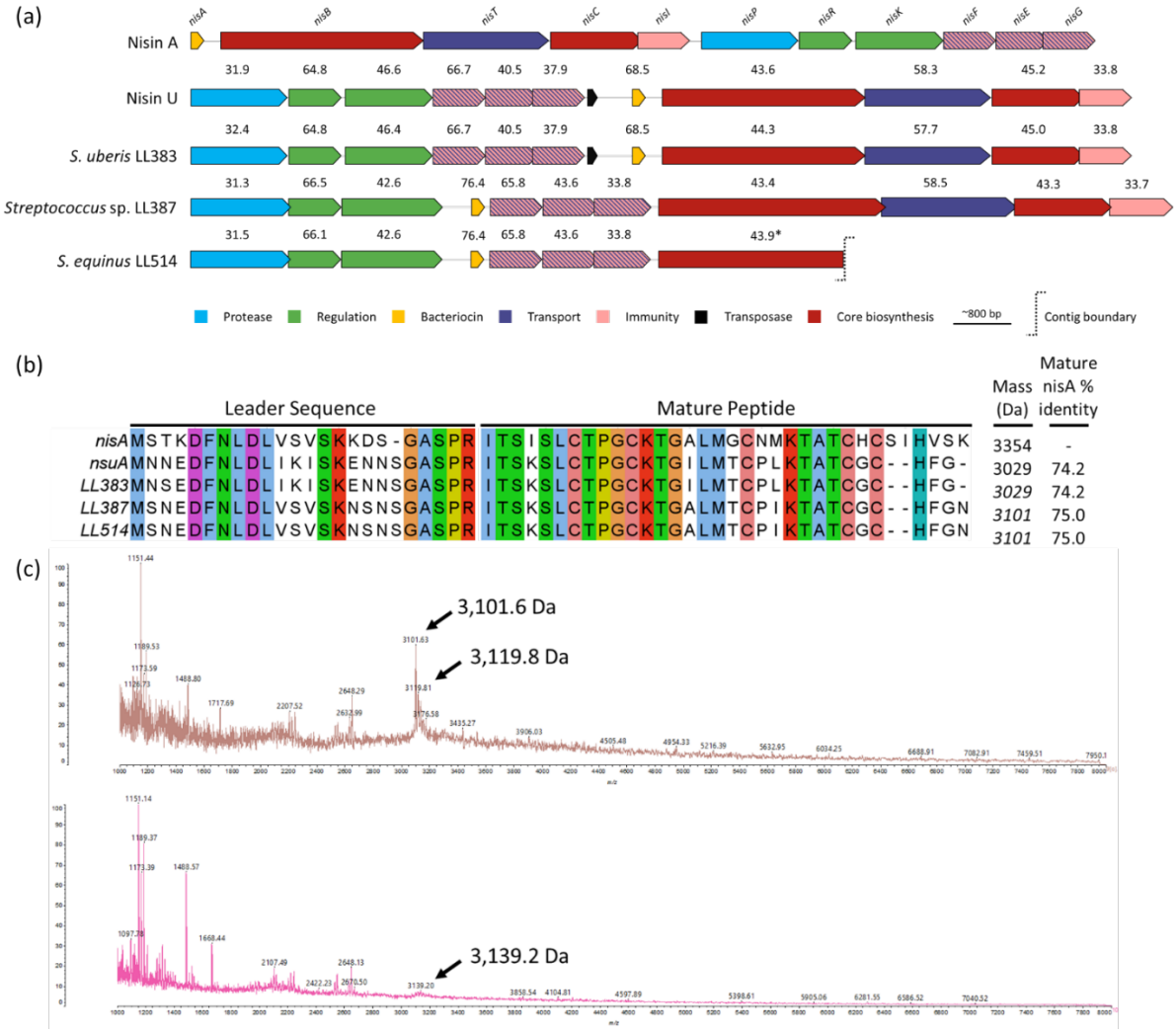
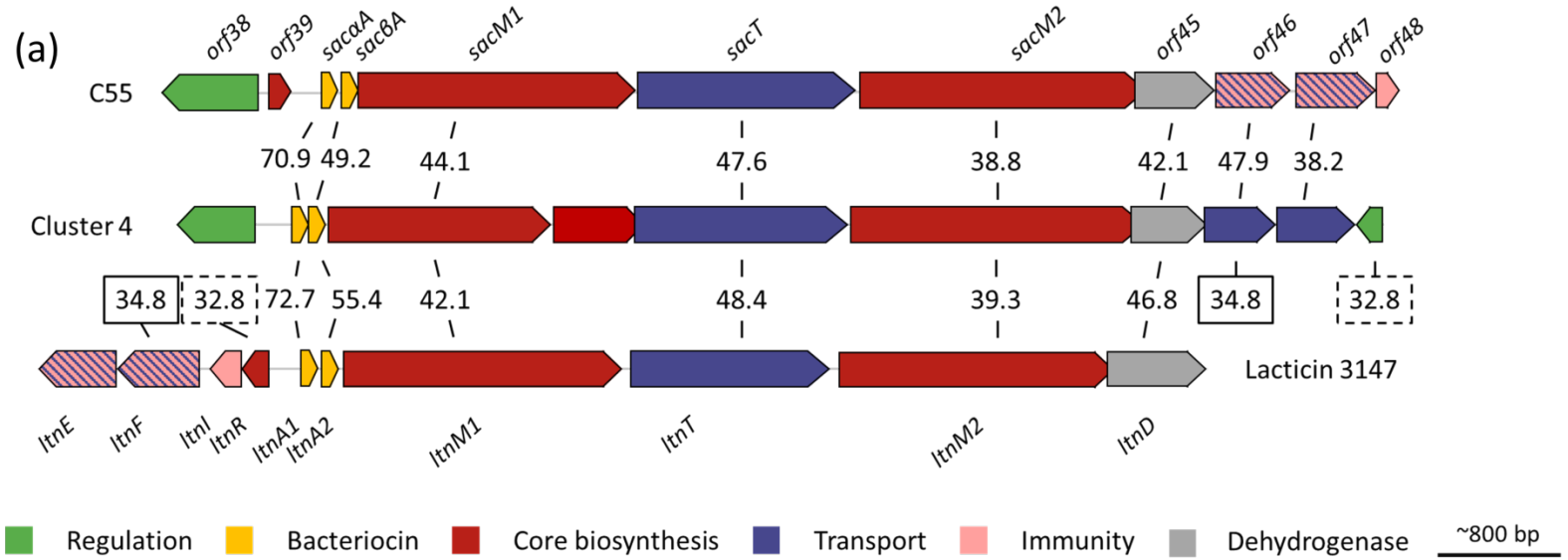


Figure 8. (a) Operon comparison of lacticin 3147 (*Lactococcus lactis* DPC3147), C55 (*Staphylococcus aureus* C55) and Cluster 4 (*S. uberis* LL-383). Amino acid percentage identity above 30% is shown between operons. Sequence alignment of predicted (a) A1 and (b) A2 mature peptides and known two component lantibiotics. Conserved residues coloured with Clustalx scheme.



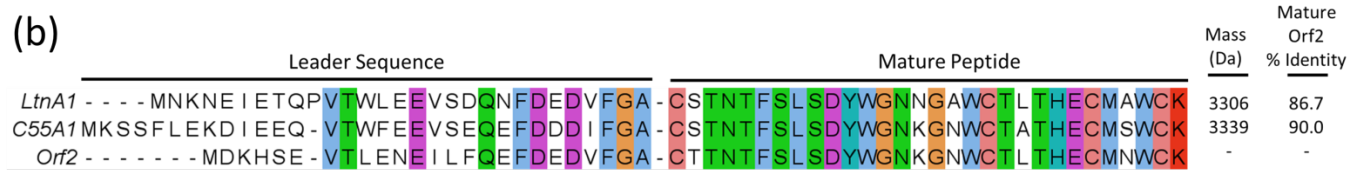




Figure 9. Multiple sequence alignment of proteins encoded by ORF4 and ORF5 from Cluster 4 (*S. uberis* LL-383) aligned to LtnM1 (*Lactococcus lactis* DPC3147) and SacM1 (*Staphylococcus aureus* C55). Residues coloured with Clustalx scheme.

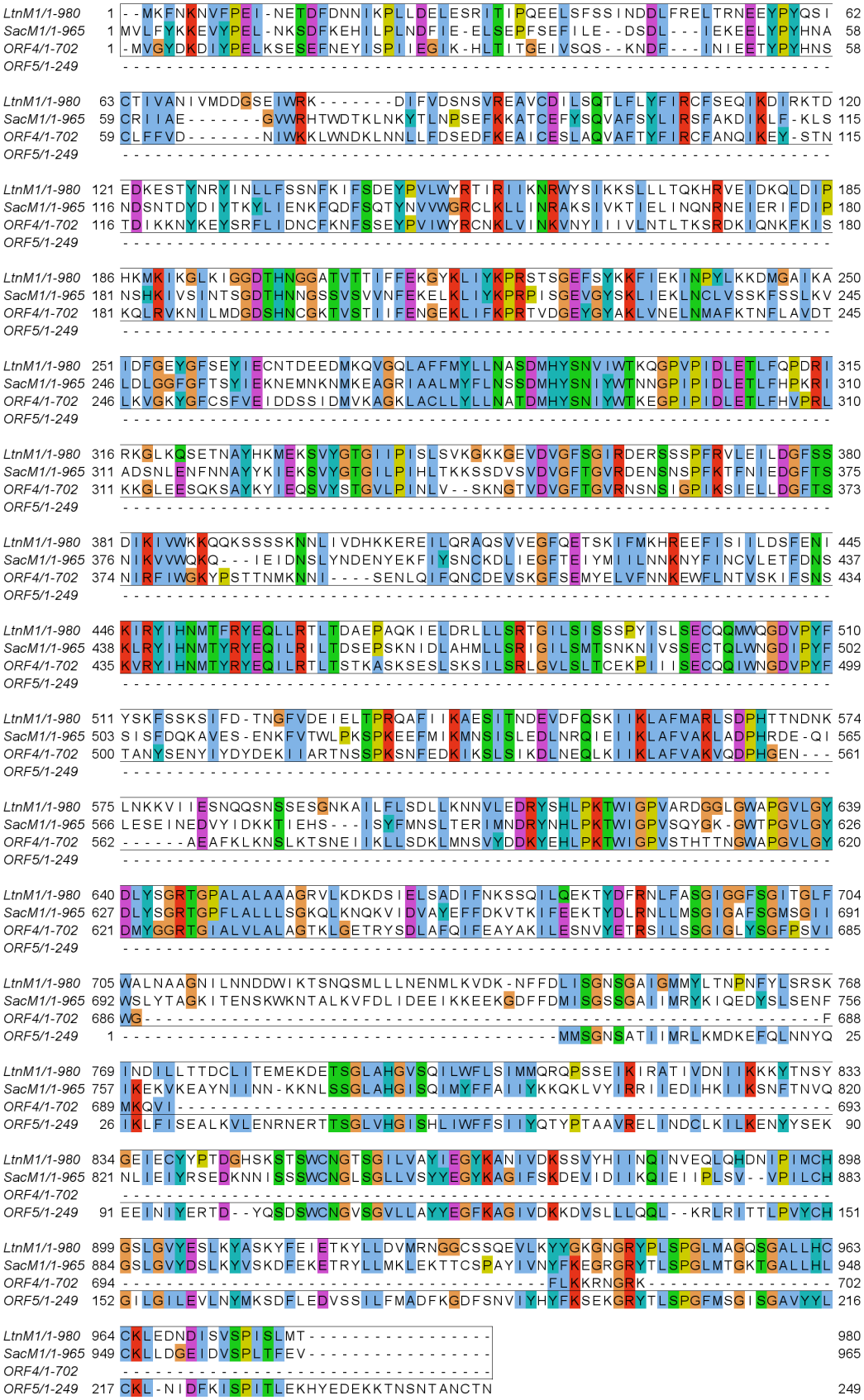
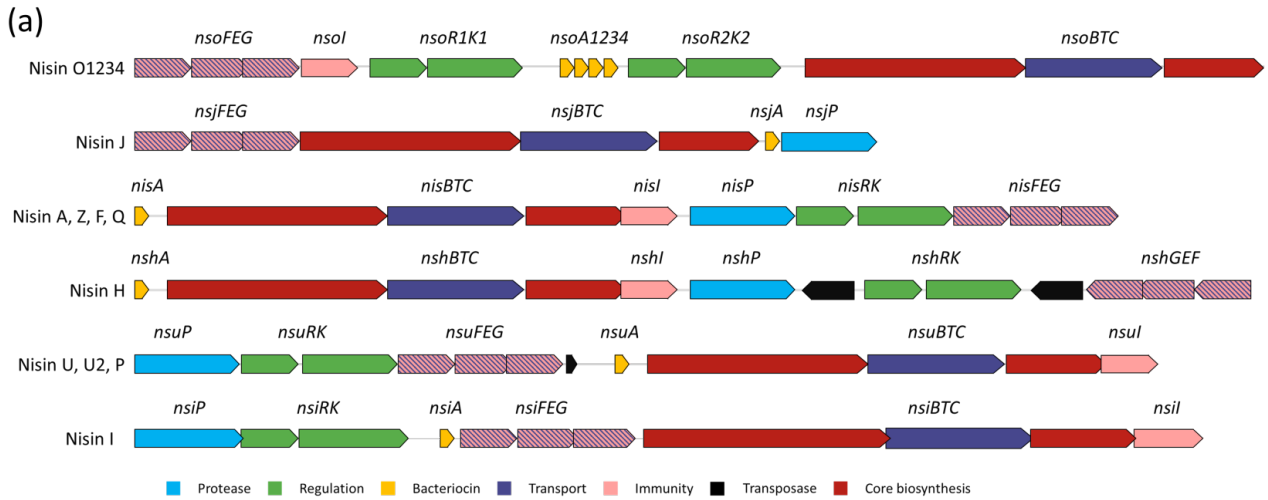


Figure 10. (a) Nisin variant operon structure comparison to nisin I . (b) Sequence alignment of known nisin variants. Residues coloured with Clustalx scheme.



(b)

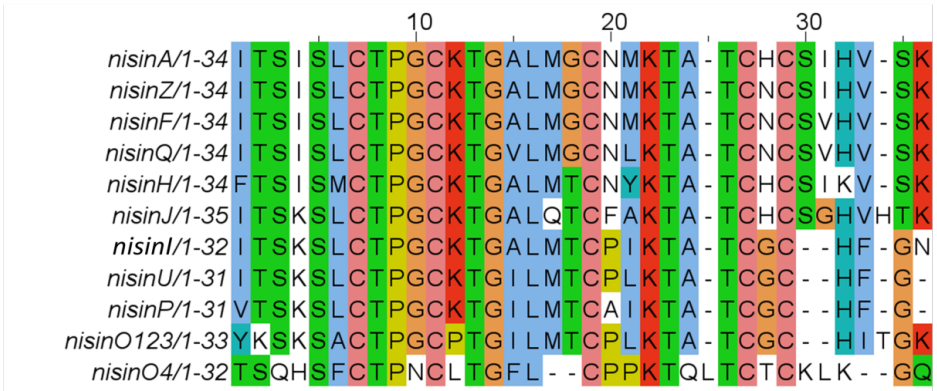


Figure 11. (a) Operon comparison of carnolysin (*Carnobacterium maltaromaticum*), and Cluster 7 (*Lactococcus lactis*. LL-427), and cytolysin (*Enterococcus faecalis*). Cluster 7 ORFs numbered 1-9 from left to right. Amino acid identity to genes shown between operons. (b) Sequence alignment of encoded structural peptides. Residues coloured with Clustalx scheme. Cleavage sites of processing proteins indicated with arrows.

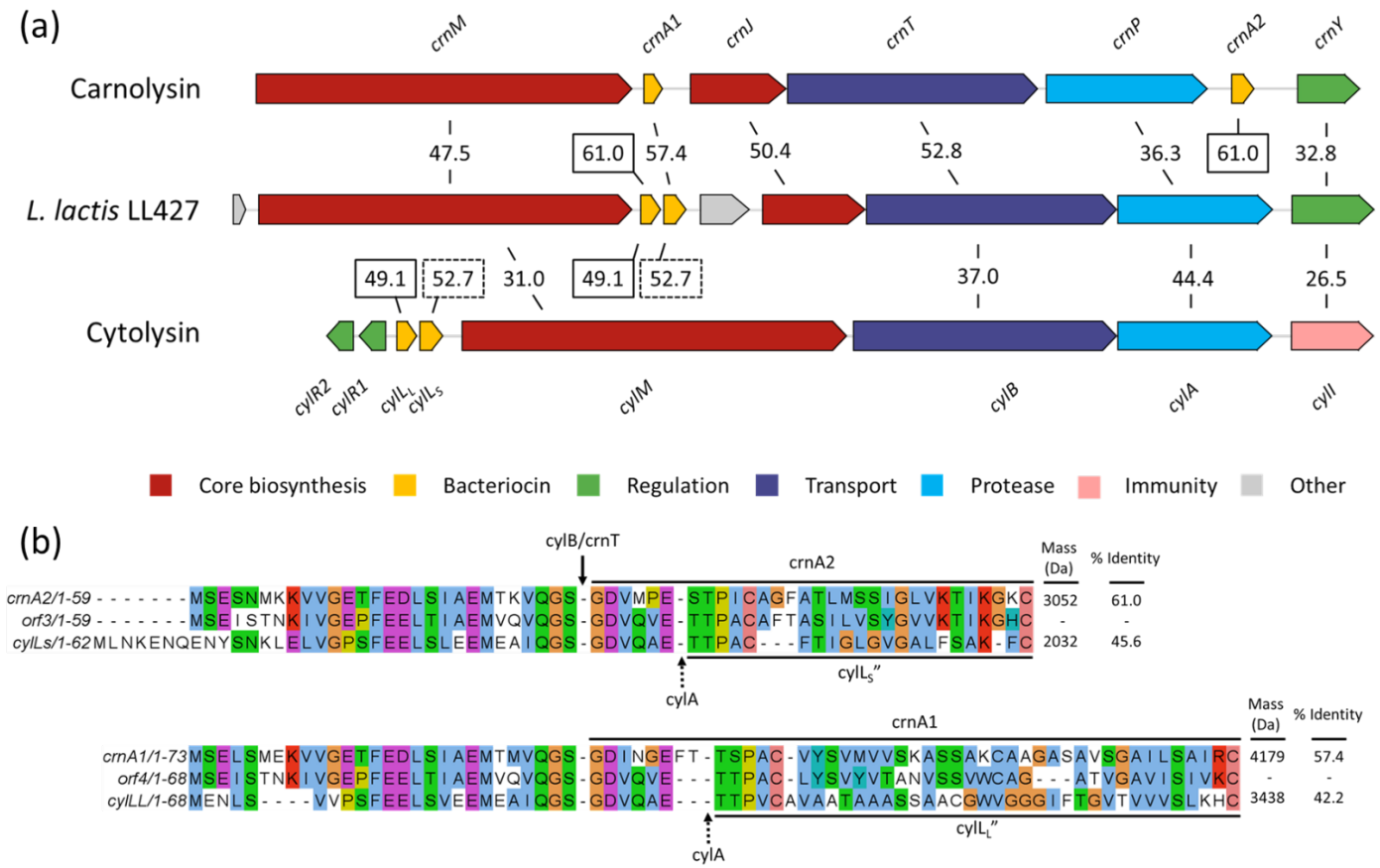


Table 1. Type and origin of samples from which strains were isolated.

Sample Type	Number of Samples	Sample Source Location	Number of Isolates	Number of 'defined inhibition' producers
Raw sheep's milk	74	New Zealand	293	28
Raw goat's milk	21	New Zealand	174	18
Raw cow's milk	9	Ireland	130	3
Sheep faeces	1	Ireland	25	1
Other*	7	-	201	0

*Unpasteurised sheep, goat, cow and buffalo cheeses, fresh honey, honeycomb, and canine saliva samples

Table 2. 16s identification of bacteriocin producers.

Strain	Source	16s rRNA identification	% identity
LL-383	Sheep's Milk	*Streptococcus uberis*	100
LL-387	Sheep's Milk	*Streptococcus lutetiensis/equinus/infantarius*	99/99/99
LL-427	Sheep's Milk	*Lactococcus lactis*	99
LL-432	Sheep's Milk	*Enterococcus faecium/durans/hirae/ratti*	99/99/99/99
LL-514	Sheep's Milk	*Streptococcus equinus/lutetiensis*	97/97
LL-519	Sheep's Milk	*Enterococcus faecium/durans/hirae/ratti*	99/99/99/99
LL-787	Sheep Faeces	*Actinomyces ruminicola*	98

Table 3. Constructed genome statistics.

Strain	# Contigs	Median coverage	N50*	N90†	L50‡	# Contigs (>10 kbp)	Median contig coverage (>10 kbp)	N50*	N90†	L50‡
LL-383	72	37 x	400,041	54,449	2	11	186.1 x	400,041	79,793	2
LL-387	114	47.2 x	1,136,482	111,075	1	6	188.7 x	1,136,482	111,075	1
LL-427	209	111.0 x	171,277	27,207	6	27	123.5 x	171,277	37,865	6
LL-432	309	146.6 x	37,402	7,239	22	71	173.4 x	43,058	15,489	18
LL-514	39	18.7 x	317,872	59,461	3	11	49.5 x	317,872	59,461	3
LL-519	1133	1.3 x	33,740	645	29	79	257.3 x	46,954	15,830	20

*†Sequence length of the shortest contig at 50% and 90% and of the total genome length, respectively. ‡Smallest number of contigs which constitute half of genome size

Table 4. Constructed genome data from bacteriocin gene cluster, prophage, and plasmid sequence prediction software.

Strain	GC Content (%)		Genome size (Mbp)		Bacteriocin Gene Cluster Predicted	Predicted Prophage (Complete/ Potential/ Incomplete)	Plasmid Sequences Detected
	Strain	Reference Genome	Strain	Reference Genome			
LL-383[a]	36.3	36.6	1.96	1.85	4	0/1/0	0
LL-387[b]	37.2	37.5	1.85	1.93	1	0/0/2	0
LL-427[c]	34.9	35.6	2.62	2.60	2	1/2/7	1
LL-514[b]	37.3	37.4	1.86	1.88	1*	0/0/0	0

Reference genomes, accession: [a]*S. uberis* 0140J, NC_012004; [b]*S. equinus* AG46, NZ_JNLO01000001; [c]*L. lactis* ssp. *lactis* A12, NZ_LT599049.1. *****Gene cluster truncated by contig boundary.

Table 5. ORF details of predicted bacteriocin gene clusters (BGCs) encoded in genomes of antimicrobial strains.

BGC	ORF	Size (bp)	Strand	Top pBLAST Result	Source	% Identity
1	1	747	-	Response regulator transcription factor	*S. uberis*	100
	2	231	+	circular bacteriocin, uberolysin family	*S. uberis*	100
	3	231	+	UblB	*S. uberis*	99.4
	4	1608	+	Stage II Sporulation protein	*S. uberis*	100
	5	546	+	ATP-binding cassette domain containing protein	*S. uberis*	100
	6	642	+	UblE	*S. uberis*	100
	7	732	+	GntR family transcriptional regulator	*S. uberis*	100
2	1	630	+	ATP-binding cassette domain containing protein	*S. uberis*	99.5
	2	117	+	Hypothetical protein	*S. uberis*	100
	3	576	+	Response regulator ComE	*S. uberis*	99
	4	1878	+	Peptide cleavage/export ABC transporter	*S. uberis*	98.7
	5	246	+	garvicin Q family class II bacteriocin	*S. uberis*	100
	6	300	+	bacteriocin immunity protein	*S. uberis*	100
	7	207	+	Hypothetical protein	*S. uberis*	100
	8	228	+	bacteriocin	*S. hongkongensis*	98.7
	9	321	+	bacteriocin immunity protein	*S. uberis*	99.1
	10	300	+	bacteriocin immunity protein	*S. uberis*	100
3	1	1374	-	NsuP	*S. uberis*	97.8
	2	699	-	NsuR	*S. uberis*	97.4
	3	1269	-	NsuK	*S. uberis*	98.3
	4	693	-	NsuF	*S. uberis*	97.8
	5	747	-	NsuE	*S. uberis*	99.6
	6	693	-	NsuG	*S. uberis*	99.6
	7	129	-	Putative transposase	*S. gordonii*	81
	8	165	-	NsuA	*S. uberis*	98.2
	9	2976	-	Lantibiotic dehydratase	*S. agalactiae*	96.8
	10	1809	-	NsuT	*S. uberis*	99
	11	1278	-	NsuC	*S. uberis*	99.5
	12	717	-	NsuI	*S. uberis*	99.6

Continued...

BGC	ORF	Size (bp)	Strand	Top pBLAST Result	Source	% Identity
4	1	852	+	Putative response regulator	*S. canis*	99.7
	2	168	-	Plantaricin C family lantibiotic	*S. dysgalactiae*	98.2
	3	201	-	Hypothetical protein	*S. dysgalactiae*	94.2
	4	2109	-	Type 2 lantipeptide synthetase LanM	*S. canis*	98.2
	5	750	-	Type 2 lantipeptide synthetase LanM	*S. anginosus*	99.6
	6	2148	-	ATP binding cassette domain protein	*S. canis*	100
	7	2787	-	DUF4135 domain-containing protein	*S. canis*	100
	8	1116	-	Zinc-binding alcohol dehydrogenase family protein	*S. dysgalactiae*	99.2
	9	855	-	ABC transporter ATP binding	*S. equi*	97.5
	10	729	-	ABC transporter permease	*S. anginosus*	99.2
	11	207	+	Helix-turn-helix domain containing protein	*S. canis*	100
5	1	1251	-	S8 family serine peptidase	*S. equinus*	98.8
	2	684	-	response regulator transcription factor	*S. equinus*	100
	3	1287	-	HAMP domain-containing histidine kinase	*S. equinus*	99.8
	4	171	-	gallidermin/nisin family lantibiotic	*S. equinus*	100
	5	693	-	ABC transporter ATP-binding protein	*S. equinus*	100
	6	735	-	lantibiotic ABC transporter permease	*S. equinus*	100
	7	651	-	lantibiotic transporter	*S. equinus*	99.5
	8	2997	-	lantibiotic dehydratase	*S. equinus*	100
	9	1809	-	ABC transporter ATP-binding protein	*S. equinus*	99.8
	10	1263	-	lanthionine synthetase C family protein	*S. equinus*	100
	11	738	-	NisI/SpaI family lantibiotic immunity lipoprotein	*S. equinus*	100
6	1	1251	-	S8 family serine peptidase	*S. equinus*	99.8
	2	684	-	response regulator transcription factor	*S. equinus*	100
	3	1287	-	HAMO domain-containing histidine kinase	*S. equinus*	99.8
	4	171	-	gallidermin/nisin familiy lantibiotic	*S. equinus*	100
	5	693	-	ABC transporter ATP-bindin protein	*S. equinus*	100
	6	735	-	lantibiotic ABC transporter permiease	*S. equinus*	99.9
	7	651	-	lantibiotic transporter	*S. equinus*	100
	8	2607	-	lantibiotic dehydratase	*S. equinus*	100

Continued...

BGC	ORF	Size (bp)	Strand	Top pBLAST Result	Source	% Identity
7	1	114	-	hypothetical protein	*L. lactis*	100
	2	3003	-	type 2 lantipeptide synthetase LanM	*L. lactis*	100
	3	180	-	type 2 lantibiotic	*L. lactis*	100
	4	207	-	type 2 lantibiotic	*L. lactis*	100
	5	342	-	No significant similarity found	-	-
	6	717	-	NAD(P)H-dependent oxidoreductase	*L. lactis*	100
	7	2121	-	peptidase domain-containing ABC transporter	*L. lactis*	99.9
	8	1290	-	S8 family serine peptidase	*L. lactis*	100
	9	600	-	XRE family transcriptional regulator	*L. lactis*	100

Table 6. Top nucleotide BLAST results of unaligned contigs from *L. lactis* LL-427.

Contig	Length (bp)	Description	Query Cover	E value	Per. Ident	Accession
13	60,893	*Lactococcus lactis* subsp. *lactis* strain 14B4 plasmid p14B4, complete sequence	48%	0	93.8%	CP028161.1
19	28,603	*Lactococcus lactis* subsp. *lactis* strain 275 plasmid p275D, complete sequence	31%	0	99.4%	CP016702.1
21	27,207	*Lactococcus lactis* subsp. *lactis* strain UC77, complete genome	46%	0	99.8%	CP015906.1
22	20, 261	*Lactococcus* phage 98201, complete genome	87%	0	93.6%	KX456213.1
23	19, 797	*Lactococcus lactis* subsp. cremoris strain NCDO712 plasmid pLP712, complete sequence	88%	0	99.4%	FJ649478.1
24	19, 339	*Lactococcus lactis* subsp. *lactis* KF147, complete genome	84%	0	95.3%	CP001834.1
25	15, 644	*Lactococcus lactis* subsp. *lactis* strain A12 genome assembly, plasmid: pA12-3	39%	0	98.0%	LT599052.1
27	14, 242	*Lactococcus lactis* subsp. *lactis* CV56, complete genome	98%	0	98.7%	CP002365.1

Table 7. Selective conditions for bacterial strain isolation.

Isolation Media	Composition (g litre^{-1})	pH	Incubation conditions	Putative Isolates
Streptococcus thermophilus selective agar (STA)	Tryptone, 10.0; dipotassium phosphate, 2.0; sucrose, 10.0; yeast extract, 5.0; agar, 15.0; bromocresol purple, 0.03	6.8	42 °C, O_2^-, 48 – 72h	*Streptococcus thermophilus*
LM17	Tryptone, 5.0; soya peptone, 5.0; meat extract, 5.0; lactose monohydrate, 5.0; meat peptone, 2.5; casein peptone, 5.0; yeast extract, 2.5; ascorbic acid, 0.5; magnesium sulphate, 0.25; di-sodium-glycerophosphate, 19.0; agar, 15.0	6.9	30 °C, O_2, 48 – 72h	*Lactococcus* spp.
Lactobacillus selective agar (LBS)	Pancreatic digest of casein, 10.0; yeast extract, 5.0; monopotassium phosphate, 6.0; ammonium citrate, 2.0; dextrose, 20.0; polysorbate 80, 1.0; sodium acetate hydrate, 25.0; magnesium sulphate, 0.575; manganese sulphate, 0.12; ferrous sulphate, 0.035; agar, 15.0	5.5	30 °C, O_2^-, 72h	*Lactobacillus* spp.
MRS, pH 5.4	Proteose peptone, 10.0; beef extract, 10.0; yeast extract, 5.0; dextrose, 20.0; polysorbate 80, 1.0; ammonium citrate, 2.0; sodium acetate, 5.0; magnesium sulphate, 0.05; dipotassium phosphate, 2.0; agar, 15.0	5.4	42 °C, O_2^-, 48h	*Lactobacillus* spp.
MRS supplemented with vancomycin	Proteose peptone, 10.0; beef extract, 10.0; yeast extract, 5.0; dextrose, 20.0; polysorbate 80, 1.0; ammonium citrate, 2.0; sodium acetate, 5.0; magnesium sulphate, 0.05; dipotassium phosphate, 2.0; agar, 15.0; vancomycin hydrochloride*, 0.03	6.5	37 °C, O_2^-, 48 – 72h	*Lactobacillaceae except Lactobacillus, Amylolactobacillus and Holzapfelia*
Transgalactosylated oligosaccharide (TOS) supplemented with mupirocin	Casein enzymic hydrolysate, 10.0; yeast extract, 1.0; potassium dihydrogen phosphate, 3.0; dipotassium hydrogen phosphate, 4.8; ammonium sulphate, 3.0; magnesium sulphate heptahydrate, 0.2; L-cysteine hydrochloride monohydrate, 0.5; sodium propionate, 15.0; galactooligosaccharide, 10.0; agar, 15.0; lithium mupirocin*, 0.05†	6.7	37 °C, O_2^-, 48 – 72h	Bifidobacteria
*supplement added after autoclaving, †concentration doubled when isolating from faecal sample				

Chapter 5. *Actinomyces* produce defensin-like bacteriocins (actifensins) with a highly degenerate structure and broad antimicrobial activity

Abstract

We identified a strain of *Actinomyces ruminicola* which produces a potent bacteriocin with activity against a broad range of Gram-positive bacteria, many of which are pathogenic to animals and humans. The bacteriocin was purified and found to have a mass of 4,091 ± 1 Da with a sequence of GFGCNLITSNPYQCSNHCKSVGYRGGYCKLRTVCTCY containing three disulphide bridges. Surprisingly, near relatives of actifensin were found to be a series of related eukaryotic defensins displaying greater than 50% identity to the bacteriocin. A pangenomic screen further revealed that production of actifensin-related bacteriocins is a common trait within the genus, with 47 being encoded in 161 genomes. Furthermore, these bacteriocins displayed a remarkable level of diversity with a mean amino acid identity of only 52% between strains/species. This level of redundancy suggests that this new class of bacteriocins may provide a very broad structural basis on which to deliver and design new broad-spectrum antimicrobials for treatment of animal and human infections.

Introduction

Novel antimicrobial compounds are increasingly important in the food, agriculture, and medical fields due to decreasing efficacies of current antimicrobial treatments. Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria which can target another bacterium of the same species (narrow spectrum) or bacteria of other species/genera (broad spectrum) (1). Bacteriocin producers are self-protected through the production of specific immunity proteins, and as bacteriocins are gene encoded, they can be genetically modified. Bacteriocins produced by Gram-positive bacteria have been grouped according to their primary structure into class I (posttranslationally modified bacteriocins) and class II (unmodified or cyclic bacteriocins) (2). Class II incorporates several subgroups, including the class IId bacteriocins, which are a heterogenous group of linear, unmodified, nonpediocin-like peptides (3).

Defensins are antimicrobial peptides ubiquitous among eukaryotes which play a role in innate immunity but have also been found to act as signalling peptides, toxins, enzyme inhibitors, and abiotic stress responders and to have anticancer properties. Defensins are small (<10 kDa) cysteine-rich (forming three to six disulphide bonds) peptides with low amino acid identity, and the two superfamilies are thought to have evolved convergently (4). Only two expressed defensin-like bacteriocins have been described; the laterosporulins were previously identified among prokaryotes and contain disulphide bonds in positions homologous to those in eukaryotic defensins (5, 6). Other disulphide bond-containing bacteriocins, such as bactofencin, have been compared with eukaryotic defensins due to their highly cationic nature (7, 8). Laterosporulin and its homolog laterosporulin10 are class IId bacteriocins produced by *Brevibacillus* spp. which have been described as broad-spectrum antimicrobials against both Gram-negative and Gram-positive bacteria. The two peptides are 5.6 kDa and 6.0 kDa and share only 57.6% amino acid sequence identity but have conserved cysteines, which are characteristic of eukaryotic defensins (6).

Actinomyces spp. are a heterogenous group of high-GC-content, Gram-positive non-spore-forming facultative or obligate anaerobes that belong to the *Actinomycetaceae* family within the phylum Actinobacteria (9). In humans, a number of species are known colonizers of hard surfaces in the oral cavity, where they play a key role in plaque biofilm formation (10, 11). They have been identified as core members of the oral bacteriome, present in moderate abundance (>0.1% to >2.0%) among geographically diverse populations (10, 12-15). *Actinomyces* spp. have been implicated in oral health as being associated in greater abundance in individuals with dental caries, one of the most prevalent chronic oral diseases worldwide (14, 15). Most characterized strains are clinical isolates of human origin, while some opportunistically pathogenic species such as *Actinomyces israelii* and *Actinomyces gerencseriae* are known to cause the uncommon infectious disease actinomycosis (16). Though *Actinomyces* spp. are abundant in the oral cavity, little is known about their presence in the gut, probably due to their low abundance (<0.1%) (10). Many *Actinomyces* spp. have been isolated from faecal material and from the gastrointestinal tracts of different animals, indicating a propensity for gastric transit survival, and their presence has also been noted in the urogenital tract (17-24).

Here, we identify a new group of bacteriocins using a pangenomic *in silico* approach paired with functional screening. Many *in silico* genome mining tools have been developed for the successful detection of novel antimicrobial-producing operons (25, 26). Obviously, these methods rely on relationships with previously known genes; therefore, functional screening is crucial for the identification of unrelated antimicrobials. In this study, we isolated a potent bacteriocin-producing strain of *Actinomyces ruminicola* from sheep faeces – the bacteriocin produced resembled eukaryotic defensins, having three characteristic disulphide bridges. A subsequent pan-genus *Actinomyces* analysis revealed that such bacteriocins are widely distributed in these bacteria, albeit with a highly variable structure.

Materials and Methods

Isolation of bacteria and identification of bacteriocin production

Samples of raw milk, unpasteurized cheeses, sheep faeces and honey were serially diluted in maximum recovery diluent (MRD; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) and plated on several medium types for the isolation of bacteriocin-producing bacteria: *Streptococcus thermophilus* selective agar incubated aerobically at 42 °C; M17 (Merck, Darmstadt, Germany) supplemented with 10% (wt/vol) lactose incubated at 30 °C aerobically; de Man, Rogosa, and Sharpe (MRS; Difco Laboratories, Detroit, MI) agar supplemented with 30 mg litre^{-1} vancomycin hydrochloride incubated at 37 °C; MRS adjusted to pH 5.4 incubated at 42 °C anaerobically; *Lactobacillus* selective agar (LBS, Difco) incubated at 30 °C anaerobically; and TOS (transgalactosylated oligosaccharide) agar (Sigma-Aldrich, Wicklow, Ireland) supplemented with 50 mg litre^{-1} lithium mupirocin incubated at 37 °C anaerobically.

Isolates were subject to an initial bacteriocin production screen by overlaying with 10 ml 'sloppy' MRS agar (7.5 g litre^{-1} agar) tempered to 50 °C and seeded with an overnight culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901 (0.25% [vol/vol]). Cultures which were found to produce distinct zones of inhibition in the agar overlay were cultured in broth for well diffusion assays. For well diffusion assays, 20 ml of sloppy MRS agar seeded with *L. bulgaricus* LMG 6901 as described above was poured and allowed to set, in which 6-mm-wide wells were then bored. Fifty microlitres of cell-free supernatant was added to each well, and plates were incubated at 37 °C overnight. Zones of inhibition were indicative of antimicrobial activity.

Bacterial strains, media, reagents

Strains used in this study and their incubation conditions are listed in Table 3. *A. ruminicola* DPC 7226 was routinely maintained in brain heart infusion (BHI) broth (Oxoid) at 37 anaerobically using an anaerobic chamber (Baker Ruskinn Concept 400, Fannin Ltd., Dublin, Ireland). Growth curves were performed in 10 ml volumes of

prewarmed BHI broth inoculated with 1% (vol/vol) of a 48-hour subculture. Oxygen tolerance and temperature experiments were performed in duplicate and measured using OD_{600}. Growth and actifensin production were determined through serial dilution plating for CFUs in triplicate. Activity units per millilitre ($AU \cdot ml^{-1}$) were calculated as shown:

$$\text{Activity units per milliltre} = \frac{\text{Highest dilution factor which retains activity}}{\text{Volume of supernatant in well (ml)}}$$

Medium reagents were sourced from Sigma-Aldrich (Wicklow, Ireland) unless stated otherwise.

Purification of actifensin

A. ruminicola DPC 7226 was streaked from -20 °C stock on BHI agar and grown at 37 °C for 72 h anaerobically. A single colony was used to inoculate 10 ml of BHI broth and incubated anaerobically for 72h. The grown broth was sub-cultured in 500 ml BHI broth (pre-equilibrated overnight in an anaerobic chamber) to a final concentration of 2 % (vol/vol), and incubated for 48 h under the same conditions. All broths were examined for purity and assayed for antimicrobial activity by well diffusion assay as outlined above, using *L. bulgaricus* LMG 6901 as an indicator species.

Following incubation the culture was centrifuged at 4,500 x g for 30 min. Cell pellets were set aside for peptide purification. CFS was applied to an Econo column containing 30 g Amberlite XAD-16N beads (Sigma Aldrich, Arklow, Co. Wicklow, Ireland) prewashed with Milli-Q water. The column was washed with 300 ml 30% ethanol and the antimicrobial activity was eluted with 300 ml 70 % 2-propanol (IPA) containing 0.1% trifluoroacetic acid (TFA). IPA (0.1% TFA) was removed from the active column eluent by rotary evaporation, and the sample was applied to a 60 ml 10 g Strata-E C_{18} SPE column (Phenomenex, Cheshire, UK) pre-equilibrated with methanol and water. The column was washed with 60 ml 25% ethanol and then eluted in 60 ml IPA (0.1 % TFA). Cell pellets were resuspended in 100 ml IPA (0.1 % TFA) and stirred at room temperature for 3 to 4 h. The resulting suspension was

centrifuged at 20,817 x g for 1 min. Supernatant was retained for analysis. IPA (0.1 % TFA) was removed from the sample by rotary evaporation, and the sample was applied to a 12 ml 2 g Strata-E C_{18} SPE column (Phenomenex, Cheshire, UK) pre-equilibrated with methanol and water. The column was washed with 15 ml 20 % ethanol and then eluted with 15 ml IPA (0.1 % TFA). Eluents from cell extract and supernatant were subjected to reversed-phase high-performance liquid chromatography (RP-HPLC). The samples were applied to a semi-preparative Proteo Jupiter (10 mm [inside diameter] x 250 mm [width], 90 Å [pore size], 4 µm [particle size]) RP-HPLC column (Phenomenex, Cheshire, UK) running a gradient of 25 to 50 % acetonitrile and 0.1 % TFA where buffer B was 90 % acetonitrile 0.1% TFA. The resulting eluent was monitored at 214 nm and fractions were collected at 1 min intervals. Column eluents and HPLC fractions were assayed for antimicrobial activity by well diffusion assay as outlined above. Eluents and HPLC fractions displaying antimicrobial activity were assayed by MALDI-TOF mass spectrometry to determine the molecular mass of antimicrobial compounds (Axima TOF^2 MALDI-TOF mass spectrometer; Shimadzu Biotech, Manchester, UK). A MALDI target plate was precoated with α-cyano-4-hydroxycinnamic acid) matrix solution, 0.5 µl of the supernatant from the cell extract was then placed on the target, and a final layer of matrix solution was added. Positive-ion linear or reflectron mode was used to detect peptide masses. Fractions containing pure actifensin (observed at 4091±1 Da) were pooled and lyophilised in a Genevac lyophiliser (Suffolk, United Kingdom) for characterisation.

Actifensin characterization

Characterization was performed using purified bacteriocin. To test protease susceptibility, 100-µl aliquots of 50 µg ml^{-1} were subjected to treatment with 20 mg ml^{-1} proteinase K (Sigma-Aldrich) and α-chymotrypsin (Sigma-Aldrich) at 37 °C for 3 h, followed by a 10 min incubation at 100 °C to denature the enzymes. Fifty-microlitre aliquots were assayed on *L. delbrueckii* subsp. *bulgaricus* LMG 6901 indicator plates. Heat stability was determined by 30 min incubations at 60 °C, 70 °C, 80 °C, 90 °C, and 100 °C and by autoclaving at 121 °C for 15 min.

For spectrum of activity, a well diffusion assay was carried out as described above with the strains in in the appropriate medium. Fifty microlitres of purified bacteriocin at a concentration of 50 μg ml $^{-1}$ was added to a well. Following overnight incubation under the appropriate conditions, zones of activity were measured and categorised as no inhibition, weak inhibition (0.5 mm to 2 mm), strong inhibition (2.5 mm to 5 mm), and very strong inhibition (>5 mm). MIC against selected pathogens was assayed as described above, starting at 100 μg ml $^{-1}$ peptide solution and serially diluted 1:2 to 0.78 μg ml $^{-1.}$

DNA was extracted using a GenElute bacterial genomic DNA kit (Sigma) and prepared for sequencing using a Nextera XT kit (Illumina) for library preparation. DNA concentration was quantified using a Qubit 2.0 fluorometer. Sequencing was carried out using an Illumina MiSeq platform with paired-end 2 x 300-bp reads by the Teagasc Sequencing Centre, Teagasc Food Research Centre, Moorepark, Fermoy, Ireland. Assembly was performed using tools available on the public server at https://usegalaxy.org (27). Assembly was performed de novo using SPADES (version 3.0.0) and resulted in 116 contigs (28). Contigs were aligned to a reference genome using Mauve (version 20150226, build 10), followed by annotation with RAST (version 2.0) (29, 30). The annotated genome was analysed for predicted bacteriocin and secondary metabolite production clusters using BAGEL4 and antiSMASH and any further annotation was carried out using Artemis genome browser (version 16.0.0) (31-33).

BAGEL screen and phylogenetic analysis of *Actinomyces* species

GenBank and FASTA assemblies of the genus *Actinomyces* were acquired from the NCBI assembly database and screened using BAGEL4 (31). Where available, corresponding 16S rRNA sequences were acquired from the RDP database (34), and where unavailable, *Actinomyces* sp. genomes were subject to analysis using RNAmmer (35). 16S rRNA sequences were aligned using MUSCLE (36), and a phylogram was generated using iTOL (37). The phylogram was then visually overlaid with the BAGEL4 screen data.

Reverse bacteriocin identification, peptide and structure prediction, and homology

Two hundred micrograms freeze-dried purified peptide was sent for N-terminal amino acid sequencing (AltaBioscience, UK). The resulting 15-residue sequence, GFGXNLITSNPYQXS, was used to search for a bacteriocin structural gene with Artemis genome browser. Following identification of the structural gene, other genomes were searched for genes homologous to the active and propeptide using BLASTp; genes on contigs consisting of less than 5 kbp were excluded. Additional actifensin homologs were identified from the study by Dash et al. (2019) among 147 nonredundant bacterial cysteine stabilised α-helix β-sheet (CSαβ) peptide motif sequences (38). Alignments were generated using Clustal Omega (39) and visualized with Jalview (40). Structural modeling was performed using SWISS-MODEL (41), and structural images were generated using PyMOL (42).

Data availability.

Genomic data analysed in this study were deposited in GenBank/EMBL under accession number SPKK00000000 and are publicly available from the NCBI database at https://www.ncbi.nlm.nih.gov/.

Results

Identification of a novel bacteriocin producing *Actinomyces* sp.

Actinomyces ruminicola DPC 7226 was isolated from sheep faeces plated on TOS agar supplemented with lithium mupirocin for the isolation of *Bifidobacterium* spp. During an initial screen of >10,000 colonies for bacteriocin producers, this strain was found to produce a large zone of inhibition when overlaid with an acid-tolerant indicator species, *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901 (Fig. 1a). The neutralized cell-free supernatant (CFS) was also found to produce a zone of inhibition against *L. delbrueckii* subsp. *bulgaricus* LMG 6901, indicating production of a soluble antimicrobial molecule (Fig. 1b). This activity was eliminated when the supernatant was treated with proteinase K, demonstrating that the antimicrobial was proteinaceous in nature (data not shown).

The growth of *A. ruminicola* DPC 7226 was assayed under a range of conditions prior to bacteriocin purification. The strain grew best anaerobically at 35 – 40 °C (Fig. 2a and b). Bacteriocin production in broth coincided with late log to early stationary phase of growth peaking at 640 AU ml^{-1} after 27 h (Fig. 2c). Antimicrobial activity was purified from pelleted bacterial cells (C_{18} SPE; reversed-phase high-performance liquid chromatography (HPLC)) and CFS (Amberlite XAD, C_{18} SPE; reversed-phase HPLC), and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) of active peaks detected a mass of 4,091 ± 1 Da (Fig. 3a and b). The mass was also detected by colony MS (Fig. 3c). The activity of the HPLC-purified fraction from CFS was assayed against *L. delbrueckii* subsp. *bulgaricus* LMG 6901 and found to be active at <1 µg ml^{-1} (Fig. 3d). The antimicrobial peptide was found to be heat stable, retaining almost all activity after treatment for 30 min at 100 °C, but was completely lost after treatment at 121 °C for 15 min (data not shown).

Spectrum of inhibition

A range of indicator organisms was tested against the purified antimicrobial to determine the spectrum of inhibition. The antimicrobial was active against a broad

range of genera, with 22 of the 27 strains screened inhibited to various degrees, including species of the genera *Lactococcus*, *Enterococcus*, *Lactobacillus*, *Streptococcus*, *Pediococcus, Bacillus, Staphylococcus*, other *Actinomyces* spp., and *Clostridium* spp. (Fig. 4). No inhibition against the Gram-negative species *Salmonella enterica* or *Escherichia coli* was observed. *Listeria* spp. and *Bacillus* spp. were inhibited weakly or not at all (Fig. 4). Inhibition against other *Actinomyces* spp. was found, and activity was particularly strong against *Staphylococcus aureus* and *Clostridium difficile*.

MICs were determined against *Enterococcus faecium* APC 1031, *E. faecium* NCDO 0942, *S. aureus* R693, *Streptococcus agalactiae* APC 1055, and *C. difficile* DPC 6534 (Fig. 5). Enterococci were inhibited at 3.05 to 6.1 µM. *S. aureus* was inhibited at 3.05 µM. *S. agalactiae* and *C. difficile* were inhibited at 0.76 µM (Fig. 5). The MIC of pure actifensin against log phase *C. difficile* DPC 6534 cells in solution was determined to be 1.6 µM (Fig. 6).

Distribution of genes encoding bacteriocins in the genus *Actinomyces*

As the active mass could not be matched to any previously known antimicrobial peptide and no antimicrobial compounds were previously described within the species, the genome of *A. ruminicola* DPC 7226 was sequenced. Following genome annotation, the draft genome was analysed using BAGEL4 to search for potential antimicrobial-encoding operons. Gene clusters were identified containing putative genes for thiopeptide production (data not shown), but the masses predicted, 2,195.4 Da and 1,152.5 Da, did not correspond with the mass detected in the antimicrobial HPLC fractions.

In conjunction with screening of the genome of *A. ruminicola* DPC 7226, we also set out to characterize the antimicrobial potential of the genus. One hundred sixty-one *Actinomyces* species genomes in various stages of assembly were screened using BAGEL4 (Table 1). The genomes belonged to isolates obtained from humans (78.2%) or other animals (16.1%) or were of unknown origin (4.9%), while one was an environmental isolate (0.6%). One hundred six areas of interest were revealed in 76 strains, covering 18 species. Ninety areas of interest contained complete operons

for antimicrobial production. Twenty-nine were predicted to encode class I bacteriocins, including 7 LanBC modified lantibiotics, 16 LanM modified lantibiotics, 1 single-peptide sactibiotic, 3 lasso peptides, and 2 thiopeptides. Thirteen operons were predicted to encode class IId bacteriocins, and a further 48 operons were predicted to encode bacteriolysins. A phylogenetic tree was generated from the 16S rRNA sequences of 142 *Actinomyces* genomes with *Bacteroides fragilis* ATCC 25285 as the root and overlaid with operon type and strain source (Fig. 7). Bacteriocin production genes were widely distributed across the *Actinomyces* pangenome, though bacteriolysin production genes were found exclusively among human isolates (Fig. 7).

Genetic and molecular characterization of the actifensin determinant

To identify the gene encoding the 4,091 (±1)-Da peptide within the genome of *A. ruminicola* DPC 7226, pure peptide was subjected to N-terminal sequencing, which revealed a primary sequence consisting of Gly-Phe-Gly-X-Asn-Leu-Ile-Thr-Ser-Asn-Pro-Tyr-Glu-X-Ser, with blanks at residue positions 4 and 14 denoted as probable cysteines (Fig. 8a). This 15-amino-acid sequence was matched to a 69-residue small open reading frame in the draft genome, capable of encoding a 37-amino-acid mature peptide (hereafter referred to as actifensin) with a predicted mass of 4,097.7 Da preceded by a 32-residue leader sequence (Fig. 8a).

The genetic locus encoding actifensin is shown in Fig. 8b, where *afnA* encodes actifensin. Within an approximately 6.5-kbp upstream region of *afnA*, genes encoding an ABC transporter permease (*afnJ*), an ATP binding ABC transporter (*afnK*), and another ABC transporter permease (*afnL*) were identified as being present. Downstream of *afnA*, three hypothetical genes of unknown function (*afnG* to *afnI*) were found, followed by genes encoding another ATP binding ABC transporter (*afnF*), a predicted α/β hydrolase superfamily protein (*afnE*), another protein of unknown function, a subtilisin-like protease, and a LuxR family transcription factor (*afnD*, *afnC*, and *afnB*, respectively). Within *afnE* is a predicted RHO-independent transcription terminator, and upstream of the structural gene are four predicted promoters. A putative ribosome binding site was also identified nine base pairs upstream of the

ATG start codon for the peptide consisting of a purine rich sequence, 5'-GAAAGG-3' (Fig. 8a).

The leaderless structural peptide was found to have a predicted mass of 4,097.7 Da. This mass was approximately 6 Da higher than detected by MALDI-TOF MS. The difference between predicted and observed masses most likely corresponds to the loss of six hydrogen atoms during the formation of disulphide bonds between the six cysteines. Short peptides with numerous disulphides in specific positions are characteristic of the defensin peptide families (4). To confirm the presence of disulphide bonds in actifensin, pure peptide was reduced and alkylated to break open the disulphide bonds and then subjected to trypsin digestion and peptide mass fingerprint analysis by MALDI-TOF MS. Reduction and alkylation of actifensin resulted in a 4,440-Da mass, which correlates with the expected increase in mass of 58 Da for each cysteine. MALDI-TOF MS analysis of the subsequent trypsin digest detected a mass of 2,257.02 Da, which corresponds to the first 19 amino acids of the peptide (Gly-1 to Lys-19) containing three alkylated cysteine residues (data not shown). Three other predicted masses for Ser-20 to Arg-24, Gly-25 to Arg-31, and Thr-32 to Tyr-37 (predicted and alkylated masses of 581.30 Da, 584.25 Da, and 803.31 Da, respectively) were not detected.

Discovery of actifensin homologs

BLASTp analysis with the mature AfnA sequence found homology to open reading frames (ORFs) encoding peptides within the fungal genera *Blastomyces*, *Emmonsia*, and *Emergomyces*, *Helicocarpus griseus*, and a defensin from the mollusc species *Ruditapes philippinarum* (58%, 58%, 55%, 52%, and 61% identity, respectively (Fig. 9). Characteristic conserved cysteines were noted, though low sequence identity was observed between the mature actifensin peptide and eukaryotic defensins. The same was found when AfnA was compared with known previously characterized arthropod, ascomycete, and mollusc defensins (Fig. 10a) with conserved secondary structures (Fig. 10b).

BLASTp analysis using the 69-residue AfnA immature sequence identified 37 homologous structural genes within the genus *Actinomyces* and one homolog from

a *Corynebacterium* sp. sequence (Fig. 11a). Further analysis indicated that the homologs were present in 15 operons from 14 strains, in addition to conserved genes for transport, transcription regulation, and proteolytic activity (Fig. 11b). *Actinomyces* sp. strain 2119, *Actinomyces oris* S64C, *Actinomyces succiniciruminis* AM4, *A. oris* CCUG 34286, *Actinomyces* sp. strain F0337 *Actinomyces* sp. strain HMSC075C01, and *A. oris* MMRCO6-1 had at least two actifensin homologs, while *Actinomyces* sp. CCUG 34286 contained an operon with seven copies, the most observed within one genome, (Fig. 11b). The genome of *A. oris* MMRCO6-1 contained six encoded actifensin homologs detectable over two contigs, but only one (contig 50) contained the other conserved ORFs (*afnB-I* and *afnJ-K*) present in the actifensin operon. Twelve of 14 operons had a highly conserved arrangement of *afnB-I*, all of which also had ABC transporter genes directly upstream of the bacteriocin ORF. The mean amino acid identity between all encoded structural peptides was 52%. The highest identity observed between actifensin and a homolog was 77% identity with AfnA in *Actinomyces* sp. strain CTC72, though higher identities were observed between other peptides (Fig. 12). We proceeded to characterise ten predicted cysteine-stabilised αβ (CSαβ) peptides predicted by Dash et al. (2019). The genes are present in five *Actinomyces* genomes bringing the total number of encoded peptides to 47 homologs from 19 strains. *Actinomyces oris* S24V, *Actinomyces denticolens* PA, *Actinomyces* sp. strain Chiba-101, *Actinomyces johnsonii* F0542, and *Actinomyces* sp. strain F0330 have genes which were not identified using BLASTp and the actifensin propeptide sequence (38). Strains S24-V, PA, and Chiba-101 display the conserved *afnB* to *afnI* ORFs following *afnA*, which are absent in strains F0330 and F0542 (Fig. 9b).

The propeptide contains a conserved G-X-E motif prior to the start of the mature peptide (Fig. 11a). In 36 of the predicted peptides, an alanine residue is present after the glycine, which may be involved in secretion and cleavage. This putative GA cleavage signal is replaced by a TS motif in 8 of the 49 encoded peptides (*A. oris* S64C AfnA5, *A. oris* CCUG 34286 AfnA7, *A. oris* MMRCO6-1 contig 75 AfnA2, *Actinomyces* sp. F0337 AfnA4, *Actinomyces* sp. HMSC075C01 AfnA4, *A.*

oris MMRCO6-1 contig 50 AfnA4 and AfnA3, and *A. oris* S24V AfnA5). A conserved Pro residue was noted following the first conserved Cys in addition to a conserved G-Y-X-G-G-X-C sequence at positions +22 to +28 in the mature peptide (Fig. 11a).

Discussion

We describe a novel group of bacteriocins with broad-spectrum inhibitory activity within the *Actinomyces* genus. Actifensin is the first such bacteriocin to be discovered, which is produced by a strain of *Actinomyces ruminicola*.

Actifensin inhibited a broad range of Gram-positive species, including notable pathogens such as vancomycin-resistant *Enterococcus* and methicillin-resistant *Staphylococcus*. Given the global challenge of the increase in antibiotic resistance, there is an urgent need for new classes of antimicrobials. Bacteriocins have been suggested as an alternative to conventional antibiotics due to their effectiveness at low concentrations and their potential to be genetically modified (2). Class II bacteriocins are diverse in sequence and structure whose mechanism of action is generally through interaction with the cell membrane, causing permeabilization and pore formation and dissipating the membrane potential (3). The defensin-like bacteriocin laterosporulin10 has been found to act on the cell membrane of *S. aureus* Mtb H37Rv, disrupting cellular homeostasis (6). Plectasin and eurocin, fungal C6 defensins, are known to bind lipid II, inhibiting bacterial cell wall biosynthesis (43, 44). Actifensin possesses an N-terminal loop extension which, in other defensin peptides, has been implicated in membrane disruptive capability (45). The loop consists of nine residues between Cys-4 and Cys-14 beginning with an Asn. In most of the other peptide sequences identified, the N loop is six residues long, beginning with a Pro (except in AfnA from *Actinomyces* sp. strain F0588 or *A. naeslundii* S44D, which has an eight-residue N loop with a serine or arginine in the first position, respectively, followed by a Pro) (Fig. 11a).

Actifensin also inhibited the growth of *C. difficile* and *Clostridium sporogenes* (Fig 4). Clostridia are known colonizers of the rumen, and as *A. ruminicola* DPC7226 was isolated from the faeces of a ruminant, actifensin production may provide a competitive advantage in the gut microbiome. Indicator species were chosen which were immediately available, though other species may be of interest for future studies, particularly oral cavity inhabitants given presence of *Actinomyces* spp. in the niche. *Actinomyces neuii* and *Actinomyces radingae* were both inhibited by

actifensin; however, it would be interesting to see if cross-resistance between actifensin and other actifensin-like producers exists.

A pangenus *in silico* screen revealed that the genus *Actinomyces* (Fig. 7) is a rich source of antimicrobials and has genes for bacteriolysin and lantibiotic production (48/90 and 29/90 operons, respectively). Thirteen class II bacteriocins were predicted by BAGEL4, but neither the actifensin operon nor its homologs were detected, likely due to lack of similarity with known bacterial systems. One previous study described odontolycin, a bacteriocin produced by an *Actinomyces odontolyticus* dental plaque isolate, though no further research on the peptide was reported (46). Our study detected no operons for bacteriocin production were found among five *A. odontolyticus* genomes screened (Fig. 7).

The actifensin structural gene encodes a 37-amino-acid mature peptide preceded by a 32-amino-acid leader sequence (Fig. 8). A GA motif at positions −3 and −2 was identified, which is a known cleavage signal used in ABC transporter-mediated secretion (47). Indeed, there are several predicted ABC transporter genes within the actifensin operon. ABC transporter genes could also play a role in self-immunity to the actifensin peptide. Unusually, an additional glutamic acid residue is present at position −1 before the mature peptide. As the purified peptide was subjected to N-terminal sequencing, we can be certain that the mature peptide begins with a glycine residue. Therefore, the additional glutamic acid residue at position −1 is most likely subject to exopeptidase cleavage prior to activity, and indeed, there are genes present with predicted protease activities (Fig. 8).

The GA cleavage motif is present in 36 of the homolog structural genes, with TS replacing the motif in eight instances, GT and GG in two cases, and GS, SA, and DA in one each (Fig. 11a). A double glycine is the most commonly found motif for ABC transporter-mediated cleavage among bacteriocins, though GA and GS have also been observed (47). It will be interesting to see if the peptides bearing other residues at this location are indeed subject to ABC-mediated transport. We note that each operon containing a gene with a nontraditional TS/GT/SA/DA signal contains at least one more structural gene than those with a GG/GA sequence. This could indicate

potential diversification of a repertoire of bacteriocins enabling improved ability to combat multiple competitors. It was also surprising that an actifensin homolog was found in a distantly related *Corynebacterium* sp., though many of the conserved genes in the *Actinomyces* sp. operons were not present (Fig. 11b). As such, this may be nonfunctional, as ABC transporter-related genes are missing upstream of the structural gene and the conserved *afnB* to *afnI* pattern is absent. The genera *Corynebacterium* and *Actinomyces* are distantly related members within the phylum Actinobacteria, and some species are known members of plaque biofilms, providing an opportunity for horizontal gene transfer (15). However, given the dissimilarity of the operons, they may have been acquired independently at some stage.

As stated above, the laterosporulins produced by *Brevibacillus* spp. are two structurally defensin-like bacteriocins with broad-spectrum inhibitory activity (5, 6). Their amino acid sequences are 57.6% similar, which is comparable to that for actifensin and its predicted homologs and they share conserved cysteine residues which form disulphide bridges. Conserved disulphide bridges are characteristic of defensins and are present in vertebrate, invertebrate, plant, fungal defensins, and defensin-like peptides (4). Actifensin has a predicted mass of 4,097.7 Da, but the actual mass is 4,091 ± 1 Da by MALDI-TOF MS. The same discrepancy in predicted and observed masses was noted with laterosporulin, where six hydrogen atoms are lost in the formation of disulphide bonds. We hypothesize that bonds in actifensin likely form in the 1-4, 2-5, and 3-6 formations, similar to that in ascomycete and arthropod C6 defensins (Fig. 13), as the amino acid motifs (C-$X_{5–12}$-C-X_3-C-$X_{9–10}$-C-$X_{4–5}$-C-X-C) are conserved (4). The structure of laterosporulin10 has been determined to be architecturally similar to human α-defensin, though its disulphide connectivity is homologous to that of β-defensins (Fig. 13) (6). The overall architecture and disulphide connectivity of actifensin are likely to be homologous to those of C6 defensins, consisting of an N-terminal α-helix followed by a two-stranded antiparallel β-sheet stabilized by disulphide bridges (Fig. 13). An actifensin homolog we identify as AfnA from *Actinomyces* sp. oral taxon 171 strain F0337 has had its three-dimensional (3D) structure determined and is publicly available under PDB accession

number 2RU0. The peptide (labelled actinomycesin) is strikingly similar to C6 fungal and arthropod defensins, which have also been characterized (Fig. 10), however, no published material is available regarding its activity, antimicrobial or otherwise. Indeed, two antiparallel beta sheets stabilised by disulphide bonds with an interposed short turn region, previously described as the γ-core motif, are a ubiquitous feature of antimicrobial peptides (48). Actifensin exhibits the highly conserved GXC (positions 26 to 28 in the mature peptide) as do all its encoded homologs.

The presence of three disulphide bonds likely contributes towards the peptide's thermal stability, as has been previously found with θ-defensins (49). Mutants of class IIa (pediocin-like) bacteriocins, pediocin PA-1 and sakacin P, containing an additional c-terminal disulphide bond increased strain specific temperature dependent potency and improved the antimicrobial spectrum of the peptides (50). In the case of lasso-peptides, class I (posttranslationally modified) bacteriocins, disulphide bonds contribute toward heat and protease resistance (51). Future structural studies may establish the role of disulphide formation in actifensin activity, as well as heat and protease resistance, relative to other defensins and class IId bacteriocins.

Cysteine stabilised α-helix β-sheet (CSαβ) motif containing peptides comprise one of the most widespread families of defensins and defensin-like peptides. A recent publication identified a number of CSαβ sequences in bacterial genomes with potential for antimicrobial, toxin, or signalling activity (38). Of 58 peptides identified within the phylum Actinobacteria by Dash et al. (2019), 34 were of the genus *Actinomyces*, 24 of which we identified using BLAST with the actifensin propeptide sequence (Table 2). A further 113 bacterial peptide sequences identified by Dash et al. remain to be characterized from a functional perspective and may be a potent source for antimicrobials. Interestingly, a bacterial defensin-like peptide, AdDLP, identified *in silico* was synthesised and recombinantly expressed, and the peptide was found to have anti-*Plasmodium* activity (52). Bacterial CSαβ peptides may be an untapped source of potential applications and have been proposed as the ancestral evolutionary origin of eukaryotic defensins (53).

In the search for novel antimicrobials for application in health and food, genomic and pangenomic approaches are becoming increasingly common (25, 26). These approaches are advantageous in that large amounts of genetic data can be analysed to identify novel antimicrobials/bacteriocins and can even allow one to "reincarnate" otherwise "dormant" genes (54). However, such analyses are dependent on the ability of programs to predict based on databases of previously identified sequences, and so peptides with novel structures and operons may not be detected. Though a number of bacteriocin operons were found in the *Actinomyces* spp. genomes using BAGEL4, actifensin was not identified by genome sequence alone, which highlights the importance of functional screening for antimicrobial compounds in addition to *in silico* screening. By using BLAST, 37 structural genes with homology to actifensin were found in *Actinomyces* spp. along with a single structural gene from a *Corynebacterium* sp. As some CSαβ peptides function as toxins, future applications will require any potential cytotoxic effects to be assayed. We propose that actifensins and the laterosporulins may constitute a new subgroup of class II bacteriocins: the defensin-like bacteriocins. These bacteriocins share only moderate identity to each other but contain highly conserved cysteine residues and are structurally related to eukaryotic defensins.

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Figures and Tables

Figure 1. Antimicrobial activity of *Actinomyces ruminicola* DPC 7226 from colonies overlaid with *L. delbrueckii* subsp. *bulgaricus* LMG 6901 in sloppy MRS (a) and in well diffusion with neutralized CFS (b).

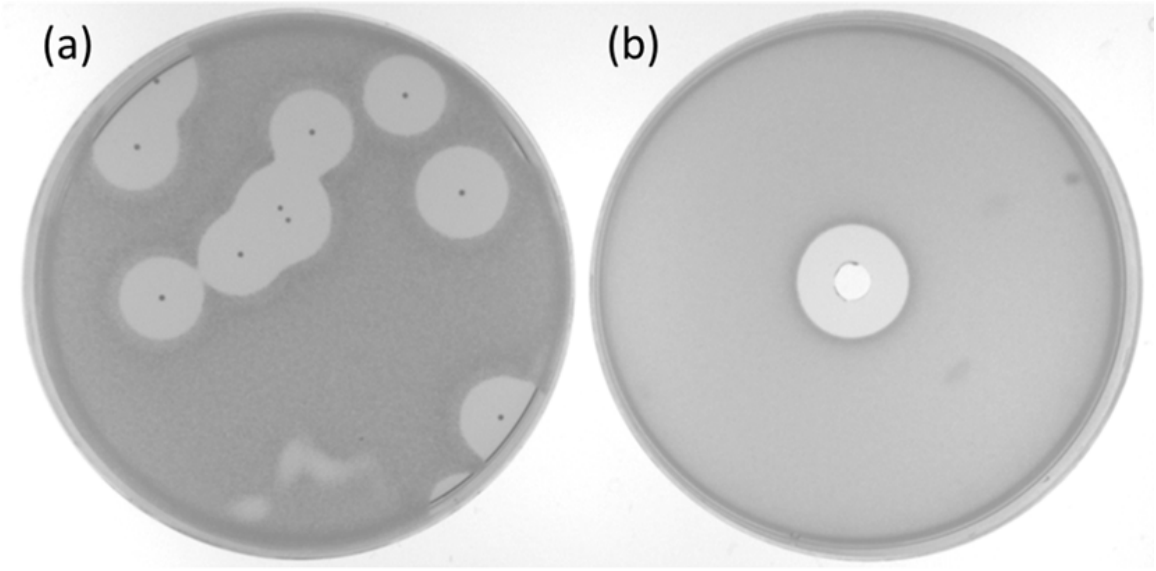


Figure 2. (a) Growth of *Actinomyces ruminicola* DPC 7226 in the presence and absence of O_2 and (b) at different temperatures. (c) Above: Bacteriocin production kinetics of *A. ruminicola* DPC7226 cultured at 37 °C anaerobically. Below: Dilution series well diffusion assays of supernatant.

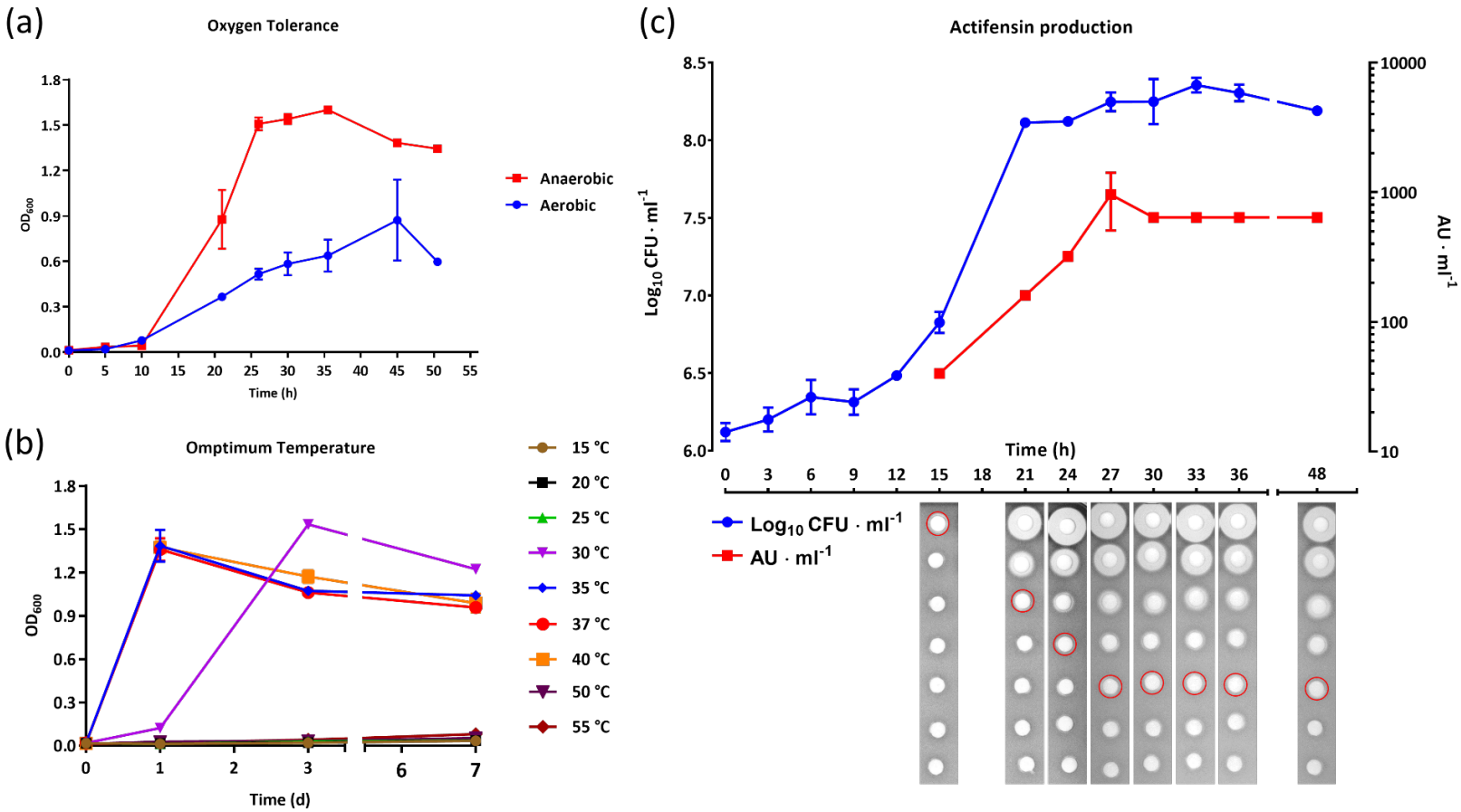


Figure 3. Detection of actifensin 4,091 ± 1 Da (indicated by arrows) by MALDI-TOF MS from cell-free supernatant (a), cell extract (b), and colonies on a plate (c). (d) The 4,091 Da compound when purified was active to <1 µg ml^{-1}; indicator, *L. bulgaricus* LMG 6901.

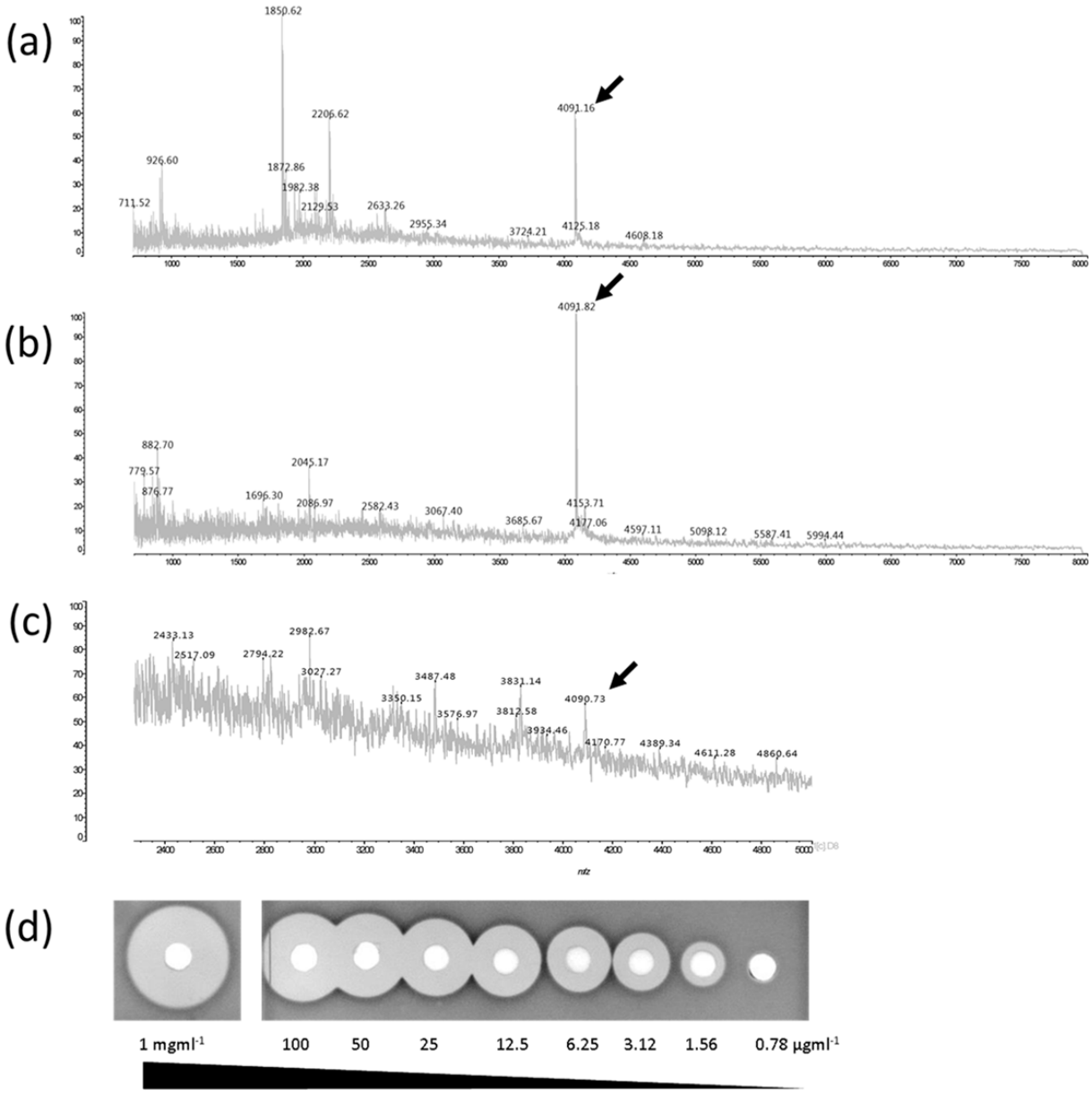


Figure 4. Inhibition of actifensin against a broad spectrum of indicator species. Weak inhibition, 0.5- to 3-mm zone; strong inhibition, 3- to 5-mm zone; very strong inhibition, >5-mm zone. VRE, vancomycin-resistant *Enterococcus*; MRSA, methicillin-resistant *Staphylococcus aureus*.

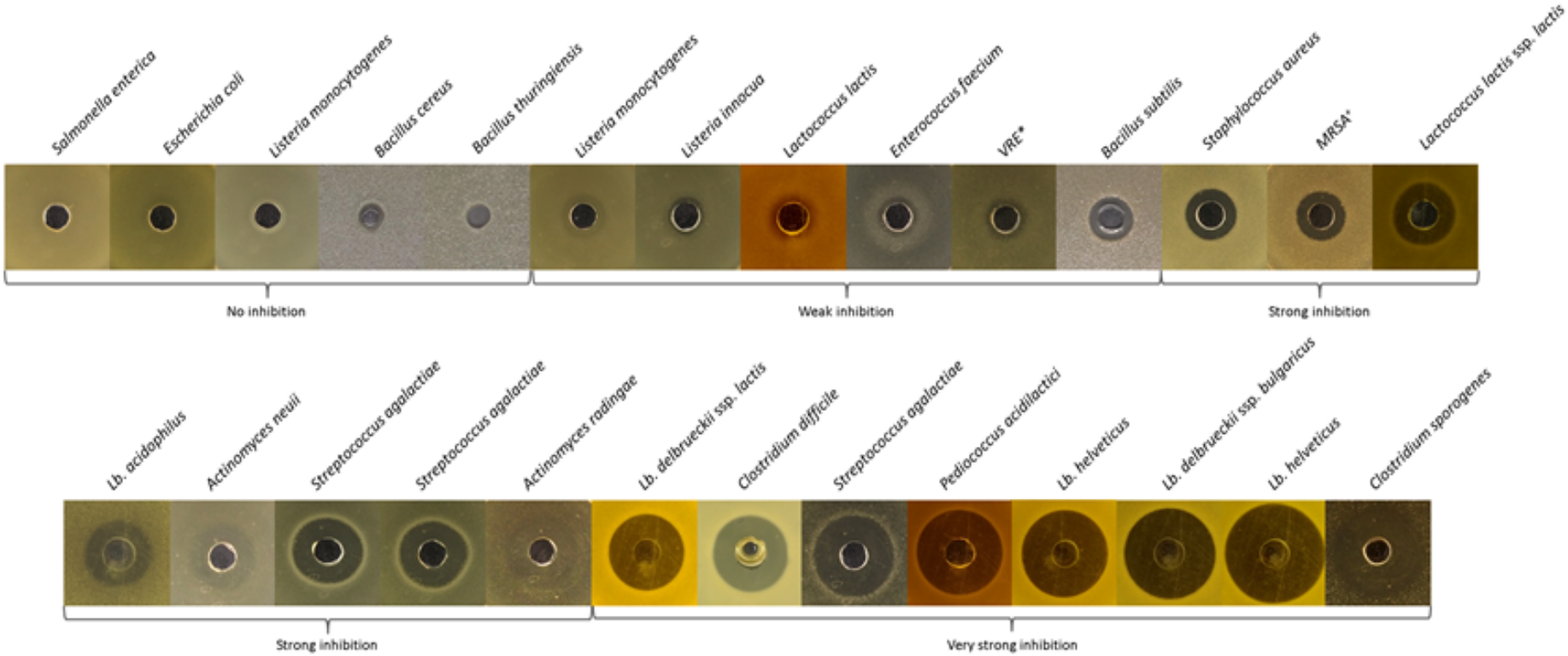


Figure 5. Minimum inhibitory concentration of actifensin peptide against Gram-positive pathogens determined by well diffusion assay.

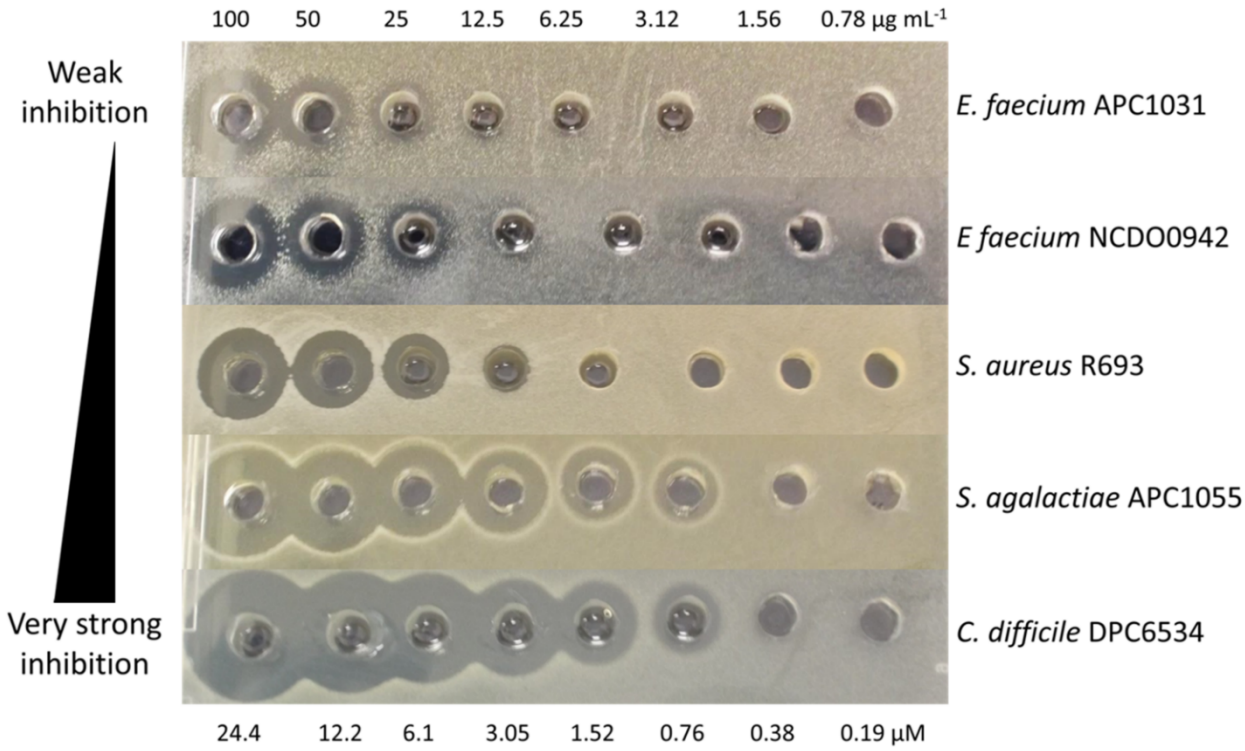


Figure 6. Minimum inhibitory concentration of actifensin peptide against *Clostridium difficile* DPC6534 in solution compared with reported MIC_{50} of Trnα, Trnβ and Trnαβ.

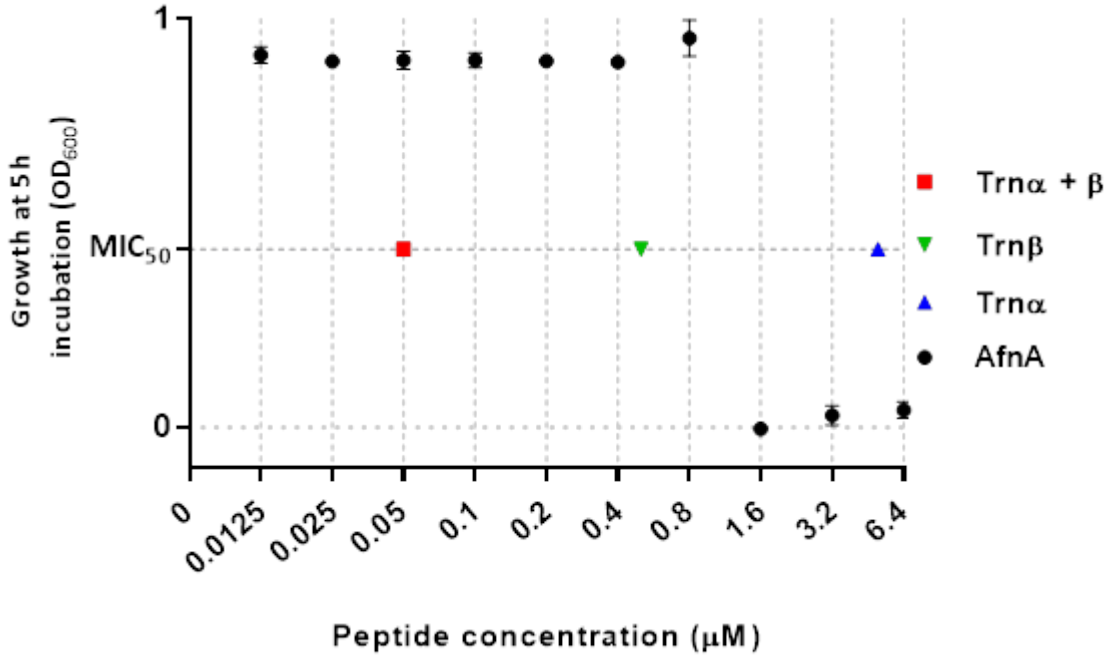


Figure 7. Phylogram of *Actinomyces* genomes using 16S sequences overlaid with BAGEL4 predictions, strain source, and presence of actifensin or predicted homolog operon.

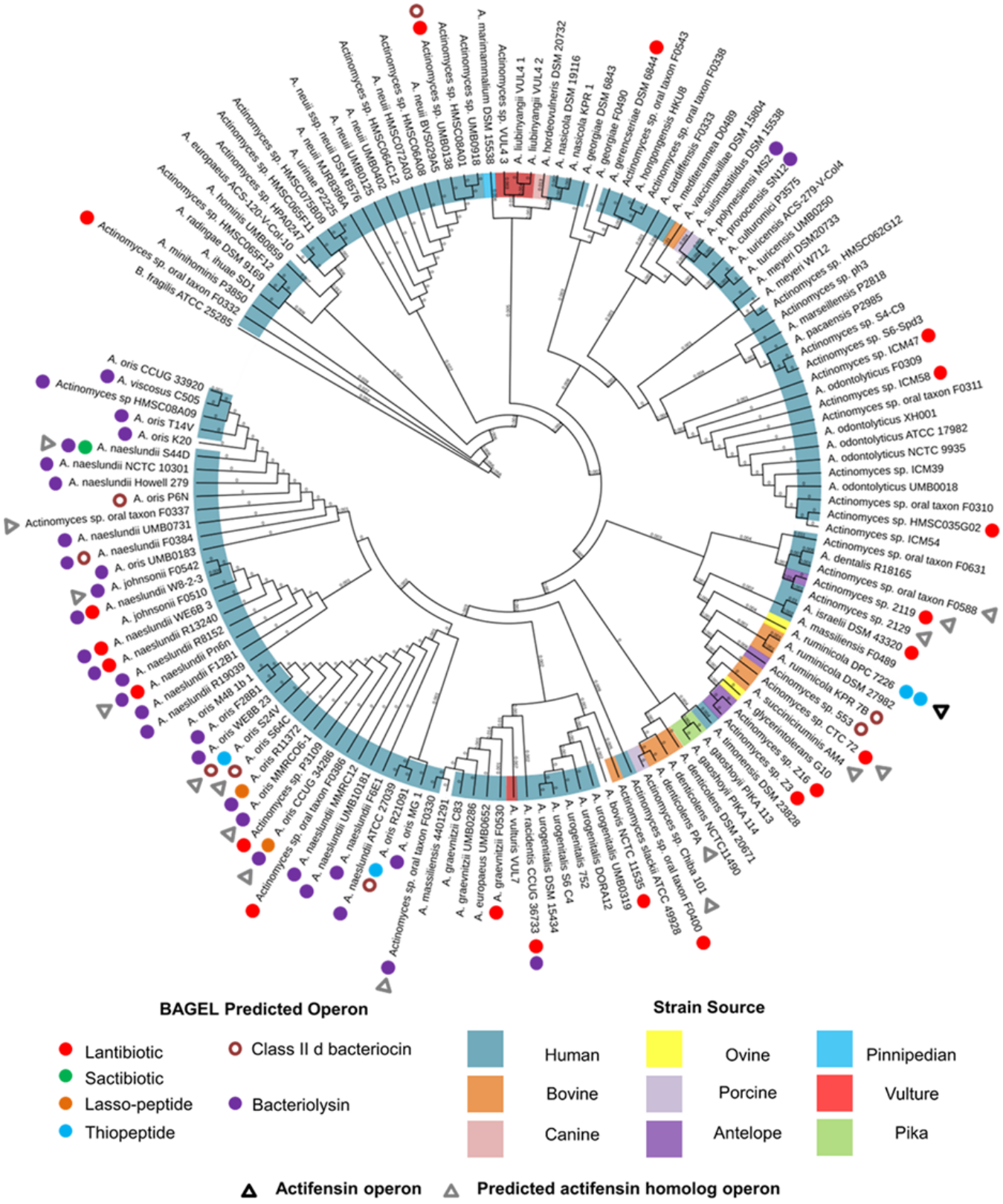


Figure 8. (a) Sixty-nine-residue propeptide identified following genome analysis using the 15-amino-acid sequence (underlined) determined by N-terminal amino acid sequencing. RBS, putative ribosome binding site highlighted 8 bp upstream of the start codon. (b) Genetic vicinity of structural gene containing nearby genes for transport, hypothetical and proteolytic proteins, and a transcription factor.

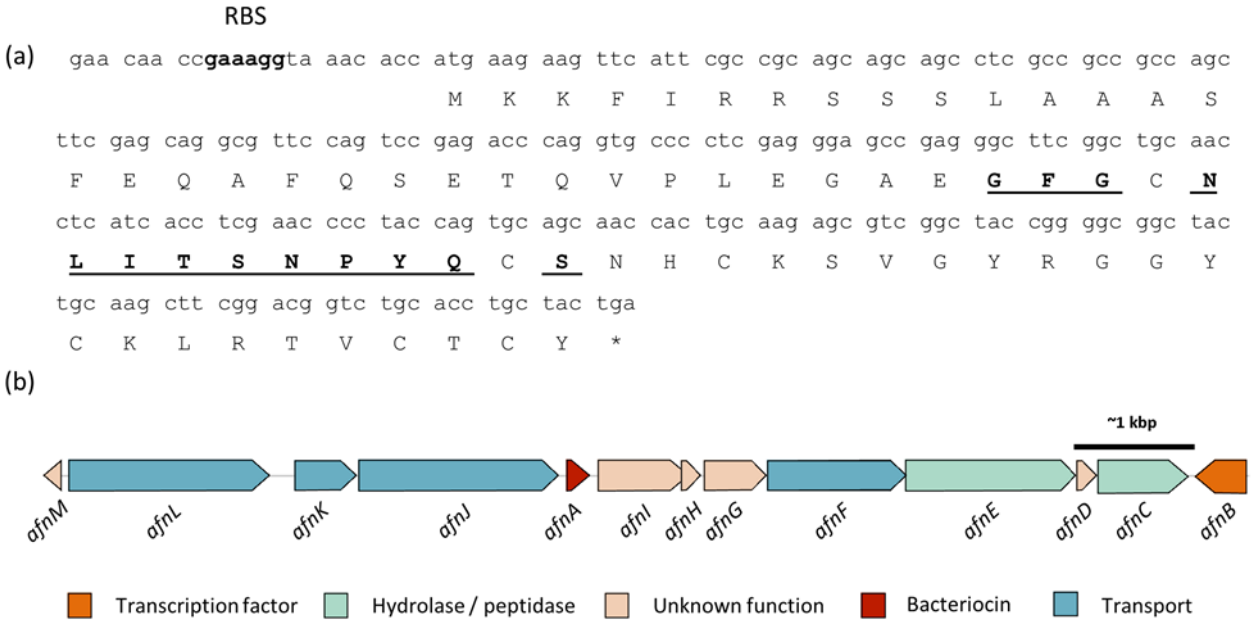


Figure 9. Eukaryotic sequences with homology to mature AfnA identified using BLASTp. Residues with 100% identity are highlighted.

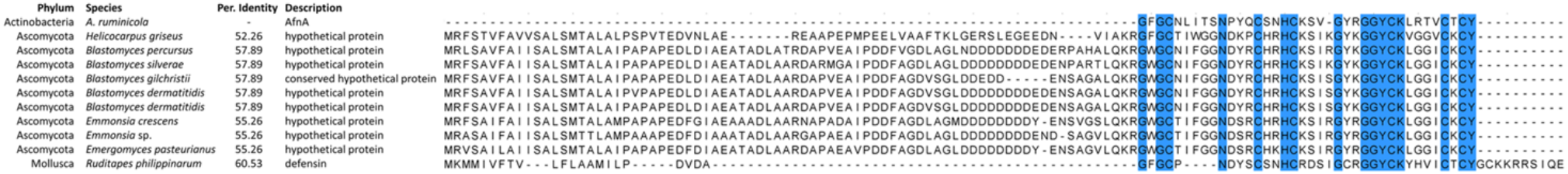


Figure 10. (a) Mature peptide sequence alignment of AfnA with characterized defensin family peptides from different phyla. Known disulphide connectivity is outlined above highlighted cysteine residues. (b) Available 3D structures of sequences in panel a. Alpha helices are coloured red, and beta sheets are shown in blue. Protein data bank accession numbers shown below the structures (in parentheses).

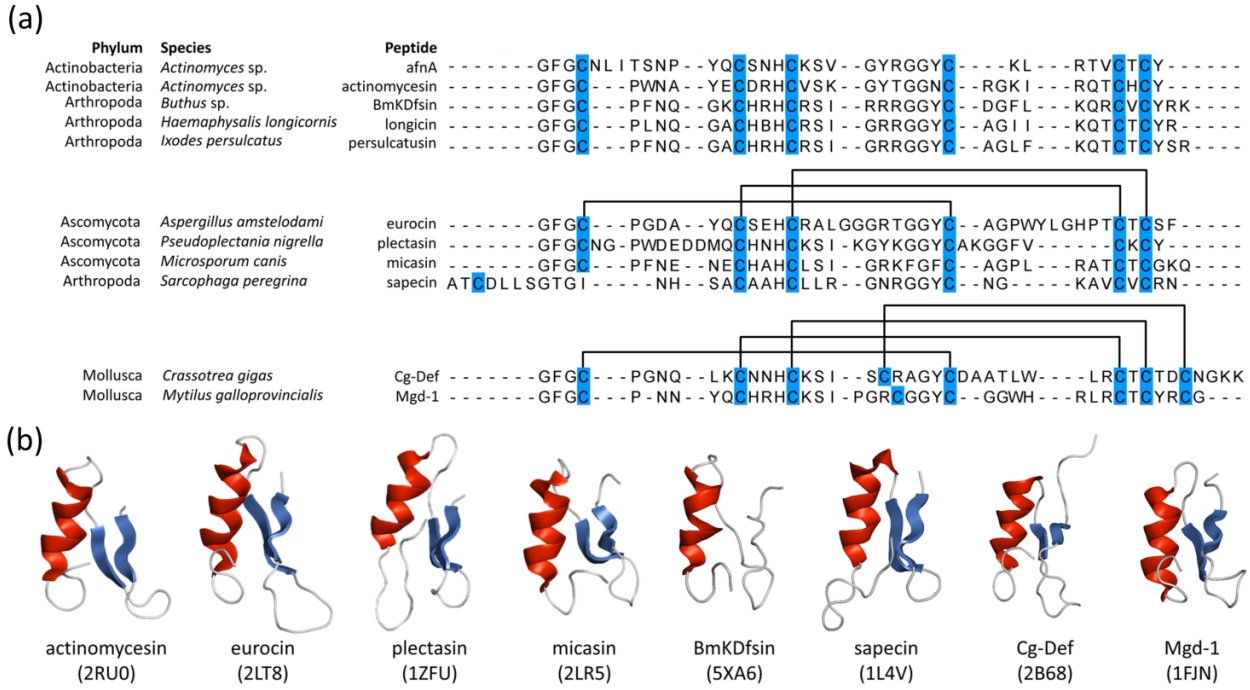


Figure 11. (a) Sequence alignment of actifensin propeptide sequence (boxed) with structural genes predicted for *Actinomyces* sp. peptides. Amino acids with greater than 80% conservation are coloured, and leader sequences and mature active peptides are indicated at the top. Putative disulphide connectivity between conserved cysteines of the mature peptide is indicated at the bottom right, and putative cleavage sites are indicated at the bottom centre. (b) Diagrams of actifensin homolog production operons. Multiple bacteriocin genes within one operon are denoted *afnA1* to *afnA7* where present.

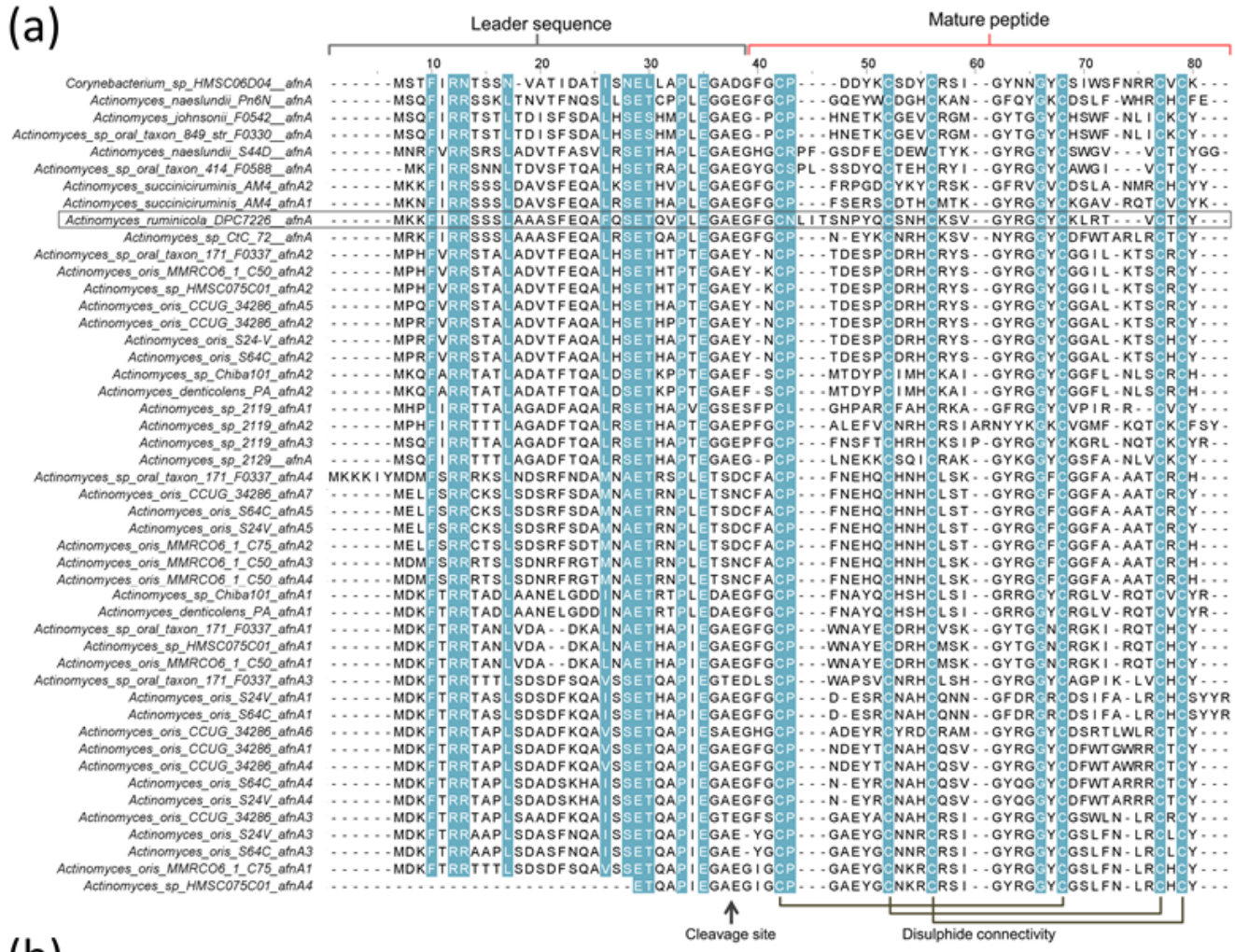
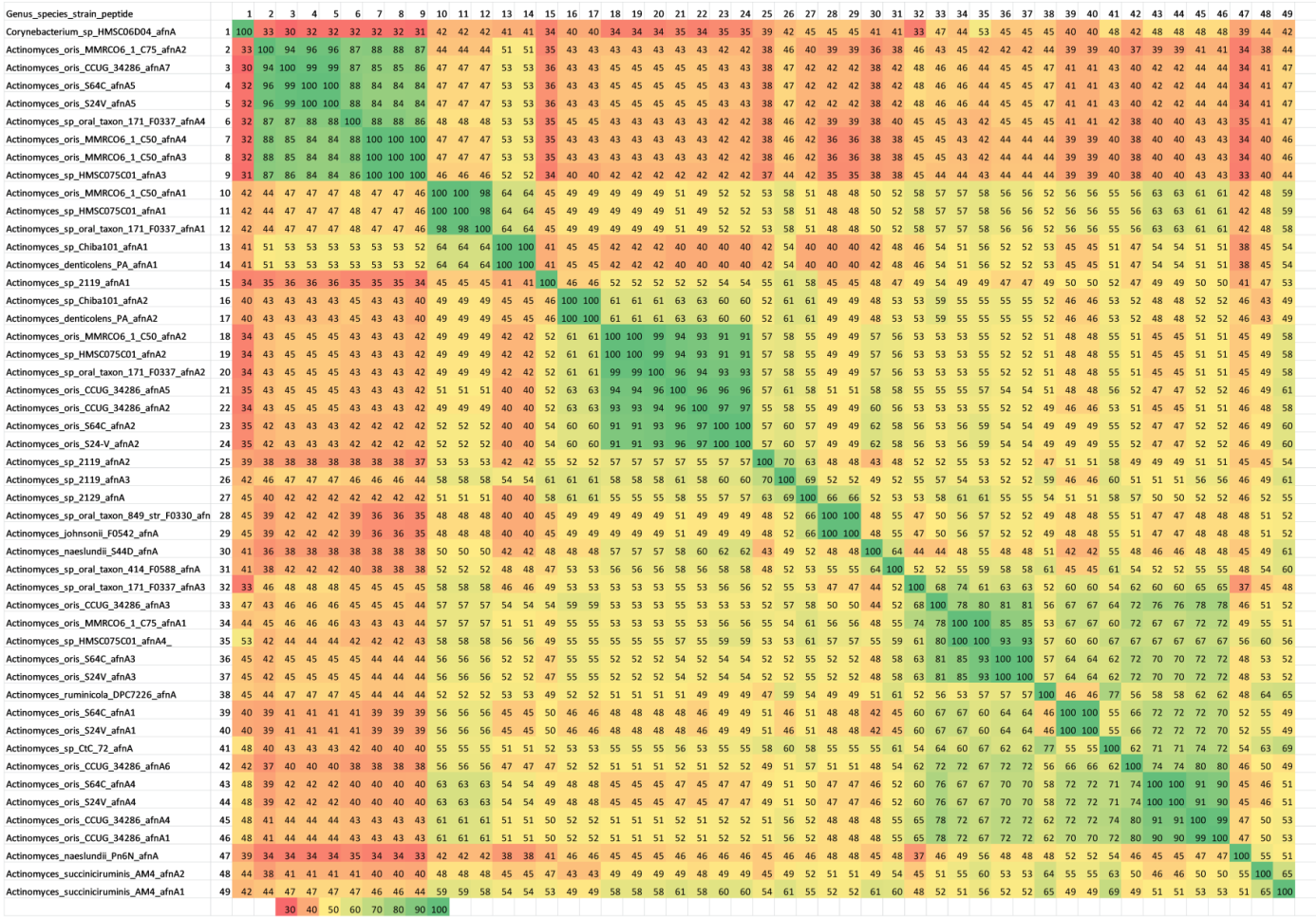

(a)
Leader sequence
Mature peptide
Cleavage site
Disulphide connectivity
(b)
Actifensin operon
Actinomyces sp. 2119
Actinomyces sp. 2129
Actinomyces sp. CtC 72
Actinomyces sp. oral taxon 414 F0588
A. oris S64C
A. naeslundii S44D
Actinomyces sp. oral taxon 171 F0337
A. succiniciruminis AM4
A. oris CCUG34286
Actinomyces sp. HMSC075C01
A. oris MMRCO6-1 contig 50
A. oris S24V
Actinomyces sp. Chiba101
A. denticolens PA
A. naeslundii Pn6N
Actinomyces sp. oral taxon 849 F0330
A. johnsonii F0542
A. oris MMRCO6-1 contig 75
Corynebacterium sp. HMSC06D04
Transcription factor
Hydrolase / peptidase
Unknown function
Bacteriocin
Transport
Other

Figure 12. Percent-identity matrix of actifensin (Actinomyces_ruminicola_DPC7226_afnA) mature peptide amino acid sequence and homologous AfnA sequences.



Genus_species_strain_peptide		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
Corynebacterium_sp_HMSC06D04_afnA	1	100	33	30	32	32	32	32	32	31	42	42	42	41	41	34	40	40	34	34	34	35	34	35	35	39	42	45	45	45	41	41	33	47	44	53	45	45	45	40	40	48	42	48	48	48	48	39	44	42
Actinomyces_oris_MMRC06_1_C75_afnA2	2	33	100	94	96	96	87	88	88	87	44	44	44	51	51	35	43	43	43	43	43	43	43	42	42	38	46	40	39	39	36	38	46	43	45	42	42	42	44	39	39	40	37	39	39	41	41	34	38	44
Actinomyces_oris_CCUG_34286_afnA7	3	30	94	100	99	99	87	85	85	86	47	47	47	53	53	36	43	43	45	45	45	45	45	43	43	38	47	42	42	42	38	42	48	46	46	44	45	45	47	41	41	43	40	42	42	44	44	34	41	47
Actinomyces_oris_S64C_afnA5	4	32	96	99	100	100	88	84	84	84	47	47	47	53	53	36	43	43	45	45	45	45	45	43	43	38	47	42	42	42	38	42	48	46	46	44	45	45	47	41	41	43	40	42	42	44	44	34	41	47
Actinomyces_oris_S24V_afnA5	5	32	96	99	100	100	88	84	84	84	47	47	47	53	53	36	43	43	45	45	45	45	45	43	43	38	47	42	42	42	38	42	48	46	46	44	45	45	47	41	41	43	40	42	42	44	44	34	41	47
Actinomyces_sp_oral_taxon_171_F0337_afnA4	6	32	87	87	88	88	100	88	88	86	48	48	48	53	53	35	45	45	43	43	43	43	43	42	42	38	46	42	39	39	38	40	45	45	43	42	45	45	45	41	41	42	38	40	40	43	43	35	41	47
Actinomyces_oris_MMRC06_1_C50_afnA4	7	32	88	85	84	84	88	100	100	100	47	47	47	53	53	35	43	43	43	43	43	43	43	42	42	38	46	42	36	36	38	38	45	45	43	42	44	44	44	39	39	40	38	40	40	43	43	34	40	46
Actinomyces_oris_MMRC06_1_C50_afnA3	8	32	88	85	84	84	88	100	100	100	47	47	47	53	53	35	43	43	43	43	43	43	43	42	42	38	46	42	36	36	38	38	45	45	43	42	44	44	44	39	39	40	38	40	40	43	43	34	40	46
Actinomyces_sp_HMSC075C01_afnA3	9	31	87	86	84	84	86	100	100	100	46	46	46	52	52	34	40	40	42	42	42	42	42	42	42	37	44	42	35	35	38	38	45	44	44	43	44	44	44	39	39	40	38	40	40	43	43	33	40	44
Actinomyces_oris_MMRC06_1_C50_afnA1	10	42	44	47	47	47	48	47	47	46	100	100	98	64	64	45	49	49	49	49	49	51	49	52	52	53	58	51	48	48	50	52	58	57	57	58	56	56	52	56	56	55	56	63	63	61	61	42	48	59
Actinomyces_sp_HMSC075C01_afnA1	11	42	44	47	47	47	48	47	47	46	100	100	98	64	64	45	49	49	49	49	49	51	49	52	52	53	58	51	48	48	50	52	58	57	57	58	56	56	52	56	56	55	56	63	63	61	61	42	48	59
Actinomyces_sp_oral_taxon_171_F0337_afnA1	12	42	44	47	47	47	48	47	47	46	98	98	100	64	64	45	49	49	49	49	49	51	49	52	52	53	58	51	48	48	50	52	58	57	57	58	56	56	52	56	56	55	56	63	63	61	61	42	48	58
Actinomyces_sp_Chiba101_afnA1	13	41	51	53	53	53	53	53	53	52	64	64	64	100	100	41	45	45	42	42	42	40	40	40	40	42	54	40	40	40	42	48	46	54	51	56	52	52	53	45	45	51	47	54	54	51	51	38	45	54
Actinomyces_denticolens_PA_afnA1	14	41	51	53	53	53	53	53	53	52	64	64	64	100	100	41	45	45	42	42	42	40	40	40	40	42	54	40	40	40	42	48	46	54	51	56	52	52	53	45	45	51	47	54	54	51	51	38	45	54
Actinomyces_sp_2119_afnA1	15	34	35	36	36	36	35	35	35	34	45	45	45	41	41	100	46	46	52	52	52	52	52	54	54	55	61	58	45	45	48	47	49	54	49	49	47	47	49	50	50	52	47	49	49	50	50	41	47	53
Actinomyces_sp_Chiba101_afnA2	16	40	43	43	43	43	45	43	43	40	49	49	49	45	45	46	100	100	61	61	61	63	63	60	60	52	61	61	49	49	48	53	53	59	55	55	55	55	52	46	46	53	52	48	48	52	52	46	43	49
Actinomyces_denticolens_PA_afnA2	17	40	43	43	43	43	45	43	43	40	49	49	49	45	45	46	100	100	61	61	61	63	63	60	60	52	61	61	49	49	48	53	53	59	55	55	55	55	52	46	46	53	52	48	48	52	52	46	43	49
Actinomyces_oris_MMRC06_1_C50_afnA2	18	34	43	45	45	45	43	43	43	42	49	49	49	42	42	52	61	61	100	100	99	94	93	91	91	57	58	55	49	49	57	56	53	53	53	55	52	52	51	48	48	55	51	45	45	51	51	45	49	58
Actinomyces_sp_HMSC075C01_afnA2	19	34	43	45	45	45	43	43	43	42	49	49	49	42	42	52	61	61	100	100	99	94	93	91	91	57	58	55	49	49	57	56	53	53	53	55	52	52	51	48	48	55	51	45	45	51	51	45	49	58
Actinomyces_sp_oral_taxon_171_F0337_afnA2	20	34	43	45	45	45	43	43	43	42	49	49	49	42	42	52	61	61	99	99	100	96	94	93	93	57	58	55	49	49	57	56	53	53	53	55	52	52	51	48	48	55	51	45	45	51	51	45	49	58
Actinomyces_oris_CCUG_34286_afnA5	21	35	43	45	45	45	43	43	43	42	51	51	51	40	40	52	63	63	94	94	96	100	96	96	96	57	61	58	51	51	58	58	55	55	55	57	54	54	51	48	48	56	52	47	47	52	52	46	49	61
Actinomyces_oris_CCUG_34286_afnA2	22	34	43	45	45	45	43	43	43	42	49	49	49	40	40	52	63	63	93	93	94	96	100	97	97	55	58	55	49	49	60	56	53	53	53	55	52	52	49	46	46	53	51	45	45	51	51	46	48	58
Actinomyces_oris_S64C_afnA2	23	35	42	43	43	43	42	42	42	42	52	52	52	40	40	54	60	60	91	91	93	96	97	100	100	57	60	57	49	49	62	58	56	53	56	59	54	54	49	49	49	55	52	47	47	52	52	46	49	60
Actinomyces_oris_S24-V_afnA2	24	35	42	43	43	43	42	42	42	42	52	52	52	40	40	54	60	60	91	91	93	96	97	100	100	57	60	57	49	49	62	58	56	53	56	59	54	54	49	49	49	55	52	47	47	52	52	46	49	60
Actinomyces_sp_2119_afnA2	25	39	38	38	38	38	38	38	38	37	53	53	53	42	42	55	52	52	57	57	57	57	55	57	57	100	70	63	48	48	43	48	52	52	55	53	52	52	47	51	51	58	49	49	49	51	51	45	45	54
Actinomyces_sp_2119_afnA3	26	42	46	47	47	47	46	46	46	44	58	58	58	54	54	61	61	61	58	58	58	61	58	60	60	70	100	69	52	52	49	52	55	57	54	53	52	52	59	46	46	60	51	51	51	56	56	46	49	61
Actinomyces_sp_2129_afnA	27	45	40	42	42	42	42	42	42	42	51	51	51	40	40	58	61	61	55	55	55	58	55	57	57	63	69	100	66	66	52	53	53	58	61	61	55	55	54	51	51	58	57	50	50	52	52	46	52	55
Actinomyces_sp_oral_taxon_849_str_F0330_afn	28	45	39	42	42	42	39	36	36	35	48	48	48	40	40	45	49	49	49	49	49	51	49	49	49	48	52	66	100	100	48	55	47	50	56	57	52	52	49	48	48	55	51	47	47	48	48	48	51	52
Actinomyces_johnsonii_F0542_afnA	29	45	39	42	42	42	39	36	36	35	48	48	48	40	40	45	49	49	49	49	49	51	49	49	49	48	52	66	100	100	48	55	47	50	56	57	52	52	49	48	48	55	51	47	47	48	48	48	51	52
Actinomyces_naeslundii_S44D_afnA	30	41	36	38	38	38	38	38	38	38	50	50	50	42	42	48	48	48	57	57	57	58	60	62	62	43	49	52	48	48	100	64	44	44	48	55	48	48	51	42	42	55	48	46	46	48	48	45	49	61
Actinomyces_sp_oral_taxon_414_F0588_afnA	31	41	38	42	42	42	40	38	38	38	52	52	52	48	48	47	53	53	56	56	56	58	56	58	58	48	52	53	55	55	64	100	52	52	55	59	58	58	61	45	45	61	54	52	52	55	55	48	54	60
Actinomyces_sp_oral_taxon_171_F0337_afnA3	32	33	46	48	48	48	45	45	45	45	58	58	58	46	46	49	53	53	53	53	53	55	53	56	56	52	55	53	47	47	44	52	100	68	74	61	63	63	52	60	60	54	62	60	60	65	65	37	45	48
Actinomyces_oris_CCUG_34286_afnA3	33	47	43	46	46	46	45	45	45	44	57	57	57	54	54	54	59	59	53	53	53	55	53	53	53	52	57	58	50	50	44	52	68	100	78	80	81	81	56	67	67	64	72	76	76	78	78	46	51	52
Actinomyces_oris_MMRC06_1_C75_afnA1	34	44	45	46	46	46	43	43	43	44	57	57	57	51	51	49	55	55	53	53	53	55	53	56	56	55	54	61	56	56	48	55	74	78	100	100	85	85	53	67	67	60	72	67	67	72	72	49	55	51
Actinomyces_sp_HMSC075C01_afnA4_	35	53	42	44	44	44	42	42	42	43	58	58	58	56	56	49	55	55	55	55	55	57	55	59	59	53	53	61	57	57	55	59	61	80	100	100	93	93	57	60	60	67	67	67	67	67	67	56	60	56
Actinomyces_oris_S64C_afnA3	36	45	42	45	45	45	45	44	44	44	56	56	56	52	52	47	55	55	52	52	52	54	52	54	54	52	52	55	52	52	48	58	63	81	85	93	100	100	57	64	64	62	72	70	70	72	72	48	53	52
Actinomyces_oris_S24V_afnA3	37	45	42	45	45	45	45	44	44	44	56	56	56	52	52	47	55	55	52	52	52	54	52	54	54	52	52	55	52	52	48	58	63	81	85	93	100	100	57	64	64	62	72	70	70	72	72	48	53	52
Actinomyces_ruminicola_DPC7226_afnA	38	45	44	47	47	47	45	44	44	44	52	52	52	53	53	49	52	52	51	51	51	51	49	49	49	47	59	54	49	49	51	61	52	56	53	57	57	57	100	46	46	77	56	58	58	62	62	48	64	65
Actinomyces_oris_S64C_afnA1	39	40	39	41	41	41	41	39	39	39	56	56	56	45	45	50	46	46	48	48	48	48	46	49	49	51	46	51	48	48	42	45	60	67	67	60	64	64	46	100	100	55	66	72	72	72	70	52	55	49
Actinomyces_oris_S24V_afnA1	40	40	39	41	41	41	41	39	39	39	56	56	56	45	45	50	46	46	48	48	48	48	46	49	49	51	46	51	48	48	42	45	60	67	67	60	64	64	46	100	100	55	66	72	72	72	70	52	55	49
Actinomyces_sp_CtC_72_afnA	41	48	40	43	43	43	42	40	40	40	55	55	55	51	51	52	53	53	55	55	55	56	53	55	55	58	60	58	55	55	55	61	54	64	60	67	62	62	77	55	55	100	62	71	71	74	72	54	63	69
Actinomyces_oris_CCUG_34286_afnA6	42	42	37	40	40	40	38	38	38	38	56	56	56	47	47	47	52	52	51	51	51	52	51	52	52	49	51	57	51	51	48	54	62	72	72	67	72	72	56	66	66	62	100	74	74	80	80	46	50	49
Actinomyces_oris_S64C_afnA4	43	48	39	42	42	42	40	40	40	40	63	63	63	54	54	49	48	48	45	45	45	47	45	47	47	49	51	50	47	47	46	52	60	76	67	67	70	70	58	72	72	71	74	100	100	91	90	45	46	51
Actinomyces_oris_S24V_afnA4	44	48	39	42	42	42	40	40	40	40	63	63	63	54	54	49	48	48	45	45	45	47	45	47	47	49	51	50	47	47	46	52	60	76	67	67	70	70	58	72	72	71	74	100	100	91	90	45	46	51
Actinomyces_oris_CCUG_34286_afnA4	45	48	41	44	44	44	43	43	43	43	61	61	61	51	51	50	52	52	51	51	51	52	51	52	52	51	56	52	48	48	48	55	65	78	72	67	72	72	62	72	72	74	80	91	91	100	99	47	50	53
Actinomyces_oris_CCUG_34286_afnA1	46	48	41	44	44	44	43	43	43	43	61	61	61	51	51	50	52	52	51	51	51	52	51	52	52	51	56	52	48	48	48	55	65	78	72	67	72	72	62	70	70	72	80	90	90	99	100	47	50	53
Actinomyces_naeslundii_Pn6N_afnA	47	39	34	34	34	34	35	34	34	33	42	42	42	38	38	41	46	46	45	45	45	46	46	46	46	45	46	46	48	48	45	48	37	46	49	56	48	48	48	52	52	54	46	45	45	47	47	100	55	51
Actinomyces_succiniciruminis_AM4_afnA2	48	44	38	41	41	41	41	40	40	40	48	48	48	45	45	47	43	43	49	49	49	49	48	49	49	45	49	52	51	51	49	54	45	51	55	60	53	53	64	55	55	63	50	46	46	50	50	55	100	65
Actinomyces_succiniciruminis_AM4_afnA1	49	42	44	47	47	47	47	46	46	44	59	59	58	54	54	53	49	49	58	58	58	61	58	60	60	54	61	55	52	52	61	60	48	52	51	56	52	52	65	49	49	69	49	51	51	53	53	51	65	100
					30	40	50	60	70	80	90	100																																								

Figure 13. Conserved structures of the defensin peptide superfamily and defensin-like bacteriocins, laterosporulin and actifensin. β sheets are coloured blue, α helices are coloured red, and disulphide bonds are shown in yellow.

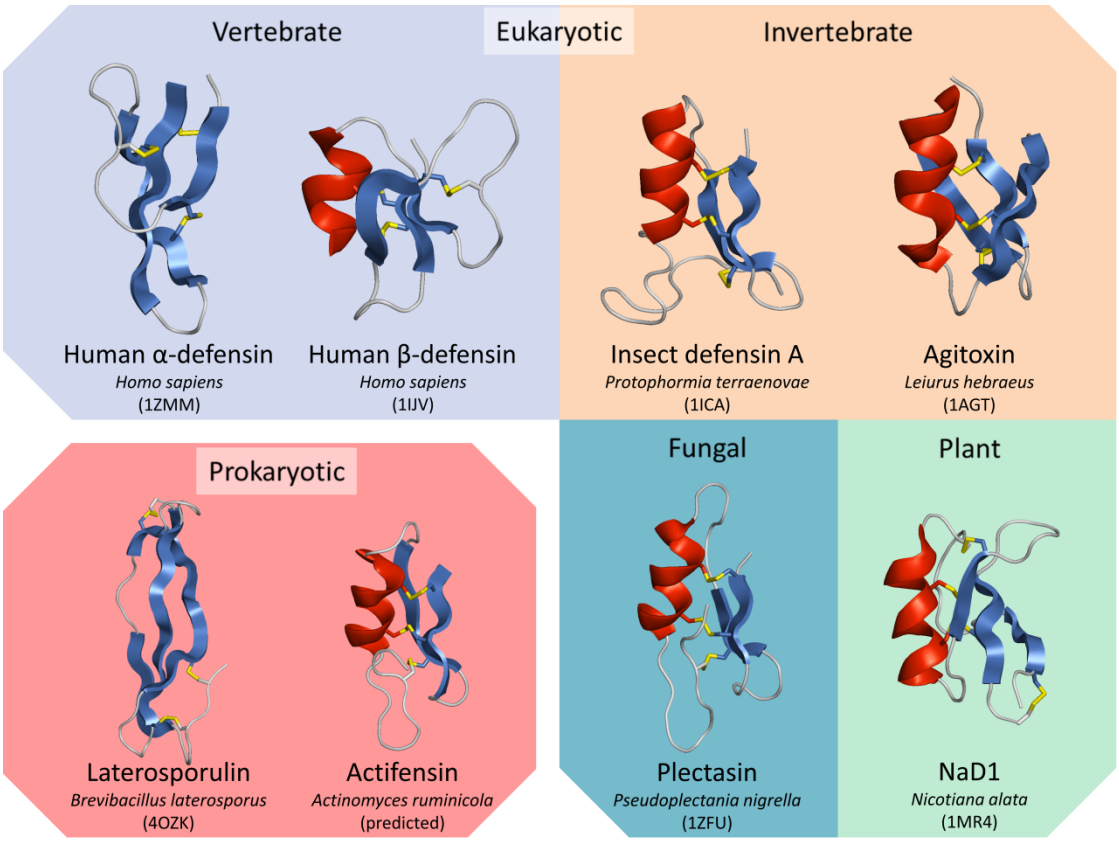


Table 1. *Actinomyces* genomes used in in silico screen.

Species	Strain ID	Source	Nucleotide Accession	Assembly Level	Bacteriocin predicted by BAGEL4
A. bouchesdurhonensis	Marseille-P2825	Human gastric liquid	FQSA00000000.1	Scaffold	No
A. bovis	NCTC11535	Unknown	UAPQ00000000.1	Contigs	LAPs, Lanthipeptide class II
A. cardiffensis	F0333	Human oral	AQHZ00000000.1	Scaffold	No
A. coleocanis	DSM15436	Urogenital tract of a Cocker Spaniel dog	ACFG00000000.1	Scaffold	No
A. culturomici	Marseille-P3575	Human sputum	UIFX00000000.1	Scaffold	No
A. dentalis	DSM 19115	Human dental abscess	AUBL00000000.1	Scaffold	No
A. denticolens	DSM 20671	Cow dental plaque	BDIO00000000.1	Contigs	No
A. denticolens	PA	Cow dental plaque	MVIW00000000.1	Scaffold	No
A. denticolens	DSM 20671	Cow dental plaque	FQYL00000000.1	Scaffold	No
A. denticolens	NCTC11490	Cow dental plaque	UFSA00000000.1	Contigs	No
A. europaeus	ACS-120-V-Col10b	Unknown	AGWN00000000.1	Scaffold	No
A. europaeus	UMB0652	Human urogenital tract	PNHW00000000.1	Scaffold	No
A. gaoshouyii	pika_114	GI tract of *Ochotona curzoniae*	MVIV00000000.1	Scaffold	No
A. gaoshouyii	pika_113	GI tract of *Ochotona curzoniae*	CP020468.1	Complete	No
A. georgiae	F0490	Unknown	AKFS00000000.1	Contigs	No
A. georgiae	DSM66843	Human gingival crevices	AUBM00000000.1	Scaffold	No
A. gerencseriae	DSM6844	Human gingival crevices	AUBN00000000.1	Scaffold	Michiganin A
A. glycerintolerans	G10	Sheep rumen	FQTT00000000.1	Scaffold	No
A. graevenitzii	C83	Human isolate	AWSC00000000.1	Scaffold	Flavucin
A. graevenitzii	F0530	Human oral cavity	ACRN00000000.1	Scaffold	Geobacillin I
A. graevenitzii	UMB0286	Human urogenital tract	PNHV00000000.1	Scaffold	No
A. hominis	UMB0859	Human urogenital tract	PNHU00000000.1	Scaffold	LAPs
A. hongkongensis	HKU8	Human isolate	CP017298.1	Complete	No
A. hordeovulneris	DSM20732	Canine ascites fluid	MQVS00000000.1	Scaffold	No
A. ihuae	SD1	Human gut microbiome	CZPX00000000.1	Contigs	No
A. israelii	DSM76651	Human brain abscess	JONS00000000.1	Scaffold	Variacin
A. johnsonii	F0510	Human gingival plaque	AWSE00000000.1	Scaffold	Linocin
A. johnsonii	F0542	Human oral cavity	AWSD00000000.1	Scaffold	No
A. liubinyangii	VUL4_1	Faeces of *Gypaetus barbatus*	MQSU00000000.1	Scaffold	No
A. liubinyangii	VUL4_2	Faeces of *Gypaetus barbatus*	MQSV00000000.1	Contigs	No
A. marimammalium	DSM15383	Samples from two dead seals and a porpoise	MPDM00000000.1	Scaffold	No
A. marseillensis	Marseille-P2818	Human sputum	FTLP00000000.1	Scaffold	No
A. massiliensis	F0489	Unknown	AKIO00000000.1	Scaffold	No
A. massiliensis	4401291	Human blood culture	AKFT00000000.1	Contigs	No
A. mediterranea	D0489	Human gut microbiome	FTPB00000000.1	Contigs	Putative bacteriocin
A. meyeri	W712	Unknown	CP012072.1	Complete	No
A. meyeri	DSM20733	Human purulent pleurisy	FNLK00000000.1	Contigs	No
A. minihominis	Marseille-P3850	Human stool	FYEG00000000.1	Contigs	No
A. naeslundii	ATCC 27039	Human abdominal abscess	MSRJ00000000.1	Scaffold	Variacin, Linocin
A. naeslundii	F6E1	Human dental plaque	MSRS00000000.1	Scaffold	Streptomonomicin , Linocin
A. naeslundii	Howell 279	Human	ALJK00000000.1	Contigs	Linocin

Species	Strain ID	Source	Nucleotide Accession	Assembly Level	Bacteriocin predicted by BAGEL4
A. naeslundii	WE6B-3	Human dental plaque	MSKZ00000000.1	Scaffold	Linocin
A. naeslundii	W8-2-3	Human dental plaque	MSLB00000000.1	Scaffold	Linocin
A. naeslundii	R19039	Human liver abscess	MSRH00000000.1	Scaffold	Linocin
A. naeslundii	R13240	Human subphrenic abscess	MSRI00000000.1	Scaffold	Linocin
A. naeslundii	R8152	IUCD	MSRL00000000.1	Scaffold	Linocin
A. naeslundii	F12B1	Human dental plaque	MSRQ00000000.1	Scaffold	Linocin
A. naeslundii	MMRC12-1	Human soft lesion	MSRR00000000.1	Scaffold	Linocin
A. naeslundii	Pn6N	Human dental plaque	PKKP00000000.1	Scaffold	Linocin
A. naeslundii	CCUG 37599	Human cerebrospinal fluid	PKKK00000000.1	Scaffold	Linocin
A. naeslundii	T23P-1	Human dental plaque	AP017894.1	Complete	Linocin
A. naeslundii	S65A	Human dental plaque	MSRK00000000.1	Scaffold	Linocin
A. naeslundii	S44D	Human dental plaque	MSRU00000000.1	Scaffold	Linocin, Variacin, LAPs
A. naeslundii	S43L	Human dental plaque	MSRO00000000.1	Scaffold	Linocin, Subtilosin A
A. naeslundii	R24330	IUCD	MSKY00000000.1	Scaffold	Linocin, Lanthipeptide class II
A. naeslundii	NCTC 10301	Human dental plaque	MSLA00000000.1	Scaffold	Linocin, Lanthipeptide class II
A. naeslundii	MB-1	Human dental plaque	MSKX00000000.1	Scaffold	Linocin, Lanthipeptide class II
A. naeslundii	G127B	Human dental plaque	MSRT00000000.1	Scaffold	Linocin, LAPs, Lactococcin 972
A. naeslundii	CCUG 35334	Human blood culture	MSRM00000000.1	Contigs	LAPs, Linocin
A. naeslundii	UMB0731	Human urogenital tract	MSRP00000000.1	Scaffold	LAPs, Linocin
A. naeslundii	UMB0181	Human urogenital tract	MSRN00000000.1	Scaffold	LAPs, Linocin
A. nasicola	KPR-1	Human nose	MQVR00000000.1	Scaffold	LAPs
A. nasicola	DSM 19116	Human anthral washout	FNQV00000000.1	Contigs	No
A. neuii	BVS029A5	Human urogenital tract	AGWP00000000.1	Scaffold	No
A. neuii	MJR8396A	Human vaginal isolate	LRPJ00000000.1	Scaffold	No
A. neuii	UMB0125	Human urogenital tract	PKKO00000000.1	Contigs	No
A. neuii	UMB0402	Human urogenital tract	PKKN00000000.1	Scaffold	No
A. neuii subsp. neuii	DSM 8576	Human mammary hematoma	ATUW00000000.1	Contigs	No
A. odontolyticus	ATCC 17982	Carious lesions of the dentine	ACYT00000000.2	Scaffold	LAPs
A. odontolyticus	F0309	Human	AAYI00000000.2	Scaffold	No
A. odontolyticus	XH001	Human oral cavity	LLVT00000000.1	Scaffold	No
A. odontolyticus	UMB0018	Human urogenital tract	PKKM00000000.1	Scaffold	No
A. odontolyticus	NCTC9935	Human dental caries	UAPR00000000.1	Contigs	No
A. oris	S24V	Human dental plaque	MSGO00000000.1	Scaffold	Lactococcin 972
A. oris	K20	Unknown	MSKL00000000.1	Scaffold	Lactococcin 972
A. oris	T14V	Human dental plaque	MSKI00000000.1	Scaffold	Lactococcin 972, Thiopeptide
A. oris	MG-1	Human dental plaque	PKKL00000000.1	Scaffold	Lactococcin 972, Linocin
A. oris	S64C	Human dental plaque	BABV00000000.1	Contigs	Linocin

Species	Strain ID	Source	Nucleotide Accession	Assembly Level	Bacteriocin predicted by BAGEL4
A. oris	CCUG 33920	Human dental plaque	CP014232.1	Complete	Linocin
A. oris	R21091	Human brain abscess	MAUB00000000.1	Contigs	Linocin
A. oris	P6N	Human dental plaque	MSQE00000000.1	Scaffold	Linocin
A. oris	MMRCO6-1	Human soft lesion	MSKM00000000.1	Scaffold	Linocin
A. oris	M48-1B-1	Human dental plaque	MSKN00000000.1	Scaffold	Linocin
A. oris	F28B1	Human dental plaque	MSKO00000000.1	Scaffold	Linocin
A. oris	CCUG 34286	Human gingival crevices	MSKV00000000.1	Scaffold	Linocin
A. oris	A19A-1	Human skin lesion	MSKW00000000.1	Scaffold	Linocin
A. oris	R11372	IUCD	MSKU00000000.1	Scaffold	Linocin, Lactococcin 972
A. oris	WE8B-23	Human dental plaque	MSKR00000000.1	Scaffold	Linocin, Streptomonomicin
A. oris	A7A-1	Human skin lesion	MSKS00000000.1	Scaffold	Linocin, LAPs,
A. oris	R23275	Human blood culture	MSKT00000000.1	Scaffold	LAPs, Linocin
A. oris	W11-1-1	Human dental plaque	MSKQ00000000.1	Scaffold	LAPs, Linocin, Lactococcin 972
A. oris	G53E	Human dental plaque	MSKP00000000.1	Scaffold	LAPs, Linocin, Streptomonomicin
A. oris	F4D1	Human dental plaque	MSKK00000000.1	Scaffold	Thiopeptide, Lactococcin 972
A. oris	UMB0183	Human urogenital tract	MSKJ00000000.1	Scaffold	No
A. pacaensis	Marseille-P2985	Human sputum	LT635457.1	Complete	No
A. polynesiensis	MS2	Human gut	CCXH00000000.1	Contigs	Linocin
A. provencensis	SN12	Human stool	FTLI00000000.1	Scaffold	Linocin
A. radicidentis	CCUG 36733	Human root canal scraping	CP014228.1	Complete	Lanthipeptide class II, Putative bacteriocin, Linocin homolog
A. radingae	DSM 9169	Human	LT629792.1	Complete	No
A. ruminicola	KPR-7B	Cattle rumen	FNHU00000000.1	Scaffold	Lactococcin 972
A. ruminicola	DSM 27982	Cattle rumen	FNIM00000000.1	Scaffold	No
A. slackii	ATCC 49928	Oral cavity	AUAK00000000.1	Scaffold	No
A. sp.	VUL4_3	Aegypius monachus	CP032514.1	Complete	LAPs
A. sp.	2129	Human faeces	CP017812.1	Complete	No
A. sp.	S2_006_000_R1_54	Hospital surfaces & sink samples	QFPC00000000.1	Contigs	No
Actinomyces sp.	ICM47	Human oral cavity	PPPL00000000.1	Scaffold	Lactococcin 972
Actinomyces sp.	ICM39	Human	CP027862.1	Complete	Staphylococcins
Actinomyces sp.	ICM58	Human	QMIO00000000.1	Scaffold	Staphylococcins
Actinomyces sp.	Chiba101	Domestic pig	OOHN00000000.1	Contigs	Variacin
Actinomyces sp.	HPA0247	Unknown	ALCA00000000.1	Contigs	Flavucin
Actinomyces sp.	ph3	Human stool	ALIY00000000.1	Contigs	Flavucin
Actinomyces sp.	Z16	Tibetan antelope (*Pantholops hodgsonii*) faeces	LTNX00000000.1	Scaffold	Flavucin
Actinomyces sp.	ICM54	Unknown	QYRR00000000.1	Contigs	Michiganin A
Actinomyces sp.	S4-C9	Human vaginal isolate	MTPX00000000.2	Contigs	Microbisporicin
Actinomyces sp.	S6-Spd3	Human vaginal isolate	PKMC00000000.1	Scaffold	Microbisporicin
Actinomyces sp.	HMSC075B09	Human tissue wound	LWNX00000000.1	Scaffold	Linocin
Actinomyces sp.	HMSC064C12	Human wound	LTXO00000000.1	Scaffold	Linocin
Actinomyces sp.	HMSC072A03	Human urine	QMIN00000000.1	Scaffold	Lanthipeptide class II
Actinomyces sp.	HMSC035G02	Human sputum	ALCB00000000.1	Contigs	No
Actinomyces sp.	HMSC065F12	Human	CAGY00000000.1	Contigs	No
Actinomyces sp.	HMSC062G12	Human abscess	ATCA00000000.1	Scaffold	No
Actinomyces sp.	HMSC065F11	Human wound	JDFI00000000.1	Contigs	No
Actinomyces sp.	HMSC075C01	Human cheek abscess	JRMV00000000.1	Contigs	No
Actinomyces sp.	HMSC08A09	Human	JRMU00000000.1	Contigs	No

Species	Strain ID	Source	Nucleotide Accession	Assembly Level	Bacteriocin predicted by BAGEL4
Actinomyces sp.	HMSC08A01	Human urogenital tract	LWOG00000000.1	Scaffold	No
Actinomyces sp.	HMSC06A08	Human urogenital tract	LWNY00000000.1	Scaffold	No
Actinomyces sp.	CtC 72	Cattle rumen	LTEW00000000.1	Scaffold	No
Actinomyces sp.	Marseille-P3109	Human sputum	LTEK00000000.1	Scaffold	No
Actinomyces sp.	UMB0138	Human urogenital tract	LTEH00000000.1	Scaffold	No
Actinomyces sp.	UMB0918	Human urogenital tract	LTXG00000000.1	Scaffold	No
Actinomyces sp.	553	Tibetan antelope (*Pantholops hodgsonii*) faeces	LTVP00000000.1	Scaffold	No
Actinomyces sp.	Z5	Tibetan antelope (*Pantholops hodgsonii*) faeces	LTUQ00000000.1	Scaffold	No
Actinomyces sp.	Z3	Tibetan antelope (*Pantholops hodgsonii*) faeces	AP017896.1	Complete	No
Actinomyces sp.	2119	Tibetan antelope (*Pantholops hodgsonii*) faeces	PNHT00000000.1	Scaffold	No
Actinomyces sp.	F0330	Human oral cavity	AFUR00000000.1	Contigs	Lactococcin 972, Linocin
Actinomyces sp.	F0332	Human oral cavity	AFBL00000000.1	Scaffold	Variacin
Actinomyces sp.	F0337	Human oral cavity	AFQC00000000.1	Scaffold	Variacin
Actinomyces sp.	F0310	Human oral cavity	ACUY00000000.2	Scaffold	Microbisporicin
Actinomyces sp.	F0338	Human oral cavity	ACTB00000000.1	Scaffold	Linocin
Actinomyces sp.	F0386	Human oral cavity	AEPP00000000.1	Scaffold	No
Actinomyces sp.	F0400	Human oral cavity	AEUH00000000.1	Scaffold	No
Actinomyces sp.	F0384	Human oral cavity	AECW00000000.1	Scaffold	No
Actinomyces sp.	F0379	Human oral cavity	AMEW00000000.1	Scaffold	No
Actinomyces sp.	F0311	Human oral cavity	AWSF00000000.1	Scaffold	No
Actinomyces sp.	F0543	Human gingival plaque	AWSG00000000.1	Scaffold	No
Actinomyces sp.	F0631	Human gingival plaque	CP027236.1	Complete	No
Actinomyces sp.	F0588	Human gingival plaque	CP012590.1	Complete	No
A. succiniciruminis	AM4	Cattle rumen	900002405.1	Scaffold	No
A. suimastitidis	DSM 15538	Pig mammary abscess	AUBF00000000.1	Scaffold	No
A. timoneneis	DSM 23828	Human osteo-articular sample	AKGF00000000.1	Scaffold	No
A. turicensis	ACS-279-V-Col4	Human urogenital tract	AGWQ00000000.1	Scaffold	No
A. turicensis	UMB0250	Human urogenital tract	PKKJ00000000.1	Scaffold	No
A. urinae	Marseille-P2225	Human urine	FPKP00000000.1	Scaffold	No
A. urogenitalis	DORA 12	Infant gut	ACFH00000000.1	Scaffold	No
A. urogenitalis	DSM 15434	Human urogenital tract	JRMT00000000.1	Contigs	No
A. urogenitalis	S6-C4	Human vaginal isolate	JUUL00000000.1	Contigs	No
A. urogenitalis	752_PACI	Human	PKHA00000000.1	Scaffold	No
A. urogenitalis	UMB0319	Human urogenital tract	AZLV00000000.1	Scaffold	No
A. vaccimaxillae	DSM 15804	Cattle jaw lesion	ATUX00000000.1	Contigs	Lanthipeptide class I
A. viscosus	C505	Human upper respiratory tract	ACRE00000000.2	Scaffold	Linocin
A. vulturis	VUL7	Faeces of vulture	LZRK00000000.1	Scaffold	No

Table 2. AfnA propeptide sequences, encoding genes, and source genomes.

Species	UniPROT Annotation	Gene annotation	*afn* gene	Peptide sequence	Residues	Source
A. naeslundii_Pn6N	hypothetical protein	-	*afnA*	MSQFIRRSSKLTNVTFNQSLLSETCPPLEGGEGFGCPGQEYWCDGHCKANGFQYGKC DSLFWHRCHCFE	69	This study (BLAST)
A. naeslundii_S44D	hypothetical protein	-	*afnA*	MNRFVRRSRSLADVTFASVLRSETHAPLEGAEGHGCRPFGSDFECDEWCTYKGYRGG YCSWGVVCTCYGG	70	This study (BLAST)
A. oris CCUG34286	Uncharacterized protein	BKH23_08770	*afnA7*	MELFSRRCKSLSDSRFSDAMNAETRNPLETSNCFACPFNEHQCHNHCLSTGYRGGFC GGFAAATCRCY	68	This study (BLAST)
A. oris CCUG34286	Arthropod defensin	BKH23_08775	*afnA6*	MDKFTRRTAPLSDADFKQAVSSETQAPIESAEGHGCPADEYRCYRDCRAMGYRGGYC DSRTLWLRCTCY	69	This study (BLAST)
A. oris CCUG34286	Arthropod defensin	BKH23_08780	*afnA5*	MPQFVRRSTALADVTFEQALHSETHAPTEGAEYNCPTDESPCDRHCRYSGYRGGYCG GALKTSCRCY	67	This study (BLAST)
A. oris CCUG34286	Arthropod defensin	BKH23_08785	*afnA4*	MDKFTRRTAPLSDADFKQAVSSETQAPIEGAEGFGCPNDEYTCNAHCQSVGYRGGYC DFWTAWRRCTCY	69	This study (BLAST)
A. oris CCUG34286	Arthropod defensin	BKH23_08790	*afnA3*	MDKFTRRTAPLSAADFKQAISSETQAPIEGTEGFSCPGAEYACNAHCRSIGYRGGYCGS WLNLRCRCY	68	This study (BLAST)
A. oris CCUG34286	Arthropod defensin	BKH23_08795	*afnA2*	MPRFVRRSTALADVTFAQALHSETHPPTEGAEYNCPTDESPCDRHCRYSGYRGGYCG GALKTSCRCY	67	This study (BLAST)
A. oris CCUG34286	Arthropod defensin	BKH23_08800	*afnA1*	MDKFTRRTAPLSDADFKQAVSSETQAPIEGAEGFGCPNDEYTCNAHCQSVGYRGGYC DFWTGWRRCTCY	69	This study (BLAST)
A. oris MMRCO6-1	Uncharacterized protein	BKH27_13445	*afnA2*	MELFSRRCTSLSDSRFSDTMNAETRNPLETSDCFACPFNEHQCHNHCLSTGYRGGFC GGFAAATCRCH	68	This study (BLAST)
A. oris MMRCO6-1	Uncharacterized protein	BKH27_11350	*afnA4*	MDMFSRRRTSLSDNRFRGTMNAETRNPLETSNCFACPFNEHQCHNHCLSKGYRGGF CGGFAAATCRCH	68	This study (BLAST)
A. oris MMRCO6-1	Arthropod defensin	BKH27_13440	*afnA1*	MDKFTRRTTTLSDSDFSQAVSSETQAPIEGAEGIGCPGAEYGCNKRCRSIGYRGGYCGS LFNLRCHCY	68	This study (BLAST)
A. oris MMRCO6-1	-	-	*afnA3*	MDMFSRRRTSLSDNRFRGTMNAETRNPLETSNCFACPFNEHQCHNHCLSKGYRGGF CGGFAAATCRCH	68	This study (BLAST)
A. oris MMRCO6-1	Arthropod defensin	BKH27_11365	*afnA1*	MDKFTRRTANLVDADKALNAETHAPIEGAEGFGCPWNAYECDRHCMSKGYTGGNC RGKIRQTCHCY	67	This study (BLAST)
A. oris MMRCO6-1	Uncharacterized protein	BKH27_11360	*afnA2*	MPHFVRRSTALADVTFEQALHSETHTPTEGAEYKCPTDESPCDRHCRYSGYRGGYCG GILKTSCRCY	68	This study (BLAST)
A. oris S64C	Uncharacterized protein	BKH30_00540	*afnA5*	MELFSRRCKSLSDSRFSDAMNAETRNPLETSDCFACPFNEHQCHNHCLSTGYRGGFC GGFAAATCRCY	68	This study (BLAST)
A. oris S64C	Arthropod defensin	BKH30_00535	*afnA4*	MDKFTRRTAPLSDADSKHAISSETQAPIEGAEGFGCPNEYRCNAHCQSVGYQGGYCD FWTARRRCTCY	68	This study (BLAST)
A. oris S64C	Arthropod defensin	BKH30_00530	*afnA3*	MDKFTRRAAPLSDASFNQAISSETQAPIEGAEYGCPGAEYGCNNRCRSIGYRGGYCGS LFNLRCLCY	67	This study (BLAST)
A. oris S64C	Arthropod defensin	BKH30_00525	*afnA2*	MPRFVRRSTALADVTFAQALHSETHAPTEGAEYNCPTDESPCDRHCRYSGYRGGYCG GALKTSCHCY	67	This study (BLAST)

Species	UniPROT Annotation	Gene annotation	*afn* gene	Peptide sequence	Residues	Source
A. oris S64C	Arthropod defensin	BKH30_00520	*afnA1*	MDKFTRRTASLSDSDFKQAISSETHAPIEGAEGFGCPDESRCNAHCQNNGFDRGRCD SIFALRCHCSYYR	70	This study (BLAST)
A. ruminicola DPC7226	-	afnA	*afnA*	MKKFIRRSSSLAAASFEQAFQSETQVPLEGAEGFGCNLITSNPYQCSNHCKSVGYRGG YCKLRTVCTCY	69	This study (BLAST)
Actinomyces sp. CtC72	Arthropod defensin	BW737_000795	*afnA*	MRKFIRRSSSLAAASFEQALRSETQAPLEGAEGFGCPNEYKCNRHCKSVNYRGGYCDF WTARLRCTCY	68	This study (BLAST)
Actinomyces sp. F0337	Arthropod defensin	HMPREF9057_00 041	*afnA4*	MKKKIYMDMFSRRRKSLNDSRFNDAMNAETRSPLETSDCFACPFNEHQCHNHCLSK GYRGGFCGGFAAATCRCH	74	This study (BLAST)
Actinomyces sp. F0338	Arthropod defensin	HMPREF9057_00 042	*afnA3*	MDKFTRRTTTLSDSDFSQAVSSETQAPIEGTEDLSCPWAPSVCNRHCLSHGYRGGYCA GPIKLVCHCY	68	This study (BLAST)
Actinomyces sp. F0339	Arthropod defensin	HMPREF9057_00 043	*afnA1*	MDKFTRRTANLVDADKALNAETHAPIEGAEGFGCPWNAYECDRHCVSKGYTGGNCR GKIRQTCHCY	66	This study (BLAST)
Actinomyces sp. F0340	-	-	*afnA2*	MPHFVRRSTALADVTFEQALHSETHTPTEGAEYNCPTDESPCDRHCRYSGYRGGYCG GILKTSCRCY	67	This study (BLAST)
Actinomyces sp. F0588	Uncharacterized protein	AM609_13910	*afnA*	MKFIRRSNNLTDVSFTQALHSETRAPLEGAEGYGCSPLSSDYQCTEHCRYIGYRGGYCA WGIVCTCY	67	This study (BLAST)
Actinomyces sp. 2119	Arthropod defensin	D4740_07960	*afnA1*	MHPLIRRTTALAGADFAQALRSETHAPVEGSESFPCLGHPARCFAHCRKAGFRGGYCV PIRRCVCY	66	This study (BLAST)
Actinomyces sp. 2119	Arthropod defensin	D4740_07965	*afnA2*	MPHFIRRTTTLAGADFTQALRSETHAPTEGAEPFGCPALEFVCNRHCRSIARNYYKGKC VGMFKQTCKCFSY	72	This study (BLAST)
Actinomyces sp. 2119	Arthropod defensin	D4740_07970	*afnA3*	MSQFIRRTTALAGADFTQALRSETHAPTEGGEPFGCPFNSFTCHRHCKSIPGYRGGYC KGRLNQTCKCYR	70	This study (BLAST)
Actinomyces sp. 2129	-	-	*afnA*	MSQFIRRTTTLAGADFTQALRSETHAPTEGAEGPCPLNEKKCSQICRAKGYKGGYCGS FANLVCKCY	67	This study (BLAST)
Actinomyces sp. HMSC075C01	-	HMPREF2883_RS 00205	*afnA3*	MKKENYMDMFSRRRTSLSDNRFRGTMNAETRNPLETSNCFACPFNEHQCHNHCLSK GYRGGFCGGFAAA	69	This study (BLAST)
Actinomyces sp. HMSC075C01	-	HMPREF2883_RS 13750	*afnA4*	ETQAPIEGAEGIGCPGAEYGCNKRCRSIGYRGGYCGSLFNLRCHCY	46	This study (BLAST)
Actinomyces sp. HMSC075C01	Uncharacterized protein	HMPREF2883_RS 00210	*afnA1*	MDKFTRRTANLVDADKALNAETHAPIEGAEGFGCPWNAYECDRHCMSKGYTGGNC RGKIRQTCHCY	66	This study (BLAST)
Actinomyces sp. HMSC075C01	-	HMPREF2883_RS 13755	*afnA2*	MPHFVRRSTALADVTFEQALHSETHTPTEGAEYKCPTDESPCDR HCRYSGYRGGYCGGILKTSCRCY	68	This study (BLAST)
A. succiniciruminis AM4	Knottin, scorpion toxin-like	AAM4_0198	*afnA2*	MKKFIRRSSSLDAVSFEQALKSETHVPLEGAEGFGCPFRPGDCYKYCRSKGFRVGVCDS LANMRCHCYY	69	This study (BLAST)
A. succiniciruminis AM4	Arthropod defensin	AAM4_0199	*afnA1*	MKNFIRRSSSLDAVSFEQALRSETHAPLEGAEGFGCPFSERSCDTHCMTKGYRGGYCK GAVRQTCVCYK	69	This study (BLAST)
Corynebacterium sp. HMSC06D04	-	-	*afnA*	MSTFIRNTSSNVATIDATISNELLAPLEGADGFGCPDDYKCSDYCRSIGYNNGYCSIWSF NRRCVCK	67	This study (BLAST)
Actinomyces sp. Chiba101	Cysteine-rich antibacterial peptide	CHIBA101_0489	*afnA1*	MDKFTRRTADLAANELGDDINAETRTPLEDAEGFGCPFNAYQCHSHCLSIGRRGGYC RGLVRQTCVCYR	70	Identified by Dash et. al (2019)
Actinomyces sp. Chiba101	Cysteine-rich antibacterial peptide	CHIBA101_0490	*afnA2*	MKQFARRTATLADATFTQALDSETKPPTEGAEFSCPMTDYPCIMHCKAIGYRGGYCG GFLNLSCRCH	68	Identified by Dash et. al (2019)

Species	UniPROT Annotation	Gene annotation	*afn* gene	Peptide sequence	Residues	Source
Actinomyces sp. F0330	Uncharacterized protein	HMPREF0975_02295	*afnA*	MSQFIRRTSTLTDISFSDALHSESHMPLEGAEGPCPHNETKCGEVCRGMGYTGGYCHSWFNLICKCY	68	Identified by Dash et. al (2019)
A. johnsonii F0542	Arthropod defensin	HMPREF1979_01045	*afnA*	MSQFIRRTSTLTDISFSDALHSESHMPLEGAEGPCPHNETKCGEVCRGMGYTGGYCHSWFNLICKCY	68	Identified by Dash et. al (2019)
A. denticolens PA	Arthropod defensin	SAMN05216246_102303	*afnA1*	MDKFTRRTADLAANELGDDINAETRTPLEDAEGFGCPFNAYQCHSHCLSIGRRGGYCRGLVRQTCVCYR	70	Identified by Dash et. al (2019)
A. denticolens PA	Arthropod defensin	SAMN05216246_102304	*afnA2*	MKQFARRTATLADATFTQALDSETKPPTEGAEFSCPMTDYPCIMHCKAIGYRGGYCGGFLNLSCRCH	68	Identified by Dash et. al (2019)
A. oris S24-V	Uncharacterized protein	BKH30_00540	*afnA5*	MELFSRRCKSLSDSRFSDAMNAETRNPLETSDCFACPFNEHQCHNHCLSTGYRGGFCGGFAAATCRCY	69	Identified by Dash et. al (2019)
A. oris S24-V	Arthropod defensin	BKH30_00530	*afnA3*	MDKFTRRAAPLSDASFNQAISSETQAPIEGAEYGCPGAEYGCNNRCRSIGYRGGYCGSLFNLRCLCY	68	Identified by Dash et. al (2019)
A. oris S24-V	Arthropod defensin	BKH30_00520	*afnA1*	MDKFTRRTASLSDSDFKQAISSETHAPIEGAEGFGCPDESRCNAHCQNNGFDRGRCDSIFALRCHCSYYR	71	Identified by Dash et. al (2019)
A. oris S24-V	Arthropod defensin	BKH30_00535	*afnA4*	MDKFTRRTAPLSDADSKHAISSETQAPIEGAEGFGCPNEYRCNAHCQSVGYQGGYCDFWTARRRCTCY	69	Identified by Dash et. al (2019)
A. oris S24-V	Arthropod defensin	BKH30_00525	*afnA2*	MPRFVRRSTALADVTFAQALHSETHAPTEGAEYNCPTDESPCDRHCRYSGYRGGYCGGALKTSCHCY	68	Identified by Dash et. al (2019)

Table 3. Growth conditions of indicator species used in this study.

Indicator Species	Growth Medium	Incubation Conditions
Actinomyces neuii LMG 19524t	BHI	37 °C, O_2^-
Actinomyces radingae LMG 15960t	BHI	37 °C, O_2^-
Bacillus cereus NCIMB700577	BHI	37 °C, O_2^+
Bacillus subtilus S249	BHI	37 °C, O_2^+
Bacillus thuringiensis DPC6431	BHI	37 °C, O_2^+
Clostridium difficile DPC6534	RCM	37 °C, O_2^-
Clostridium sporogenes LMG10143	RCM	37 °C, O_2^-
Enterococcus faecium APC1031	TSY	37 °C, O_2^-
Enterococcus faecium NCDO942	TSY	37 °C, O_2^-
Escherichia coli DPC6054	BHI	37 °C, O_2^+
Lactobacillus acidophilus DPC5377	MRS	37 °C, O_2^-
Lactobacillus delbrueckii ssp. *bulgaricus* LMG6901	MRS	37 °C, O_2^-
Lactobacillus delbrueckii ssp. *lactis* DPC5387	MRS	37 °C, O_2^-
Lactobacillus helveticus DPC5353	MRS	37 °C, O_2^-
Lactobacillus helveticus DPC5385	MRS	37 °C, O_2^-
Lactococcus lactis ATCC11454	GM17	30 °C, O_2^+
Lactococcus lactis ssp. *lactis* DPC3147	GM17	30 °C, O_2^+
Listeria innocua DPC1768	BHI	37 °C, O_2^+
Listeria monocytogenes DPC3572	BHI	37 °C, O_2^+
Listeria monocytogenes DPC6893	BHI	37 °C, O_2^+
Pediococcus acidilactici LMG2351	MRS	30 °C, O_2^+
Salmonella enterica ser. Typhimurium DPC6046	BHI	37 °C, O_2^+
Staphylococcus aureus DPC5645	BHI	37 °C, O_2^+
Staphylococcus aureus R963	BHI	37 °C, O_2^+
Streptococcus agalactiae APC1055	BHI	37 °C, O_2^+

Chapter 6. Actifensin homologs are widely distributed across the *Actinomyces* genus and in some distantly related organisms

Abstract

The aim of this study was two-fold; firstly, to examine the distribution of bacterial gene homologs of the antimicrobial peptide, actifensin, produced by *Actinomyces ruminicola* DPC 7226; and secondly, to determine the activity of the synthesised peptide as compared to its natural form. *Actinomyces oris* CCUG 34286 contains seven homologs of the actifensin structural gene, *afnA,* but was found to be inactive in agar overlay and well diffusion assays against *Lactobacillus delbrueckii* ssp. *bulgaricus* LMG 6901. However, it exhibited the same level of immunity (6.1 µM) to the purified peptide as the producer organism *A. ruminicola* DPC 7226. The AfnA peptide was successfully synthesised in the linear form (4,097 Da) using microwave-assisted solid phase peptide synthesis (MW-SPPS), but formation of three disulphide bonds was not observed as evidenced by a higher molecular weight of 6 Da when compared to that naturally produced. Further bacterial *afnA* homologs were identified using the conserved gene neighbours encoding proteins of unknown function (AfnGHI) and position-specific iterative (PSI) BLAST. Twenty-nine new gene clusters were identified across the phylum Actinobacteria - separated into three distinct groups based on operon arrangement and encoded peptide sequence structure. Groups I and II share characteristic features of AfnA, while group III was found to be similar to eukaryotic inhibitor cysteine knot (ICK) peptides. This data builds upon our previous knowledge of actifensin and show further links between actifensin and eukaryotic peptide structures.

Introduction

Actifensin (AfnA) is a class IId broad spectrum bacteriocin (bacterial ribosomally produced antimicrobial peptide) produced by *Actinomyces ruminicola* DPC 7226 that was isolated from sheep faeces (1). The peptide bears a strong resemblance to cis-defensins, an ancient group of antimicrobial peptides that are ubiquitous in eukaryotes as host-defence peptides (2, 3). Similar to defensins, actifensin is small (<10 kDa), cationic, cysteine rich (forming three characteristic disulphide bonds) and contains a γ-core motif (4). Following identification of actifensin, a group of 47 homologous encoded peptides were detected throughout the genus *Actinomyces* and within a single *Corynebacterium* species (1). The peptides display high levels of sequence diversity and redundancy, with up to seven copies of the structural gene (*afnA*) encoded by a single strain, *Actinomyces oris* CCUG 34286. Actifensin production was traced to a conserved operon within *Actinomyces* spp. encoding genes for transport (*afnFJKL*), proteolytic cleavage (*afnCE*), regulation (*afnB*) and nearby genes of unknown function (*afnDGHI*). The mature peptide inhibits a broad range of bacteria including such notable pathogens as *Streptococcus agalactiae, Staphylococcus aureus*, and *Clostridioides difficile* (formerly *Clostridium difficile*). Given the global crisis of antimicrobial resistance, the natural diversity of actifensin family peptides makes them interesting candidates for further investigation, as single amino acid substitutions in bacteriocins have been found to impact on their antimicrobial activity (5, 6).

Defensins are amongst the most well studied group of antimicrobial peptides and are divided into two super-families (cis- and trans-defensins) which are thought to have evolved convergently (2). Defensin structures have been adapted to numerous functions in many organisms including antibacterial, antifungal, antiprotozoal, anticancer and cell signalling (7-12). There is also a structural and evolutionary relationship between the structures of antimicrobial invertebrate defensins and some animal toxins which target ion channels, such as scorpion toxins (13, 14). Defensins contain highly conserved cysteine residues, with distinct spacing within the peptides influencing disulphide bond orientation and defining the group

(3). The evolutionary ancestor of eukaryotic cysteine-stabilised α-helix β-sheet (CSαβ) cis-defensins has been suggested to be bacterial in nature, since similar encoded peptide sequences have been detected from myxobacterial soil species, albeit with fewer cysteine residues than typical defensin peptides (15). With the increased availability of genome sequence data, the evolutionary history of defensin and disulphide-rich peptides is being further investigated revealing widespread new related peptides which potentially share common ancestors (16). Regardless of their origin, defensin-like peptides have applications as antibacterial and antifungal agents for food preservation owing to their stability, heat tolerance and antimicrobial activity at low concentrations (17, 18).

In this study, we investigated the antimicrobial potential of *A. oris* CCUG 34826 which encodes seven actifensin gene homologs. Following this, we describe the characteristics of synthesised active linear AfnA from *A. ruminicola* DPC 7226 for the first time. Finally, using gene neighbouring and position specific iterative (PSI) BLAST, we describe the identification of *afnA* homologs outside the genus *Actinomyces* and a group of distantly related bacterial genes encoding cysteine-rich peptides which share homology with eukaryotic toxin peptides.

Materials and Methods

Bacterial culture, media reagents, and antimicrobial assays

The source of strains used in this study and their incubation conditions are listed in Table 1. Medium reagents were sourced from Sigma-Aldrich (Wicklow, Ireland) unless stated otherwise. Antimicrobial activity was assayed by overlaying strains grown on agar with 10 ml 'sloppy' agar (7.5 g litre^{-1} agar) tempered to 46 °C and seeded with a grown culture indicator organism (0.25% [vol/vol]). For well diffusion assays with cell free supernatant (CFS), 20 ml of sloppy agar seeded with an indicator species as described above and was poured and allowed to set, in which 6-mm-wide wells were then bored. Fifty microlitres of CFS was added to each well, and plates were incubated for 24-48 h. Zones of inhibition were indicative of antimicrobial activity. Minimum inhibitory concentrations (MICs) of purified/synthesised peptide were assayed by well diffusion as described, with peptide in aqueous solution in twofold serial dilutions. MIC was defined as the lowest concentration at which any inhibition around a well was observed.

A. oris CCUG 34286 AfnA induction experiment

Colonies of *A. oris* CCUG 34286, *A. ruminicola* DPC 7226, and *A. ruminicola* DSM 27982 were inoculated in duplicate into 10 ml BHI broths containing 0%, 1%, and 5% (vol/vol) active CFS from a previous 48 h culture of *A. ruminicola* DPC 7226. After 24 and 48 h of incubation, 1 ml was removed from the broth. Five microlitres was spotted onto agar for incubation and subsequent antimicrobial assay. The remaining broth was centrifuged at 12,000 x g for 5 min to sediment cells, and the supernatant was filtered (0.02 µm filter) for well diffusion assay. After antimicrobial assay of grown spots, the agar overlay was removed, and the spots were subject to colony mass spectrometry.

Colony mass spectrometry

Bacterial colonies were mixed with 50 µl of 70% isopropanol (IPA) containing 0.1% (v/v) trifluoroacetic acid (TFA). The bacterial IPA suspension was vortexed and

centrifuged at 20,817 x g for 1 min at 21 °C. Supernatant was retained for analysis. Mass spectrometry was performed in all cases with an Axima TOF^2 matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometer (Axima TOF^2 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) TFA) was placed onto the target. This matrix solution was left for 30 sec and then removed. The residual solution was then air dried and the previously prepared supernatant was positioned onto the pre-coated sample spot. Positive-ion linear mode was used to detect peptide masses.

Peptide synthesis

Actifensin active peptide was synthesised from the C terminus to the N terminus using microwave-assisted solid phase peptide synthesis (MW-SPPS) on a Liberty Blue microwave peptide synthesizer (CEM Corporation. Mathews, North Carolina, USA). Following deprotection the exposed amino group was coupled conventionally with the carbonyl group of the next amino-protected amino acid at 75 °C, 0W for 3600 sec in the presence of the activator, 0.5MN,N'-diisopropylcarbodiimide in DMF, and activator base, 1.1M hydroxybenzotriazole in DMF. The subsequent amino acids were added using microwave deprotection at 75 °C, 60W for 600 sec and microwave coupling at 75°C, 35W for 600 sec. Arginines were double coupled at 75 °C, 35W, 300 sec and histidine and cysteine coupled at 25 °C, 0W, 300 sec and then 50 °C, 35W for 900 sec. Following synthesis, the peptide was cleaved from the resin by adding a cleavage mix containing 9.25ml TFA, 250 µl water, 250 µl 2'2-(ethylenedioxy)-diethanethiol and 500 µl tri-isopropylsilane. This mixture was then heated at 37 °C for 1 h to cleave the peptide from the resin. Resin was removed from the cleavage mix using an Accent Cleavage system (CEM, Corporation. Mathews, North Carolina, USA) and the TFA evaporated by bubbling with nitrogen. Peptide was precipitated from the remaining solution by adding 45ml of diethyl ether pre-cooled to −20 °C and centrifuging at 1000 g for 3 min at 4 °C. The precipitated peptide was washed free of scavengers by resuspending in a second aliquot of 45ml ice cold diethyl ether and the centrifugation step repeated. Synthesis was performed

in duplicate and activity of synthesised actifensin was assayed by well diffusion as outlined above.

To determine disulphide formation over time, 100 µl of peptide in aqueous solution was stored at room temperature for seven days, and 0.5 µl was taken for MALDI-TOF MS fingerprint analysis as described above on days 0, 1, 2, 4, and 7.

In silico analyses

In order to identify distantly related homologs of actifensin, the NCBI bacterial database of non-redundant protein sequences was interrogated with position-specific iterative (PSI-)BLAST on default settings using the amino acid sequences of AfnG, AfnH, and AfnI from *A. ruminicola* DPC 7226 as input. Results above the default threshold (0.005) were checked for a source genome sequence. The genomes were then investigated for a similar genetic architecture (gene size and order) to actifensin and the presence of a nearby cysteine rich (>4 Cys residues) peptide ORF. Results positive for both were added to the search to generate the position-specific scoring matrix (PSSM) for the next iteration. The iterative process continued until there were no more new results above the cut-off threshold. Bacterial genome sequences of positive results were acquired from the NCBI GenBank database and visualised using Artemis genome browser (19). Sequence alignments and percent identity matrices were generated using MUSCLE (20). Alignments were adjusted and visualised using Jalview (21). Intrinsic transcription terminators were predicted using PePPER software (22).

Data availability.

Genomic data analysed in this study are publicly available from the NCBI GenBank database at https://www.ncbi.nlm.nih.gov/.

Results

Actinomyces oris CCUG 34286 encodes seven *afnA* homologs

Actinomyces oris CCUG 34286 was previously found to encode a gene cluster homologous to the actifensin operon in *A. ruminicola* DPC7226, but containing seven potential structural genes (*afnA1-7*) (1). The operons were largely similar apart from the number of *afnA* structural genes (Fig. 1a). Homologous genes were detected for *afnBCDEFGHIAJK* but not the predicted transport gene *afnL*. Identity between the encoded proteins ranged from 43.7% with AfnI (unknown function) to 70.6% with AfnK (predicted transporter) (Fig. 1a). The levels of identity between the AfnA propeptide and AfnA1-7 from *A. oris* CCUG 34286 ranged from 47.0% to 62.1%, with a mean identity of 54.2% (Fig. 1a).

A. oris CCUG 34286 was acquired from the American Type Culture Collection (strain designation ATCC 49340). In an initial screen, *A. oris* CCUG 34286 colonies produced small diffuse zones of inhibition against *Lactobacillus delbrueckii* ssp. *bulgaricus* in agar overlay (Fig. 1b). The phenotype was distinct from *A. ruminicola* DPC 7226 which potently inhibited indicator growth with large clear zones, and from *A. ruminicola* DSM 27982 which lacks genes for actifensin production and caused no inhibition (Fig. 1b). Using well diffusion assays, we also sought to determine the immunity of *A. oris* CCUG 34286 to purified AfnA compared with *A. ruminicola* DPC 7226 and *A. ruminicola* DSM 27982. *A. ruminicola* DSM 27982 was inhibited at 0.76 µM whereas *A. oris* CCUG 34286 exhibited the same level of immunity as the producer organism *A. ruminicola* DPC 7226 at 6.1 µM.

As *A. oris* CCUG 34286 lacked an antimicrobial phenotype, we cultured *A. oris* CCUG 34286 in the presence of cell-free supernatant (CFS) of *A. ruminicola* DPC 7226 to induce expression. *A. oris* CCUG 34286 cultured in broth with 1% and 5% CFS and spotted on agar exhibited the same negative production phenotype when overlaid with *L. bulgaricus* LMG6901 (Fig 2a). Well diffusion assays using CFS of *A. oris* CCUG 34286 cultured with *A. ruminicola* DPC 7226 CFS also failed to inhibit *L. bulgaricus* LMG6901 (data not shown). When *A. ruminicola* DSM 27982 was used as the

indicator organism, 2mm diffuse zones of inhibition were observed from the *A. oris* CCUG 34286 cultured without CFS, which increased in size to 5mm and 6mm with 1% and 5% CFS, respectively (Fig. 2a). Conversely, zones of inhibition produced by *A. ruminicola* DPC 7226 against *A. ruminicola* DSM 27982 were reduced from 8 mm to 7 mm when cultured with CFS (Fig. 2a). Mass spectrometry conducted on cells collected from under the overlay detected the ~4091 Da mass corresponding to actifensin from *A. ruminicola* DPC 7226 cultured with and without CFS (Fig. 2b). No mass corresponding to an AfnA homolog was detected from *A. oris* CCUG 34286 cells inhibiting the growth of *A. ruminicola* DSM 27982 (Fig. 2b). An investigation of intergenic sequences upstream of *afnA* and *afnK* in both strains detected no clear promoter sequences (data not shown). A 37 bp potential transcription terminator sequence (left stem: 5'- TGTGGGCTGTCAGATGC – 3', loop: 5' - CGA - 3', right stem: 5' – GCATCTGACAGCCCACA – 3') was detected between *afnA5* and *afnA6* in *A. oris* CCUG 34286.

Active actifensin can be produced by chemical synthesis

Active AfnA from *A. ruminicola* DPC 7226 could be chemically synthesised and inhibited *L. bulgaricus* LMG 6901 in well diffusion assay at 762 nM (Fig. 3). The MALDI-TOF MS mass fingerprint of synthesised peptide showed a mass between 4096 and 4097 Da, corresponding to an actifensin peptide lacking disulphide bonds, and a smaller peak at 3993 Da which could not be separated during the purification of the synthesised peptide (Fig. 3). To determine if disulphide bridges would form spontaneously, 1.0 and 0.1 mg ml^{-1} concentration solutions of natural and synthesised peptide were assayed by mass spectrometry after 0, 1, 2, 4, and 7 days stored at room temperature (Fig. 4). Natural actifensin at both concentrations was detected as a mass of 4091±1 Da which remained stable until day 7. Synthesised peptide at 1 mg ml^{-1} was detected as 4096 – 4097 Da at day 0 (Fig. 4). At 0.1 mg ml^{-1} the peptide was detected as 4094.7 Da on day 1 followed by 4095.9 Da on day 2, 4094.2 on day 4 and 4094.5 Da on day 7, potentially indicating the formation of a single disulphide bond (Fig. 4).

Gene clusters homologous to the actifensin operon in the phylum Actinobacteria

Gene clusters homologous to the actifensin production operon were found by gene neighbouring analysis and position specific iterative BLAST (PSI-BLAST) using amino acid sequences of the hypothetical proteins of unknown function AfnG, AfnH, and AfnI. Twenty-nine gene clusters throughout the phylum Actinobacteria were identified from the genera *Actinomyces, Corynebacterium, Micromonospora, Stackebrandtia, Nocardia, Kribella, Amycolatopsis, Streptomyces, Nonomuraea, Actinoplanes, Actinophytocola,* and *Actinomadura*. Twenty-two of the 29 clusters also encoded nearby genes with predicted transport and peptidase functions, albeit in alternate positions. The gene clusters were organised into three groups based on overall gene arrangement (Groups I-III) (Fig. 5).

Group I is most like the actifensin consensus operon previously found in the genus *Actinomyces*, with each cluster encoding homologs of *afnCDEFGHIA* in the same positions (Fig. 5). The group contains two *Actinomyces* spp. (*Actinomyces* sp. 340 and *Actinomyces* sp. 299), one unspeciated *Actinomycetaceae* isolate and *Corynebacterium* sp. HMSC06D04. Both *Actinomyces* genomes encode three copies of *afnA* that were not identified by BLAST analysis using the structural peptide sequence. Group II consists of clusters from the three strains *Micromonospora rosaria* DSM 803, *Stackebrandtia albiflava* DSM 45044, and *Nocardia pseudobrasiliensis* DSM 44290. In group II, homologs of *afnFGHIA* were detected in the same order as the actifensin operon. Downstream genes corresponding to *afnBCDE* are absent, but a predicted serine peptidase gene is encoded upstream from *afnA* which may serve the function of *afnE/C*. Group III is characterized by the presence of an additional ORF between *afnGHI* homologs and the structural gene. The group contains clusters from 22 strains, including 11 *Streptomyces* spp., 3 *Amycolatopsis* spp., 3 *Actinomadura* spp., *Kribella albertanoniae* JCM 30547, *Micromonospora citrea* DSM 43903, *Nonomuraea* sp. KC401, *Actinoplanes* sp. LAM 7112, and *Actinophytocola oryzae* DSM 45499. Of the 22 gene clusters, 15 contain a gene predicted to encode protease activity and another encoding transport,

immediately downstream from an *afnG* homolog, albeit in the reverse order of *afnEF* (Fig. 5).

The groups were further subdivided by position of cysteine residues within the ORF which encodes a cysteine rich peptide upstream of *afnGHI* homologs (Fig. 6). Group I encoded peptides exhibit a pattern of six cysteines with specific spacing, [C-$X_{(5-8)}$-C-$X_{(3)}$-C-$X_{(9)}$-C-$X_{(5-8)}$-C-$X_{(1)}$-C](where X is any non-Cys residue), which is almost identical to the previously identified AfnA peptide and gene homologs, [C-$X_{(3-8)}$-C-$X_{(3)}$-C-$X_{(9)}$-C-$X_{(5-8)}$-C-$X_{(1)}$-C]. Group II sequences contain eight cysteine residues, with two distinct patterns. The encoded peptides in *M. rosaria* DSM 803 and *S. albiflava* DSM 45044 (group IIa) have a similar pattern to group I and AfnA but with an additional two N-terminal Cys residues, [CC-$X_{(1)}$-C-$X_{(5)}$-C-$X_{(3)}$-C-$X_{(10)}$-C-$X_{(7)}$-C-$X_{(1)}$-C] (Fig 6a). Group IIb consists solely of the sequence from *N. pseudobrasiliensis* DSM 44290 which does not conform to the same pattern with [C-$X_{(3)}$-C-$X_{(9)}$-C-$X_{(8)}$-C-$X_{(5)}$-C-$X_{(4)}$-C-$X_{(1)}$-CC] (Fig. 6a). Group III is divided into three subgroups, IIIa and IIIb containing 6 cysteines, and IIIc consisting of a single encoded peptide from *Actinophytocola oryzae* DSM 45499 containing 11 Cys residues which does not align to the others (Fig 9b). Subgroup IIIa exhibits a [C-$X_{(6)}$-C-$X_{(5)}$-CC-$X_{(12)}$-CC] pattern in encoded peptides from *Streptomyces albidochromogenes* DSM 41800 and *Streptomyces helvaticus* DSM 40431. Subgroup IIIb contains 20 encoded peptides with a [C-$X_{(6)}$-C-$X_{(5-6)}$-CC-$X_{(3-8)}$-C-$X_{(4-12)}$-C] pattern (Fig 6b).

Group III sequences were analysed using BLASTp to identify defensin or defensin-like relatives. Two sequences (*Streptomyces* sp. WAC01529 and *Streptomyces* sp. NL152K) share homology with arthropod inhibitor cysteine knot peptides, Asilidin-Mar1A from *Machimus arthriticus* (Breck robberfly), Asilidin-Eru1A from *Eutolmus rufibarus* (Golden-tabbed robberfly) (Fig. 6c). The *Streptomyces* sequences share a mean identity of 33.0% and 50.0% with the arthropod propeptides and mature peptides, respectively. Mean identity between all encoded peptides was highly variable, ranging from 9.6% to 100.0% between sequences (Fig. 7).

Twelve of the non-*Actinomyces* strains containing actifensin operon homologs were acquired from the DSMZ culture collection (Table 1). Three of the

strains acquired (*Amycolatopsis alba* DSM 44262, *Streptomyces cyaneus* JCM 4220, and *Streptomyces albidochromogenes* DSM 41800) inhibited the growth of *L. bulgaricus* LMG 6901 in agar overlay (Fig. 8). Colony mass spectrometry of the inhibiting strains failed to detect masses corresponding to predicted peptide masses (Fig. 8).

Discussion

The genome of *A. oris* CCUG 34286 contains seven homologs of the actifensin structural gene, *afnA*, potentially coding for peptides (immature) with 47.0% to 62.1% identity to AfnA from *A. ruminicola* DPC 7226 (Fig. 1). However, the strain did not exhibit the same level of antimicrobial activity against the Gram-positive indicator species *L. delbrueckii* ssp. *bulgaricus* as the actifensin producer *A. ruminicola* DPC 7226 (Fig. 1c). *A. oris* CCUG 34286 cells had a similar immunity to purified actifensin peptide in solution as *A. ruminicola* DPC 7226 (6.1 µM). Self-immunity of a producer to their bacteriocin by encoding specific immunity proteins is characteristic of bacteriocin producers, and cross-immunity is known to occur between similar peptides (23-25). However, the mode of action and mechanism of immunity of actifensin are yet to be determined. The difference in the antimicrobial phenotype between *A. oris* CCUG 34286 and *A. ruminicola* DPC 7226 could potentially be attributed to downregulated expression of *afn* genes or the choice of indicator (insensitive) species, as bacteriocins can have a broad or narrow spectrum of inhibition (26). As none of the seven encoded *A. oris* CCUG 34286 peptides could be detected using MALDI-TOF mass spectrometry (Fig. 2b), it is likely genes encoding them were not expressed. Indeed, the finding that there appears to be a strong transcription terminator sequence preceding *afnA5* with no obvious promoter downstream could explain why at least five of the peptides could not be detected. In many species, bacteriocin expression is induced by the presence of the peptide itself or a specific inducer, but this can be affected by a variety of growth conditions such as temperature, pH, and salt concentration (27). Culturing *A. oris* CCUG 34286 with CFS of *A. ruminicola* DPC 7226 failed to induce production of detectable quantities of any of the seven encoded AfnA peptide homologs (Fig. 2).

AfnA from *A. ruminicola* DPC 7226 could be synthesised and was found to be active against *L. bulgaricus* LMG 6901, though a smaller 3,993 Da product could not be separated from the desired 4,097 Da peptide during the purification process (Fig. 5). The synthesised peptide also failed to form disulphide bonds as would be evidenced by a loss of 6 Da (-2 Da per bond) from ~4,097 Da to ~4,091 Da in the

observed mass. As such, the reduced activity of the synthesised peptide could be a result of the lack of disulphide bond formation. This is in contrast to previous research which has found that the linear form of bacteriocins, such as the class IIa bacteriocin Pediocin P-A1, can retain similar activity to the purified natural peptide (28). Bacteriocins containing disulphide bonds, such as bactofencin A, can rely on the activity of an accessory protein which ensures correct formation of a disulphide bond *in vivo* (29). Spontaneous bond formation has been observed in solution post-synthesis (30), however in this study, synthesised actifensin did not form the three disulphides observed in the natural peptide (Fig. 4). Recent studies have synthesised diverse defensins such as rattusin, and lucifensin, and successfully allowed correct disulphide bond formation through oxidative refolding (31, 32). However, such techniques have typically yielded low amounts of folded compound from large quantities of starting peptide can result in incorrect disulphide pairing. With optimisation, chemical synthesis could be a means for large scale production of linear active peptide as has been successful for several class II bacteriocins (33).

Given the ubiquity of defensins, and defensin-like structures in nature, we sought to identify actifensin-like genes in other bacterial clades. Nearby hypothetical proteins (AfnGHI) were used due to their larger size and proximity of their genes to the structural gene *afnA*, on the assumption that their conserved nature and unknown function was specific to the production of actifensin. The use of gene order and gene neighbours to identify genes of similar function and to unravel evolutionary history of proteins has been established as an effective method (34, 35). PSI-BLAST employs an iterative process using multiple inputs of related sequences to generate a position specific scoring matrix which is then used to identify distantly related sequences based on high-scoring conserved residues (36). Having a number of known gene homologs greatly increases the accuracy of PSI-BLAST, and so we used multiple AfnG, AfnH, and AfnI sequences from across the genus *Actinomyces* (37). We found 29 gene clusters throughout the Actinobacteria phylum, from species of the genera *Actinomyces, Corynebacterium, Micromonospora, Stackebrandtia, Nocardia, Kribella, Amycolatopsis, Streptomyces, Nonomuraea, Actinoplanes, Actinophytocola,* and *Actinomadura*, several of which are commensals of the skin and oral microbiota,

notable soil inhabitants, secondary metabolite producers, and pathogens (Fig. 5) (38). Gene neighbouring analysis revealed new *Actinomyces* strains (*Actinomyces* sp. 340, and *Actinomyces* sp. 299) harbouring multiple *afnA* genes which were not previously identified using BLAST and the AfnA propeptide sequence, increasing the number of *Actinomyces* spp. actifensin homologs by six (1). This search additionally revealed the presence of another *afnA* gene homolog within the genome of *Corynebacterium* sp. HMSC06D04 together with homologs of the related genes *afnCDEFGHI*, providing further evidence that actifensin production may occur outside the genus *Actinomyces.* It remains to be determined whether the genes have been recently transferred from one genus to another, or if they share a common ancestor. Clinically-relevant *Actinomyces* spp. and *Corynebacterium* spp. have been co-isolated from infection wounds and fluids, an environment which would enable them to interact (39). They are also members of the oral microbiome and there is evidence of a shared evolutionary ancestor for pilus assembly genes between the two genera (40, 41). The more distantly-related strains of group II, *M. rosaria* DSM 803 and *S. albiflava* DSM 45044 also contained likely homologs of the AfnA propeptide, as they shared many of the consistent features of AfnA sequence, including homologous leaders, conserved disulphide positions and a GXC motif (γ-core) (Fig. 6a). Both are soil microbes and *Micromonospora* spp., in particular, are of interest for the production of secondary metabolites due to their influence on plant growth (42, 43). The conserved peptide sequences within these strains as well as *Corynebacterium* sp. HMSC06D04 are highly similar to AfnA and structurally related to eukaryotic CSαβ defensins, though further studies may elucidate whether the relationship is divergent from an ancient bacterial precursor, or by convergent evolution which is thought to have occurred previously in eukaryotic defensins (2, 3).

Group III clustered separately from groups I and II based on operon arrangement, sequence structure and identity (Fig.5 and 6b, Fig. 7), and display a distinct cysteine banding pattern similar to that of inhibitory cysteine knot (ICK or knottin) peptides (Fig.6c). Like defensins, ICK peptides are a family of peptides containing a stable structural motif consisting of β-sheets stabilised by disulphide bonds in the form of a knot (44). ICK peptides are produced by animals, plants and

fungi, where they have been adapted to numerous functions, but are prominent in the venom of cone snails and spiders (45). The two near identical encoded peptide sequences we identified in *Streptomyces* sp. WAC01529 and *Streptomyces* NL15-2K were found to be 50 % identical to the mature sequences of the venom peptides of predatory robber flies, Asilidin1-Mar1A and Asilidin1-Eru1A (46). Antimicrobial activity of *A. alba* DSM 44262, *S. cyaneus* JCM 4220, and *S. albidochromogenes* DSM 41800 could not be traced to the ICK-like peptides by mass spectrometry (Fig. 8). This is unsurprising given the degree of difference between the encoded peptides and actifensin, as well as the propensity of soil actinomycetes to produce antimicrobial compounds (47, 48). The encoded peptides have unknown activity and may not be expressed, explaining the lack of corresponding masses by mass spectrometry. The function of *afnGHI* and distantly related homologs remains to be determined, yet they appear to be a conserved feature downstream from a disulphide rich ORF containing a γ-core motif. Future knockout and cloning experiments may help to determine if these genes are essential to produce correctly folded AfnA (and homologs) or are involved in immunity. ICK peptides are being investigated as bio-insecticides (49), therefore the encoded group III peptides identified here could be expressed and assessed for insecticidal activity and anti-eukaryotic activity as well as determining their antimicrobial activity.

This study further elaborates on the biology of actifensin, the antimicrobial produced by *A. ruminicola* DPC 7226, and describes the characteristics of a synthetic peptide. We also identify homologous ORFs in the genomes of high-GC content non-*Actinomyces* species, expanding the family of known potential actifensin-like peptides. These data raise further questions regarding the evolutionary history between defensin and toxin structures in prokaryotes and eukaryotes and illustrate their conserved trans-kingdom nature.

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Figures and Tables

Figure 1. (a) Gene comparison of actifensin operon and homologous cluster containing seven peptides in *A. oris* CCUG 34286. Colour blocks connecting genes indicate degree of % amino acid identity of encoded proteins. (b) Multiple sequence alignment of AfnA1-7 from *A. oris* CCUG 34286 with AfnA from *A. ruminicola* DPC 7226 and predicted mass based on sequence (with 6 Da deducted to account for 3 disulphide bonds). Predicted cleavage site indicated by arrow. (c) Agar overlays of *A. ruminicola* DSM 27982 (negative control), *A. ruminicola* DPC 7226 (actifensin producer), and *A. oris* CCUG 34286. Lighter colour = zone of clearing in overlay. Indicator organism: *L. delbrueckii* ssp. *bulgaricus* LMG6901.

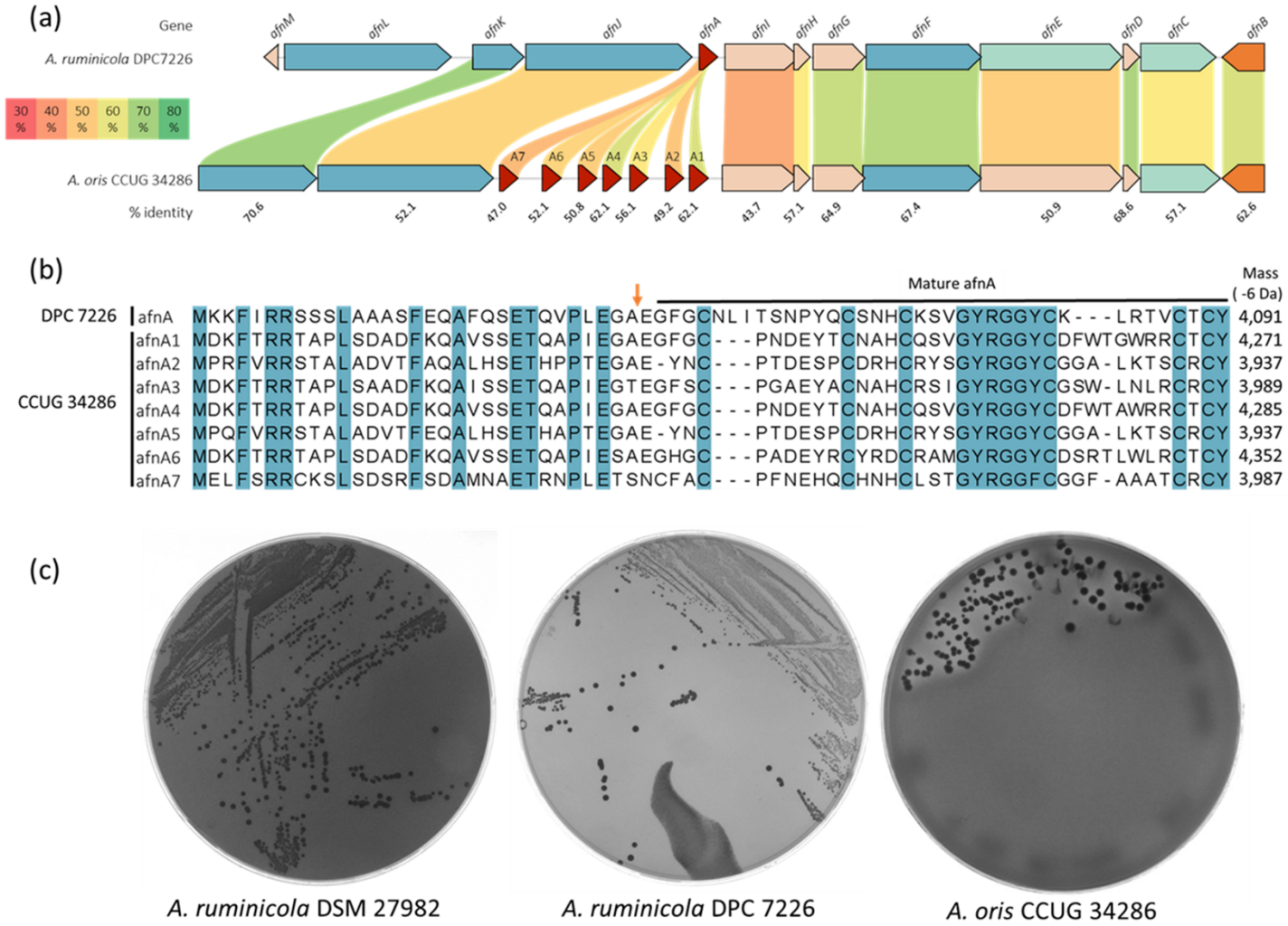

(a)
Gene
A. ruminicola DPC7226
30 %
40 %
50 %
60 %
70 %
80 %
A. oris CCUG 34286
% identity
afnM
afnL
afnK
afnJ
afnA
afnI
afnH
afnG
afnF
afnE
afnD
afnC
afnB
A7
A6
A5
A4
A3
A2
A1
70.6
52.1
47.0
52.1
50.8
62.1
56.1
49.2
62.1
43.7
57.1
64.9
67.4
50.9
68.6
57.1
62.6
(b)
Mature afnA
Mass (-6 Da)
DPC 7226
CCUG 34286
afnA MKKFIRRSSSLAAASFEQAFQSETQVPLEGAEGFGCNLITSNPYQCSNHCKSVGYRGGYCK---LRTVCTCY 4,091
afnA1 MDKFTRRTAPLSDADFKQAVSSETQAPIEGAEGFGC---PNDEYTCNAHCQSVGYRGGYCDFWTGWRRCTCY 4,271
afnA2 MPRFVRRSTALADVTFAQALHSETHPPTEGAE-YNC---PTDESPCDRHCRYSGYRGGYCGGA-LKTSCRCY 3,937
afnA3 MDKFTRRTAPLSAADFKQAISSETQAPIEGTEGFSC---PGAEYACNAHCRSIGYRGGYCGSW-LNLRCRCY 3,989
afnA4 MDKFTRRTAPLSDADFKQAVSSETQAPIEGAEGFGC---PNDEYTCNAHCQSVGYRGGYCDFWTAWRRCTCY 4,285
afnA5 MPQFVRRSTALADVTFEQALHSETHAPTEGAE-YNC---PTDESPCDRHCRYSGYRGGYCGGA-LKTSCRCY 3,937
afnA6 MDKFTRRTAPLSDADFKQAVSSETQAPIESAEGHGC---PADEYRCYRDCRAMGYRGGYCDSRTLWLRCTCY 4,352
afnA7 MELFSRRCKSLSDSRFSDAMNAETRNPLETSNCFAC---PFNEHQCHNHCLSTGYRGGFCGGF-AAATCRCY 3,987
(c)
A. ruminicola DSM 27982
A. ruminicola DPC 7226
A. oris CCUG 34286

Figure 2. (a) Spots of *A. ruminicola* DPC 7226 (1, 4), *A. ruminicola* DSM 27982 (2, 5), and *A. oris* CCUG 34286 (3, 6) cultured with 0, 1, or 5% DPC 7226 CFS, overlaid with *L. bulgaricus* LMG6901 or *A. ruminicola* DSM 27982. (b) Mass spectrometry results of corresponding numbered spots from (a). .

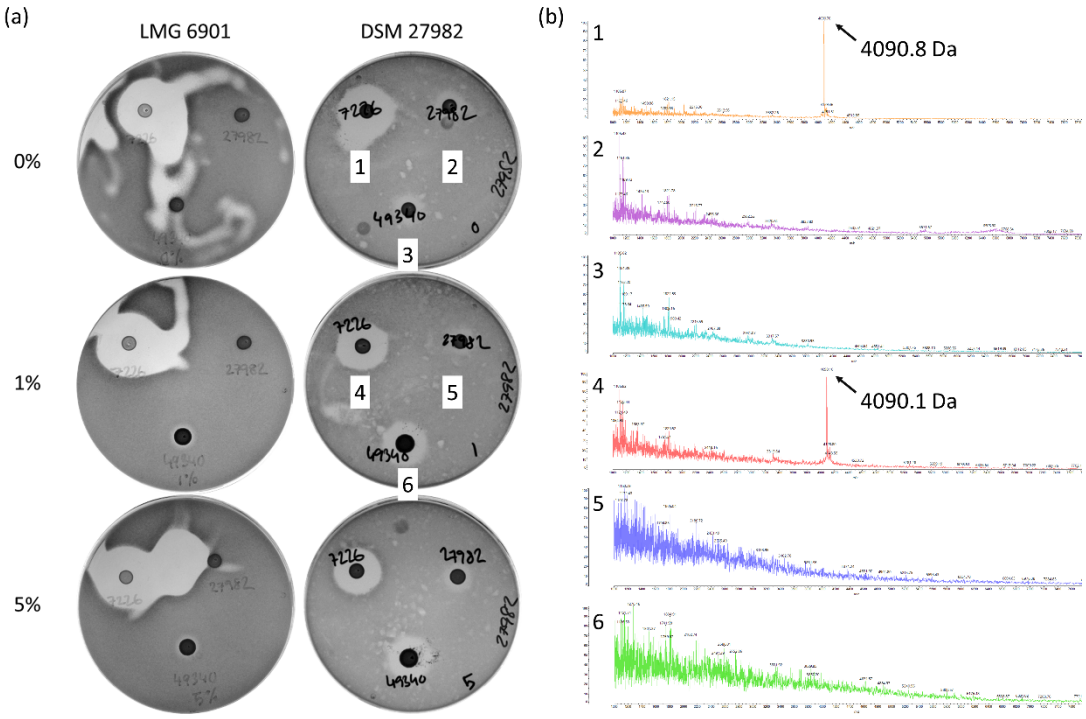


Figure 3. Activity and mass spectra of natural purified (P) and chemically synthesised (S) actifensin.

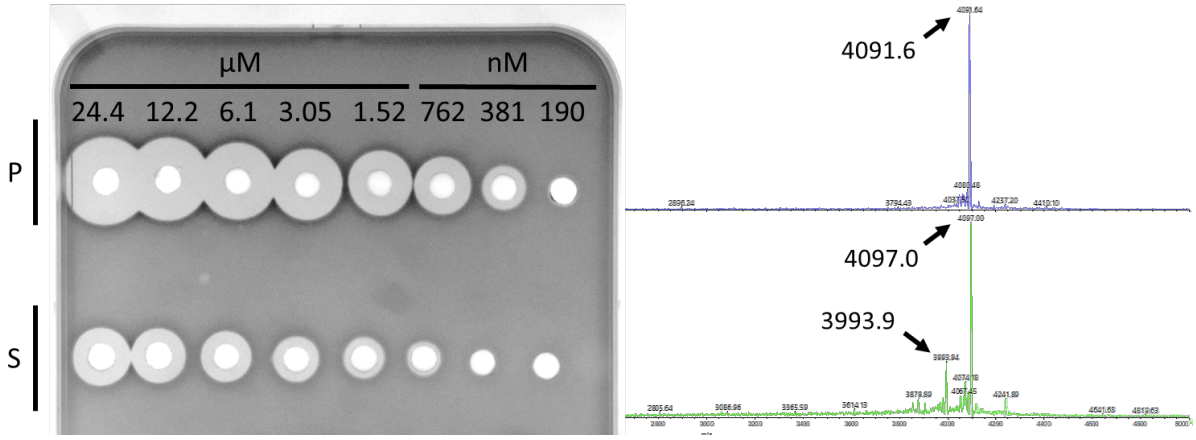


Figure 4. Mass spectra (contracted) of natural and synthesised actifensin solutions stored at room temperature for 7 d. Arrows: Synthesised peptide with a mass below 4095 Da which may have formed a single disulphide bond.

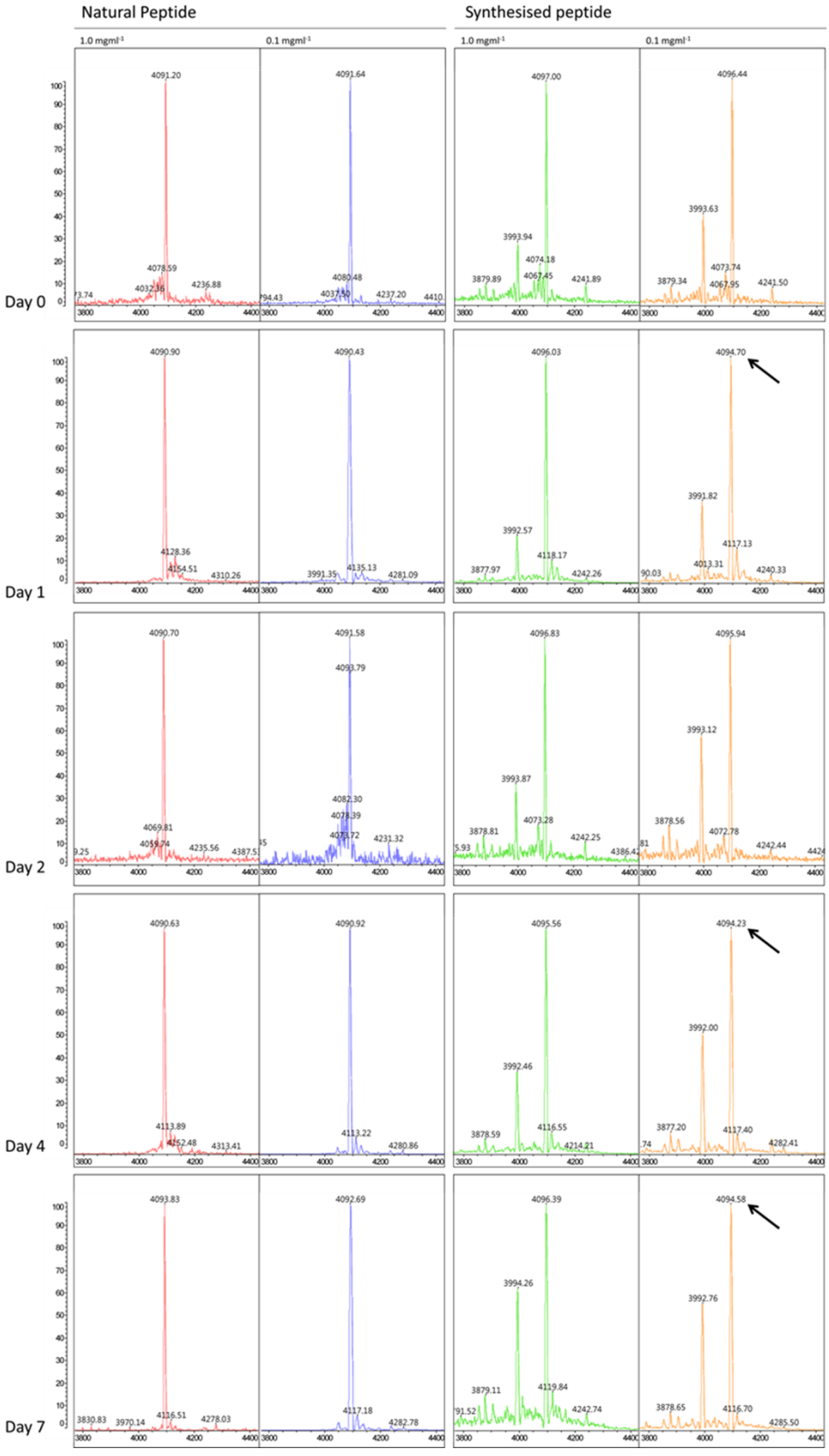


Figure 5. (a) Gene cluster comparison of actinobacterial actifensin homologs identified by gene neighbouring. Underlined strains were acquired for characterization.

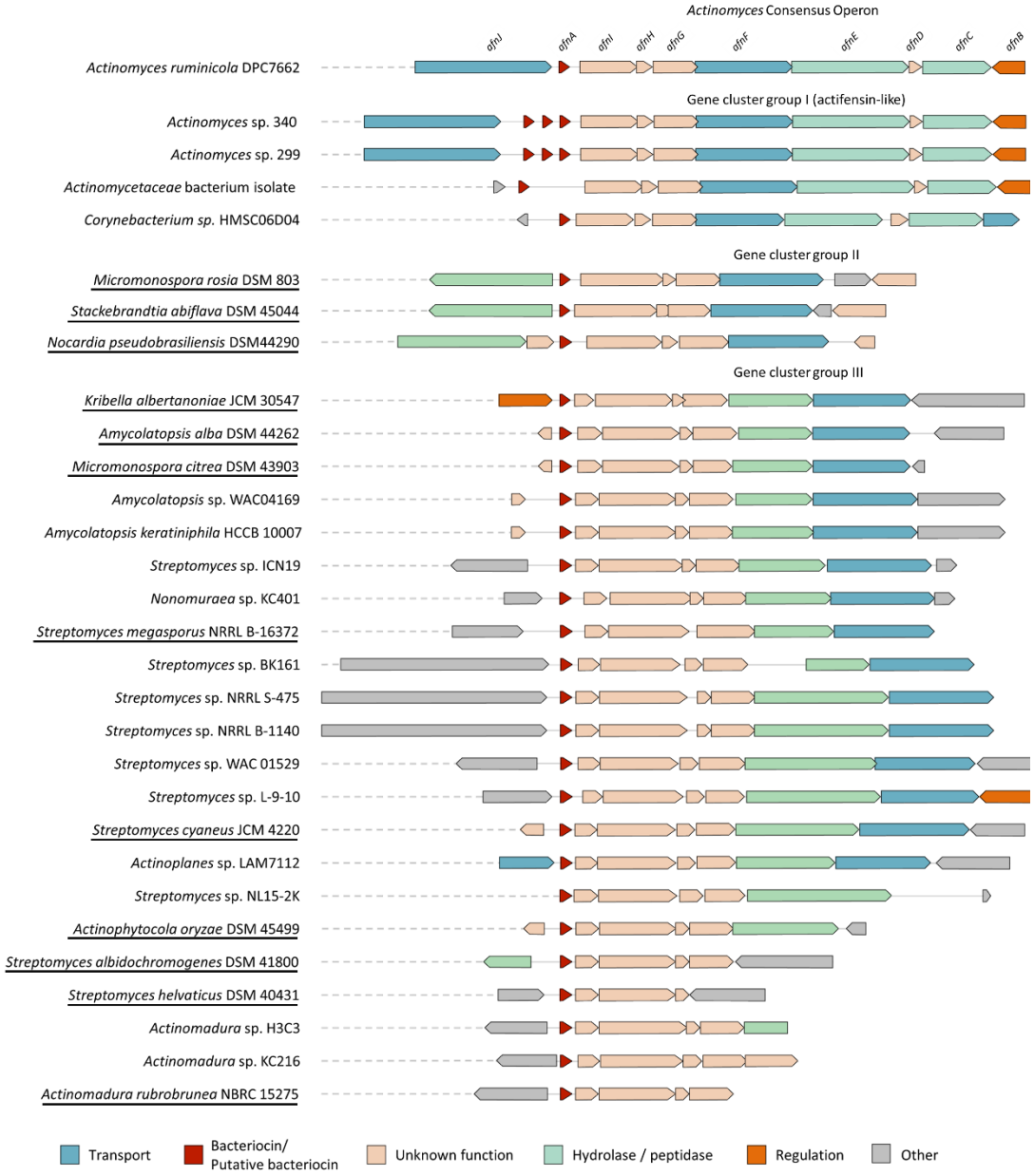


Figure 6. Multiple sequence alignments of cysteine rich ORFs from (a) Group I, Group II, and (b) Group III identified in gene neighbouring analysis. (c) Alignment of group III peptides with homologous eukaryotic inhibitor cysteine knot peptides (ICKs).

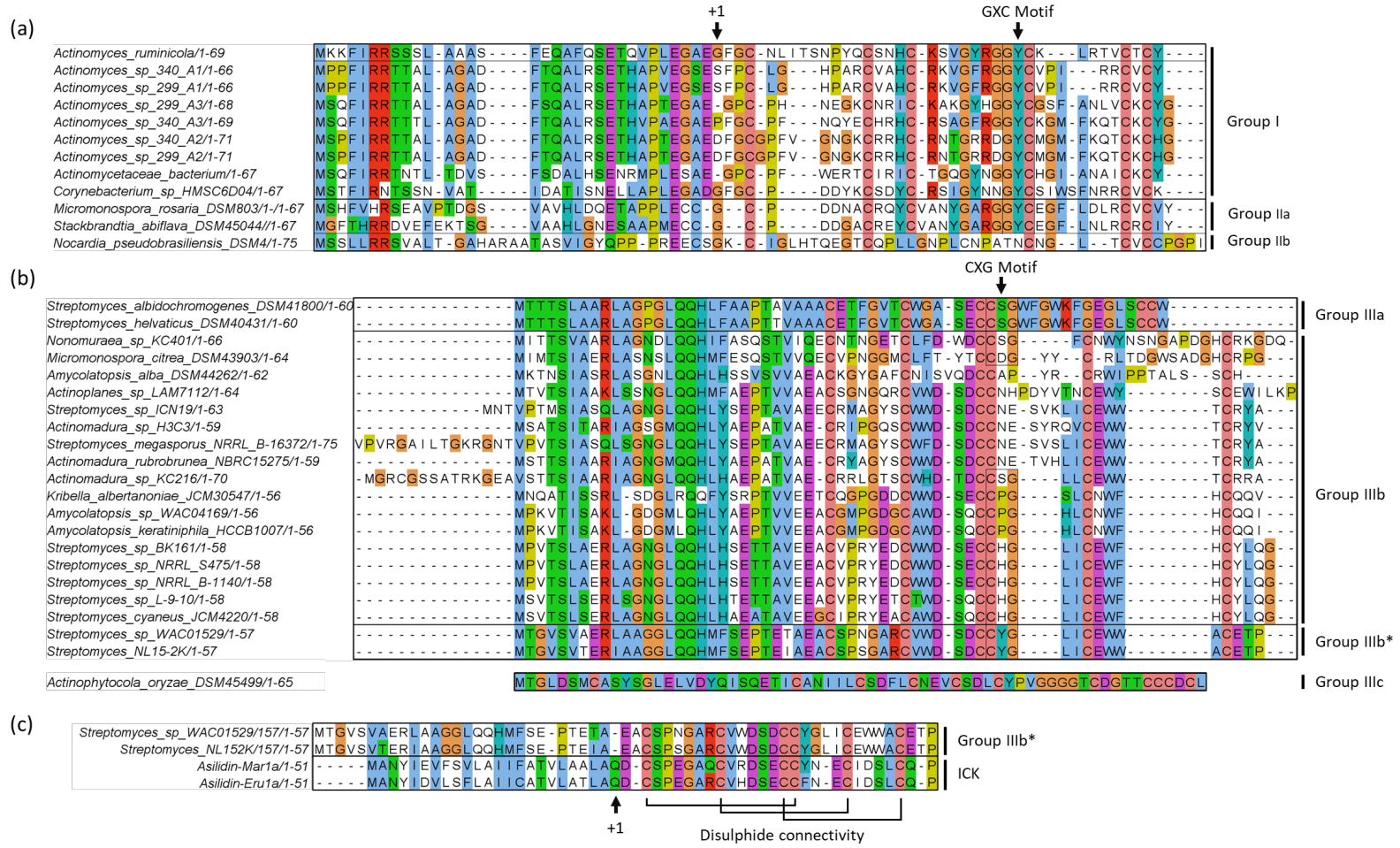


Figure 7. Percent identity matrix of cysteine rich ORFs identified by gene neighbouring analysis and AfnA from *Actinomyces ruminicola* DPC 7226

Order	Family	Genus_species_strain		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
Pseudonocardiales	Pseudonocardiaceae	Actinophytocola_oryzae_DSM45499	1	100	17	19	23	20	22	22	18	23	21	21	17	17	19	16	16	16	20	20	17	13	18	19	21	17	19	19	19	19	18	20	19	15	15
Corynebacteriales	Nocardiaceae	Nocardia_pseudobrasiliensis_DSM4	2	17	100	25	24	25	27	27	30	28	26	26	20	18	9.6	13	13	17	18	16	11	10	15	15	13	17	13	13	13	17	18	18	15	14	18
Corynebacteriales	Corynebacteriaceae	Corynebacterium_sp_HMSC6D04	3	19	25	100	45	38	39	39	42	42	41	41	26	29	12	19	19	17	13	11	16	16	17	19	20	19	17	17	17	17	19	19	17	18	16
Actinobacteriales	**Actinomycetaceae**	**Actinomyces_ruminicola**	4	23	24	45	100	45	53	53	54	59	47	47	33	28	25	27	27	27	14	14	16	21	18	25	23	25	23	23	23	23	21	19	19	16	16
Actinobacteriales	Actinomycetaceae	Actinomycetaceae_bacterium	5	20	25	38	45	100	43	43	60	52	43	43	32	27	19	21	21	19	13	13	13	19	16	20	20	20	22	22	22	20	19	17	19	18	16
Actinobacteriales	Actinomycetaceae	Actinomyces_sp_340_A1	6	22	27	39	53	43	100	100	60	64	64	64	31	28	18	22	22	17	18	16	20	20	14	20	20	25	29	29	29	24	24	22	22	19	18
Actinobacteriales	Actinomycetaceae	Actinomyces_sp_299_A1	7	22	27	39	53	43	100	100	60	64	64	64	31	28	18	22	22	17	18	16	20	20	14	20	20	25	29	29	29	24	24	22	22	19	18
Actinobacteriales	Actinomycetaceae	Actinomyces_sp_299_A3	8	18	30	42	54	60	60	60	100	65	65	65	30	30	17	25	25	21	21	20	19	19	15	22	22	31	33	33	33	29	24	22	22	21	20
Actinobacteriales	Actinomycetaceae	Actinomyces_sp_340_A3	9	23	28	42	59	52	64	64	65	100	74	74	31	28	19	24	24	20	19	19	17	17	17	22	22	25	29	29	29	23	24	22	24	23	21
Actinobacteriales	Actinomycetaceae	Actinomyces_sp_340_A2	10	21	26	41	47	43	64	64	65	74	100	100	31	30	17	22	22	15	18	16	17	14	15	18	18	25	29	29	29	23	25	24	20	21	19
Actinobacteriales	Actinomycetaceae	Actinomyces_sp_299_A2	11	21	26	41	47	43	64	64	65	74	100	100	31	30	17	22	22	15	18	16	17	14	15	18	18	25	29	29	29	23	25	24	20	21	19
Micromonosporales	Micromonosporaceae	Micromonospora_rosaria_DSM803	12	17	20	26	33	32	31	31	30	31	31	31	100	63	11	25	25	18	9.1	9.1	10	17	13	19	19	21	21	21	21	22	13	13	18	19	15
Glycomycetales	Glycomycetaceae	Stackbrandtia_abiflava_DSM45044	13	17	18	29	28	27	28	28	30	28	30	30	63	100	11	27	27	16	15	13	12	13	16	16	16	21	21	21	21	21	20	18	23	22	20
Pseudonocardiales	Pseudonocardiaceae	Amycolatopsis_alba_DSM44262	14	19	9.6	12	25	19	18	18	17	19	17	17	11	11	100	30	30	34	40	40	35	38	38	39	35	33	37	37	37	33	38	34	38	32	35
Pseudonocardiales	Pseudonocardiaceae	Amycolatopsis_sp_WAC04169	15	16	13	19	27	21	22	22	25	24	22	22	25	27	30	100	100	64	44	44	35	29	50	41	39	52	48	48	48	50	46	45	42	49	47
Pseudonocardiales	Pseudonocardiaceae	Amycolatopsis_keratiniphila_HCCB	16	16	13	19	27	21	22	22	25	24	22	22	25	27	30	100	100	64	44	44	35	29	50	41	39	52	48	48	48	50	46	45	42	49	47
Propionibacteriales	Nocardiodaceae	Kribella_albertanoniae_JCM30547	17	16	17	17	27	19	17	17	21	20	15	15	18	16	34	64	64	100	40	40	31	32	43	39	38	43	48	48	48	43	43	41	36	42	38
Streptomycetales	Streptomycetaceae	Streptomyces_albidochromogenes_D	18	20	18	13	14	13	18	18	21	19	18	18	9.1	15	40	44	44	40	100	98	52	41	49	53	49	53	59	59	59	53	50	48	56	47	53
Streptomycetales	Streptomycetaceae	Streptomyces_helvaticus_DSM40431	19	20	16	11	14	13	16	16	20	19	16	16	9.1	13	40	44	44	40	98	100	54	43	49	53	49	51	57	57	57	51	48	46	58	49	55
Streptosporangiales	Streptosporangiaeceae	Nonomuraea_sp_KC401	20	17	11	16	16	13	20	20	19	17	17	17	10	12	35	35	35	31	52	54	100	63	45	41	36	42	42	42	42	40	43	45	49	44	49
Micromonosporales	Micromonosporaceae	Micromonospora_citrea_DSM43903	21	13	10	16	21	19	20	20	19	17	14	14	17	13	38	29	29	32	41	43	63	100	42	42	37	40	44	44	44	40	37	39	41	36	39
Micromonosporales	Micromonosporaceae	Actinoplanes_sp_LAM7112	22	18	15	17	18	16	14	14	15	17	15	15	13	16	38	50	50	43	49	49	45	42	100	65	60	47	48	48	48	48	48	52	50	51	51
Streptomycetales	Streptomycetaceae	Streptomyces_sp_WAC01529	23	19	15	19	25	20	20	20	22	22	18	18	19	16	39	41	41	39	53	53	41	42	65	100	93	51	56	56	56	51	54	51	48	48	52
Streptomycetales	Streptomycetaceae	Streptomyces_NL15-2K	24	21	13	20	23	20	20	20	22	22	18	18	19	16	35	39	39	38	49	49	36	37	60	93	100	49	53	53	53	49	51	47	48	52	52
Streptomycetales	Streptomycetaceae	Streptomyces_cyaneus_JCM4220	25	17	17	19	25	20	25	25	31	25	25	25	21	21	33	52	52	43	53	51	42	40	47	51	49	100	84	84	84	88	51	51	52	48	54
Streptomycetales	Streptomycetaceae	Streptomyces_sp_BK161	26	19	13	17	23	22	29	29	33	29	29	29	21	21	37	48	48	48	59	57	42	44	48	56	53	84	100	100	100	88	58	58	52	46	54
Streptomycetales	Streptomycetaceae	Streptomyces_sp_NRRL_S475	27	19	13	17	23	22	29	29	33	29	29	29	21	21	37	48	48	48	59	57	42	44	48	56	53	84	100	100	100	88	58	58	52	46	54
Streptomycetales	Streptomycetaceae	Streptomyces_sp_NRRL_B-1140	28	19	13	17	23	22	29	29	33	29	29	29	21	21	37	48	48	48	59	57	42	44	48	56	53	84	100	100	100	88	58	58	52	46	54
Streptomycetales	Streptomycetaceae	Streptomyces_sp_L-9-10	29	19	17	17	23	20	24	24	29	23	23	23	22	21	33	50	50	43	53	51	40	40	48	51	49	88	88	88	88	100	49	53	48	45	50
Streptomycetales	Streptomycetaceae	Streptomyces_sp_ICN19	30	18	18	19	21	19	24	24	24	24	25	25	13	20	38	46	46	43	50	48	43	37	48	54	51	51	58	58	58	49	100	90	64	64	78
Streptomycetales	Streptomycetaceae	Streptomyces_megasporus_NRRL_B-1	31	20	18	19	19	17	22	22	22	22	24	24	13	18	34	45	45	41	48	46	45	39	52	51	47	51	58	58	58	53	90	100	56	63	75
Streptosporangiales	Thermonosporaceae	Actinomadura_sp_KC216	32	19	15	17	19	19	22	22	22	24	20	20	18	23	38	42	42	36	56	58	49	41	50	48	48	52	52	52	52	48	64	56	100	70	79
Streptosporangiales	Thermonosporaceae	Actinomadura_sp_H3C3	33	15	14	18	16	18	19	19	21	23	21	21	19	22	32	49	49	42	47	49	44	36	51	48	52	48	46	46	46	45	64	63	70	100	80
Streptosporangiales	Thermonosporaceae	Actinomadura_rubrobrunea_NBRC152	34	15	18	16	16	16	18	18	20	21	19	19	15	20	35	47	47	38	53	55	49	39	51	52	52	54	54	54	54	50	78	75	79	80	100
										10	20	30	40	50	60	70	80	90	100																		

Figure 8. Antimicrobial activity and mass spectrometry results of strains containing actifensin homolog gene clusters.

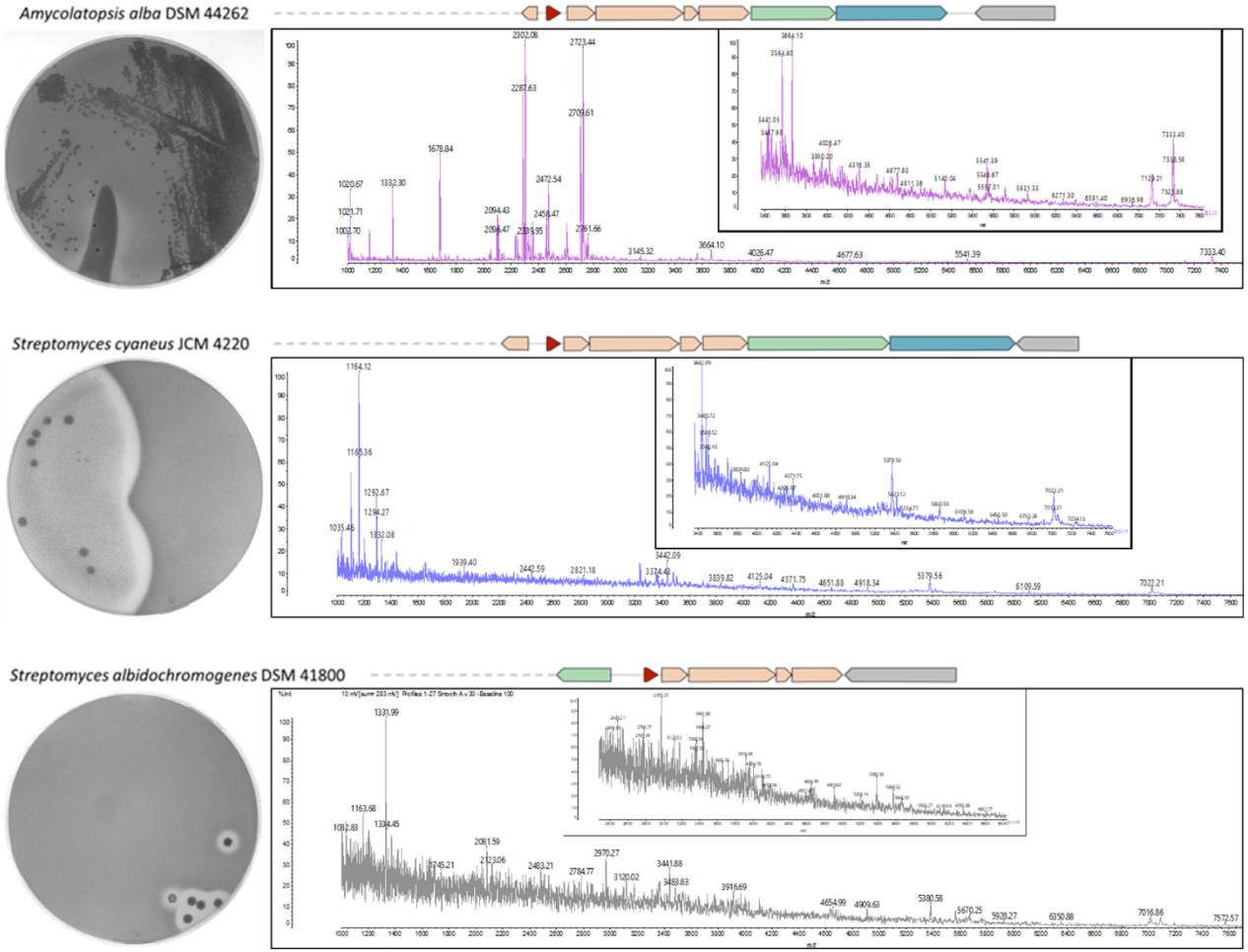


Table 1. Strains and culture conditions used in this study.

Species	Strain	Alternate code	Temp. (°C)	Medium	O_2
Actinomadura rubrobrunea	NBRC 15275	DSM 43750	55	ROMM*	+
Actinomyces oris	CCUG 34286	ATCC 43930	37	BHI	-
Actinomyces ruminicola	DPC 7226	-	37	BHI	-
Actinomyces ruminicola	DSM 27982	-	37	BHI	-
Actinophytocola oryzae	DSM 45499	-	28	TSB	+
Amycolatopsis alba	DSM 44262	-	28	GYM†	+
Kribella albertananoniae	JCM 30547	DSM 26744	28	GYM†	+
Lactobacillus delbrueckii ssp. *bulgaricus*	LMG 6901	-	37	MRS	-
Micromonospora citrea	DSM 43903	-	28	GYM†	+
Micromonospora rosaria	DSM 803	-	28	GYM†	+
Nocardia pseudobrasiliensis	DSM 44290	-	37	TSB	+
Stackebrandtia albiflava	DSM 45044	-	28	GYM†	+
Streptomyces albidochromogenes	DSM 41800	-	28	GYM†	+
Streptomyces cyaneus	JCM 4220	DSM 40108	28	GYM†	+
Streptomyces helvaticus	DSM 40431	-	28	GYM†	+
Streptomyces megasporus	NRRL B-16372	DSM 41476	45	GYM†	+

*Rolled oats mineral medium (International Streptomyces project (ISP) medium 2), and †glucose, yeast, maltose medium (ISP medium 3) from Shirling and Gottlieb (1966).

Chapter 7. Discussion

Chapter 1 provides a summary of food-borne pathogens associated with raw milk and the risks associated with its consumption, including spore-forming bacteria which are hazardous to human health and resistant to pasteurisation. An overview of recent research on microbial by-products of dairy fermentation describes a range of metabolites which affect the shelf life and quality of the product and the impact of said metabolites on consumer health. Bacterial metabolites such as biogenic amines, bioactive peptides, and antimicrobial peptides known as bacteriocins, and recent methods of identification and characterization are described.

Chapter 2 describes the production and techno-functional characterization of yoghurt produced using bovine milk derived from different diets, perennial ryegrass (GRS) and total mixed ration (TMR). This study expands upon a number of publications in recent years describing the effects of bovine diet on milk and products thereof, which have mainly highlighted the advantageous aspects of a grass based diet (1-6). By analysing several quality indicating characteristics such as yoghurt composition, texture, culture viability, and volatiles, we determined that yoghurt manufactured using bovine milk from a GRS based diet contained more protein, less lactose, altered colour and improved late shelf life texture characteristics compared with yoghurt manufactured using bovine milk from the TMR diet. TMR yoghurts had increased quantities of many volatile compounds associated with typical natural yoghurt odour and flavour. Within these data are several new findings and scope for future studies.

To expand upon these data, further research would benefit from sensory analysis to determine if the differences found in volatiles between yoghurts from the different diets can be perceived by the consumer, and if dairy products derived from animals on TMR or GRS diets are preferred. This could also be investigated in relation shelf life, to establish if the increased protein content of the yoghurt manufactured using bovine milk from a GRS based diet has perceptively improved texture after long periods of cold storage. As we investigated the impact of the two diets on an artisanal style yoghurt (pasteurised whole milk without standardising for fat and protein content) further research is needed to determine if the effects observed in this study also apply to a commercially-produced alternative. In addition, future studies should

investigate other compositional attributes which are affected by diet, such as mineral, amino acid, and fatty acid content. Indeed, milk, cheese, and butter manufactured using bovine milk from a GRS based diet can be considered healthier due to higher levels of (mono- and poly-) unsaturated fats and, improved protein content relative to their TMR-derived counterparts (1-3), so too could a yoghurt containing 'healthier fat' be produced. Not only can dairy products manufactured using milk from a GRS-based diet be considered beneficial to consumers, but also, due to improved colour attributes (which is strongly correlated with increased trans-β-carotene content) can be more pleasing to the consumer's eye. These studies could also be complemented through addition of adjunct cultures capable of producing compounds that confer desirable flavour and texture enhancing, and shelf life extending functions, to develop a true long-life (both to consumer and fermentate) product.

In Chapter 3, the application of a well-studied lantibiotic, nisin, to preserve and extend the shelf life of commercial pasteurised milk is described. This study was planned as part of the European funded Joint Programming Initiative – A Healthy Diet for a Healthy Life (JPI-HDHL) LONGLIFE project which aimed to investigate the application of beneficial LAB and their fermentation by-products to develop new foods and ingredients with improved functionality and shelf life. A range of nisin concentrations were tested to determine the minimum amount of nisin in milk which would inhibit spoilage organism growth. Nisin A impacted spoilage organisms at 1 - 10 μg ml^{-1} and completely prevented growth at 1 mg ml^{-1} for up to 49 days in cold storage. This study, though limited in scope, determined the efficacy of using a bacteriocin for shelf life extension of pasteurised milk, establishing a nisin concentration around which to carry out future studies and to investigate cost/benefit analysis of addition. Nisin has previously been investigated extensively in various dairy products, including for the elimination of pathogenic organisms in milk (7). However, few studies have investigated the effect of nisin (and other bacteriocins) beyond the typical shelf life of milk. Nisin is uniquely positioned to be included as a milk preservative, given its clean label and long history of safe use in

food. It is also particularly effective at inhibiting the growth of Gram-positive spore-formers which are a leading cause of milk spoilage.

Many factors, however, must be considered to implement the application of nisin, and other bacteriocins in liquid milk for direct consumption. A major limitation includes cost, as the cost of additional processing, wherein nisin must be produced, purified, and added to milk, will likely be passed on to the consumer. Therefore, the impact on shelf life must be substantial, and the sensory effects must be minimal for an increased cost product to be desired and/or tolerated by the consumer. The market placement for an extended shelf life milk without the sensory attributes of UHT treatment should also be considered, as consumers in countries where UHT milk is popular may be accustomed to, and even prefer UHT milk. The cost of an extended shelf life milk must also be compared to alternatives, as in many countries UHT milk is available to the consumer at a lower cost than standard HTST pasteurised whole milk. Using advertised costs of Nisaplin® and the lowest determined concentration at which an effect was observed, an additional manufacturing cost of €0.011 to €0.035 per litre was estimated.

Future work should investigate the effects of nisin addition to milk on the sensory attributes such as flavour, odour, mouthfeel, and colour. Optimisation of methods to include nisin in milk must be investigated in order to achieve optimum activity. Novel delivery methods into the milk could be investigated such as generation of nisin coated packaging on the interior of containers, enabling minimal disruption to the milk production line by inclusion in the final step. Studies could also investigate addition of nisin to raw bulk-tank milk prior to pasteurisation which may reduce initial microbial load and continue to be active post-pasteurisation. Other milk types should also be considered for application of nisin, such as skim, low-fat and flavoured milks (e.g. chocolate, vanilla), as composition can affect the activity of the peptide. Should nisin in milk impact the sensory attributes it may prove more useful in flavoured milks which could mask any potential negative side effects of nisin addition.

The data presented in Chapter 3 indicate nisin can preserve milk, though at relatively high concentrations of peptide. Future work may investigate the

incorporation of a combined hurdle antimicrobial approach, in order to reduce the required concentration of peptide which may reduce the monetary cost of addition. Other bacteriocins, compounds (e.g. organic acids, essential oils), or physical treatments (e.g. high pressure), which can sensitize Gram negative spoilage organisms to nisin may be of interest. A study to identify compounds which synergise with nisin may involve the generation of a contemporary biobank of milk spoilage organisms against which to screen a panel of combined compounds which can then be tested in milk. As bacteriocins are frequently isolated with activity against closely related species, compounds from spoilage organisms are not to be overlooked as they may be uniquely positioned to inhibit a broad range of spoilage organisms.

In Chapter 4, the identification of novel bacteriocin producing LAB isolated from diverse, but mainly raw milk sources, is described. Of 823 strains, seven strains were identified as putative bacteriocin producers. Following whole genome sequencing and predictive analysis, four high quality genomes were generated containing gene clusters for the production of diverse bacteriocin types, including class I (posttranslationally modified, type I and II lantibiotics) and class II (unmodified) peptides. There is further characterization to be performed with regard to the strains isolated in this study, and the bacteriocin encoding genes identified in their genomes.

The pathogenic species *Streptococcus uberis* is one of the leading causes of bovine mastitis worldwide and in turn, one of the leading bacterial causes of milk losses (8). As such, *Streptococcus uberis* LL-383 is of interest as an antimicrobial producing isolate encoding multiple bacteriocin gene clusters (BCGs). The strain can be further characterized by elucidating which, if not all, of the compounds are produced and under what conditions. Future studies may determine the abundance of bacteriocin production gene clusters in the *S. uberis* pan/core genome and establish if the bacteriocin production can act as a virulence/niche factor which enables improved colonisation relative to competing species. Spectra of inhibition by well diffusion assays, purification via HPLC and MALDI-TOF MS along with structural analysis of novel peptides could be utilized to further characterise the strain's antimicrobial potential.

Streptococcus spp. LL-387 and LL-514 which were isolated from sheep milk of different New Zealand farms, are closely related strains of the taxonomically complicated *Streptococcus bovis/equinus* complex (SBSEC). Both strains were found to encode genes for the same novel nisin variant, we named nisin I. Further research is required to purify the peptide and establish its spectrum of inhibition and activity relative to other nisin variants. The isolation of two strains encoding the same novel nisin variant from separate farms could imply a wide prevalence of the BCGs among raw sheep milk isolates. Further analysis may reveal how pervasive the nisin I operon is among milk isolate genomes and metagenomes of milk samples.

Lactococcus lactis LL-427 was found to encode a novel BCG similar to carnolysin (*Carnobacterium maltaromaticum*) and enterococcal cytolysin (*Enterococcus faecalis*). As such, future research should determine if the novel bacteriocin acts as a toxin, as does cytolysin (9). Any potential toxin activity would limit future food applications of the strain regardless of its GRAS status. A range of experiments could investigate bacteriocin expression, purification, spectrum of activity and safety. Based on the data in this thesis there is also evidence that the bacteriocin is plasmid-encoded, and as such antibiotic resistance should be investigated, as well as the production of other compounds, such as biogenic amines, exo-polysaccharides, CRISPR-Cas9, and phage. Following a comprehensive analysis and depending on the data, the strain could be investigated for application in dairy fermentations.

Chapter 5 describes the characterization of a novel bacteriocin producing strain, *Actinomyces ruminicola* DPC 7226, which was isolated from sheep faeces during the screen described in the previous chapter, and the identification of a new family of related and diverse bacteriocins dispersed across the genus *Actinomyces.* During this study the pan-genome of the genus *Actinomyces* was subject to predictive analysis using the BCG prediction program BAGEL, identifying 90 complete gene clusters for antimicrobial production, including 29 class I bacteriocins, 13 class II(d) bacteriocins and 48 operons for bacteriolysin production. Many of the genomes of *Actinomyces* spp. which are publicly available are human clinical isolates from rare cases of actinomycosis, and as such an investigation into the antimicrobial potential of these pathogens may be warranted. Certainly, the nature of the bacteriocins themselves

are of interest as they may have useful properties such as broad/narrow spectra of inhibition and/or high activity against pathogenic species.

The mechanism of action (MOA) of actifensin could be established using a range of experiments, such as those previously used to determine the MOA of the structurally-related fungal defensin, Plectasin (10). Determining the growth kinetics of a susceptible indicator species with actifensin in comparison with diverse types of well characterized antibiotics would provide an indication of its MOA. Combined with cell-wall biosynthesis, potassium release to determine pore-forming ability, and equi-molar lipid II binding experiments, it could be ascertained if actifensin acts in a similar manner to plectasin, binding to lipid II and preventing cell wall biosynthesis. Combined with nuclear magnetic resonance imaging of actifensin in a putative complex with lipid II, specific residues could be elucidated which contribute towards lipid II binding, and the data could therefore be extrapolated, based on amino acid conservation, to the encoded actifensin homologs detected in other *Actinomyces* spp.

Future experiments could also ascertain the functions of other genes present in the actifensin operon. Currently the operon is annotated according to predicted function and its limits were determined by gene synteny. Generation of a range of plasmid constructs containing a various *afn* operon gene may elucidate the necessity and function of said genes, particularly those of unknown function which are conserved. In this manner, a strain containing the actifensin operon under an inducible or constitutive promoter could be generated for production of large quantities of actifensin for purification and further characterization.

In addition to MOA, and operon characterization, the function and safety of the peptide itself could be further investigated. Actifensin shares the features of defensins, many of which are involved in a variety of biological functions. *In vitro c*ell culture experiments may establish any potential cytotoxic effects which would prevent its use as an antimicrobial for human health applications, to be further detailed in *in vivo* animal models of infection. *In vitro* gastric transit and digestion paired with faecal fermentation experiments and sequencing analysis could also be

utilised to determine the resistance of the peptide to degradation following ingestion and the impact on the human gut microbiota. As some *Actinomyces* sp. are primary colonizers of hard oral surfaces, leading to plaque formation on teeth, there could be an application for actifensin against other *Actinomyces* sp. and LAB which are undesirable in the oral cavity.

Finally, chapter 6 expands on actifensin in closely related organisms and the distribution of actifensin related genes in distantly related organisms. The chapter describes the characterization of the *Actinomyces oris* CCUG 34286 which encodes homologous genes for actifensin production that are not expressed. As with heterologous expression experiments described above for constitutive and/or inducible expression of high quantities of AfnA, so too could a similar set of experiments be employed for *A. oris* CCUG 34286 encoded homologs, following successful experiments with AfnA as a positive control. The diverse peptides encoded by *A. oris* CCUG 34286 if expressed and purified would warrant examination of spectra of inhibition, and minimum inhibitory concentration against pathogenic species. Combined with data from potential MOA studies designed for AfnA from *A. ruminicola* DPC 7226, a larger picture of the functional impact of different structures (specific residues, and secondary structure) of the peptides on activity and safety could be established and related to known defensins. Continuation of experiments involving the synthesis of AfnA would require the production of large quantities of synthetic peptide, which would require subsequent oxidative refolding. Synthesis may be utilised as an alternative, but sub-optimal method for the production of AfnA variants to test activity and stability. The genomic findings described in this chapter also raise interesting questions regarding the evolutionary relationship between defensins, toxins, and conserved disulphide-rich peptides. Functional characterization of the conserved hypothetical protein encoding genes, *afnGHI*, in *A. ruminicola* DPC 7226 would provide valuable information with regard to their activity in actifensin production, be it in regulation, self-immunity or other. These data could then be used to help identify the role of gene homologs in the distantly related organisms encoding diverse cysteine rich peptides.

In summary, as part of the EU- Joint Programming Initiative 'A Healthy Diet for a Healthy Life' (JPI HDHL) funded LONGLIFE project, this thesis covers a broad range of topics. The thesis initially investigated safety and shelf life of yoghurt fermented using commercial starter culture. In Chapter 2 the combined properties of the base material with the starter culture determined factors which are desirable in a fermented product. Chapter 3 examined the potential and limitations of bacteriocins as biopreservatives to ensure safety and shelf life of dairy products, and is followed by a screen for novel bacteriocins with intended applications in food preservation. Within Chapter 4, the isolation of bacteriocin producers is described from both milk-associated pathogenic LAB and a potential fermentation organism. Subsequently, Chapters 5 and 6 determined the potential of non-food associated bacteriocin producers and characterised a novel group of antimicrobial peptides. The studies described range from factors which can affect and improve the quality and safety of dairy products, the isolation and characterization of novel antimicrobial peptide producing bacteria isolated for use in dairy fermentations and products, and the description of novel bacteriocins and distantly-related homologs to be investigated for future applications in food and health.

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