Antimicrobial potential of *Clostridium* and closely related species derived from farm environmental samples

A thesis presented in partial fulfilment of the

requirements for the degree of

Doctor of Philosophy

in

Food Technology

at Massey University, Manawatū, New Zealand

Amila Srilal Nawarathna Weligala Pahalagedara

2021

Abstract

The exploration of antimicrobial compounds from natural sources such as bacteria, has been fast tracked by the development of antimicrobial resistance to existing antimicrobials and the increasing consumer demand for natural food preservatives. So far, antimicrobial discovery has been biased towards aerobic and facultative anaerobic bacteria and fungi. Strict anaerobes such as *Clostridium* species have not been thoroughly investigated for their antimicrobial potential. The objective of the current study was to evaluate the antimicrobial potential of *Clostridium* and closely related species against bacteria associated with food spoilage, food safety, and human health.

Tests on culture media inoculated with *Clostridium* and closely related species from farm samples (conditioned media/CMs) showed various degrees of antimicrobial activity. Farm 4 soil conditioned medium (F4SCM) showed potential for further investigation in the search for potent antimicrobials with its promising antimicrobial activity. Bacterial isolates (FS01, FS2.2, FS03, and FS04) belonging to *Clostridium* and closely related spp. associated with F4SCM showed antimicrobial potential as evident by culture-based and genome-based methods.

F4SCM and FS03CM (CM prepared from FS03) metabolomes showed the presence of several putative antimicrobial metabolites. Among them, 2-hydroxyisocaproic acid (HICA) showed antimicrobial activity against a wide range of bacteria associated with food spoilage and safety indicating its potential as a bio-preservative agent in food products. The cell cytoplasmic membrane is a likely target of the HICA's antimicrobial activity.

Overall, this study demonstrates that anaerobic bacterial species, *Clostridium,* and closely related species can produce antimicrobial metabolites, that have potential applications in food preservation and human health. The knowledge obtained in this study will help future investigations to identify and characterize antimicrobials from these *Clostridium* and closely related bacteria and expands the understanding of the potential to produce antimicrobial compounds from the genus *Clostridium* and closely related species.

Dedication

This thesis is dedicated to my loving parents, Nandani and Nawarathna, who first taught me the value of education and for their love, encouragement, and consistent support throughout my journey.

This thesis is also dedicated to my loving wife Roshana for her love, tremendous sacrifice, patience, understanding, encouragement, and continuous support throughout my PhD journey. I'm truly thankful for having you in my life.

Acknowledgements

First and foremost, I would like to thank my supervisors, main AgResearch supervisor Dr. Tanushree B Gupta, main Massey supervisor Prof. Steve Flint, and co-supervisor Dr. Jon Palmer, for their invaluable guidance and constant encouragement throughout my studies. I'm highly grateful to Dr. Tanushree B Gupta for initiating this project, obtaining funds, and the invaluable inputs in formulating the research. Your insightful feedback pushed me to sharpen my thinking and brough my work to a higher level. I'm also highly grateful to Prof. Steve Flint, and Dr. Jon Palmer for their invaluable inputs in conceiving the project, constant support, guidance, and valuable discussions, which helped me to widen my research from various perspectives.

I would also like to thank Dr. Gale Brightwell, the science team leader of Food System Integrity team, at AgResearch Ltd., for your insightful comments, encouragement, and support. My sincere thank also goes to Dr. Arvind Subbaraj for your valuable input, guidance, and support in my metabolomics work. I also had the pleasure of working with Dr. Ruy Jauregui, Dr. Paul Maclean, and Dr. Eric Altermann. Thank you for your support, guidance, and feedback on my genomics work. I'm grateful to Dr. Sandeep Gupta for your support in the lab with the freeze dryer and other advice.

I gratefully acknowledge the financial support from Strategic Science Investment Fund (SSIF), AgResearch Ltd., New Zealand in the form of research fund, stipend, and tuition fees.

My deep gratitude goes to the AgResearch Food System Integrity team: I greatly appreciate Dr. Adrian Cookson, Dr. Delphine Rapp, Dr. Ashwathi Soni, and Mr. John Mills for reviewing my journal manuscripts and conference proceedings. Many thanks also go to laboratory manager, Faith Palevich for your support in the lab and out of the lab in many ways. I'm also especially grateful to laboratory services assistant, Tania Buwalda for the great support offered to me during my laboratory experiments. Thank you to Kwang Subharat, Amanda Gardner, Colleen Ross, Marie Moinet, and Shuyan Wu for your support during my PhD journey. I appreciate Food Microbiology and Biofilm Research team at Massey University for the support extended to me during my PhD.

I would also like to thank Massey Genome Services for their support with whole genome sequencing work, Manawatu Microscopy and Imaging Centre (MMIC) at Massey University, for their support for electron microscopy imaging, and Metabolomics Innovation Centre (TMIC) at University of Alberta for their support with metabolomics work.

Last but not the least, I extend my sincere gratitude to my family and friends for their continuous encouragement and support. Specially, my family; Amma, Thaththa, Achala, Rasangi, Duleesha, Sampath, my nieces, and nephew, I would never have made it this far without the support of you.

I'm very much thankful to my wife, Roshana, who has been by my side throughout my PhD journey despite her hard time without being able to live with me in New Zealand during my PhD. I cannot be grateful enough for your love, patience, understanding, encouragement, and amazing support.

My friends, Rose, Alexis, Soundarya, and Eden, I truly believe that I'm very fortunate to be doing my PhD with all of you as my colleagues. I learnt many things from you, and you all have inspired me to do better. You all made my PhD journey much more enjoyable and exciting. Thank you very much for your support, encouragement, and positivity. A very special thank goes to Rose's family, Mike, Louis, Ash, Tracey, Deana, Grace, Lucy, Vivian, and Graeme for making me feel so welcome and becoming my kiwi family.

Table of Contents

List of Figures

List of Tables

List of Appendices

List of Abbreviations

3-HPAA	3-hydroxyphenylacetic acid
AMR	antimicrobial resistance
amu	atomic mass units
ANI	average nucleotide identity
antiSMASH	antibiotics & secondary metabolite analysis shell
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photo-ionisation
ATP	adenosine triphosphate
BGC/BGCs	biosynthetic gene cluster/s
CAWG	chemical analysis working group
CDC	the centre for disease control and prevention
CDS	coding sequence
CFU	colony forming unit
CIL	chemical isotope labelling
CM/CMs	conditioned medium/ conditioned media
CMGS	cooked meat glucose starch medium
dDDH	digital DNA:DNA hybridization
DmPA	^{12}C-/^{13}C-dimethylaminophenacyl
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EICs	extracted ion chromatograms
EMBL	the European molecular biology laboratory
EPA	environmental protection agency
ESBL	extended-spectrum β-lactamase
ESI	electrospray ionisation
ESR	environmental science and research
FDA	U.S. food and drug administration

FGD	functional genome distribution
GABA	γ-aminobutyric acid
GBDP	genome BLAST distance phylogeny
GRAS	generally recognise as safe
HICA	2-hydroxyisocaproic acid
HicD	hydroxyisocaproic/ hydroxyisocaproate acid dehydrogenase
HILIC	hydrophilic interaction liquid chromatography
HMDB	human metabolome database
HPLC	high performance liquid chromatography
KICA	ketoisocaproic acid
LAB	lactic acid bacteria
LAP	linear azol(in)e-containing peptides
LC-MS	liquid chromatography coupled to mass spectrometry
MBC	minimum bactericidal concentration
MHB	muller-hinton broth
MIBiG	minimum information about a biosynthetic gene cluster
MIC	minimum inhibitory concentration
MRSA	methicillin-resistant *S. aureus*
MS	mass spectrometry
NAM	*N*-acetylmuramic acid
NCBI	the national centre for biotechnology information
NCTC	national collection of type cultures
NMR	nuclear magnetic resonance
NPN	1-N-phenylnaphthylamine
NRPs	non-ribosomal peptides
NRPSs	non-ribosomal peptide synthetases
OD	optical density
PBPs	penicillin-binding proteins
PCA	principal component analysis
PCR	polymerase chain reaction

PG	peptidoglycan
pHMMs	profile hidden Markov models
PKs	polyketides
PKSs	polyketide synthases
PVDF	polyvinylidene fluoride
QC	quality control
Q-TOF	quadrupole time-of-flight mass spectrometry
RFU	relative fluorescence units
RiPPs	ribosomally synthesized post-translationally modified peptides
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SAM	*s*-adenosylmethionine
SBA	sheep blood agar
SCFAs	short chain fatty acids
SCIFF	six cysteines in forty-five residues
SCM	scanning electron microscopy
SFP	shahidi ferguson perfringens
SN	spore number
TEM	transmission electron microscopy
TMN	total microbial number
tRNA	transfer ribonucleic acid
TSB	tryptic soy agar
TYGS	type (strain) genome server
UHPLC	ultra high-performance liquid chromatography
WHO	world health organization

List of Publications

Journal articles

Pahalagedara ASNW, Flint S, Palmer J, Brightwell G, Gupta TB: Antimicrobial production by strictly anaerobic *Clostridium* spp. *International Journal of Antimicrobial Agents* 2020:105910.

Pahalagedara ASNW, Flint S, Palmer J, Subbaraj A, Brightwell G, Gupta TB: Antimicrobial activity of soil *Clostridium* enriched conditioned media against *Bacillus mycoides*, *Bacillus cereus*, and *Pseudomonas aeruginosa*. *Frontiers in Microbiology* 2020, 11(3113).

Pahalagedara ASNW, Ruy J, Maclean P, Altermann E, Flint S, Palmer J, Brightwell G, Gupta TB: Culture and genome-based analysis of four soil *Clostridium* isolates reveal their potential for antimicrobial production. *BMC Genomics* 2021, 22, 686.

Pahalagedara ASNW, Flint S, Palmer J, Brightwell G, Gupta TB: Antimicrobial efficacy and possible mechanism of action of 2-hydroxyisocaproic acid (HICA). (Under review).

Pahalagedara ASNW, Flint S, Palmer J, Brightwell G, Gupta TB: Non-targeted metabolite profiling of conditioned medium resulted by the growth of anaerobic bacterium closely related to *Terisporobacter glycolicus*, identifies metabolites with antimicrobial potential. (In preparation).

Conference proceedings and workshops

Pahalagedara ASNW, Flint S, Palmer J, Subbaraj A, Brightwell G, Gupta TB: Antimicrobial activity of *Clostridium* enriched conditioned media. *Poster session presented at the New Zealand microbiological society (NZMS) conference*, Palmerston North, New Zealand, November 2019.

Pahalagedara ASNW, Flint S, Palmer J, Subbaraj A, Brightwell G, Gupta TB: Antimicrobial potential of *Clostridium* enriched conditioned media. *Three-minute oral presentation presented at the New Zealand microbiological society (NZMS) online conference*, November 2020.

Meat industry innovation workshop, organized by AgResearch and Meat Industry Association (MIA), Palmerston North, New Zealand. March 2021. Oral presentation titled on “Antimicrobials from *Clostridium* and closely related species”.

Chapter 1

Project Introduction

1.1 Background and origin of the research

Antimicrobial agents are natural or synthetic compounds, which have a capacity to kill or inhibit the growth of microorganisms such as bacteria and fungi [1-4]. This chemically and structurally heterogeneous group of compounds are widely used in medicine, food and agriculture to control harmful and undesirable microorganisms [5-7]. Identification and characterization of antimicrobial compounds from plants, animals, and microorganisms have been studied extensively, and as a result many antimicrobials have been added to the current list over the time. However, due to the new immerging and changing infections, global spread of antimicrobial resistant bacteria, and the consumer demand for natural food preservatives, undoubtedly there is a requirement for novel and natural antimicrobials and strategies, that can combat these emerging resistant bacteria [8]. Bacterial diversity is important in the quest for novel antimicrobial compounds as different bacteria may have diverse metabolism leading to the production of variety of small metabolites. Microorganisms such as bacteria are not distributed consistently within their community. Their distribution is affected by the interactions with surrounding microorganisms and the surrounding physical environments [9]. Their metabolism and chemical diversity of the metabolites has most likely been developed as a result of mutual interactions with neighbouring microorganisms and adaptations to challenging physiological environments. However, bacterial diversity does not seem to be well considered in antimicrobial discovery as only the members of a few bacterial groups such as actinomycetes and lactic acid bacteria have been extensively screened for their potential to produce antimicrobials [10].

Until recently, microbial bioactive compound discovery has been biased towards aerobic and facultative anaerobic bacteria. Obligately anaerobic bacteria have been largely neglected in terms of using their metabolic potential to produce bioactive compounds, including antimicrobials. *Clostridium* spp., which are ubiquitously present in soil and intestines of humans and animals, and play an important roles in natural processes such as degradation of waste, fixation of carbon dioxide and fermentation of organic matters, have not been thoroughly investigated for their potential to produce antimicrobial compounds [11]. This might be due to the long-standing assumption that anaerobes were not capable of producing secondary metabolites such as polyketides and non-ribosomal peptides [12]. Perhaps, the pathogenicity of some members of the genus attracted more attention than beneficial effects of non-pathogenic *Clostridium* species. Their metabolic diversity could be of great importance to explore novel secondary metabolites having antimicrobial properties. In recent years, computational genomic approaches such as genome mining have shown that *Clostridium* species possess the genetic content to produce various groups of secondary metabolites and peptides. For instance, genome mining of publicly available genomes revealed the presence of biosynthesis gene clusters (BGCs) tentatively encoding for non-ribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) in *Clostridium* species [13].

Although most of the *Clostridium* spp. are saprophytes and not involved in a disease process, pathogenic species have received the most attention owing to their detrimental effects on humans, animals, and food systems. To date, only limited information is available on antimicrobial potential of *Clostridium* spp. due to low scientific interest in this topic. Nevertheless, the limited studies conducted thus far have provided some indication of the antimicrobial potential of *Clostridium* species [14-16]. In this context, this study aimed to investigate the antimicrobial potential of *Clostridium* and closely related spp. derived from farms in New Zealand. The focus was to explore the antimicrobial efficacy against bacterial species of significant health, food quality, and safety concern and identify potent antimicrobials produced by *Clostridium* species under the given growth conditions.

Origin of the study

Preliminary unpublished studies demonstrated that when microbial spores isolated from farm environments (soil, feed, and dairy effluent) were enriched in cooked meat glucose starch (CMGS) medium under anaerobic growth conditions, only *Clostridium* and closely related spp. could thrive, and the growth of other facultative anaerobic microorganisms such as *Bacillus* spp. were inhibited. Closely related species referred here were genetically closely related bacteria to the genus *Clostridium* such as *Paraclostridium* and *Terrisporobacter* species. Further studies demonstrated that this growth inhibition was not a result of CMGS medium (Identification and characterization of antimicrobial 1.1) and suggested that this growth inhibition could be due to antimicrobial compounds produced by *Clostridium* and closely related spp. during their growth in CMGS under anaerobic conditions.

Hypothesis

It was hypothesised that *Clostridium* and closely related spp. derived from farm samples produce compounds with antimicrobial activities against other bacteria such as *Bacillus* species during their growth in CMGS medium.

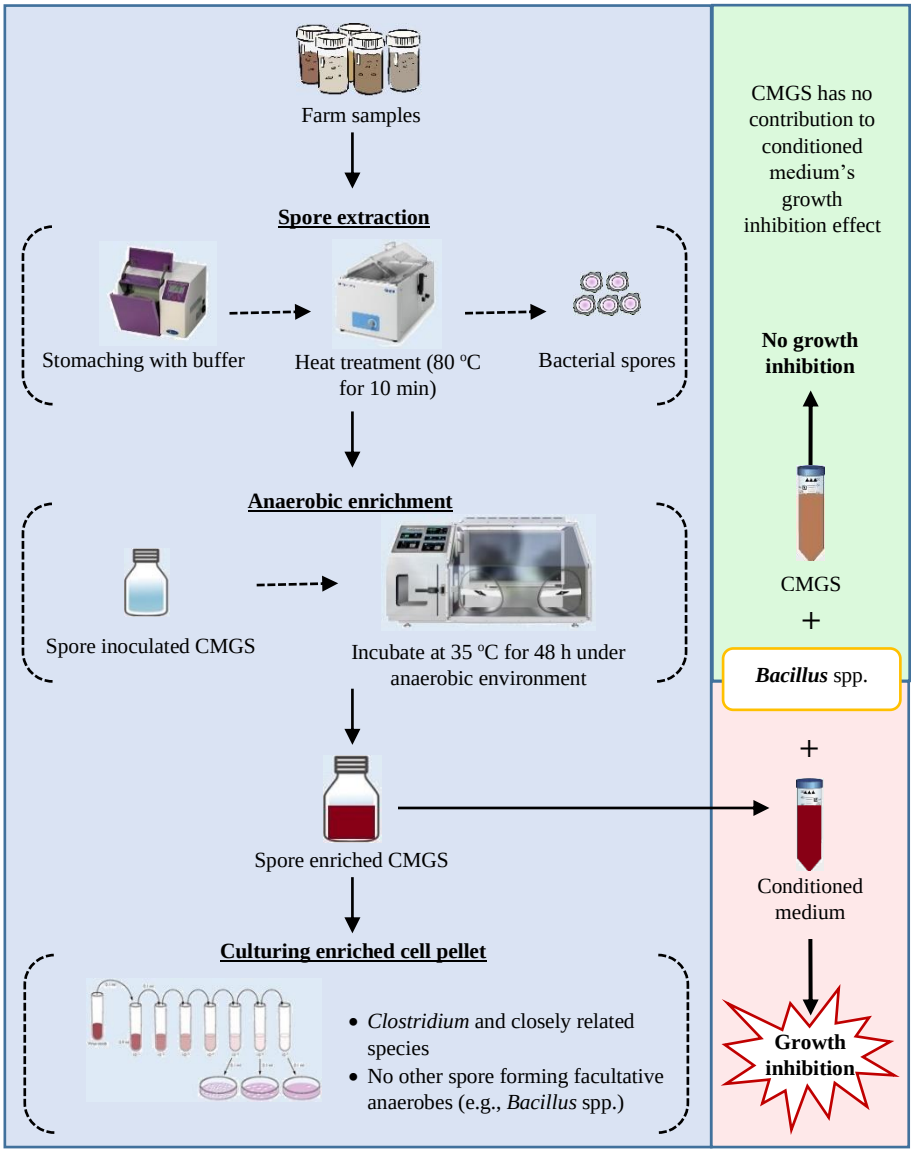


Figure 1.1: Schematic diagram showing the preliminary observations of growth inhibition activity of conditioned media.

1.2 Project objectives

The overall aim of the research presented in this thesis was to gain an understanding of the antimicrobial potential of *Clostridium* and closely related spp. derived from farm environments. As very little information can be found in the literature on this topic, the new knowledge obtained from this study will extend the current knowledge on the antimicrobial potential of anaerobic bacteria and will encourage consideration of *Clostridium* and closely related spp. as a viable source of antimicrobial compounds and may help to develop potent antimicrobial compounds/strategies against health, food quality and safety concern bacteria.

The key research questions and the corresponding research objectives can be summarised as follows:

1) Conceivably, conditioned media prepared from various farm samples will possess various degrees of growth inhibition potential based on the activity of associated *Clostridium* and closely related species populations in each sample. What are the antimicrobial profiles of conditioned media prepared from farm soil, feed, and dairy effluent samples against select bacterial species of human health, food quality and safety concern? What conditioned media/medium will have relatively high antimicrobial profiles?

   Objective 1: To screen conditioned media prepared from soil, feed, and dairy effluent samples for antimicrobial activity to select the conditioned medium with the best antimicrobial profiles for further studies.

2) What *Clostridium* and closely related spp. are responsible for the antimicrobial activities of select conditioned media?

   Objective 2: To isolate and identify bacteria from select samples and confirm the involvement of *Clostridium* and closely related spp. in conditioned medium's antimicrobial activity.

3) Are there significant changes in the metabolite composition of CMGS growth medium after the growth of *Clostridium* and closely related species? What secondary metabolites are produced during the growth of *Clostridium* and closely related spp. under anaerobic conditions and what metabolites might be associated with antimicrobial activity?

   Objective 3: To investigate the metabolite profiles of select conditioned media using metabolomics and further analysis to identify putative antimicrobial compounds from conditioned medium of interest.

4) What is the antimicrobial potential of select isolates of *Clostridium* and closely related species? What is the genetic potential of select bacterial isolates for the synthesis of secondary metabolites belonging to antimicrobial compound groups?

   Objective 4: To evaluate the antimicrobial potential of select bacterial isolates using genome-based and culture-based approaches.

5) What bacterial isolates are involved in the production of 2-hydroxyisocaproic acid (HICA) in CMGS. What is the antimicrobial efficacy of HICA against bacterial species of human health, food quality, and safety concern? How does HICA exert its effect on bacterial cells?

   Objective 5: To investigate the HICA production by relevant bacterial isolate/s, the antimicrobial efficacy of HICA and its possible antimicrobial mechanism/s.

Chapter 2

Literature review

The literature review has been divided into six sub-sections based on the content. The first part discusses the general information about antimicrobial compounds such as definitions, the importance of antimicrobials with their various applications, microorganisms as a promising source of antimicrobials and the need for the discovery of novel antimicrobial compounds or new applications of currently known compounds. Overall, this section shows the challenges and opportunities in this field highlighting the need for more studies on antimicrobial compounds.

The second sub-section aims to provide some understanding of common antimicrobial compound groups produced by microorganisms, highlighting their chemical nature, producers, applications, and some history. This knowledge certainly assists new antimicrobial studies such as the current study for the identification of genetic and metabolic potential of unexplored bacteria to produce known/unknown compounds belonging to antimicrobial compound groups.

The third sub-section discusses common mechanisms of action of antimicrobial compounds. This knowledge is important to develop novel antimicrobial compounds targeting specific cell functions or to understand the possible antimicrobial mechanisms of novel antimicrobial compounds.

The objective of the sub-section four is to highlight the applications of two omics platforms, metabolomics, and genomics in recent antimicrobial studies. Since these two platforms are employed in the current study, this information assists in understanding the principals and different techniques/tools applicable to this study.

The fifth sub-section has reviewed the current knowledge and understanding of antimicrobial potential of strictly anaerobic bacteria giving special attention to the genus *Clostridium*. This knowledge creates the background of the current study supporting the concept of the research and encouraging further studies.

In the last sub-section, an introduction to 2-hydroxyisocaproic acid (HICA) identified in this study, has been provided based on the current knowledge on its biochemistry, distribution, and biological activities. This information is vital to understand its production and potential applications as an antimicrobial compound.

2.1 Antimicrobial compounds

The environment is inhabited with a massive group of small molecular weight organic compounds with a large diversity of molecular structures produced by various living organisms [17]. Some of these molecules are reported to have various beneficial effects in humans, animals, and agriculture [18]. One of the key recognized group of bioactive compounds is antimicrobials. Antimicrobials are chemical compounds, which have a capacity to kill or inhibit the growth of microorganisms such as bacteria and fungi [1-4, 19]. They can be either natural, semi-synthetic or synthetic compounds based on their origin [20]. Natural antimicrobials are chemical substances with antimicrobial properties produced by living organisms or found in nature. Antibiotics are medicine used to prevent or treat bacterial infections. On the other hand, antimicrobials can be a food preservative or inhibitor of plant disease. Presently, natural antibiotics are frequently used as starting materials for semi-synthetic antimicrobial compound discovery followed by synthetic modifications to increase efficiency, bioavailability, and to decrease side effects. Fully synthetic antimicrobial compounds are produced by fully synthetic routes [21]. Natural products have been, and continue to be, the most important sources of novel pharmaceutical compounds [22]. Antimicrobial compounds are critically important due to their demonstrated therapeutic and protective properties against various harmful and unfavourable microorganisms.

2.1.1 Significance of antimicrobial compounds with their applications

Antimicrobials, primarily antibiotics, have contributed to the control of infectious diseases throughout history [7]. Antibiotics have saved lives of millions by contributing to the control of many infectious diseases, which were leading causes of human morbidity and mortality. In the US, after the discovery of antibiotics, the main causes of death changed from infectious diseases to non-communicable diseases such as cancer and cardiovascular diseases with increased average life expectancy at birth and older population [23]. For instance, beta lactam antibiotics, such as natural penicillin and aminopenicillin have a spectrum of activity against both selected Gram-positive and Gram-negative infections including *Streptococcus pneumonia, Enterococcus* spp., *Neisseria meningitides, Enterococcus faecalis, Haemophilus influenza, Listeria monocytogenes* and *Escherichia coli* [24, 25]. Antimicrobial compounds are used in agriculture, especially in livestock, poultry, and aquaculture to prevent or treat bacterial infections and to improve animal growth and feed efficiency [5, 26]. The use of antimicrobials in plant disease control is limited due to lack of efficacy for numerous plant diseases and environmental concerns. Only streptomycin and oxytetracycline are approved to use in plant agriculture by Environmental Protection Agency (EPA) in the US [27]. Unfortunately, the increase in antimicrobial resistance among various pathogenic bacteria is now challenging the effectiveness of antimicrobial use in therapeutic and other applications [28]. To preserve the effectiveness of medically important antimicrobials for humans, the World Health Organization (WHO) has recommended to reduce the overall use of all medically important antimicrobials in food-producing animals with complete restriction on growth promotion and prevention of infectious diseases not diagnosed clinically [29].

Pathogenic bacteria cause a wide variety of infections and poisonings through contaminated foods [30]. Food spoilage microorganisms impact on food quality leading to reduced shelf life and food loss particularly during distribution and storage. Certain antimicrobial compounds such as bacteriocins, phytochemicals, benzoates, sorbates, nitrites, nitrates, and other antimicrobial peptides, are used in controlling food borne pathogenic and spoilage microorganisms [31-35]. Food antimicrobial compounds may be natural or synthetic in nature. However, natural antimicrobials have gained attention due to consumer concerns over adverse health effects of synthetics. For instance, although nitrites are permitted in foods as preservatives, there have been concerns about toxic

effects to humans [36, 37]. Lactic acid bacteria (LAB), used in food bio-preservation, may produce a variety of antimicrobial metabolites such as organic acids, hydrogen peroxide, ethanol, antifungal peptides and bacteriocins [38]. To date, nisin, produced by certain *Lactococcus* spp. is the most widely used bacterial antimicrobial compound (bacteriocin) in the food industry as a food preservative. Nisin shows antibacterial effect against a broad range of Gram-positive bacteria but shows little or no effect against Gram-negative bacteria [39, 40].

2.1.2 Need for novel antimicrobial compounds

Antimicrobial resistance (AMR) has been recognized as one of the largest health threats that mankind faces now and in the future. Therefore, new antimicrobials active against resistant bacteria are needed. Most of the natural antimicrobials developed during last few decades have lost their effectiveness against many bacteria [41]. The threat is not just bacterial resistance against one antibiotic but also multi-drug resistance. The term 'superbugs' is generally used for organisms that are emerging at a frightening rate and resistance to most/all clinically used antibiotics [41]. The Centre for Disease Control and Prevention (CDC) has reported the occurrence of more than 2.8 million antibiotic resistant infections each year including more than 35,000 deaths as a result in the US [42]. Many bacteria are able to grow as biofilm in which the cells are enclosed and protected from the environment. Bacteria within a biofilm demonstrate resistance to commonly used antimicrobial therapies [43]. Nosocomial infections/hospital acquired infections caused by Gram-negative bacteria are of particular concern due to antimicrobial drug resistance. Among them, *Pseudomonas aeruginosa* presents a serious threat to patients with cystic fibrosis, cancer, and burns accounting for a higher percentage of opportunistic infections [44].

In food applications, although, natural antimicrobials are favoured over synthetic ones, synthetic antimicrobials are still widely used in the food industry; out of 35 antimicrobials approved by the European Food Safety Authority, 32 are synthetic agents, and only 3 (nisin, lysozyme, and natamycin) are natural [45]. Therefore, there is a demand for novel natural antimicrobials that can be safely used in food preservation. Furthermore, the effectiveness of existing food antimicrobials has been challenged by the development of antimicrobial resistant bacterial strains. For instance, nisin which is the most widely used

FDA approved bacteriocin has lost its efficacy due to the development of nisin resistance in several strains of bacteria [46, 47].

Another reason for developing novel antimicrobials is associated with the toxicity. The use of some antimicrobial agents may cause adverse side effects in patients as with other therapeutic compounds or harmful side effects to consumers when added to foods [48]. Therefore, it is a challenge to introduce novel antimicrobial compounds with minimal side effects to patients/consumers.

2.1.3 Microorganisms as a source of natural antimicrobials

Studies have investigated the antimicrobial compound producing potential of various microorganisms, plants, and animals. They principally produce these compounds using their biosynthetic and metabolic routes during the growth and development, similar to the production of other bioactive compounds.

Microorganisms are not distributed consistently within their community, their distribution is affected by the interactions with surrounding microorganisms and the surrounding physical environment [9]. They make antagonistic or symbiotic relationships often mediated via small molecules with their neighbours in this competitive environment [49, 50]. Interestingly, many of these compounds including ribosomal and non-ribosomal peptides, polyketides, terpenes, and alkaloids are shown to have bioactivities that are antimicrobial, anticancer, immunomodulatory, and cholesterol lowering [51, 52] and have been exploited for human benefits. Microorganisms produce antimicrobial compounds that can fight with competitors as a survival strategy in their antagonistic relationships [49]. It has also been suggested that the true function of antimicrobials in the natural environment is to work as signal molecules [53].

Bacteria and fungi are popular antimicrobial compound producing microorganisms and they have yielded a considerable number of antimicrobial agents currently used in medicine, agriculture, and food sector. The first natural antibiotic discovered is mycophenolic acid isolated from a fungi called *Penicillium glaucum* in 1893 [54]. In 1929, Fleming observed *Penicillium notatum* has antibacterial activity and named the biological activity ‘penicillin’ [55]. Since then, antimicrobial secondary metabolite search has continued and many antimicrobial compounds, particularly antibiotics have been

discovered from bacteria and fungi. To date, β-lactam antibiotic penicillin produced by *Penicillium* spp. is the most important antimicrobial metabolite discovered from fungi [51]. Nevertheless, there are many other antimicrobial compounds discovered from different fungus species such as pneumocandin B0, arthrobotrisins A-C, and catenarin [56].

Bacteria residing in soil and fermentations have been extensively screened for their potential to produce antimicrobial compounds. A number of natural antibiotics have been isolated from the bacterial genus *Streptomyces*, which has been reported to be the largest antibiotic producing genus so far [10]. The well-known antimicrobial producing bacterial groups include Actinomycetes [10], myxobacteria [57], cynobacteria [58], *Bacillus* spp. [59] and *Pseudomonas* species [60]. Lactic acid bacteria (LAB) are well known to produce proteinaceous antimicrobial peptides called bacteriocins, which have been mainly considered to use in food preservation [61]. As an approach to novel antimicrobial compound discovery, previously overlooked bacterial groups and bacteria from previously unexplored habitats are considered in recent antimicrobial discovery efforts. For instance, bacteria from the genus *Burkholderia*, *Janthinobacterium*, and *Lysobacter* were investigated for antimicrobial compound production and they were found to produce a number of antimicrobial compounds with various chemical structures [62]. Plant [63] and insect associated bacteria [64] and marine bacteria [65] have been recognized as viable sources of antimicrobial compounds. Another less explored bacterial group is the strictly anaerobic bacteria and recently there has been an increasing interest to explore the antimicrobial potential of strictly anaerobic bacteria such as *Clostridium* spp. to identify novel antimicrobials [66]. More information on the antimicrobial potential of *Clostridium* spp. is discussed in the literature review chapter 2.5.1.

2.2 Antimicrobial compound groups derived from microorganism

2.2.1 Peptides

Peptides produced by microorganisms can be categorized into two main groups as ribosomal peptides and non-ribosomal peptides based on the synthesis mechanisms. Ribosomally synthesized peptides are low molecular weight, cationic, and hydrophobic peptides synthesized in the ribosome of microorganisms, some are post-translationally modified (RiPPs) and some are not (unmodified bacteriocins) [67, 68]. Non-ribosomal peptides are synthesized by specialized non-ribosomal peptide synthetases. They are built by over 500 different monomers including 20 proteinogenic and many different non-proteinogenic amino acids making them more diverse in their structures and properties [69, 70].

2.2.1.1 Ribosomally synthesized peptides (Bacteriocins)

Many Gram-positive and Gram-negative bacteria and a few archaea are known to produce bacteriocins [71]. These proteinaceous compounds kill or inhibit the growth of either or both Gram-positive and Gram-negative bacteria and some are active against viruses and fungi [72, 73]. However, most bacteriocins possess a relatively narrow spectrum of activity, affecting genera or species closely related to the producer organism. Bacteriocins exhibit their activity by primarily targeting the cytoplasmic membrane. However, there are different mechanisms of action and a single bacteriocin can have more than one mechanism to attack the target bacteria [74, 75].

The first bacteriocin, colicin was identified as a heat-labile compound from *Escherichia coli* V by Gratia in 1925 [76]. Whitehead [77] reported the presence of proteinaceous inhibitory substances produced by two strains of lactic streptococci in milk and few years later this substance was identified and named ‘Nisin’, a 34 amino acid long cyclic peptide. [78]. Nisin was acknowledged as a food preservative by Food and Agriculture Organization (FAO) and World Health Organization (WHO) in 1969 and approved by the US Food and Drug Administration (FDA) as an additive in canned products in the US in 1988 [79]. Today, nisin has been approved as a food additive in over 50 countries. This

remarkable commercial success stimulated further studies to investigate other antimicrobial agents equivalent to nisin. Three decades later, many bacteriocins have been identified and characterized from diverse bacterial species and sources [80-84]. Particularly, lactic acid bacteria (LAB) can produce a number of bacteriocins having antimicrobial activity to compete with other bacteria. But none of the identified compounds has been approved by FDA as a bio preservative.

Bacteriocins have become a very diverse and heterogenous group of compounds with the discovery of new bacteriocins having unique characteristics. Despite the high level of diversity, most of them share some common characteristics such as low molecular weight, cationic, and hydrophobic nature. However, there are exceptions to these shared characteristics; for instance, subtilosin A is an anionic bacteriocin produced by *Bacillus subtilis* 168 [85], and diffocins are R-type bacteriocin particles of high molecular weight isolated from different strains of *Clostridium difficile* [86]. Bacteriocins have been mainly considered for use in food bio-preservation, controlling food pathogenic and spoilage microorganisms either alone or together with other preservation methods [87, 88]. Their potential applications in other fields such as medical, horticulture, and the personal care industry have also been investigated [89].

2.2.1.2 Non-ribosomal peptides

Non-ribosomal peptides (NRPs) are a diverse group of secondary metabolites synthesized by dedicated non-ribosomal peptide synthetases (NRPSs) independent of ribosomal protein synthesis machinery, mainly in bacteria and fungi. NRPSs are modular multi-domain enzyme complexes, which serve as templates and biosynthetic machinery for non-ribosomal peptide synthesis [70, 90, 91]. NRPs are produced by soil bacteria including *Actinomycetes, Bacillus,* and filamentous fungi and recently marine bacteria have emerged as a good source of NRPs with the discovery of large numbers of NRP compounds [92, 93]. In recent years, bioinformatic techniques such as genome mining have given insight into the occurrence and distribution of NRPs and their pathways in a wide range of bacteria. Wang, Fewer [94] examined a total of 2,699 genomes from 991 organisms and demonstrated the occurrence of NRP biosynthetic pathways in *Proteobacteria, Actinobacteria, Firmicutes* and *Cyanobacteria*.

NRPs are a predominant group of drugs used in human medicine, primarily as antibacterial, followed by antifungal, anti-tumour compounds, and immunosuppressants [70, 95, 96]. They have potential applications in environmental remediation and food processing as biosurfactants and as bio control agents in agriculture [97-99]. Non-ribosomal peptides include many clinically important antibiotics such as β-lactams, daptomycin, and cyclosporin A [70, 100-102]. Some of the antimicrobial NRPs have been modified with multiple variations, e.g. various derivatives of β-lactam antibiotics (penicillins and cephalosporins) have been developed and used as clinical antibiotics [103]. Despite NRP's use as medical antibiotics, their potential applications in food preservation have not been properly investigated.

2.2.2 Polyketides

Polyketides (PKs) are a structurally and functionally diverse group of secondary metabolites mainly produced by bacteria, fungi, and plants [104]. Polyketide biosynthesis is initiated from simple carboxylic acid derivatives such as malonyl-CoA, acetyl-CoA, propionyl-CoA or methylmalonyl-CoA and they acquire their structural diversity by processing under a series of modular enzymes called polyketide synthases (PKSs), in which they are condensed and modified primarily *via* reduction, dehydration, cyclization and aromatization reactions [104-106]. This synthesis process is similar in many ways to fatty acid synthesis in bacteria and mammals [107].

The biological functions of these molecules in producing organisms are believed to be as virulence factors, toxins, pigments, or signalling molecules [108, 109]. The polyketide compounds such as polyenes, polyethers, polyphenols, macrolides, and endiines possess biological functions including immunosuppression, anticancer, and antimicrobial activities [110-113]. A few polyketide antibiotics used in medicine to treat bacterial and fungal infections are erythromycin, tetracycline, and amphotericin B. Macrolide compounds sharing lactone or lactam rings are considered as a major class of antimicrobial compounds [113].

Despite many reports on the use of polyketides as clinical antibiotics, their potential use in improving quality and safety of food products has been poorly investigated. To date, the only accepted polyketide originated food preservative is natamycin (pimaricin). Natamycin is a macrolide antifungal antibiotic containing 26-member tetraene moiety

synthesized by strains of *Actinomycetes* including *Streptomyces natalensis* and *Streptomyces chattanogenesis* [114, 115]. Natamycin is active against a wide range of molds and yeasts exerting their detrimental effect through disturbing mold and yeast cell membranes by binding to membrane ergosterol without permeabilizing the membrane [116]. Natamycin has been approved for use as an antifungal agent in many countries in a variety of food products. It has been identified as a GRAS (Generally Recognise as Safe) compound by FDA for use in food industry and considered as a natural preservative by European Union [114].

2.2.3 Other microbial compounds with antimicrobial properties

Microorganisms produce a variety of compounds, which may have antimicrobial properties, through their metabolic pathways other than through the direct regulation of gene clusters such as NRPS, PKS or RiPP. Microorganisms produce hydrogenated, carbonated, sulphur or nitrogen containing compounds (H_2, HCN, H_2S, CO_2, CO, N_2, NH_3 and NO) as by-products of primary metabolism. Some nitrogen containing compounds produced by bacteria were reported to have antimicrobial activities. For instance, ammonia was found to be active against soil-borne *Pythium* species [117]. HCN, a product of glycine catabolism demonstrated antagonistic activity against aerobic microorganisms [118]. Some bacteria living under low oxygen environments such as soil were reported to synthesize H_2 and H_2S. Hydrogen sulphite produced as a by-product of L-cysteine and L-methionine catabolism showed antifungal activities against plant pathogens such as *Penicillium italicum* and *Aspergillus niger* as well as against several human and food-borne bacteria [119].

Microorganisms including bacteria and fungi produce a broad range of volatile organic compounds as side products of their primary and secondary metabolism [120]. Bacterial organic volatiles mainly include alcohols, aldehydes, ketones, alkenes, terpenes, benzenoids, pyrazines, acids, and esters [121]. Benzenoids were reported to possess fungicidal, antibacterial, and nematicidal activities [122]. Nitrogen containing volatile organic product, pyrazines produced by *Bacillus subtilis* were found to show antibacterial, antifungal, and nematicidal activities [123, 124]. *Clostridium difficile* was reported to produce *para*-cresol through the fermentation of tyrosine, which showed antimicrobial activity against several Gram-negative bacteria [125]. *Streptomyces*

albidoflavus isolated from corn seed was reported to produce an antimicrobial sesquiterpene named albaflavenone [126]. The mechanisms of action of antimicrobial volatile organic products have not been studied in detail. However, the hydrophobicity of some metabolites such as monoterpenes were shown to perturbate the lipid layer of the cell plasma membrane, resulting in alteration of cell membrane permeability and leakage of intracellular compounds [127].

Another group of antimicrobials is antimicrobial peptides (AMPs) derived from animal venoms. They have been identified from invertebrates such as insects, marine organisms and vertebrates including fish, amphibians, and mammals [128]. These antimicrobials are found to be active against a wide range of microorganisms with less resistance and can provide synergistic effect with antibiotics [128].

2.3 Mechanisms of action of antibacterial agents

This section describes the common mechanisms of action of antibacterial compounds. The antibacterial activity of a compound is primarily attributed to the interfering of the biosynthesis and the assembly of vital structural components of target bacteria. Activity of many antimicrobials generally falls within one of five mechanisms, two of them involves the interference of bacterial cell wall biosynthesis and bacterial cell membrane destruction. The other three are interfering with the bacterial protein synthesis, bacterial DNA replication and repair, and inhibition of metabolic pathways such as fatty acid synthesis [129, 130].

2.3.1 Interference with cell wall synthesis

The bacterial cell wall is primarily composed of the peptidoglycan (murein), which is constructed with a network of glycan strands cross-linked by peptides generating a covalent mesh. This structure encloses the bacterial cell and maintains the shape by preventing them from lysis *via* osmotic pressure [131]. The cell wall synthesis is a good target for antimicrobials due to its absence in mammalian cells providing targeted selectivity. There is a difference in size and the location of this polymer within the cell envelop between Gram-positive and Gram-negative bacteria [132]. The glycan chain is built up by altering disaccharide subunits of β-1,4-linked *N*-acetylglucosamine (NAG)

and *N*-acetylmuramic acid (NAM) [132, 133]. There are two enzyme families that have critical roles in the formation of the peptidoglycan layer, transglycosylases and transpeptidases. Transglycosylase enzymes catalyse glycosidic linkages between the NAM and NAG components and transpeptidase enzymes cross-link the peptide side chains with pentaglycine bridges and cleave the terminal 2 D-alanines in the peptide side chain, resulting the mature peptidoglycan [130]. The biosynthesis of peptidoglycan (PG) can be divided into four major steps; synthesis of precursor molecules in the cytoplasm (1), transportation of lipid bound precursors from cytoplasm across the cytoplasmic membrane (2), incorporation of glycan units into growing PG (3) and transpeptidation linking and maturation (3) [132, 134].

Antimicrobials act at different stages of PG synthesis affecting the structure of the cell wall. Antibiotics such as D-cyclosering and fosfomycin interfere with the first step of the cell wall biosynthesis through the inhibition of D-Ala:D-Ala ligase and Mur enzymes respectively [135, 136]. Lipid II is a peptidoglycan precursor molecule, that can be targeted by some antimicrobial compounds such as lantibiotics (nisin and ramoplanin), mannopeptimycins, defensins (plectasin) and glycopeptide antibiotics [137]. Some highly successful and widely used cell wall biosynthesis inhibitors such as *β*-lactams and glycopeptides target stages 3 and 4. β-lactam antibiotics such as penicillins, cephalosporins and carbapenems act as traspeptidase inhibitors, hindering the conversion of nascent to mature peptidoglycan [137]. They act by forming covalent complexes with enzymes called penicillin-binding proteins (PBPs) [132]. Glycopeptide antibiotics such as vancomycin and teicoplanin are large complex heterocyclic molecules and usually too large to pass through the Gram-negative outer membrane, thus their activity is mostly restricted to Gram-positive bacteria. Glycopeptides act by binding to terminal D-alanyl-D-alanine dipeptide side chain of nascent PG and subsequent blocking of its transition to mature peptide. Therefore, glycopeptides interfere with the same step as the β-lactams although they bind to a different target from the β-lactams [130, 132].

2.3.2 Interference with cell membranes

The cell membrane architectures of Gram-positive and Gram-negative bacteria are different. Gram-negative bacteria have two distinct lipid membranes as an outer membrane and inner membrane, and they are separated by a thin layer of peptidoglycan.

Gram-positive bacteria being far less complex, comprising a cytoplasmic plasma membrane and surrounded by a thick layer of peptidoglycan and lipoteichoic acids. Due to the presence of porin proteins in the outer membrane, it is more permeable than cytoplasmic membrane allowing small molecules (500 Da or less) to go through [138]. Generally, these bacterial membranes maintain a certain degree of permeability allowing the exchange of small molecules and ions between cytoplasm and external environment [139].

Certain antimicrobial agents cause cell toxicity by altering the permeability of the outer membrane. Polymyxin B and colistin (polymyxin E) are two commercially available antibiotics having similar spectrum of activity and mechanisms of action [140]. Polymyxins exert their antimicrobial effect through the interaction with both outer and inner membrane of bacteria [141]. Positively charged polymyxins bind with the lipid A component of the bacterial outer membrane and displace Mg^{2+} or Ca^{2+}, which leads to destabilization and disruption of the outer membrane, subsequently penetrating into the inner membrane and entering the cytoplasm [142].

There are other mechanisms by which antimicrobial compounds can alter the bacterial cell membrane properties. Some antimicrobial agents can alter one or more of the bulk properties of the membrane without binding to a specific membrane component. These alterations could be changes in the spatial distribution of cell membrane molecules within the membrane or changes in the bulk physical properties including fluidity and intrinsic curvature [139]. There are many cationic antimicrobials (mainly peptides), which interact with the anionic lipids on the bacterial membrane. Antimicrobials with several cationic charges can induce the formation of domains in the membranes composed of both anionic and zwitterionic or neutral lipids leading to cell growth inhibition or cell death [143]. Some antimicrobial peptides were found to act by disturbing membrane morphology/ properties and membrane curvature [144, 145]. There are other antimicrobial agents, that can target specific lipid components in the bacterial cell membrane, and they may or may not exert their activity modifying the bulk membrane properties. The indirect way of targeting specific lipids is the inhibition of enzymes essential for their synthesis [139]. The mechanism of action of the antibiotic daptomycin is associated with redistribution of the bacterial cell membrane phospholipid cardiolipin on the membrane surface and localization of proteins involved in cell division [146].

The many roles of bacterial membrane can be affected by antimicrobial compounds. The bacterial membrane itself acts as a barrier for antimicrobials to access intracellular targets. However, its main role is to establish concentration and electrical gradients between the cytoplasm and external environment. This homeostasis in the cell is disrupted through the damage to the cell membrane by antimicrobial agents leading to growth inhibition or cell death.

2.3.3 Interference with essential cell functions

The essential cell functions including protein synthesis, DNA replication and repair, and metabolic pathways such as fatty acid synthesis are targets of some antimicrobial compounds.

The synthesis of DNA and RNA are essential functions in growing and multiplying cells. The cell division is rapidly inhibited by the inhibition of DNA synthesis. The termination of protein synthesis is a result of inhibition of RNA synthesis. Generally, antimicrobials that interfere with DNA and RNA synthesis exert their effect through either nucleotide or nucleic acid biosynthetic processes in the cell. Some antimicrobials disrupt the supply of nucleoside triphosphates needed for nucleic acid synthesis blocking the synthesis of macromolecules. Structural analogues of nucleosides such as purines and pyrimidines, interrupt the right supply of nucleotides for DNA/RNA synthesis and may directly interfere with the nucleic acid polymerization. Direct interference is achieved by inhibiting DNA and RNA dependant polymerase activity or initiating early chain termination [147]. Compounds that interrupt the supply of folate can also inhibit the nucleotide and nucleic acid synthesis. Another group of antimicrobial compounds can bind to the DNA template and block the synthesis of nucleic acids. These types of molecules can prevent both DNA replication and transcription. Topoisomerase inhibitors are another type of antimicrobials, which interfere with topological changes in DNA, that are crucial for the function and organization of DNA in bacterial cells [147].

There are antimicrobial agents that directly inhibit the synthesis of proteins by targeting steps in the protein synthesis. These antimicrobials tightly bind to the ribosome to cause changes in the protein sequence whenever ribosome synthesizes the protein. Most of antimicrobials, which inhibit protein synthesis are bacteriostatic preventing the growth of bacteria. Nevertheless, aminoglycosides show bactericidal effect through inhibition of

protein synthesis [148]. Polycationic aminoglycosides bind to the negatively charged phosphate groups at the A site of rRNA, which leads to interfere with the correct recognition of related tRNA during translation. However, based on the structural features, different classes of aminoglycosides bind to various sites on the rRNA [149]. Similarly, tetracyclines inhibit protein synthesis through binding to the 30S ribosomal subunit of rRNA [150]. The bacteriostatic antibiotic, chloramphenicol acts by binding to the 50S ribosomal subunit and subsequent inhibition of protein synthesis [148].

Metabolic pathways such as bacterial fatty acid synthesis and folic acid synthesis have been recognized as desirable targets for antimicrobial compounds. The sulfonamides inhibit enzymes involved in the folic acid pathway and subsequently blocking nucleic acid synthesis [151]. Bacteria use Type II fatty acid synthesis (FASII) to generate fatty acid components of phospholipids. Some antimicrobials specifically target the regulatory steps such as acetyl CoA carboxylase complex and condensation reactions in FASII process [152].

2.4 Genome mining and metabolomics as strategies used in the search of antimicrobial compounds

Traditional strategies used in antimicrobial discovery, that involve the isolation of antimicrobial compounds from cultured microorganisms, have successfully isolated and identified many antimicrobials used today. However, the increasing decline in the antimicrobial discovery during the last three decades has asked for novel strategies that can overcome the limitations of traditional methods [153]. Traditional methodologies may detect those molecules most readily identifiable due to their abundance in the environment. The development of ‘omics technologies’ has transformed natural product discovery to a new level. For instance, genomics has enabled information on the genetic potential of various microorganisms to produce antimicrobial metabolites in a culture-independent manner. Genome sequencing together with the identification of biosynthetic gene clusters and powerful, high sensitivity, and high throughput metabolomics techniques such as mass spectrometry (MS) and nuclear magnetic resonance (NRM) have advanced and continue to benefit antimicrobial discovery efforts.

2.4.1 Genome mining approach

In recent years, identification of biosynthetic gene clusters (BGCs) through genome mining is considered as a successful approach, that can identify novel bioactive molecules. Several computational tools have been developed to analyse microbial genomes for the synthesis of specific groups of secondary metabolites. These bioinformatic tools permit a reasonable prediction of pathway products in a culture independent manner. Under standard cultivation conditions, accessing the full genomic potential for production of bioactive molecules has been blocked due to the silent/cryptic nature of some biosynthesis gene clusters. Therefore, the traditional culture-based approach has a limited ability to induce different metabolite synthesis pathways. The genome mining approach can overcome this limitation of culture-based approaches by enabling the detection of silent metabolic pathways, which are not active under standard laboratory conditions. For instance, genome mining approaches have revealed that *Streptomyces* species, which have been extensively explored for antimicrobials, possess a higher potential to produce various secondary metabolites than previously reported through culture-based methods [154].

Many antimicrobial compounds belong to three main natural compound groups; RiPP, PK and NRP (see the literature review chapter 2.2). The biosynthetic mechanisms of these compounds are highly conserved, and a group of enzyme families are regularly and precisely involved in the biosynthesis of these molecules. Therefore, sequence information of known gene families can be used to predict the genetic potential for the biosynthesis of RiPPs, PKs and NRPs in various bacterial genomes. Several computational tools including antiSMASH [155], ARTS [156], BAGEL 3 [157], Np.searcher [158], RODEO [159], and SMURF [160] have been developed to predict BGCs in bacterial and fungal genome sequences. Two principal approaches are used in the implementation of these genome mining tools. Rule-based algorithms can precisely detect BGCs encoding known biosynthetic pathways based on the similarity to reference genes/protein domain composition [161]. The limitation of these algorithms is not being able to detect novel pathways, that have different biochemistry and enzymes. Therefore, rule-independent more generalizable machine learning approaches are used to provide a higher capability to identify novel BGCs. One such machine learning algorithm is ClusterFinder, which uses observed local protein domain frequencies (Pfam) [162].

2.4.1.1 Identification of biosynthetic gene clusters by antiSMASH

antiSMASH (antibiotics and Secondary Metabolite Analysis Shell) is a bioinformatics pipeline, which can be used for identification, annotation, and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genomes [163]. This has become a very popular tool, which is being used by many scientists to identify secondary metabolite BGCs in their genomes of interest [155].

The prediction of secondary metabolite biosynthesis by antiSMASH starts through the identification of conserved biosynthetic genes. Annotation of the genome of interest is necessary to detect conserved biosynthetic genes. antiSMASH accepts input genomes in various formats such as GenBank (NCBI), EMBL (EBI) or FASTA [164]. The gene finding tool Prodigal [165] is used by the antiSMASH bacterial version, if no annotations are present in query genomes. antiSMASH utilizes pHMMs (profile Hidden Markov Models) signature genes and PKS/NRPS signature domains for the putative detection of biosynthetic gene clusters in the query genomes. First, core enzymes involved in the biosynthesis of secondary metabolites are identified and then co-located core genes are compared with a group of manually curated cluster rules [164]. In addition to rule-based approaches, antiSMASH uses ClusterFinder algorithm to predict unknown BGCs using protein domain signatures (Pfam) [162].

The first version of antiSMASH was released in 2011 [166], since then many improvements have been added to detect varied classes of secondary metabolites. antiSMASH is equipped with other tools to provide additional information on all detected cluster types. The ‘ClusterBlast’ tool compares the similarity of individual genes and their arrangement in the query cluster with a comprehensive database of predicted BGCs of publicly available genomes. This comparison identifies other microorganisms harbouring similar BGCs as the query cluster [164]. ‘KnownClasterBlast’ module compares predicted BGCs with the known/ characterized BGCs in the Minimum Information about a Biosynthetic Gene cluster (MIBiG) database to identify closest compound/ product [167]. Moreover, antiSMASH generated results are directly used by several other independent tools including ARTS (Antibiotic Resistance Target Seeker) and Big-SCAPE (BGC clustering and classification platform) [168].

2.4.2 Metabolomics approach

Antimicrobial discovery efforts pose some challenges, frequently associated with the presence of active compounds in complex matrices with all types of compounds. Therefore, an elaborative and high-throughput analytical technique is required to isolate and identify active compounds. Metabolomics is defined as the identification and quantification of metabolites produced as end products of a biological process in cells, tissues or an organism [169]. It is a high-throughput analytical technique that allows global analysis of low molecular weight molecules of all cellular metabolic reactions, irrespective of the reactions. This is considered as the preferred method to identify compounds in complex mixtures [170].

The two main analytical platforms employed in metabolomics are mass spectrometry (MS) [171] and Nuclear Magnetic Resonance (NMR) spectroscopy [172] and they have contributed to its rapid growth. NMR has several beneficial features for the analysis of metabolites in complex biological samples such as no sample preparation, highly quantitative, highly reproducible, enables unknown metabolite identification, and non-destructive to samples. The challenges of NMR technique include relatively poor sensitivity compared to MS, and limited spectral resolution as chromatography is not used as in Liquid Chromatography coupled to Mass Spectrometry (LC-MS) [173]. Mass spectrometry provides information on the exact mass and the fragmentation patterns of metabolites. Unlikely NMR, MS requires sample preparation and chromatographic separation for the analysis of complex biological samples [174]. However, there is no one single analytical technique available to identify all metabolites in a biological sample due to the complexity of a biological system and the chemical diversity of the metabolome. The combination of several analytical tools and conditions can increase the coverage of metabolite detection in LC-MS.

Metabolomics have found a variety of applications in antimicrobial compound discovery, from the active compound identification, production optimization, and investigating mechanisms of action to the toxicity testing [175, 176]. This is an effective tool for the chemical screening and comparison of the metabolomes of various bacterial fermentations highlighting the differently produced molecules between samples, which facilitates the identification of active compounds. The structural information of the molecules of interest obtained from the NMR or mass spectrometry can be used to compare with literature and databases. This helps to identify known

structures/compounds in the samples without time consuming isolation processes. On the other hand, some novel compounds may have a known structural core with unknown functional groups, and they can be structurally characterized with the help of various 2D NMR techniques [177, 178]. Furthermore, MS/MS spectra/fragmentation patterns in combination with molecular networking analysis is capable of identifying previously unknown neighbouring compounds within the same chemical groups/families using spectral correlations [179]. For compounds with unknown nuclei, it is inevitable to isolate them using chromatography followed by full *de novo* structural characterization and these compounds can be considered as truly new antimicrobials [180].

There are two metabolomics approaches, targeted and non-targeted, that can be applied in research studies. Each approach has its own advantages and disadvantages.

2.4.2.1.1 Non-targeted approach

The non-targeted approach is the comprehensive analysis of all the measurable analytes in a sample, that includes both chemically known and unknown compounds. This approach is always attached to chemometric techniques, such as multivariate analysis, to decrease the extensive amount of data obtained into a smaller set of manageable data [181]. Non-targeted data processing consists of several defined steps such as noise filtering, peak detection, peak deconvolution, retention time alignment, and finally annotation of features [182].

Non-targeted or metabolite profiling is used in inductive research studies with an objective to obtain metabolomics information relating to a wide range of metabolites in the sample. Therefore, this workflow is utilized when there is no/low information regarding the metabolite composition of the sample. This metabolomics approach has been applied in studies focusing on metabolic biomarker identification [183], bioactive compound identification [184], antimicrobial mechanisms [185], environmental health [186], nutrition [187], and human diseases [188]. The limitations of this approach are no/limited absolute quantitative data, reduced precision and accuracy to detect a wide range of metabolites, and not feasible to identify all metabolites detected [189].

Non-targeted metabolomics is suitable for microbial cell culture studies as it can provide valuable information on the metabolite profiles of cell cultures grown under various growth conditions. These experiments can be complemented with functional assays (e.g.,

antimicrobial activity assay) designed to test the bioactivities (e.g., antimicrobial activity) of different broth cultures/fractions/metabolites. If the non-targeted approach leads to the identification of a novel metabolite, subsequent experiments can be performed to characterize it structurally.

2.4.2.1.2 Targeted approach

The targeted approach is the measurement of defined groups of metabolites, which have been chemically characterized and biochemically annotated [181]. In other words, the focus of this approach is a pre-defined group of metabolites using the former knowledge of metabolites and their metabolic pathways. This clear definition of the chemical species lowers the possibility of analytical artifacts developed during experiment and the data analysis [181]. These analyses can be carried out in a quantitative or semi-quantitative way with the use of isotopically labelled internal standards. Metabolite extraction procedure can be designed specifically targeting the metabolites of interest, which lowers the interference by other high abundant chemical species in the sample. Moreover, the specificity, accuracy, and precision of the analysis can be improved by developing and validating highly specific analytical methods for targeted molecules. A higher level of metabolite separation is typically employed in targeted analysis compared to the non-targeted workflow [181].

This metabolic workflow is used in hypothesis testing or deductive studies. In other words, targeted metabolomics analysis is employed when there is an identified rationale for selecting chemical species under study. Therefore, this workflow can be applied in antimicrobial research when the chemical compounds under study are defined/known.

2.4.2.2 Liquid chromatography coupled to mass spectrometry (LC-MS)

Liquid chromatography coupled to mass spectrometry (LC-MS) is an important analytical tool in metabolomics. LC-MS technique provides the advantages of its both components, LC as a powerful separation technique of a wide range of molecules and MS as a powerful and sensitive detection, and identification technique. There are numerous LC-MS methods, which have been developed for various applications based on their requirements.

The chromatographic separation of as many as analytes in the samples is important in non-targeted workflow. There is no single LC method, that can analyse all the metabolites. Therefore, the optimal coverage is achieved by the use of multiple LC-MS methods [190]. In many non-targeted metabolomics studies, hydrophilic interaction liquid chromatography (HILIC) is used to enhance the retention and separation of polar metabolites [191]. A C18 column has been employed to analyse semi-polar compounds in biological samples [192].

Analytes coming from the LC system are ionized in the mass spectrometer, and the resulting ions and fragmented ions are analysed on the basis of their mass/charge (*m/z*) ratio [193]. Mass spectrometers with different technologies of both ionization and ion analysis are available. A few ionization technologies available are electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI). Commonly used mass analysers in mass spectrometers are quadrupole analysers, time-of-flight analysers, and ion trap ionisers [193]. In non-targeted workflow, mass detection is carried out in full scan mode, usually from 50 to 1200-1500 atomic mass units (amu) for detecting as many compounds as possible.

2.4.2.3 Chemical isotope labelling (CIL) liquid chromatography mass spectrometry (LC-MS).

As described above, the conventional LC-MS based non-targeted workflow uses multiple LC techniques to separate as many as compounds in the sample since a single extraction and separation method is not optimal for all the metabolites. Alternatively, chemical isotope labelling (CIL) liquid chromatography coupled to mass spectrometry can be performed for metabolome profiling. This technique is based on grouping metabolites into several subgroups depending on the availability of common functional moieties and carrying out in-depth analysis of individual chemical group sub-metabolomes [194]. In this way, better coverage of the overall metabolome of the biological sample can be achieved. Chemical isotope labelling (CIL) is used to introduce a mass tag to all the metabolites containing common functional groups in the sample and the standards are also tagged using the same chemical derivatization reaction, but with a different mass tag (e.g. samples are labelled with ^{12}C and standards are labelled with ^{13}C) [195]. The properties of labelling reagents such as chemical structure and reactivity are important for

the detection sensitivity of all metabolites in relevant submetabolomes [194]. Differential isotope labelling of individual samples and a reference sample with light and heavy isotope reagents respectively, followed by LC-MS analysis allows precise quantification of metabolites in the metabolome [196]. The peak intensity ratio of the light and heavy isotope labelled metabolite pair is the basis for relative quantification of metabolites in two comparative samples.

For chemical labelling of functional groups, various isotope labelling methods can be used. Several isotope reagents with various labelling chemistries have been developed for both targeted and non-targeted metabolomics approaches [194, 197]. An isotope reagent ^{12}C-/^{13}C-dansyl chlorite is used to label metabolites containing primary amines, secondary amines, or phenolic hydroxyl group(s) (Figure 2.1). Previously, the amine/phenol submetabolome has been analysed with high coverage using dansylation LC-MS [196]. An isotope reagent ^{12}C-/^{13}C-dimethylaminophenacyl (DmPA) bromide is used to label carboxylic acid containing metabolites. Figure 2.2 shows the isotope labelling reaction of metabolites having carboxylic functional group/s using isotope reagent ^{12}C-/^{13}C-dimethylaminophenacyl bromide [195].

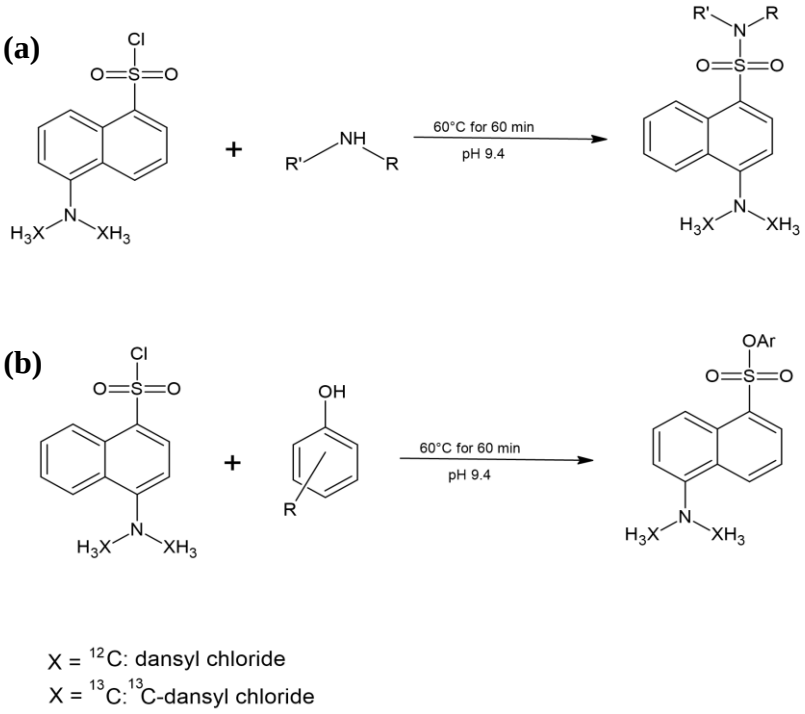
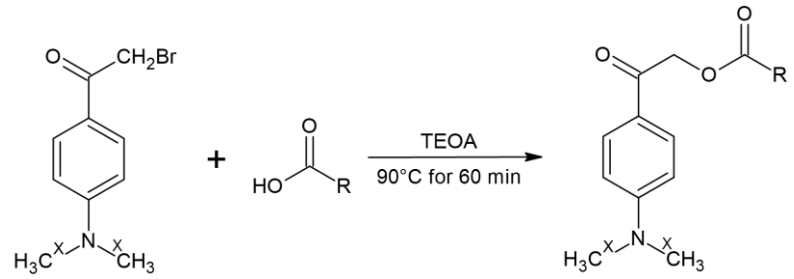


Figure 2.1: Reactions of dansylation derivatization. Dansylation of amine-containing **(a)**, and phenol-containing **(b)** compounds. The drawing was adapted from Guo and Li [196] using ChemSketch software version C15E41.



X = 12 or 13

Figure 2.2: Isotope labelling reaction of metabolites containing carboxylic acid using isotope reagent ^{12}C-/^{13}C-dimethylaminophenacyl (DmPA) bromide. The drawing was adapted from Guo and Li [195] using ChemSketch software version C15E41.

2.4.2.4 Metabolite identification

Metabolite identification is the conversion of unidentified metabolite features to known chemical entities or metabolites. The identification of metabolites is often considered as the major challenge in non-targeted metabolomics. Simple and automated techniques are required for identifying thousands of metabolite features generated in a single sample from powerful metabolomics tools such as MS and NMR. But metabolite identification techniques are still manual or semi-automated and only metabolite features of biological interest are included for identification instead of all metabolites detected in the study [189].

During the identification process, it is common to assign a large number of tentative structures to a one metabolite feature (mass to charge ratio and retention time) and some features may not have any candidate match in curated databases as they are considered incomplete. In addition, metabolite features obtained from untargeted metabolomics experiments are not always metabolites, they could be related species such as isotopes, adducts or neutral losses of a single metabolites which may have a different *m/z* value.

Metabolite identification is carried out by searching the experimental mass measurements (MS1 or MS/MS data) through public databases within a mass tolerant window to obtain potential candidates. In 2007, the Chemical Analysis Working Group (CAWG) suggested the minimum reporting standards for metabolite identification with different confidence levels [198]. Their proposed guidelines consist of four metabolite identification confidence levels as outlined in Figure 2.3.

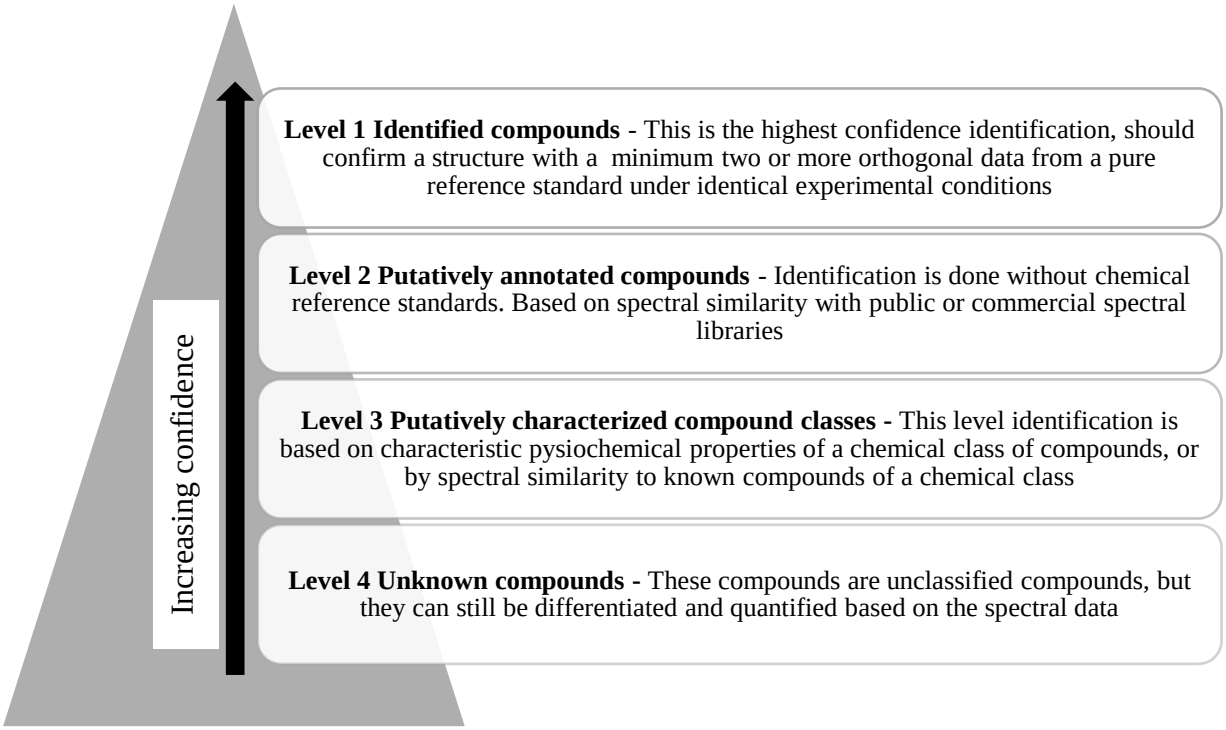


Figure 2.3: Proposed metabolite identification confidence levels by Chemical Analysis Working Group (CAWG) [198].

As given in Figure 2.3, the highest identification confidence (level 1) is achieved by confirming the structure through minimum two or more orthogonal data from a reference standard under identical experimental conditions. Level 2 identification is done based on spectral similarity with public or commercial spectral libraries without reference standard acquisition. Public libraries, which can be used for metabolite identification with level 2 confidence are METLIN [199], MassBank [200], and Human Metabolome Database (HMDM) [201].

In general, a variety of metabolomics techniques and procedures developed so far have found applications in various fields of study including antimicrobial discovery. Non-targeted and targeted workflows can be used based on the objectives of the antimicrobial research. However, there are advantages and drawbacks inherent to every technique used in metabolomics. The principal challenge in non-targeted approach is the identification of detected metabolite features.

2.5 Anaerobic bacteria as a source of antimicrobial compounds

Most of the identified natural products with antimicrobial, antiviral, and antitumor activity are of bacterial origin [202]. Many of these compounds have been isolated from fewer bacterial groups such as actinomycetes and Myxobacteria. Nevertheless, in recent years opportunities for new antimicrobial compound discovery from well-known bacterial groups have fallen considerably [203]. Microbial diversity presents a great opportunity for the search for novel antimicrobial compounds [204]. Physiochemical parameters in natural ecosystems determine the assembly of microbial communities and these microorganisms within these communities have developed metabolic mechanisms to produce and consume numerous metabolites allowing them to cross-feed one another and survive in highly specific niches, yet we know very little about these interactions [205]. Therefore, diverse bacteria living in various ecosystems are of great interest in antimicrobial discovery. In recent years, microorganisms living in various environments such as desert soil, freshwater sediments, endophytes and symbiotic microbes from marine environments, humans, insects, and nematodes have been explored for new antimicrobials [14, 206-210]. However, obligately anaerobic bacteria, which occur ubiquitously in various soil environments and the intestine of humans and animals, are yet to be explored for bioactive molecules such as antimicrobial compounds.

Anaerobic bacteria thrive in many natural environments, where oxygen is depleted and have a vital role in carbon circulation. Examples of such environments are soil, sediments from rivers and lakes, and gastrointestinal tracts of animals. They play an important role in natural processes such as degradation of waste, fixation of carbon dioxide, and fermentation of organic matters [11]. Anaerobic bacteria have found their way into several industrial applications such as production of fuels and chemicals. The first industrial fermentation process was the acetone-butanol-ethanol process by *Clostridium acetobutylicum* in 1920 [211].

Microorganisms living under extreme environmental conditions have distinctive properties, and potential to yield unique bioactive molecules. Conceivably, the secondary metabolism of obligate anaerobes such as *Clostridium* spp. has evolved giving arise to various adaptations to survive and proliferate in oxygen limited environments [212].

Therefore, the synthesis of specialized secondary metabolites should be more relevant to these bacteria.

The healthy gastrointestinal microbiota is composed of obligate anaerobes such as *Clostridium* Cluster IV and XIV species together with other well-known beneficial microorganisms and these obligately anaerobic microorganisms are also likely to produce health promoting and antimicrobial compounds [213]. Specific *Clostridium butyricum* strains have been commercialized as probiotic organisms for humans and animals mainly in Japan, Korea, and China [214]. *Clostridium butyricum* MIYAIRI has been reported to have antagonistic interaction against enterohemorrhagic *E. coli* O157:H7 in infected mice and their germinating spores are found to inhibit *Clostridium difficile* spore germination [215, 216].

2.5.1 *Clostridium* spp. as antimicrobial producers

Bacteria of the genus *Clostridium* are Gram-positive, rod-shaped, endospore-forming, and obligately fermentative anaerobic bacteria with over 200 known *Clostridium* species [217]. Different species are capable of fermenting sugars, amino acids, purines and pyrimidines, and other substrates. Several saccharolytic Clostridia produce butyric acid as a main fermentation product together with other neutral compounds such as butanol and acetone. Some proteolytic Clostridia ferment individual amino acids including glycine, glutamate, alanine, histidine, and serine while others ferment only an amino acid pairs such as glycine and alanine [218].

In this genus, pathogenic species have received the most attention owing to their harmful effects on humans, animals, and food systems. Pathogenic *Clostridium* spp. are known to cause human diseases such as tetanus, botulism, gas gangrene, and pseudomembranous colitis [219]. Mesophilic spore forming anaerobic bacteria such as *Clostridium putrefaciens*, *Clostridium estertheticum*, and *Clostridium gasigenes* are involved in spoilage of refrigerated vacuum-packed meat products [220]. *Clostridium botulinum* and *Clostridium perfringens* are two of the most common foodborne pathogens [221]. Despite the pathogenic members, most of the *Clostridium* spp. are saprophytes, not involved in disease. The non-pathogenic majority of *Clostridium* spp. may have beneficial effects on human health and food systems through the production of bioactive compounds.

In recent years, there is an increasing scientific interest to explore the antimicrobial potential of *Clostridium* and closely related spp. with the intention of finding secondary metabolites with potent antimicrobial applications in medicine and food preservation [62]. This has been encouraged by genomic studies on *Clostridium* spp. showing the genetic potential to produce various secondary metabolites belonging to known antimicrobial compound groups. But, still limited chemical and genetic information is available on the clostridial secondary metabolites and their bioactivities. The limited studies conducted thus far focusing on the antimicrobial potential of *Clostridium* spp. have provided some positive outcomes as described in the following section.

2.5.1.1 Identification of antimicrobials from *Clostridium* spp. using culture-based methods

Traditionally, the antimicrobial compound discovery has relied upon the testing of bacteria cultivated under standard laboratory conditions for antimicrobial activity and isolating active compounds from them. This method has been successfully used in the discovery of many antimicrobial compounds currently approved for various applications. The idea of *Clostridium* spp. as potential antimicrobial producers is not novel even though they have not received much attention compared with other bacterial groups such as *Bacillus* species, which produce a wide variety of antimicrobial secondary metabolites (mainly antimicrobial peptides). These include polymyxin, bacitracin, gramicidine, and subtilosin [222-225].

During 1960-1980, a few research groups worked on the screening *Clostridium* spp. for bacteriocin production (Table 2.1). In 1968, Hongo, Murata [226] described the isolation of four bacteriocins named as clostocins A, B, C, and D including some of their properties and the activity against a wide range of *Clostridium* and *Bacillus* species. Two other studies conducted by Mahony and Butler [227] and Mahony [228] focused on the screening of *Clostridium perfringens* strains for bacteriocin production and found that 3 out of 33 and 10 out of 94 strains were capable of producing bacteriocins. A few parallel studies demonstrated the isolation and characterization of three bacteriocins, namely bacteriocin 28, bacteriocin N5, and Perfringocin 11105, from *C. perfringens* strains [229-231]. *Clostridium* spp. such as *Clostridiurn butyricum* and *Clostridium botulinum* were also investigated for bacteriocin production (Table 2.1) [229, 232]. Since then, not much

attention has been given to the genus *Clostridium* as a potential source of antimicrobials. Now, there is a renewed interest in this subject particularly with new genomic knowledge and understanding of strict anaerobes.

Table 2.1: Antimicrobial compounds identified from *Clostridium* species.

Name of the compound	Producing organisms	Microorganisms inhibited	Description	Reported year	Reference
Clostocins A, B, C, D	*Clostridium* spp.	*Clostridium* spp. and many strains in the family *Bacillaceae*	Bacteriocins-insensitive to UV light, ribonuclease and deoxyribonuclease. A and D – thermostable (100 ^{o}C for 30 min) and B and C – relatively thermo-labile (started to lose the activity at 60 ^{o}C after 30 min. treatment)	1968	[226]
Clostocins O	*Clostridium saccharoperbutylacetonicum* Nl-4 (ATCC13564)	*Clostridium* spp.	Phage-tail-like bacteriocin.	1968	[226]
Perfringocin 11105	*Clostridium perfringens* type A NCIB 11105	*Clostridium pasteurianum*	Bacteriocin-Amphiphilic peptide having molecular mass of around 76 kDa	1974	[229]
Butyricin 7423	*Clostridium butyricum* NCIB7423	*Clostridium pasteurianum*	Bacteriocin-Trypsin sensitive amphiphilic protein. Its action appears to be at cell membrane leading to altered permeability	1974	[229]
Boticin P	*Clostridium botulinum* PM-15, type E	Other *Clostridium botulinum* strains	Bacteriocin having phage tail-like structure	1974	[232]
Bacteriocin N5	*Clostridium perfringens* BP6K-N5	*Clostridium* spp.	Bacteriocin-single polypeptide chain with a molecular mass approximately 82 kDa. Inhibits the synthesis of DNA,	1975	[230, 233]

			RNA and protein in sensitive cells		
Bacteriocin 28	*Clostridium perfringens* strain 28	*Clostridium* spp.	Bacteriocin-heat-stable glycoprotein peptide containing 15 amino acids with molecular mass around 84 kDa	1982	[231]
BCN5	*Clostridium perfringens* CPN50	*Clostridium* spp.	Bacteriocin-expression can be stimulated by UV light. Molecular mass is approximately 96.5 kDa	1986	[234]
Boticin B	*Clostridium botulinum* 213B	*Clostridium botulinum* and related *Clostridium* spp.	Bacteriocin-heat stable small peptide with a predicted size of 50 amino acid residues	2000	[235]
Closticin 574	*Clostridium tyrobutyricum* ADRIA932	*Clostridium tyrobutyricum* and *some Lactobacillus strains*	Bacteriocin-synthesized as a preprotein containing 310 amino acid residues and subsequently processed to 82 amino acid peptide	2003	[236]
Circularin A	*Clostridium beijerinckii* ATCC25752	*Clostridium tyrobutyricum strains* and *Lactococcus, Enterococcus,* and *some Lactobacillus strains*	Bacteriocin-circular peptide having 69 amino acid residues and three amino acid leader sequence	2003	[236]
Closthioamide	*Clostridium cellulolyticum* DSM5812 and pSB050	Methicillin-resistant *Staphylococcus* aureus and vancomycin-resistant enterococci (VRE) strains	Antibiotic-belonging to a new class of natural products, the polythioamides	2010	[14]
Diffocins	*Clostridium difficile*	Other *Clostridium difficile* strains	Bacteriocin-R-type phage tail-like high molecular weight peptide	2012	[86]

Perfrin	Necrotic enteritis-associated *n etB*-positive *Clo stridium perfringens* strain	Other *Clostridium perfringens* strains	Bacteriocin- 11.5 kDa C-terminal fragment of a 22.9 kDa protein	2014	[237]
Clostrubin	*Clostridium beijerinckii*	*Escherichia coli, Bacillus subtilis,* Methicillin-resistant *Staphylococcus* aureus, *Enterococcus faecalis,* and *Mycobacterium* spp.	Antibiotic-polyphenolic polyketide antibiotic, first reported polyketide from anaerobic bacteria	2014	[15]
Clostrindolin	*Clostridium beijerinckii* HK1805	*Mycobacterium vaccae*	Pyrone alkaloid	2019	[16]

Until recently, no clostridial secondary metabolite having antimicrobial activity was identified other than bacteriocins. The discovery of three novel antimicrobial metabolites, closthioamide, clostrubin, and clostrindolin (Table 2.1, Figure 2.4) from *Clostridium cellulolyticum* DSM5812, *Clostridium beijerinckii* ATCC25752, and *Clostridium beijerinckii* HK1805 respectively are examples of current efforts exploring the antimicrobial potential of *Clostridium* species. Closthioamide possesses antimicrobial activity against a wide range of Gram-positive bacteria and partial activity against some *E. coli* strains. It is effective against Methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant enterococci (VRE) strains. This potent antibiotic exerts its activity by interfering with DNA replication and DNA gyrase activity [238]. Clostrubin is the first polyketide discovered from a strict anaerobe and shows antimicrobial activity against MRSA, VRE strains and mycobacteria [15]. Clostrindolin is a pyron alkaloid produced by *Clostridium beijerinckii*. It was found to show antimicrobial activity against *Mycobacterium vaccae* [16]. Several other recent papers have reported the identification, purification and characterization of antimicrobial peptides from *Clostridium* species (Table 2.1). Kemperman, Kuipers [236] identified two novel bacteriocins namely closticin 574 and circularin from *Clostridium tyrobutyricum* and *Clostridium*

beijerinckii respectively. Perfrin was identified as a novel bacteriocin from a *Clostridium perfringens* strain, which was active against other *Clostridium perfringens* strains [237]. In their study, Gebhart, Williams [86] reported diffocins, R-type phage tail-like bacteriocins from *Clostridium diffcile* strains, active against other *Clostridium diffcile* strains.

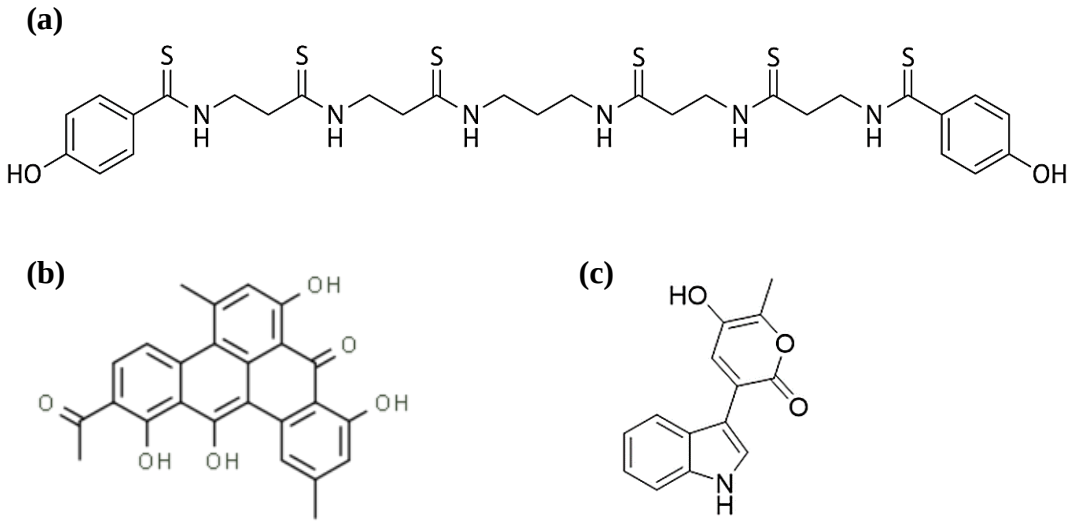


Figure 2.4: Structures of three antimicrobial metabolites identified from *Clostridium* species. Closthiamide **(a)** [238], Clostrubin **(b)** [239], and Clostrindolin **(c)** [16]. Structures were drawn by ChemSketch software version C15E41.

2.5.1.2 Genome-based identification of secondary metabolite gene clusters in *Clostridium* species

The growing number of bacterial genome sequences together with the understanding of the genetic basis of secondary metabolites may provide information on the potential of bacteria to synthesize a variety of secondary metabolites. This information can be utilized in the development of novel antimicrobial compounds. In recent years, computational strategies have shown the genetic capacity of various overlooked bacterial groups to produce a variety of secondary metabolites [62]. Genome mining has been employed in identifying the antimicrobial potential of obligate anaerobes including *Clostridium* spp. by predicting the genomic potential to produce diverse natural products including ribosomally synthesized and post-translationally synthesized peptides (RiPPs),

polyketides (PKs) and non-ribosomal peptides (NRPs) [240]. More information on RiPPs, PKs, and NRPs compounds and the use of genome mining approach in antimicrobial discovery can be found in the literature review chapter 2.2 and 2.4.1 respectively.

2.5.1.2.1 The occurrence of RiPP gene clusters in the genus **Clostridium**

There are over 20 different RiPP families, which have been described based on the structural characteristics and biosynthetic machinery [241]. Recently, a wide range of bacteria have been studied for the presence of RiPP gene clusters using genome mining [240, 242-244]. But still limited information is available on the presence of RiPP gene clusters in the genomes of *Clostridium* species. A recent study assessed 224 complete/partial genomes of ruminal bacteria and showed that out of 33 ruminal bacterial strains harbouring bacteriocin gene clusters, 7 were *Clostridium* species. Another study conducted by Letzel, Pidot [245] investigated complete and published genomes of 211 anaerobic bacteria including 35 *Clostridium* strains for the occurrence of RiPP encoding genes and gene clusters and found that over 25% of test anaerobic genomes had the genetic basis for producing RiPPs either alone or in combination with other secondary metabolites including PKs and NRPs. This study also highlighted the capability of *Clostridium* spp. to synthesize a variety of RiPP classes such as lanthipeptides, sactipeptides, thiopeptides, lasso peptides, linear azol(in)e-containing peptides (LAP), lacto-coccins, and head to tail cyclic peptides. Nevertheless, lanthipeptides, sactipeptides, and LAP were the dominating RiPP classes detected in *Clostridium* species (Table 2.2). Tushar, Sasi Jyothsna [246] described the presence of gene clusters responsible for the synthesis of four microcins in the genome of a newly identified *Clostridium* spp. JC272.

Table 2.2: Putative RiPP gene clusters detected in *Clostridium* species.

Bacterial species	Strains	Accession No.	BGC type
C. cellulovorans	ATCC35296/ 743B	CP002160.1	Lanthipeptide I & II & Thiopeptide
C. kluyveri	DSM555	CP000673.1	Lanthipeptide I & sactipeptide
C. acetobutylicum	ATCC824 DSM1731 EA2018	AE001437.1 CP002660.1 CP002118.1	Lanthipeptide II & sactipeptide
C. beijerinckii	NCIMB8052	CP000721.1	Lanthipeptide II
C. botulinum	H04402065	FR773526.1	Lanthipeptide II & LAP
C. botulinum	A2 Kyoto-F A1 ATCC19397 A1 Hall B1 Okra A3 Loch Maree Ba4 str. 657 F230613 A ATCC3502 F str. Langeland	AY497358 CP000726.1 CP000727.1 CP000939.1 CP000962.1 CP001083.1 CP002011.1 AM412317.1 CP000728.1	LAP
C. cellulolyticum	H10	CP001348.1	Sactipeptide
C. difficile	630	AM180355.1	Sactipeptide
C. lentocellum	DSM5427	CP002582.1	Sactipeptide
C. thermocellum	ATCC27405	CP000568.1	Sactipeptide
C. perfringens	13	BA000016.3	Lasso peptide
C. perfringens	SM101	CP000312.1	Lactococcin-like RiPP & head to tail cyclized peptide

2.5.1.2.2 The occurrence of non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) gene clusters in the genus Clostridium

Recent genomic studies rejected the previous misconception of strict anaerobes not carrying genes for NRPs and PKs by showing the genetic potential of *Clostridium* spp. for production of NRPs and PKs. A study conducted by Seedorf, Fricke [247] evaluated the metabolic capabilities of *Clostridium kluyveri* using its whole genome sequence and found four coding sequence (CDS) clusters predicted to encode NRPS and NRPS-PKS hybrids. Another study evaluated 211 published anaerobic genomes in search of specifically putative NRPS and PKS and revealed that 33% of test genomes possessed PKS and NRPS genes. In addition, there was an association between the secondary metabolic potential and habitat of the organism. Anaerobic microorganisms isolated from soil had three times higher genetic content to produce secondary metabolites than all other anaerobes collective from different habitats [13].

Behnken and Hertweck [248] investigated the presence of PKS gene clusters in the genomes of 31 *Clostridium* spp. and found a wide distribution of putative PKS gene clusters only in non-pathogenic strains. All 26 pathogenic strains of *Clostridium botulinum, Clostridium sporogenes, Clostridium tetani, Clostridium novyi, Clostridium perfringens and Clostridium difficile* involved in the study did not have PKS genes. They also used degenerate primers based on the KS domains to screen 22 non-sequenced strains for the occurrence of modular type I PKS genes. Only three species, *Clostridium hungatei, Clostridium chartatabidum, and Clostridium akagii* were detected with PKS genes. Another study, which examined 223 complete bacterial genomes (five from the class Clostridia) for the presence of putative PKS and NRPS gene clusters, reported that *Clostridium acetobutylicum* held a putative gene cluster encoding for PKS [249] (Table 2.3).

Table 2.3: Putative PKS, NRPS, and PKS-NRPS gene clusters detected in *Clostridium* species.

***Clostridium* spp.**	**Strains**	**Accession No.**	**BGC types**	**Reference**
C. acetobutylicum	ATCC824	AE001437.1	PKS	[249], [248]
C. kluyveri	DSM555	CP000673.1	NRPS PKS-NRPS	[247], [248]
C. beijerinkii	NCIMB8052	CP000721.1	PKS-NRPS	[248]
C. cellulolyticum	H10	CP001348.1	PKS-NRPS	[248]
C. cellulovorans	743B	CP002160.1	PKS	[248]
C. papyrosolvens	DSM2782	GCA_000175795.2	PKS, PKS-NRPS	[248]
C. thermocellum	ATCC27405	CP000568.1	PKS	[248]
C. hungatei	DSM14427	MZGX00000000.1	PKS	[248]
C. chartatabidum	DSM5482		PKS	[248]
C. akagii	DSM12554	KK366007.1	PKS	[248]

2.6 2-hydroxyisocaproic acid (HICA)

2.6.1 Biochemistry and its distribution

2-hydroxyisocaproic acid (HICA), which is also called 2-hydroxy-4-methylvaleric acid, DL-leucic acid, 2-hydroxy-4-methylpentanoic acid and alpha-hydroxyisocaproic acid, is an analogue of leucine with hydroxy substituent at the 2-position and a methyl substituent at the 4-position [250]. This compound is produced as a by-product of the leucine degradation pathway in humans and certain microorganisms (Figure 2.5). 2-ketoisocaproic acid (KICA) is the central intermediate in the metabolism of leucine. It can be transaminated back to the leucine amino acid, hydrogenated to its hydroxy acid (HICA), attached to CoA to produce isovaleric acid or decarboxylated to aldehyde (3-methylbutanal) [251]. HICA is produced by transamination of leucine to KICA followed by reduction of KICA to HICA. The reduction reaction is catalysed by the hydroxyisocaproic/hydroxyisocaproate acid dehydrogenase (HicD) enzyme [252].

HICA is produced by leucine metabolism in human tissues including connective tissues and muscles [253]. It is considered as a physiological agent present in the human body at low levels. The healthy adult usually contains around 0.1-0.25 mmol/L HICA in plasma. The plasma HICA level increases after exercise or during prolonged fasting due to the use of proteins for energy [254]. HICA has also been reported to be a microbial metabolite produced during fermentation of animal proteins [255]. Mainly lactic acid bacteria such as *Lactobacillus plantarum*, *Lactococcus lactis*, *Lactobacillus plantarum*, *Lactobacillus brevis,* and *Leuconostoc mesenteroides* have been reported to produce HICA. Other microorganisms, which are able to produce HICA are *Clostridium difficile* and *Peptostreptococcus anaerobicus* [251, 256-261]. Fermented food products such as certain cheeses, soy sauce, wines, and kimchi have been found to contain HICA [261, 262]. Cytotoxicity and genotoxicity of HICA has been tested using human periodontal ligament fibroblasts and reported that it is safe for humans at concentrations < 10 mg/mL [263].

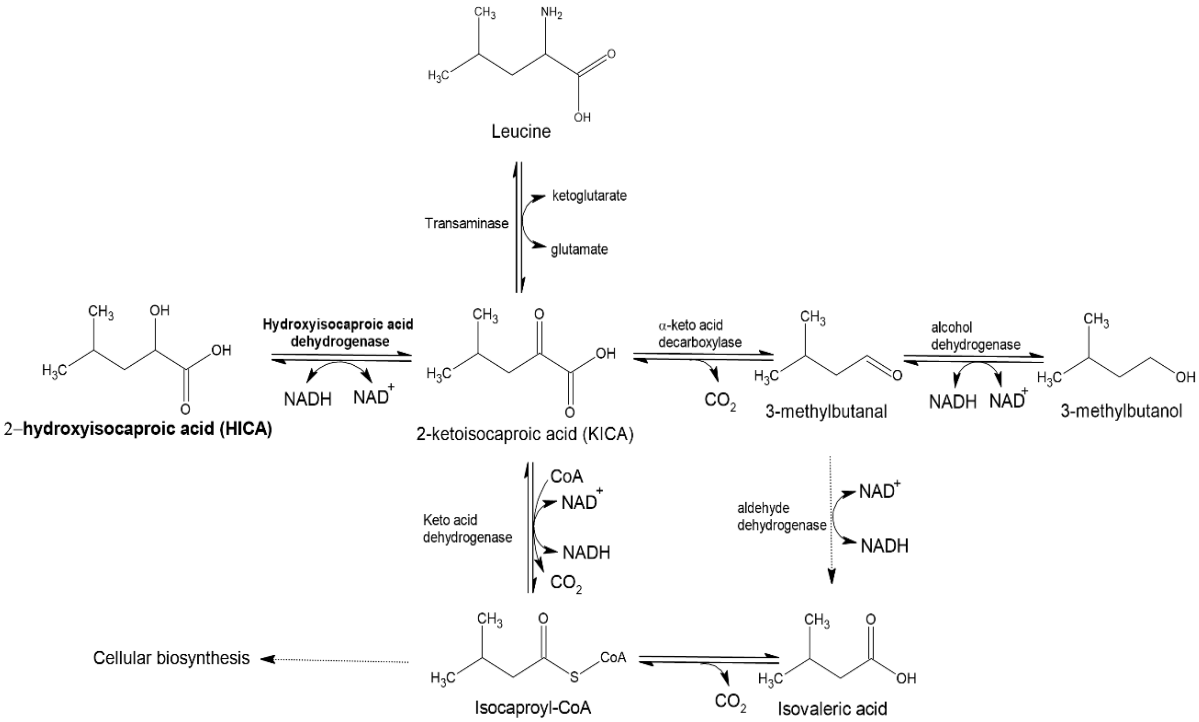


Figure 2.5: Schematic illustration of reaction scheme of the leucin degradation pathway. The drawing was adapted from Smit, Engels [251] using ChemSketch software version C15E41.

2.6.2 Antimicrobial properties of HICA

HICA has shown antimicrobial activity against certain bacteria and fungi. Sakko, Tjäderhane [252] investigated the antimicrobial activity of HICA against several Gram-positive and Gram-negative human pathogenic bacteria including multi-drug resistant strains. Their results suggested that HICA was active against all tested bacteria with minimum bactericidal concentration (MBC) ranging from 4.5 – 36 mg/mL. HICA was highly active against *Pseudomonas aeruginosa* and *Fusobacterium nucleatum* strains showing 4.5 mg/mL MBC, while *Enterococcus faecalis* and *Lactobacillus rhamnosus* isolates showed the greatest MBC (36 mg/mL) among tested bacteria. HICA was also active against methicillin-resistant *S. aureus* (MRSA) and extended-spectrum β-lactamase (ESBL) positive *E. coli* strains with MBC values of 9 mg/mL and 18 mg/mL respectively. HICA demonstrated broad antifungal activity against *Candida* and *Aspergillus* species associated with systemic fungal infections [264]. Another study showed that HICA inhibited *Candida albicans* biofilm formation with significant

reduction of the mutagenic potential of the biofilms [265]. To date, HICA has only been considered for use as an interappointment medication in the treatment of root canal infections [266].

2.6.3 Other benefits of HICA

Nieminen, Hernandez [267] studied anti-inflammatory potential of HICA using *C. albicans* biofilm in a murine model and found that the HICA attenuated inflammatory response together with reduced expression of immune mediators. Other than the bioactive properties, HICA has been investigated as a nutritional supplement for athletes. HICA has been reported to improve the muscle mass and speed muscle recovery after exercise [253, 268].

2.7 Summary

Antimicrobial compounds are natural or synthetic compounds, which have a capacity to kill or inhibit the growth microorganisms. These compounds play vital roles in medicine, food, and agriculture. The exploration of novel natural antimicrobial compounds has become important due to several reasons, in particular, consumer demand for natural food preservatives over synthetic ones due to concerns over adverse health effects of synthetic chemicals. The development of microbial resistance to current antimicrobials has fast tracked the exploration of novel antimicrobial compounds. Microorganisms, mainly bacteria and fungi, have been recognized as promising sources of natural antimicrobial compounds. Extensive work has been done to identify and characterize bacterial antimicrobial compounds. However, antimicrobial discovery has been biased towards aerobic and facultative anaerobic bacteria. Strict anaerobes such as *Clostridium* spp. have been largely neglected. In recent years, previously overlooked bacterial groups and bacteria from previously unexplored habitats are considered in antimicrobial discovery efforts in search for novel antimicrobials.

Antimicrobial compounds are a chemically and structurally diverse group of compounds, but many belong to three prominent compound classes: ribosomally synthesized peptides, non-ribosomal peptides, and polyketides. Other than compounds belonging to these groups, which are produced through the direct regulation of gene clusters, bacteria are

also able to produce a variety of small molecules, which may have potent bioactivities including antimicrobial activity through different metabolic pathways. Therefore, the metabolic diversity of various bacteria could be of interest in exploring novel secondary metabolites with antimicrobial properties.

Genome mining and metabolomics have facilitated a new era of antimicrobial compound discovery. Genome mining is a valuable tool for assisting in the identification of the antimicrobial potential of previously overlooked bacteria by providing insight into the genomic potential for the production of various secondary metabolites. This approach can unveil the hidden biosynthesis capacity of bacteria by detecting biosynthetic pathways that cannot be detected using standard culture conditions. Metabolomics is a high-throughput analytical technique, which can facilitate identifying compounds in complex mixtures. Spectroscopic methods such as mass spectrometry and nuclear magnetic resonance can be used to chemically profile the metabolites produced by bacteria in search of active compounds.

In recent years, there has been renewed interest in exploring the antimicrobial potential of *Clostridium* and closely related species. With the advancement of computational genome technologies such as genome mining, *Clostridium* spp. were identified as capable secondary metabolite producers, and this has inspired natural product discoveries including potential antimicrobials from the genus *Clostridium*. Recent studies have shown the genetic potential of several *Clostridium* spp. for production of secondary metabolites belonging to known antimicrobial compound groups and a few secondary metabolites with potent antimicrobial activities have been identified from *Clostridium* species. These limited studies conducted thus far have identified the genus *Clostridium* as a viable source of antimicrobial compounds. However, this large genus of obligately anaerobes needs to be well studied to understand its actual antimicrobial potential. Their metabolic diversity with their specialization to live under harsh low oxygen environmental conditions is of interest to explore novel secondary metabolites having antimicrobial properties.

2-hydroxyisocaproic acid (HICA) is a side-product of the leucin degradation pathway and produced by the human body and certain bacteria, mainly lactic acid bacteria. A previous study has reported its antimicrobial activity against certain bacteria and fungi. As HICA can be metabolized by the human body and is a natural compound in some food products,

it has a good biocompatibility as well as a safety profile. Therefore, it may have potential applications in food preservation and human health. In this context, it is worth investigating the antimicrobial efficacy of HICA against significant bacteria associated with food quality, safety, and human health. So far, no information is available on the possible antimicrobial mechanisms of HICA against bacteria.

Chapter 3

Materials and methods

Materials and methods used throughout this study are included in this section. They have been separated based on research chapters (Chapter 4 – 8) for easy referring.

3.1 Materials and methods for Chapter 4

3.1.1 Bacterial strains and growth conditions

Bacillus mycoides ATCC6462, *Bacillus pumilus* ATCC14884, *Bacillus cereus* NZRM5, *Salmonella* Hadar NZRM4206, *Salmonella* Typhimurium NZRM3970, and *Pseudomonas aeruginosa* ATCC25668 were purchased from Environmental Science and Research (ESR), New Zealand. *Escherichia coli* O157:H7 NCTC12900 was obtained from the National Collection of Type Cultures (NCTC), Public Health England. All the frozen glycerol stocks were revived on Sheep Blood Agar (SBA) plates (Fort Richard Laboratories, New Zealand) by incubating at 35 °C overnight. All microorganisms were grown in Tryptic Soy Broth (TSB) and incubated at 35 °C overnight as required for further experiments.

3.1.2 Farm environmental samples

Farm environmental samples previously collected in a research project were used in this study. They were collected from three farm sources (soil, feed/silage, and dairy effluent) of three dairy bovine farms in the Manawatu region, New Zealand during the winter season and stored at -20 ^{o}C until use. These farm sources were selected for this study

based on the result of preliminary studies in our laboratory. These nine samples were used in stage I screening study. In addition to the three soil samples from above three farms, two additional soil samples collected from dairy bovine farms in the Manawatu region, New Zealand during the winter season were also used in stage II screening study. Farms were assigned a number for easy recording and to maintain confidentiality. More information about samples is given in the antimicrobial potential of conditioned media chapter 4.2.

3.1.3 Preparation of conditioned medium (CM)

Conditioned medium is spent medium harvested after enriching bacterial spores extracted from environmental samples. They were prepared according to the procedure described by Gupta and Brightwell [269] with minor modifications as outlined below.

3.1.3.1 Isolation of bacterial spores from farm environmental samples

Fifteen grams of the sample was weighed into a blender filter bag (BagPage, Interscience, France) and suspended in sterile 50 mL Butterfield's diluent [270] under aseptic conditions. It was then blended in a laboratory stomacher (Seward Stomacher 400, UK) at high-speed setting for 2 min. Blended liquid was transferred into a 50 mL falcon tube (Corning, USA) followed by centrifugation at 3,472 x g for 1h at 4 ^{o}C (Thermo Scientific, USA). The pellet was re-suspended in 5 mL Butterfield's diluent and heated at 80 ^{o}C for 10 min in a water bath (Grant, UK) to kill vegetative cells.

3.1.3.2 Spore enrichment

A three millilitre aliquot of heat treated sample was added to 27 mL of Cooked Meat Glucose Starch (CMGS) medium (Fort Richard Laboratories, New Zealand) supplemented with yeast extract (0.0005%), hemin (0.1%), and vitamin K (1%) and incubated in a 35 ^{o}C anaerobic chamber (Don Whitley Scientific, UK) at an atmosphere of 5 % CO_2, 10 % H_2, and 85 % N_2 in air for 48 h. Incubating under anaerobic conditions refers to the same atmospheric condition stated above throughout all experiments unless otherwise mentioned.

3.1.3.3 Extraction and sterilization of conditioned media

Enriched culture medium was well mixed and transferred into a 50 mL falcon tube. After centrifugation at 10,000 x g for 40 min at 4 ^{o}C, the supernatant was filter sterilized by passing through a polyvinylidene fluoride (PVDF) filter membrane with 0.22 µm pore size (Millipore, Ireland). Sterile conditioned medium was aliquoted and stored frozen at -20 ^{o}C until use. The sterility of conditioned medium (CM) was verified by plating 100 µL of filter sterilised sample on SBA plates.

3.1.4 Microplate turbidimetric growth inhibition assay

The bacterial growth was measured by the optical density (OD) of each culture at 595 nm measured periodically using the Multiskan GO microplate spectrophotometer with Skanlt software version 3.2 (Thermo Scientific, USA) as described by Vijayakumar and Muriana [271] with some modifications. The test bacteria were grown in TSB and the overnight bacterial culture was diluted to achieve ~ 1×10^7 CFU/mL cell density with fresh TSB. Adjusted bacterial culture (50 µL) was combined with the growth medium (CMGS, 50 µL) and various conditioned media (100 µL) or buffer (blank) in a 96-well flat bottom microtiter plate (Thermo scientific, Denmark) and mixed well by aspiration using a pipette. After covering with a Breathe-Easy® sealing membrane (Diversified Biotech, USA), the 96-well plate was incubated in a microplate spectrophotometer at 35 ^{o}C and bacterial growth in each well was determined by measuring the OD at 595 nm for 24 h (shake duration: 10 s before each measurement, kinetic interval: 1200 s). Three biological replicates (with three technical replicates for each biological replicate) were carried out for each sample. Most widely accepted and used natural food preservative, Nisin was used to compare its activity with the activity of CMs. Nisin was prepared at final treatment concentration of 45 µM using sterile distilled water. The optical density values were plotted against time to obtain bacterial growth curves.

3.1.5 Isolation of anaerobic spore forming bacteria from farm soil samples

Anaerobic spore forming bacteria were isolated from all five farm soil samples as described by Gupta and Brightwell [269] with some modifications. Bacterial spores were isolated from farm soil samples and enriched in cooked meat glucose starch medium

supplemented with yeast extract (0.0005%), hemin (0.1%), and vitamin K (1%) as described in the section 3.1.3. After 48 h anaerobic enrichment, cultures were transferred into 50 mL falcon tubes and centrifuged at 10,000 x g for 20 min. The cell pellet was re-suspended in 5 mL of Butterfield's diluent followed by ten-fold serial dilutions of the enriched cell suspension. Each serial dilution was plated on Shahidi Ferguson Perfringens (SFP) base agar (BD Difco, France) containing 50 % egg yolk emulsion (BD Difco, USA), kanamycin (12 µg/mL), and polymyxin B (BD Difco, France) and overlaid with SFP agar with no supplements followed by incubation at 35 ^{o}C for 24 h under anaerobic conditions. All individual colonies were picked and sub-cultured on fresh SBA plates and incubated at 35 ^{o}C for 24 h under anaerobic conditions. Colonies were carefully checked for their morphologies and sub-cultured to obtain pure cultures (from a single isolate) on the plate. All pure isolates were grown in TSB under anaerobic conditions and stock cultures were prepared with 25% glycerol and maintained at -80 ^{o}C.

3.1.6 Identification of farm soil isolates by 16S rRNA gene sequence analysis

3.1.6.1 Selection of representative isolates for identification

All the pure bacterial isolates obtained from five soil samples were grown in SBA plates to assess colony morphologies. All isolates in each sample were separated into groups based on the similarity of colony morphology observed by eye and representative isolates from each different colony morphology were selected for 16S rRNA gene analysis. The colonies of five soil samples were considered separately and when the same morphology was found in different samples, they were included in identification. Isolates with a slight visual difference in their morphologies to others were included in the 16S rRNA gene analysis to avoid missing the identification of bacteria. Isolation and identification of bacteria from the five soil samples was repeated using independent samples to ensure that no isolate had been missed from the identification.

3.1.6.2 16S rRNA gene amplification and sequencing

Each representative bacterial isolate was inoculated into pre-reduced TSB and incubated anaerobically at 35 ^{o}C for 48 h. Bacterial culture (1.5 mL) was centrifuged at 13,000 x g for 5 min to get the cell pellet by discarding the supernatant. The cell pellet was re-

suspended with 10 mg/mL lysozyme solution (400 µL) (Sigma-Aldrich, USA) and incubated at 37 ºC for 1 h. The genomic DNA from each isolate was extracted using a genomic DNA extraction kit (Roche diagnostics, Germany) according to the manufacturer's instructions. The 16S rRNA gene sequences were amplified from extracted DNA using primers, forward: PA 5'-AGA GTT TGA TCC TGG CTC AG-3' (Invitrogen) and reverse: PH* 5'- AAG GAG GTG ATC CAG CCG CA-3' (Invitrogen) as described by Böddinghaus, Wolters [272]. DNA amplification was conducted in a thermal cycler (Eppendorf Mastercycler pro, Germany) using the following PCR conditions: initial denaturation at 93 ºC for 3 min, followed by 30 individual cycles consisting of denaturation at 92 ºC for 1 min, annealing at 55 ºC for 1 min, and extension at 72 ºC for 1 min. The final products were extended at 72 ºC for 2 min and stored at 4 ºC. Amplified products were separated by 0.8 % gel electrophoresis and visualized by Gel Doc XR system (Bio-Rad Laboratories, USA). The PCR products were purified using QIAquick PCR purification kit (Qiagen®, Germany) and sequenced at Massey University genome service, New Zealand. All the 16S rRNA gene sequences were processed using Geneious software version 10 [273]. The consensus sequences obtained were searched against the RDP 16S rRNA sequences database version 11.5 (http://rdp.cme.msu.edu/index.jsp) [274] to identify closest taxonomically described species.

3.1.7 Spiking of Farm 4 soil isolates in sterile soil

Farm 4 soil was transferred into two 100 mL Schott bottles (10 g each) and autoclaved at 123 ºC for 30 min followed by cooling down to room temperature (25 ºC). The sterility of soil was verified by plating on SBA plates and incubating them under anaerobic and aerobic conditions. All four bacterial species isolated and identified from Farm 4 soil (FS01, FS2.2, FS03, and FS04) were inoculated into separate TSB tubes and incubated anaerobically at 35 ºC for 24 h. The sterile soil (10 g) was spiked with a cocktail of all four soil isolates (200 µL culture from each isolate) and mixed well. A negative control preparation consisted of autoclaved soil mixed with 800 µL TSB. Control and bacteria-spiked soil samples were then incubated at 35 ºC for 48 h under anaerobic conditions. After the incubation period, conditioned media were prepared from both bacteria-spiked and control soil samples as outlined in section 3.1.3. Antimicrobial activities of

conditioned media prepared from bacteria-spiked and control soil samples were assessed by the growth inhibition assay as described in section 3.1.4.

3.1.8 Effect of heat, pH, and protease enzyme on the antimicrobial activity of Farm 4 soil conditioned medium

The influence of heat on the antimicrobial activity of Farm 4 soil conditioned medium (F4SCM) was assessed by exposing it to various heat treatments (50 ^{o}C, 70 ^{o}C, 80 ^{o}C, 90 ^{o}C for 10 min and 90 ^{o}C for 20 min). Following these treatments, antimicrobial activities of F4SCM against *B. mycoides* ATCC6462, *B. cereus* NZRM5, and *P. aeruginosa* NZRM981 were assessed using the microplate turbidimetric growth inhibition assay. The influence of pH and protease enzyme on F4SCM's antimicrobial activity was assessed by the method described by Lasik-Kurdyś and Sip [275] with some modifications. For the pH stability assay, the pH of F4SCM was adjusted to acidic pH (2, 4, 6) using 5M hydrochloric acid (HCl) (Thermo Fisher Scientific, USA) and basic pH (8, 10, 12) using 5M sodium hydroxide (NaOH) (Merck, Germany) and incubated at room temperature (25 ^{o}C) for 1 h. All samples were re-adjusted to the original pH value (pH 7.2 ± 0.2) and the residual antimicrobial activities were determined by the turbidimetric growth inhibition assay. To investigate the susceptibility of F4SCM to protease enzyme, F4SCM was treated with protease enzyme (Sigma-Aldrich, USA) at a final concentration of 1 mg/mL and incubated at 37 ^{o}C for 1 h. The residual antimicrobial activities against *B. mycoides* ATCC6462, *B. cereus* NZRM5 and *P. aeruginosa* NZRM918 were determined using the microplate turbidimetric growth inhibition assay. Controls consisting of 1 mg/mL protease enzyme and each test bacteria, ensured that there was no effect of the protease on the growth of bacteria.

3.1.9 Statistical analysis

All bacterial growth experiments were carried out in triplicates and data were presented as mean ± standard deviation (S.D). The area under the experimental growth curve, which provides information on the overall bacterial growth under given growth conditions, was computed using the R package 'growthcurver' (R script is given in Appendix A1.1) [276]. Subsequent single factor Analysis of Variance (ANOVA) was performed to check the statistical significance of growth inhibition of CM by comparing the growth in the

presence and absence of CM. The values with $p < 0.05$ were considered statistically significant.

3.2 Materials and methods for Chapter 5

3.2.1 Samples and liquid-liquid extraction procedure

Farm 1 soil conditioned medium (F1SCM), Farm 2 soil conditioned medium (F2SCM), Farm 3 soil conditioned medium (F3SCM), Farm 4 soil conditioned medium (F4SCM), and Farm 5 soil conditioned medium (F5SCM) were selected for a non-targeted metabolomics study. Five replicates of CMs prepared from each soil sample together with five replicates of CMGS (growth medium) were included in this study.

Extracellular polar and non-polar metabolites present in soil CMs and CMGS were separated using liquid-liquid extraction. First, CM/CMGS (1 mL) was centrifuged at 11,900 x g for 10 min and the supernatant (200 µL) was transferred to a clean microcentrifuge tube. Eight hundred micro litres of pre-chilled chloroform:methanol (1:1, v/v) containing 1.6 mg/L of internal standards (d_5-L-tryptophan, d_4-citric acid, d_{10}-leucine, d_2-tyrosine, d_{35}-stearic acid, d_5-benzoic acid, $^{13}C_2$-glucose, and d_7-alanine) were transferred to the same tube and mixed thoroughly. Then, 400 µL of Milli Q water (Milli-Q®, Germany) was also added to the mixture and mixed thoroughly. The final mixture was centrifuged at 11,900 x g for 15 min and upper aqueous phase was transferred to two clean microcentrifuge tubes (each 200 µL). Tube contents were evaporated under a N_2 stream at 30 ^oC. One of the dried upper phase residues was reconstituted in 200 µL of acetonitrile:water (1:9, v/v) for C18 liquid chromatography mass spectrometry analysis. The second tube with dried upper phase residues was reconstituted in 200 µL of acetonitrile:water (1:1, v/v) for hydrophilic interaction liquid chromatography (HILIC) mass spectrometry. The same procedure was followed for all CMs as well as CMGS, which was used as negative control for comparing metabolite profiles. All organic solvents used for liquid-liquid extraction were obtained from Thermo Fisher Scientific (Auckland, New Zealand).

3.2.2 Liquid chromatography mass spectrometry conditions

For semi-polar compounds, 2 µL of metabolite extract was injected into a 100 mm x 2.1 mm Hypersil Gold C18 column with 1.9 µm particle size (Thermo Fisher Scientific, USA) and eluted over a 16 min gradient with a flow rate of 400 µL/min. The mobile phase was a mixture of Milli Q water with 0.1% formic acid (solvent A), and acetonitrile with 0.1% formic acid (solvent B). Gradient and other LC-MS conditions were set as described

by Fraser, Lane [19]. For polar compounds, HILIC conditions were set as described by Subbaraj, Kim [277]. Two microliters of metabolite extract were injected into a 100 mm x 2.1 mm ZIC-pHILIC column with 5 µm particle size (Merk, Germany) and eluted over a 24 min gradient with a flow rate of 250 µL/min. Mobile phase solvent A was a mixture of acetonitrile with 0.1% formic acid and solvent B consisted of Milli Q water with 16 mM ammonium formate. C18 and HILIC column effluents were connected to the Exactive Orbitrap™ (Thermo Fisher Scientific, USA) mass spectrometer with electrospray ionization. All samples were analysed in negative and positive ionization modes. Pooled sample from all extracts and internal standards were used to maintain the data quality in both positive and negative mode analyses. Samples were randomized prior to injection to avoid any systematic effects. HPLC grade solvents (Thermo Fisher Scientific, New Zealand) were used in all LC-MS analyses and all other reagents used were analytical grade.

3.2.3 Metabolomics data processing

Data processing involved a series of steps aimed at converting raw mass spectrometry data to data matrices suitable for statistical analyses such as univariate and multivariate analysis. The steps that involved in data processing were elimination of background noise, discriminating signal from noise, correcting retention time shifts, and normalising data for analytical drifts and sample variation to retain uniformity in the final data matrix.

Raw data files were converted to mzML files using the MSConvert function of ProteoWizard™ [278]. Peak detection, retention time alignment, grouping, and gap filling were performed using XCMS version 3.0.2 [279] and in-house scripts in R [280] with suitable parameters. The comparison between quality control (QC) vs blanks done using the 'diffreport' function of XCMS, was used to identify non-significant *m/z* features (FDR p value < 0.05) between the two groups. These features were removed from the QC vs samples list of *m/z* features as they correspond to background noise contributed by blanks. The QC vs samples list was further cleaned by browsing all extracted ion chromatograms (EICs) generated by 'diffreport' and removing *m/z* features representing background noise. The resulted data matrix was then normalized by a QC based robust LOESS signal correction (QC-RLSC) [281] and run-order effects were evaluated before and after normalization. Relative standard deviation (RSD) of all *m/z* features in the QC was

determined and features in the normalized data matrix with RSD > 0.3 were eliminated [282].

3.2.4 Statistical analysis

Metabolomics data were further subjected to multivariate and univariate analyses using the MetaboAnalyst 5.0 web tool (https://www.metaboanalyst.ca) [283]. Peak intensity data were checked for integrity and normalized using MetaboAnalyst's normalization protocols (autoscaling) to improve the downstream statistical analysis. Principal component analysis (PCA) was performed using all detected metabolite features to find the directions of maximum variance in the dataset. F4SCM and CMGS data sets were used to calculate fold-change values (F4SCM/CMGS) and *p*-values using the t-test, which were used to determine the statistical significance of each metabolite feature in two groups. Volcano plots were used to visualize both *p*-values and fold change values showing significantly discriminating metabolites in F4SCM and CMGS groups.

3.2.5 Metabolite identification

In the present study, metabolite identification was carried out at level two identification confidence according to Metabolomics Standard Initiative (MSI), which was a comparison of parent mass (MS1) and diagnostic source-induced fragment/ daughter ions (MS2) with corresponding matches in public spectral libraries. The online METLIN database (https://metlin.scripps.edu/landing_page.php?pgcontent=mainPage) and MassBank (https://massbank.eu/MassBank/Index) were used to putatively identify metabolite features by matching detected molecular mass data (*m/z*) with those from the database (10 ppm error tolerance).

3.3 Materials and methods for Chapter 6

3.3.1 Bacterial strains and growth conditions

Frozen glycerol stocks of Farm 4 soil isolates (FS01, FS2.2, FS03, and FS04) were revived on SBA plates following incubation at 35 °C overnight under anaerobic conditions. Each isolate grown on SBA plates was then used to inoculate TSB for genomic DNA extraction.

For growth inhibition activity tests, *Bacillus mycoides* ATCC6462, *Bacillus cereus* NZRM5, *E. coli* O157:H7 NCTC12900, *Salmonella* Typhimurium NZRM3970, and *Pseudomonas aeruginosa* ATCC25668 were revived on SBA plates from frozen glycerol stocks. They were subsequently grown in TSB at 35 °C overnight.

3.3.2 Preparation of conditioned media from Farm 4 isolates

Conditioned media were prepared from all four individual soil isolates as described in section 3.1.3 with some modifications. Briefly, each isolate grown on SBA plates was used to inoculate CMGS medium (45 mL) supplemented with yeast extract (0.0005%), hemin (0.1%) and vitamin K (1%) and incubated in a 35 °C anaerobic chamber for 48h. After the incubation period, culture media were centrifuged at 10,000 x g for 40 min at 4 °C and supernatants were filter-sterilized using 0.22 µm polyvinylidene fluoride syringe filters. Sterile conditioned media were aliquoted and stored frozen at -20 °C until use. Conditioned medium prepared from FS01 isolate was denoted as FS01CM and the other three as FS2.2CM, FS03CM, and FS04CM

3.3.3 Effect of FS03CM on spore germination and vegetative growth

3.3.3.1 Preparation of bacterial spores

Sporulation of *B. cereus* NZRM5 was carried out in TSB medium according to the method described by Soni, Oey [284] with some modifications. Bacteria were streaked on SBA plates from the -80 °C freezer stock and incubated at 30 °C for 24 h. A single colony was inoculated into TSB (400 mL) followed by incubation at 30 °C on an orbital shaker at 95 rpm. *B. cereus* NZRM5 was incubated for at least 7 days. Sporulation of bacteria was monitored by phase-contrast microscopy and calculating total microbial number (TMN) and spore number (SN) (See section 3.3.3.2) during the sporulation period. Bacterial

cultures were subsequently transferred into 50 mL falcon tubes and heat treated at 80 ^{o}C for 15 min in a water bath. Heat treated culture tubes were immediately cooled down on ice followed by centrifugation at 10,000 x g for 10 min at 4 ^{o}C. Resulting spore pellets were cleaned at least three times by washing with sterile Milli Q water (Milli-Q®, Germany) with repeated centrifugation at 10,000 x g for 10 min. After cleaning steps, spores were resuspended in sterile Milli Q water and stored at -80 ^{o}C until use. The spore density of the spore suspension was calculated by serial dilution with 0.1% peptone water and plating on SBA plates.

3.3.3.2 Assessment of total microbial number (TMN) and spore number (SN)

Total microbial number includes both the vegetative cells and spores present in the sporulating media, while the spore number is only spores. These two numbers were assessed during the sporulation time for *B. cereus* as described by Soni, Oey [284]. The sporulating culture was serially diluted in 0.1% peptone water and plated on SBA plates to estimate the TMN. To estimate the SN, the sporulating culture was heat treated at 80 ^{o}C for 15 min in a water bath then immediately cooled down in an ice slurry before serial dilution followed by plating on SBA plates. Colonies were counted after incubation of the plates at 30 ^{o}C for 48 h and was also verified after 72 h to rule out the possibility of slow growing spores. The difference between TMN and SN represents the number of vegetative cells and partially germinated spores present in the sporulating media and was used to monitor the sporulation level during the sporulation period.

3.3.3.3 Spore germination and vegetative growth assay

The effect of FS03CM on *B. cereus* NZRM5 spore germination and subsequent vegetative growth was monitored using the method described by Artíguez and Martínez de Marañón [285] with some modifications. The spore suspension was adjusted to achieve final spore densities of 1×10^4 CFU/mL and 1×10^3 CFU/mL in the assay media. Spore suspension (50 µL) was combined with CMGS (50 µL) and FS03CM (100 µL) or water (untreated control) or 0.1 mg/mL chloramphenicol (positive control) in a 96-well flat bottom microtiter plate and mixed well by aspiration using a multi-channel pipette. The plate was covered with a Breathe-Easy® sealing membrane and incubated in a microplate spectrophotometer at 35 ^{o}C. The bacterial spore outgrowth and subsequent

vegetative growth in each well was monitored by measuring OD at 595 nm for 24 h (shake duration: 10 s before each measurement, kinetic interval: 1200 s). The optical density values were plotted against time to obtain spore germination and vegetative growth curves.

3.3.4 DNA extraction and purification

Genomic DNA from pure cultures grown in TSB was extracted by using the phenol-chloroform extraction method as described by Yu, Gunn [286] with minor modifications. Bacterial cultures (5 mL) were centrifuged at 10,000 x g for 2 min and the cell pellets were resuspended in 1 mL TE buffer (composed of 10 mM Tris-HCl and 1 mM EDTA, pH 08) (Thermo Fisher Scientific, Lithuania). Resulting cell suspensions were washed twice with TE buffer by centrifuging at 10,000 x g for 2 min. After washing, harvested cell pellets were resuspended in 200 µL genomic DNA solution composed of 1 M sucrose (Fluka, Germany) in TE buffer. Fifty microliters of freshly prepared lysozyme (50 mg/mL) were then added to the cell suspensions and the resulting mixtures were incubated at 37 ^{o}C for 30 min. Following the incubation, 100 µL of 20% (w/v) sarkosyl (sodium lauroyl sarcosinate) (Sigma-Aldrich, UK) and 15 µL of RNase A (DNase free, 10 mg/mL) (Thermo Fisher Scientific, Lithuania) were added and incubated at 37 ^{o}C for another 30 min. Then, 7.5 µL of proteinase K (10 mg/mL) (Ambion, USA) was added and incubated again at 37 ^{o}C for 30 min. After digestion steps, the final volume of the crude mixtures of nucleic acids was adjusted to 600 µL using TE buffer. The adjusted solutions were then mixed with 600 µL of phenol/chloroform/isoamyl alcohol (25:24:1) (Sigma-Aldrich, USA) and centrifuged at 20,000 x g for 20 min. The resulting upper aqueous layers were transferred to new microcentrifuge tubes (1.5 mL). Phenol/chloroform/isoamyl alcohol extraction was further repeated twice following the same procedure outlined above. Subsequent final upper aqueous solutions were mixed with 50 µL of 3 M sodium acetate (pH 5.2) (BDH, UK) and three volumes of ice-cold absolute ethanol (Thermo Fisher Scientific, New Zealand) and incubated at -20 ^{o}C overnight to precipitate genomic DNA. Following incubation, all solutions were centrifuged at 17,000 x g for 20 min and supernatants were discarded. Resulting genomic DNA pellets were washed with 750 µL of 70% ethanol followed by centrifugation at 17,000 x g for 5 min. After removing supernatants, genomic DNA pellets were air-dried for about 15 min and re-suspended in 100 µL of sterile DNase free water. All samples

were stored overnight at 4 ^{o}C, separated by 0.8 % gel electrophoresis, and visualized by Gel Doc XR system (Bio-Rad Laboratories, USA). DNA quality and quantity were assessed using Qubit 4 fluorometer (Thermo Fisher Scientific, USA) and NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA).

3.3.5 Whole genome sequencing, assembling, and annotation

High quality gDNA extracted from four soil isolates was sequenced using Illumina MiSeq version 3 sequencing platform at Massey Genome Services, New Zealand. DNA library preparation was carried out with the Celero PCR workflow with enzymatic fragmentation DNA-seq library preparation kit (NuGEN, USA). The quality and quantification assessments of DNA libraries were carried out as a quality control check before sequencing. The resulting reads were quality trimmed, filtered, and *de novo* assembled with the A5-miseq assembler version 20160825 under the default settings [287]. The genome completeness was assessed using BUSCO version 1.22 [288]. The genome sequences and associated data of FS01, FS2.2, FS03, and FS04 isolates reported in this study were deposited in NCBI under the accession numbers PRJNA705805, PRJNA642910, PRJNA706025, and PRJNA605262 respectively.

3.3.6 Phylogenetic analysis using TYGS server

The 16S rRNA gene sequence based phylogenetic tree and the whole-genome sequence based phylogenetic tree were constructed using the Type (Strain) genome server (TYGS) [289]. TYGS workflow consists of several software modules called services, which are important for data processing and various analyses. Briefly, TYGS extracted available 16S rRNA gene sequences from query genomes using RNAmmer [290] and then performed NCBI BLAST+ analyses using all possible pairs of 16S rRNA gene sequences in the TYGS database. The genomes with top 50 BLAST bitscores were used for the calculation of GBDP (Genome BLAST Distance Phylogeny) values. GBDP values were calculated between the selected 16S rRNA gene sequences under the algorithm 'coverage' and distance formula d_5 [291], and the closest relatives were defined as the genomes with the lowest ten 16S rRNA gene GBDP values between each query genome and type strains. These top 10 type strain genome sequences obtained for all query genome sequences were included in both 16S rRNA gene sequence and whole-genome

sequence based phylogenetic analyses. Accurate intergenomic distances were calculated between selected genomes using the GBDP method [291]. The resulted GBDP distances were used to construct both whole-genome and 16S rRNA gene GBDP phylogenetic trees using FastME 2.1.6.1 with a BioNJ phylogeny inferring tools [289, 292, 293]. The tree was visualized using MEGA X version 10.2.2 [294]. The digital DNA:DNA hybridization (dDDH) values for each genome of the soil isolates and their closest neighbours were computed using the Genome-to-Genome Distance Calculator (GGDC version 2.1) in TYGS analysis with default parameters [289, 291]. The average nucleotide identity (ANI) values between each soil isolate genome and their closest neighbours were calculated using the ANI calculator developed by Kostas lab with default parameters (http://enve-omics.ce.gatech.edu/ani/) [295].

3.3.7 Functional genome distribution (FGD) analysis

Functional genome distribution (FGD) analysis investigates the genomic differences between bacteria and interprets the genetic diversity [296]. FGD analysis was carried out using all four soil isolates, their closest neighbours identified from TYGS phylogenetic analysis. All the genomes required for the analysis except the four soil isolates were downloaded in FASTA format from NCBI database. They were concatenated using a universal spacer-stop-spacer sequence and automatically annotated using GAMOLA 2 software package [297]. FGD analysis was performed using the predicted open reading frames (ORFeomes) of all genomes. The resulting distance value matrix was imported into MEGA X version 10.2.2 and visualized using unweighted pair group method with the arithmetic mean (UPGMA).

3.3.8 Detection of virulence genes

Known/potential virulence factors were predicted in the genomes of four soil isolates using the virulence factor data base (VFDB) integrated automatic pipeline, VFanalyzer [298].

3.3.9 Analysis of putative biosynthetic gene clusters (BGCs)

Biosynthetic gene clusters encoding for secondary metabolites were predicted and annotated using the web server version of antiSMASH 5.0 [155] with default parameters and a combination of ClusterFinder algorithm, which is an hidden Markov model based probabilistic algorithm to detect BGCs of both known and unknown metabolites [162]. antiSMASH 'ClusterBlast' and 'KnownClasterBlast' modules were used for identifying other microorganisms harbouring similar BGCs and associations to characterized/known compounds respectively.

3.3.10 Hydroxyisocaproate dehydrogenase (HicD) sequence analysis

The presence of homologous coding regions for HicD enzymes associated with the production of 2-hydroxyisocaproic acid (HICA) in the genomes of all four soil isolates was investigated by sequence similarity searching. Amino acid sequences of HicD enzymes annotated in *Clostridium* spp. were obtained from the UniProt protein sequence collection (https://www.uniprot.org) [299]. Six clostridial HicD enzyme sequences were used to identify homologous HicD enzyme-coding regions in the draft whole genomes of all four soil isolates using TBLASTN module of BLAST [300]. TBLASTN module translated nucleotide sequence of the genome to hypothetical amino acid sequence and then aligned the hypothetical amino acid sequences to the query HicD enzyme sequences.

3.3.11 Quantification of 2-hydroxyisocaproic acid

3.3.11.1 Preparation of samples and standard solution

HICA production by FS01 was verified by measuring the HICA content in conditioned medium prepared from FS01 (FS01CM). As HICA is a by-product of leucine degradation pathway, the contribution of leucine in growth medium to produce HICA by FS01 was also assessed by the addition of extraneous leucine to the growth medium. FS01CM and FS01CM enriched with leucine ($FS01CM_{Leu+}$) were prepared from the FS01 isolate as described in section 3.3.2. The only difference between two sample preparation procedures was the addition of 0.2% leucine (Merk, Germany) into the initial CMGS growth medium of $FS01CM_{Leu+}$. Three replicates each of FS01CM and $FS01CM_{Leu+}$ together with three replicates of CMGS were included in this study. CMGS was included for the comparison of HICA concentrations before and after the FS01 growth. Liquid-

liquid extraction was performed for all samples as described in section 3.2.1. The dried upper phase residue was reconstituted in 200 µL of acetonitrile:water (1:9, v/v) for subsequent C18 liquid chromatography mass spectrometry analysis. A stock solution of HICA was used to obtain standard solutions with concentrations of 10, 1, 0.1, 0.01 µg/mL HICA (Sigma-Aldrich, USA) in water. They were used to generate a standard curve for subsequent quantification. All solvents used in this study were LC-MS grade purchased from Thermo Fisher Scientific (Auckland, New Zealand).

3.3.11.2 UPLC-MS/MS conditions

Nexera X2 ultra high-performance liquid chromatography (UHPLC) system (Shimadzu, Japan) consisting of a SIL-30AC autosampler coupled to a LCMS-9030 quadrupole time-of-flight (Q-TOF) mass spectrometer (Shimadzu, Japan) equipped with an electrospray ionization source was used to analyse the HICA content in all samples. Two microliters of sample/standard were injected into a reverse phase Ascentis® Express C18 UHPLC column (2.1 x 100 mm, 2 µm particle size; Sigma, USA) and eluted at 30 ^{o}C over a 20 min gradient with a flow rate of 400 µL/min. The mobile phase solvent A was a mixture of Milli Q water and 0.1% formic acid (v/v), and solvent B consisting of acetonitrile with 0.1% formic acid (v/v). The solvent gradient program started at 5% solvent B from 0 to 0.5 min, increasing to 99% B within 12.5 min, held at 99% B for 2 min, decreasing to 5% B within 1 min and held at 5% B until the end of the elution run. MS results were obtained at *m/z* 55 – 600 in negative ionization mode with a spray voltage of -3.0 kV at a scan rate of 10 spectra/s. The ion source was operated under optimal conditions: nebulizing gas flow, 3.0 L/min; heating gas flow; 10.0 L/min; interface temperature, 300 ^{o}C; drying gas flow, 10.0 L/min; desolvation line temperature, 250 ^{o}C and heat block temperature, 400 ^{o}C. Data independent acquisition (DIA) MS/MS scan for precursor *m/z* 131.0700 with 20 Da *m/z* width was used to confirm the fragmentation data of HICA. Data analysis i.e., standard curves and HICA quantification, was done using LabSolutions Insight software version 3.50SP2 (Shimadzu, Japan).

3.3.12 Statistical analysis

All bacterial growth experiments were conducted in triplicates and the data were presented in mean ± (S.D). The area under the experimental growth curve and single factor Analysis of Variance (ANOVA) were performed to check the statistical significance of growth inhibition of CMs as described in 3.1.9.

The single factor Analysis of Variance (ANOVA) was performed to assess the statistical significance of HICA concentration between CMGS and FS01CM, CMGS and $FS01CM_{Leu+}$, and FS01CM and $FS01CM_{Leu+}$. The values with $p < 0.05$ were considered statistically significant.

3.4 Materials and methods for Chapter 7

3.4.1 Bacterial strains and growth conditions

Pseudomonas aeruginosa NAZRM4034 and *Staphylococcus aureus* NZRM917 were purchased from Environmental Science and Research (ESR), New Zealand. Purchasing information of other reference strains used in this section was given in section 3.1.1. *Bacillus cereus* M4 and *Escherichia coli* AGR3789 were milk and soil isolates respectively. *Shewanella putrefaciens* SM 26 and *Serratia proteamaculans* ENT 68 were meat isolates. *Bacillus subtilis* F2MCUH1, *Paenibacillus odorifer* F1OSP28, *Pseudomonas lundensis* F2MCUH2, and *Pseudomonas fragi* F1NBUH38 were farm environmental isolates (isolated in our laboratory). All bacteria were revived on SBA plates from frozen glycerol stocks. They were subsequently grown in Muller-Hinton broth (MHB) (Fort Richard Laboratories, New Zealand) and incubated at optimum growth temperature for each bacterium overnight.

3.4.2 Determination of minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of HICA

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of HICA against a range of Gram-positive and Gram-negative bacteria were determined by the microplate turbidimetric assay method as described earlier [301] with some modifications. An overnight bacterial culture grown in MHB was diluted with fresh MHB to achieve final inoculum of 5×10^5 CFU/mL. A two-fold dilution series of HICA (Sigma-Aldrich, USA) was prepared to achieve final assay concentrations ranging from 0.5-32 mg/mL. Bacterial culture (50 µL) was added to MHB (50 µL) and a twofold-dilution series of HICA (100 µL) or water (untreated control) in a 96-well flat bottom microtiter plate (Thermo scientific, Denmark) and covered with a Breathe-Easy® sealing membrane. The microtiter plate was incubated in a spectrophotometer at 35 ^{o}C for *Bacillus mycoides* ATCC6462, *Bacillus cereus* NZRM5, *Bacillus cereus* M4, *Pseudomonas aeruginosa* ATCC25668, *Pseudomonas aeruginosa* NAZRM4034, *Escherichia coli* O157:H7 NCTC12900, *Escherichia coli* AGR3789 , *Staphylococcus aureus* NZRM917, *Bacillus subtilis* F2MCUH1 and at 25 ^{o}C for *Shewanella putrefaciens* SM26, *Serratia proteamaculans* ENT68, *Paenibacillus odorifer* F1OSP28, *Pseudomonas lundensis* F2MCUH2, and *Pseudomonas fragi* F1NBUH38. The bacterial growth was

determined by measuring the optical density (OD) at 595 nm for 24 h. MIC was the lowest concentration of HICA at which there was no growth of test bacteria (no OD change during 24 h incubation). The MBC was assessed by removing the media from each well after 24 h assay and sub-culturing them on SBA plates. SBA allows maximum recovery of bacteria after exposing to HICA including bacteria with sub-lethal injuries. MBC was the minimum concentration of HICA required to completely kill test bacteria (no colonies on SBA plates).

3.4.3 Spore germination and vegetative growth assay

B. cereus NZRM5 spores were prepared and the effect of HICA on the *B. cereus* NZRM5 spore germination and subsequent vegetative growth was monitored as described in section 3.3.3. Briefly, the spore suspension was adjusted to achieve final spore densities of 1×10^4 CFU/mL and 1×10^3 CFU/mL in the assay media. The adjusted spore suspension (50 µL) was combined with MHB (50 µL) and various concentrations of HICA (100 µL) or water (untreated control) or 0.1 mg/mL chloramphenicol (positive control) (Sigma-Aldrich, USA) in a 96-well flat bottom microtiter plate and mixed well by aspiration using a multi-channel pipette. The plate was covered with a Breathe-Easy® sealing membrane and incubated in a microplate spectrophotometer at 35 ^{o}C. The spore outgrowth and subsequent vegetative growth in each well was monitored by measuring the OD at 595 nm for 24 h (shake duration: 10 s before each measurement, kinetic interval: 1200 s). The optical density values were plotted against time to obtain spore germination and vegetative growth curves.

3.4.4 Bacterial cell viability assay for both Gram-positive and Gram-negative bacteria

Bacteria were grown in MHB and overnight cultures were adjusted to achieve a treatment cell density of approximately 1×10^7 CFU/mL using fresh MHB. HICA (4 mg/mL) was added to the adjusted bacterial culture and incubated at 35 ^{o}C for various time intervals (0-180 min). Bacterial culture added with sterile water served as the untreated control. The bacterial cell viability was determined by BacTiter-Glo microbial cell viability assay kit (Promega, USA) according to manufacturer's instruction at various treatment times. Briefly, BacTiter-Glo reagent was prepared by combining lyophilized BacTiter-Glo™

enzyme/substrate mixture with the buffer provided in the assay kit at room temperature. One hundred microlitres of HICA treated/untreated bacterial culture were transferred to an opaque-walled 96 well plate with clear bottoms and combined with 100 µL of BacTiter-Glo reagent. After mixing the contents by shaking the plates on an orbital shaker (500 rpm) for 5 min in the dark, the luminescence intensity was measured using a Varioskan™ LUX multimode microplate reader (Thermo Fisher, USA) with luminescence detection at an integration time of 1 s. The relative cell viability at various treatment times was calculated as the percentage of untreated cells.

3.4.5 Evaluation of the loss of cell membrane integrity of Gram-positive and Gram-negative bacteria

Bacterial cell membrane integrity was assessed by CellTox™ green cytotoxicity assay kit (Promega, USA) according to manufacturer's instruction at various HICA treatment periods. Briefly, bacteria were grown overnight in MHB, and the overnight cultures were harvested by centrifugation at 10,000 x g for 10 min at room temperature (25 ^{o}C). The cell pellets were re-suspended in MHB to achieve 1×10^7 CFU/mL. HICA (4 mg/mL) was added to the bacterial cultures and incubated at 35 ^{o}C for various time intervals (15-120 min). Sterile water was used for untreated control. 2X CellTox™ green reagent was prepared by combining 30 µL of CellTox™ green dye with 15 mL of assay buffer provided in the assay kit. One hundred micro-litres of HICA treated/untreated bacterial culture were transferred to an opaque-walled 96 well plate with clear bottoms at various treatment times and combined with 100 µL of 2X CellTox™ green reagent. After shaking the plates on an orbital shaker (500 rpm) for 1 min, they were incubated in the dark for 15 min at room temperature (25 ^{o}C). The fluorescence intensities of bacterial cultures were measured at 490 nm excitation and 520 nm emission wavelengths using a Varioskan™ LUX multimode microplate reader (Thermo Fisher, USA).

3.4.6 Outer membrane permeability of Gram-negative bacteria

The effect of HICA on the permeability of outer membrane of Gram-negative bacteria was investigated using the non-polar fluorescence probe 1-N-phenylnaphthylamine (NPN) as described by Muheim, Götzke [302] with minor modifications. Gram-negative bacteria were grown in MHB at 35 ^{o}C and the overnight bacterial culture was diluted with

fresh MHB to achieve 0.5 OD_{595nm}. The cells were harvested by centrifugation at 10,000 x g for 3 min at room temperature, washed twice with assay buffer (HEPES, pH 7.2) (Gibco, UK), and resuspended in half of the original volume with the assay buffer. For the treatment groups, various concentrations of HICA (100 µL) were transferred to an opaque-walled 96 well plate with clear bottoms and combined with 40 µM NPN (50 µL) (Sigma-Aldrich, USA). Bacterial cell suspension (50 µL) was added to the wells immediately before the fluorescence measurements. For the control group, HICA was replaced by assay buffer, and the background control was assay buffer with NPN. Assay buffer was used to prepare various concentrations of HICA solutions (1–8 mg/mL working concentrations) and NPN solution (40 µM working concentration). The fluorescence intensities were measured at an excitation wavelength of 350 nm and emission wavelength of 420 nm for 10 min at 30 s intervals using a Varioskan™ LUX multimode microplate reader (Thermo Fisher, USA). The NPN uptake was calculated using the formula below and the values obtained for 10 min were averaged to get the normalized fluorescence intensity for the NPN. F_{obs} is the observed fluorescence intensity after HICA treatment, F_{con} is the fluorescence intensity of NPN with bacterial cells and F_B is the fluorescence intensity of NPN without bacterial cells in the presence of assay buffer.

$$NPN\ uptake\ =\ (F_{obs}\ -F_B)\ -\ (F_{con}\ -F_B)$$

3.4.7 Cytoplasmic membrane depolarization assay

The cytoplasmic membrane depolarization activity of HICA was determined using the voltage sensitive fluorescence dye 3,3′-Dipropylthiadicarbocyanine iodide [$DiSC_3(5)$] (Sigma-Aldrich, Czech Republic) according to the method described by Te Winkel, Gray [303] with minor modifications. Bacteria were grown overnight in MHB at 35 ^oC and diluted to 0.3 OD_{595} with fresh MHB supplemented with 0.5 mg/mL Bovine Serum Albumin (BSA) (Sigma-Aldrich, USA). The addition of BSA to the assay medium was important to reduce the absorption of $DiSC_3(5)$ to the polystyrene 96-well plate surface [301]. The diluted bacterial cell suspension (130 µL) was transferred to an opaque-walled 96 well plate with clear bottoms and combined with 20 µL of $DiSC_3(5)$ dissolved in 1% DMSO (BDH, UK) to a final concentration of 6 µM. Using 1% DMSO was important for

good solubility and the fluorescence of $DiSC_3(5)$. After $DiSC_3(5)$ addition, the fluorescence quenching was measured using the Varioskan™ LUX multimode microplate reader (Thermo Fisher, USA) at an excitation wavelength of 610 nm and emission wavelength of 660 nm, until a stable fluorescence signal was achieved. Following the addition of 4 mg/mL HICA (50 µL) or sterile water (untreated control) to corresponding wells, fluorescence was immediately measured and monitored for 25 min at 60 s intervals with 10 s of vigorous shaking before each measurement.

3.4.8 Electron microscopy

3.4.8.1 Sample preparation

3.4.8.1.1 For bacterial vegetative cells

Bacteria were grown in MHB till the mid-exponential growth phase and diluted with fresh MHB to achieve 1×10^7 CFU/mL. Bacterial cultures were then treated with 4 mg/mL HICA and incubated at 35 ^oC. HICA was replaced by sterile water in the untreated control group. Bacterial cultures treated with HICA for various time intervals were harvested by centrifugation at 10,000 x g for 6 min at 4 ^oC. The resulting cell pellets were washed twice with 0.1 M phosphate-buffered saline (PBS) (BD, USA) and used for both scanning electron and transmission electron microscopy.

3.4.8.1.2 For bacterial spores

B. cereus NZRM5 spores were prepared as described in section 3.3.3.1. The spore suspension was adjusted to achieve a final treatment spore density of 1×10^4 CFU/mL. The adjusted spore suspension was combined with HICA (4 mg/mL) or water (untreated control) or 0.1 mg/mL chloramphenicol (positive control). Treated spore cultures were harvested at different time points (0 – 24 h) by centrifugation at 10,000 x g for 6 min at 4 ^oC. The resulting pellets were washed four times with 0.1 M phosphate-buffered saline (PBS) and used for scanning electron microscopy.

3.4.8.2 Scanning electron microscopy (SEM)

The primary fixation was done by resuspending the cells/spores in 0.1 M phosphate-buffered saline (PBS) containing 3% glutaraldehyde (Merck, Germany) for at least 8 h. The cell/spore suspensions were passed through 0.4 µm Isopore™ membrane filters (Millipore, Ireland) to place the cells/ spores on the membrane. The membranes were washed three times with 0.1 M PBS for 15 min each. Dehydration was performed as an incubation series with rising ethanol concentrations as follows; 25%, 50%, 75%, 95%, and 100% for 15 min each and a final 100% for 1 h. All the samples were dried in a Polaron E3000 series II critical point drying apparatus (Quorum technologies, UK) using liquid CO_2 as the critical point fluid and 100% ethanol as the intermediary. The samples were mounted on the aluminium stubs using double-sided tapes and sputter coated with approximately 100 nm of gold using a SCD 005 sputter coater (Bal-Tec, USA). Bacteria were imaged using the FEI Quanta 200 scanning electron microscope (ThermoFisher, USA) at an accelerating voltage of 15 kV.

3.4.8.3 Transmission electron microscopy (TEM)

Bacterial samples for TEM were also prepared as described in the section 3.4.8.1.1. The primary fixation step was carried out by suspending the cells in 0.1 M PBS containing 3% glutaraldehyde. The fixed suspending cells were injected into 3% agarose tubes (HydraGene, USA) to make enclosed capsules. They were stored in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) (Merck, Germany) for at least 24 h. Enclosed capsules were subsequently washed three times with 0.1 M sodium cacodylate buffer for 45 min each, followed by post fixation with 1% osmium tetroxide (ProSciTech, Australia) in 0.1 M sodium cacodylate buffer for 1 h at room temperature and overnight at 4 ^{o}C. Samples were washed three times again with 0.1M sodium cacodylate buffer for 45 min each. Dehydration was performed as an incubation series with rising acetone (Miltonadams, New Zealand) concentrations as follows; 25%, 50%, 75%, 95%, 100%, 100%, 100% for 45 min each. Enclosed capsules were then incubated in a 1:1 mixture of resin and acetone on a stirrer overnight. The resin acetone mixture was then replaced by fresh 100% resin (812 resin, ProSciTech, Australia) without catalyst and incubated for 8 h on the stirrer. This step was repeated for four more times as 100% resin overnight, 100% resin 8 h, 100% overnight, and 100% resin 8 h. Samples were then embedded in moulds with fresh resins and cured in an oven at 60 ^{o}C for 48 h. One-micron thin layers of

embedded samples were cut using a glass knife on the ultramicrotome (Leica, Germany) and heat fixed onto glass slides. They were stained with 0.05% toluidine blue (Sigma-Aldrich, USA) for around 12 s. Regions of interest were selected by observing the embedded bacterial layers under a light microscope prior to thin sectioning. Ultrathin sections (70 nm) were cut parallel to the bacterial layer using a diamond knife (Diatome, Switzerland), stretched with chloroform, and mounted on copper grids. Copper grids were stained with saturated uranyl acetate (Thermo Fisher Scientific, USA) in 50% ethanol for 4.5 min, washed with 50% ethanol and MilliQ water followed by staining with lead citrate (Thermo Fisher Scientific, USA) for another 4.5 min and washing with MilliQ water. Bacteria were imaged using FEI Tecnai™ G^2 Spirit BioTWIN (Czech Republic) transmission electron microscope using a side mounted TEM CCD camera (Olympus, Germany).

3.4.9 Statistical analysis

All assays were conducted in triplicates and the data were presented in mean ± (S.D). The single factor Analysis of Variance (ANOVA) was performed to check the statistical significance between the untreated control and various treatment groups. The values with $p < 0.05$ were considered statistically significant.

3.5 Materials and methods for Chapter 8

3.5.1 Sample preparation

Three replicates of FS03CM were prepared following the method described in section 3.3.2. FS03CM (10 mL from each replicate) and cooked meat glucose starch medium supplemented with yeast extract (0.0005%), hemin (0.1%), and vitamin K (1%) (10 mL from each of three replicates) were lyophilized (Labconco, USA) and sent to the metabolomics innovation centre (TMIC) at University of Alberta, Canada for high performance chemical isotope labelling LC-MS metabolomics.

Briefly, lyophilized samples were re-dissolved in 5 mL water and centrifuged at 10,000 x g for 10 min. One microlitre from each sample was transferred to a new vial and diluted 100-fold for quantification. The total metabolite concentration of each sample was determined by the NovaMY sample normalization kit (Nova Medical Testing, Canada). Samples were diluted to achieve 2 mM concentration with water based on the corresponding total metabolite concentration of each sample. Each concentration adjusted sample was centrifuged at 10,000 x g for 10 min and the supernatant was aliquoted for isotope labelling reactions, preparation of pooled sample, and a backup. A pooled sample was prepared by mixing the same amount from each of the concentration adjusted samples.

3.5.2 Chemical isotope labelling

3.5.2.1 Dansylation labelling

Amino/ phenol labelling of the sample was performed using the dansyl-labelling kit (Nova Medical Testing, Canada) according to manufacturer's instructions. Briefly, the buffer reagent (12.5 µL) and $^{12}C_2$-labelling (37.5 µL) were added to individual samples. The buffer reagent (12.5 µL) and $^{13}C_2$-labelling (37.5 µL) were added to the pooled sample. All samples were mixed well, followed by centrifugation at 10,000 x g for 10 min. The mixtures were then incubated at 40 ^{o}C for 45 min. After incubation, quenching reagent (7.5 µL) was added and incubated at 40 ^{o}C for another 10 min to quench the excessive labelling reagent. Lastly, pH adjusting reagent (30 µL) was added to neutralize the solution.

3.5.2.2 DmPA labelling

Carboxyl labelling was performed using the DmPA-labelling kit (Nova Medical Testing, Canada) according to manufacturer's instructions. Briefly, the catalysing reagent (10 µL) and $^{12}C_2$-labelling (25 µL) were added to individual samples. The catalysing reagent (10 µL) and $^{13}C_2$-labelling (25 µL) were added to the pooled sample. All samples were mixed well, followed by centrifugation at 10,000 x g for 10 min. The mixtures were then incubated at 80 ^{o}C for 60 min. After the incubation period, quenching reagent (40 µL) was added and incubated at 80 ^{o}C for 30 min to quench the excessive labelling reagent. Finally, the mixtures were incubated at 80 ^{o}C for another 30 min.

3.5.3 LC-MS analysis

The equal volumes of $^{12}C_2$-labelled individual sample and corresponding $^{13}C_2$-labelled reference/pooled sample were mixed before injecting to the LC-MS. The quality control (QC) sample was prepared by mixing equal volumes of a $^{12}C_2$-labelled and a $^{13}C_2$-labelled pooled sample. QC samples were injected after every 5 samples to monitor instrument performance.

LC-MS experiments were performed using an Agilent 1290 LC linked to Bruker Impact HD quadrupole time-of-flight (Q-TOF) mass spectrometer (Bruker, Billerica, MA) with electrospray ionization (ESI). Labelled metabolites were separated using reversed phase liquid chromatography with an Agilent Eclipse Plus C18 column (150 x 2.1 mm, 1.8 µm particle size). The mobile phase A consisted of 0.1% (v/v) formic acid in water and the mobile phase B was made up of 0.1% (v/v) formic acid in acetonitrile. The mobile phase gradient started from 25% B at 0 min, increasing to 99% B within 10 min, held at 99% B for 3 min, decreased to 25% B within 0.1 min, and held at 25% B until the end of the elution run (16 min). The flow rate was 400 µL/min. The positive ion mode at a spectral acquisition rate of 1 Hz was used to collect all MS spectral information within 220-1000 *m/z* mass range.

3.5.4 Data processing

A total of 18 LC-MS data sets were uploaded to Bruker data analysis software version 4.4 to extract MS spectral peaks (9 LC-MS data form each channel). IsoMS Pro software version 1.2.9. was used for data quality check and data processing including peak picking,

peak pairing, peak-pair filtering, and peak pair intensity ratio calculation [304]. The same peak pairs detected from FS03CM and CMGS samples were aligned to obtain a single file containing the metabolite information and peak ratios (relative to the pooled sample). A zero-fill program was used to fill missing peak pair intensity values [305].

3.5.5 Statistical analysis

Peak pair intensity data were subjected to multivariate and univariate analyses using the MetaboAnalyst web tool (https://www.metaboanalyst.ca) [283]. Principal component analysis (PCA) and hierarchical clustering analysis were carried out using all detected peak-pairs in FS03CM and CMGS groups with auto data scaling. A hierarchical clustering dendrogram was built using a Euclidean distance and ward clustering algorithm. FS03CM and CMGS data were used to calculate the fold-change values (FS03CM/CMGS) and *p*-values using t-test for all detected peak pairs, and they were used to determine the statistical significance of each peak pair in two groups. A volcano plot was used to visualize both *p*-values and fold change values showing significantly discriminating metabolites in FS03CM and CMGS groups.

3.5.6 Metabolite identification

Metabolite identification was done using a three-tier ID approach based on the accurate mass and retention time. Chemical isotope labelling (CIL) library, linked identity (LI) library and Mycompound (MCID) library were used to identify metabolites at different confidence levels [306]. The parameters used in metabolite identification are given in Table 3.1.

Table 3.1: The parameters used in three-tier metabolite identification approach.

Level of identification	Parameters
Tier 1 positive identification	Retention time tolerance for CIL library: 10 s for carboxyl channel and 30 s for amino/phenol channel. Mass tolerance for CIL library: 10 ppm.
Tier 2 high confidence positive identification	Retention time tolerance for LI library: 205 s for amino/phenol channel and 75 s for carboxyl channel. Mass tolerance for LI library: 10 ppm.
Tier 3 putative identification	Mass tolerance for MCID library: 10 ppm.

Chapter 4

Antimicrobial potential of conditioned media

4.1 Introduction

Farm samples can have different diversities of *Clostridium* and closely related spp. depending on the farm source, location, and the season. A previous study carried out by our group has shown the prevalence of spore-forming bacteria on four dairy farms in New Zealand including seasonal and farm/location variation of *Clostridium* species [269]. Therefore, the metabolite composition of conditioned media prepared from various farm samples could be different based on the *Clostridium* and closely related bacterial population in each farm sample, which may lead to various degrees of antimicrobial activity.

Throughout this study, conditioned medium (CM) is referred to spent medium harvested from cultured *Clostridium* and closely related species, which contains metabolites, growth factors, and other extracellular molecules secreted by the bacterial cells. Various laboratory techniques are used to screen or assess the *in vitro* antimicrobial activity of spent medium/extract or a pure compound. Among them, a turbidimetric growth inhibition assay is a high-throughput method, that permits the precise and reproducible analysis of bacterial inhibition. This method captures the growth dynamics of bacteria over the time by measuring optical turbidity in real time [307]. This technique was employed in the current study to evaluate the interaction between conditioned medium/ compound and test microorganism. In antimicrobial studies, this method provides valuable information on the changes in growth dynamics of bacteria, such as generation time and lag phase [307].

The work presented in this chapter is associated with the first objective of this research project, which was screening conditioned media (CMs) prepared from various farm samples that may harbour *Clostridium* and closely related species, for their antimicrobial activity to select a CM with relatively high antimicrobial potency for further studies (Figure 4.1).

It is also important to identify *Clostridium* and closely related species, associated with the underlying antimicrobial potential of conditioned media for further investigations. Therefore, this chapter reports on isolation and identification of anaerobic spore forming bacteria from select farm samples which produced CMs with promising antimicrobial potential (Figure 4.1).

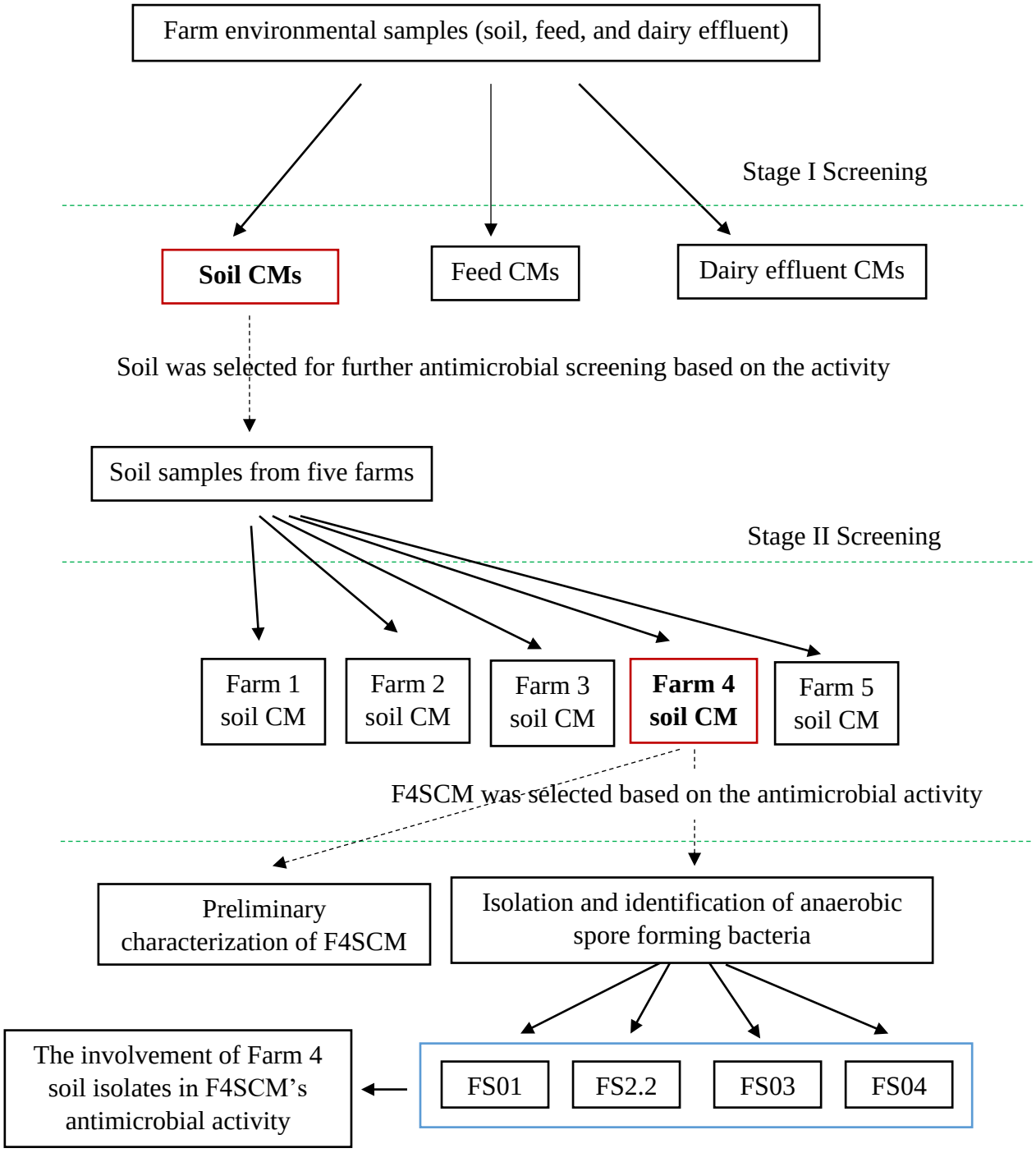


Figure 4.1: Schematic diagram showing the summery of the work presented in Chapter 4.

4.2 Results and discussion

4.2.1 Antimicrobial activity of conditioned media prepared from soil, feed (silage), and dairy effluents (Stage I screening)

The screening study of CMs for antimicrobial activity can be divided into two stages as stage I and stage II. In the stage I screening, CMs prepared from three farm sources, soil, feed (silage), and dairy effluent, which potentially had different *Clostridium* and closely related species diversities, were included to identify the best farm source that produced CMs with relatively high antimicrobial potency.

Table 4.1: Conditioned media prepared from soil, feed (silage), and dairy effluent from three farms.

Farm ID	**Farm sources**		
	Soil	**Feed**	**Dairy effluent (DE)**
Farm 1	F1SCM	F1FCM	F1ECM
Farm 2	F2SCM	F2FCM	F2ECM
Farm 3	F3SCM	F3FCM	F3ECM

As shown in Table 4.1, nine CMs were prepared from soil, feed, and dairy effluent of three farms by the method described in the materials and methods chapter 3.1.3. The CMs were named as in the Table 4.1 for easy identification and reference. Nisin was used in this study for comparison as this is an extensively used food preservative [308].

Three *Bacillus* species of food quality and safety concern were included as test microorganisms in the stage I antimicrobial activity screening study. They were particularly selected for stage I screening based on the observation that *Clostridium* and closely related spp. could inhibit the growth of *Bacillus* spp., made during preliminary studies (See the project introduction chapter Figure 1.1). *Bacillus cereus*, *Bacillus pumilus*, and *Bacillus mycoides* are ubiquitous in the environment and commonly associated with compromising food quality and safety. *B. cereus* is recognized as a significant cause of food poisoning and commonly associated with contaminated milk, cereals, and various other foods, whereas *B. pumilus* and *B. mycoides* are mainly

associated with food spoilage and their food poisoning potential appears to be low [309, 310].

4.2.1.1 Antimicrobial profiles of CMs prepared from Farm 1

The antimicrobial potential of F1SCM (Farm 1 soil CM), F1FCM (Farm1 feed CM), and F1ECM (Farm 1 dairy effluent CM) is discussed in this section. The area under the experimental curve, which provides information on the overall bacterial growth under the given growth conditions was computed using the R package 'growthcurver' [276]. Statistical analysis (one-way ANOVA, 95% CI) was used to check the statistical significance between the treatments and relevant controls.

Three CMs showed different levels of growth inhibition against three *Bacillus* species. All three CMs significantly inhibited the growth of *B. mycoides* compared to the untreated control ($p < 0.05$). Nevertheless, F1FCM and F1ECM lost their activity over the time reaching a similar level of growth as the untreated control after 24 hours. F1SCM exhibited the strongest growth inhibition among three farm sources against *B. mycoides* with an activity equal to nisin (Figure 4.2a).

The activity of CMs prepared from Farm 1 soil, feed, and dairy effluent against *B. cereus* was different from their activity against *B. mycoides*. Only F1SCM significantly reduced the growth of *B. cereus* ($p < 0.05$), while the other two CMs (F1FCM and F1ECM) had no effect on *B. cereus* growth (Figure 4.2b).

Similarly, the growth of *B. pumilus* was significantly inhibited by F1SCM while there was no significant growth inhibition by F1FCM and F1ECM compared to the untreated control ($p < 0.05$) (Figure 4.2c). In general, CM prepared from Farm 1 soil demonstrated promising antimicrobial activities against all three test bacteria.

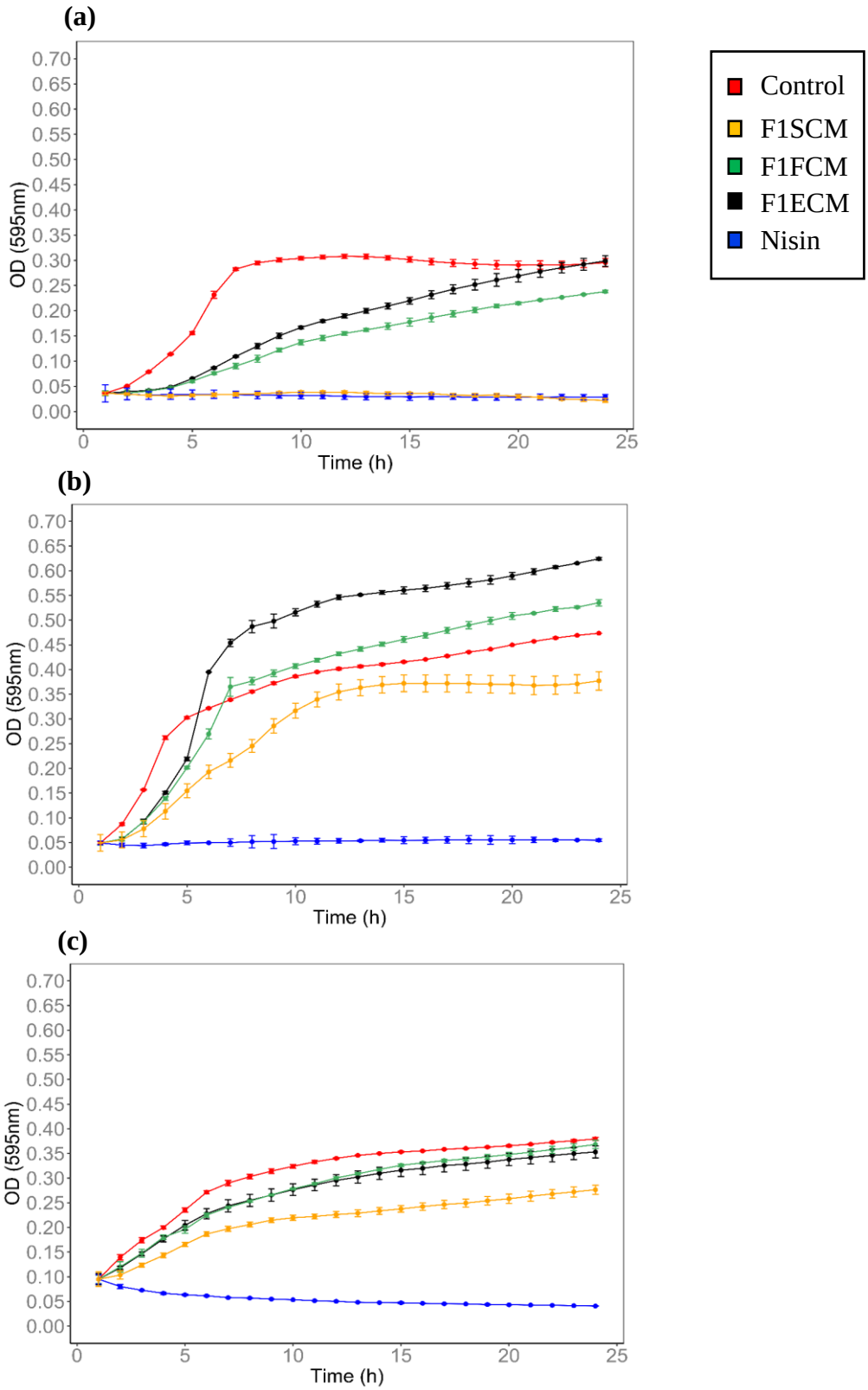

(a)
(b)
(c)
Control
F1SCM
F1FCM
F1ECM
Nisin
OD (595nm)
Time (h)
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
5
10
15
20
25

Figure 4.2: Effect of CMs prepared from Farm 1 soil, feed, and dairy effluent on the growth of three *Bacillus* species. *B. mycoides* ATCC6462 **(a)**, *B. cereus* NZRM5 **(b)**, and *B. pumilus* ATCC14884 **(c)**. Bacteria were grown in the presence of butterfield's diluent (red), F1SCM (Farm 1 soil conditioned medium; yellow), F1FCM (Farm 2 feed conditioned medium; green), F1ECM (Farm 1 dairy effluent conditioned medium; black), and nisin (blue) in the growth medium (CMGS). Each curve represents the mean growth ± S.D (n = 3).

4.2.1.2 Antimicrobial profiles of CMs prepared from Farm 2

This section describes the antimicrobial potential of CMs prepared from Farm 2 soil (F2SCM), feed (F2FCM), and dairy effluents (F2ECM). They exhibited various degrees of growth inhibition against three test bacteria. All three CMs prepared from Farm 2 significantly inhibited the growth of *B. mycoides* ($p < 0.05$), but F2SCM showed the highest level of growth inhibition compared to the untreated control (Figure 4.3a).

Similarly, the growth of *B. pumilus* was significantly affected by all three Farm 2 CMs ($p < 0.05$) (Figure 4.3c). On the other hand, only F2SCM and F2FCM significantly reduced the growth of *B. cereus* compared to the untreated control ($p < 0.05$) and there was no inhibition from F2ECM (Figure 4.3b). Overall, F2SCM and F2FCM demonstrated promising antimicrobial activity against the three test *Bacillus* species.

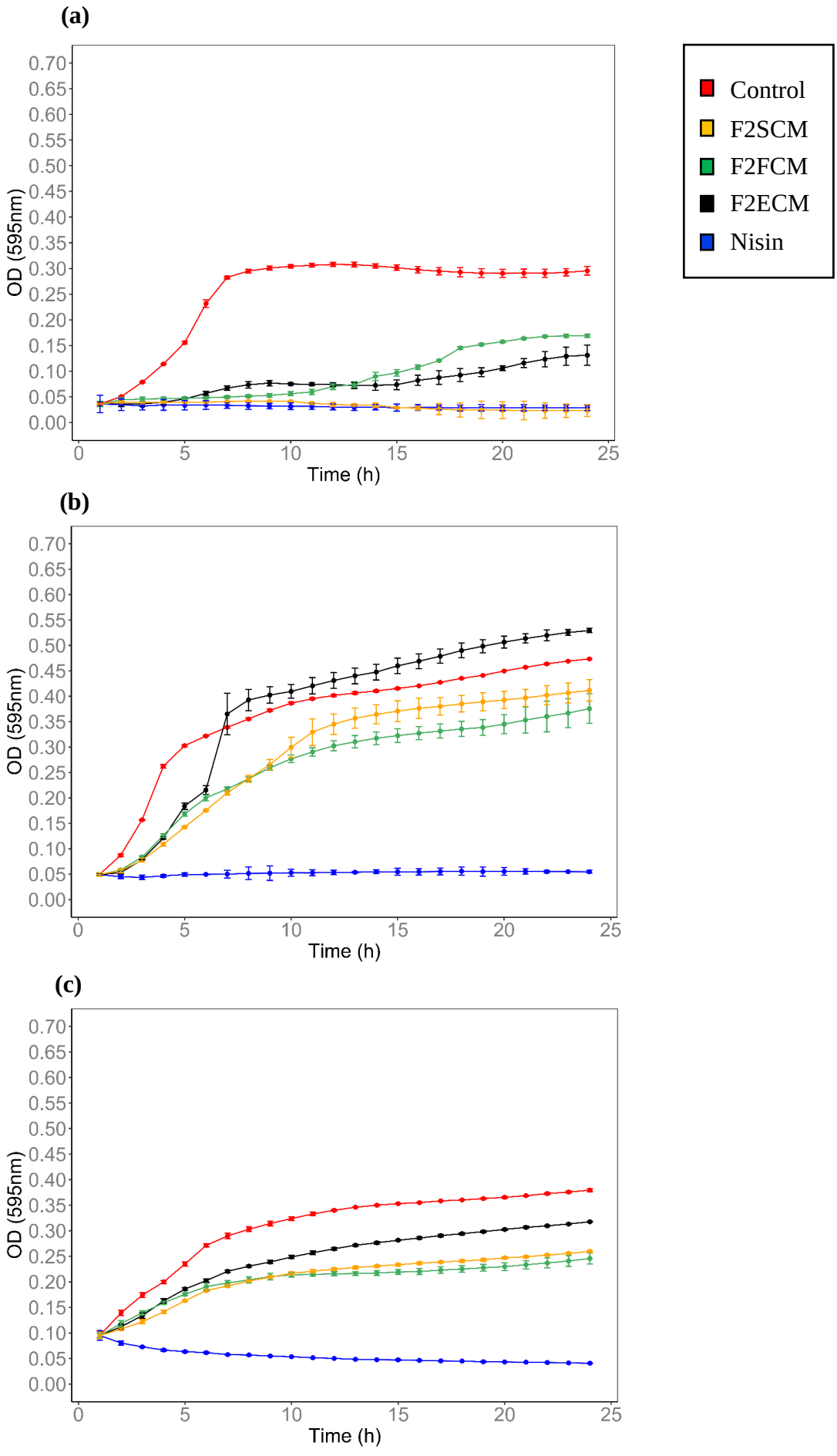

(a)
(b)
(c)
OD (595nm)
Time (h)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50
0.55
0.60
0.65
0.70
0
5
10
15
20
25
Control
F2SCM
F2FCM
F2ECM
Nisin

Figure 4.3: Effect of CMs prepared from Farm 2 soil, feed, and dairy effluent on the growth of three *Bacillus* species. *B. mycoides* ATCC6462 **(a)**, *B. cereus* NZRM5 **(b)**, and *B. pumilus* ATCC14884 **(c)**. Bacteria were grown in the presence of butterfield's diluent (red), F2SCM (Farm 2 soil conditioned medium; yellow), F2FCM (Farm 2 feed conditioned medium; green), F2ECM (Farm 2 dairy effluent conditioned medium; black), and nisin (blue) in the growth medium (CMGS). Each curve represents the mean growth ± S.D (n = 3).

4.2.1.3 Antimicrobial profiles of CMs prepared from Farm 3

Antimicrobial activities of conditioned media prepared from soil (F3SCM), feed (F3FCM), and dairy effluent (F3ECM) of Farm 3 have been described in this section. The antimicrobial potential of Farm 3 CMs varied between three farm sources like the antimicrobial profiles of Farm 1 and Farm 2 CMs. CMs prepared from all three Farm 3 sources significantly inhibited the growth of *B. mycoides* and *B. pumilus* in comparison to the untreated control ($p < 0.05$) while F3SCM exhibited the best growth inhibition activity (Figure 4.4a and Figure 4.4c). In contrast, *B. cereus* was more resistant to F3FCM and F3ECM showing strong growth reduction only with F3SCM (Figure 4.4b).

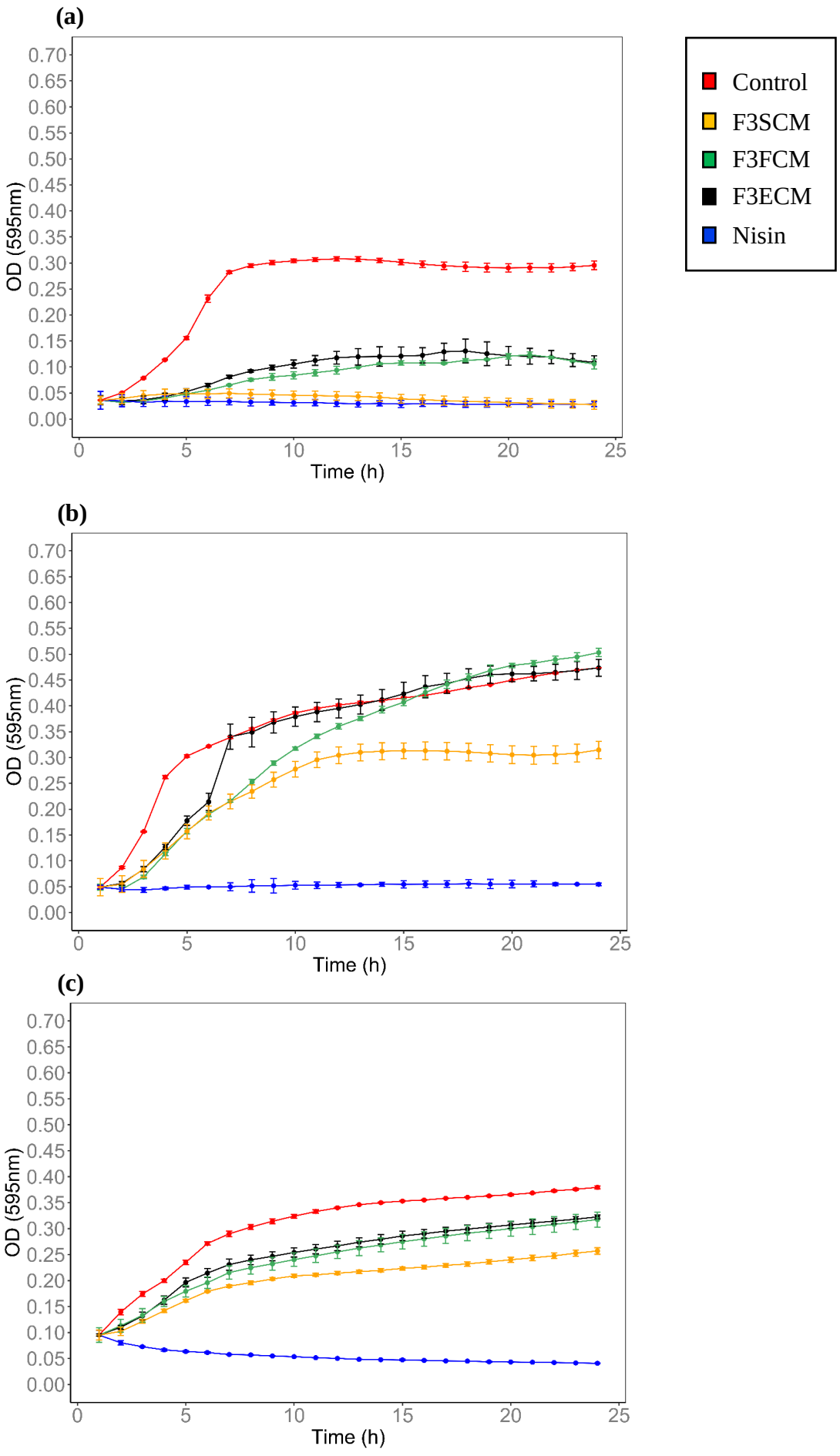

(a)
(b)
(c)
OD (595nm)
Time (h)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50
0.55
0.60
0.65
0.70
0
5
10
15
20
25
Control
F3SCM
F3FCM
F3ECM
Nisin

Figure 4.4: Effect of CMs prepared from Farm 3 soil, feed, and dairy effluent on the growth of three *Bacillus* species. *B. mycoides* ATCC6462 **(a)**, *B. cereus* NZRM5 **(b)**, and *B. pumilus* ATCC14884 **(c)**. Bacteria were grown in the presence of butterfield's diluent (red), F3SCM (Farm 3 soil conditioned media; yellow), F3FCM (Farm 3 feed conditioned media; green), F3ECM (Farm 3 dairy effluent conditioned media; black), and nisin (blue) in the growth medium (CMGS). Each curve represents the mean growth ± S.D (n = 3).

Above findings demonstrated that antimicrobial activity of CMs prepared from soil, feed, and dairy effluent from the same farm varied against the same test bacteria. Interestingly, CMs prepared from soil samples from all three farms showed superior antimicrobial activities than the other two sources against all three test bacteria. Particularly, the growth of *B. mycoides* was strongly inhibited by soil CMs from all three farms. Among three test bacteria, *B. cereus* was the most resistant bacterium showing relatively less/ no inhibition by most of CMs.

4.2.2 Antimicrobial activity of conditioned media prepared from soil samples (Stage II screening)

The stage I screening confirmed that CMs prepared farm soil samples possessed higher antimicrobial potency than CMs prepared from other two farm sources. Therefore, further screening focused only on soil CMs. In the stage II screening, five soil CMs were prepared from five different farm soils (Table 4.2) including three farms used in stage I screening and two additional farms (Farm 4 and Farm 5) to select one soil CM for further studies based on their antimicrobial activities against both Gram-positive and Gram-negative bacteria. Their antimicrobial activities were evaluated using the same assay under the same conditions to compare their activities against each test bacteria.

Table 4.2: Conditioned media prepared from five farm soils.

Farm ID	**Soil CM ID**
Farm 01	F1SCM
Farm 02	F2SCM
Farm 03	F3SCM
Farm 04	F4SCM
Farm 05	F5SCM

Gram-positive and Gram-negative test bacteria were selected based on their association with food safety or quality. Most *Escherichia coli* strains are harmless, but the presence of some *E. coli* strains can cause serious foodborne illness [311]. *P. aeruginosa* has been reported as a spoilage bacterium in dairy products [312]. *Salmonella enterica* serovars such as Typhimurium and Hadar are Gram-negative foodborne pathogens associated with human infections [313]. The association of Gram-positive test bacterial species used in this study with food safety or quality, has been discussed in section 4.21.

As shown in Figure 4.5a, all five soil CMs significantly inhibited the growth of *B. mycoides* ($p < 0.05$). F4SCM and F5SCM showed the strongest antimicrobial activities against *B. mycoides* among the five CMs keeping the bacterial growth to the minimum during the 24 h of incubation (Figure 4.5a). The growth of *Bacillus cereus* was significantly reduced by all five soil CMs ($p < 0.05$). However, *B. cereus* was more resistant to F2SCM over 24 h. F4SCM displayed a relatively stronger activity than the other four soil CMs against *B. cereus* throughout the incubation time (Figure 4.5b). The growth of *B. pumilus* was also significantly inhibited by all five soil CMs ($p < 0.05$), but F4SCM was the most active CM (Figure 4.5c). Nisin (45 µM) showed a strong antimicrobial activity against all three *Bacillus* spp. by completely inhibiting the growth (Figure 4.5). These findings highlight the antimicrobial potential of soil conditioned media against Gram-positive *Bacillus* species. Stage II screening established F4SCM as the most promising CM with antimicrobial activity against all three Gram-positive bacteria.

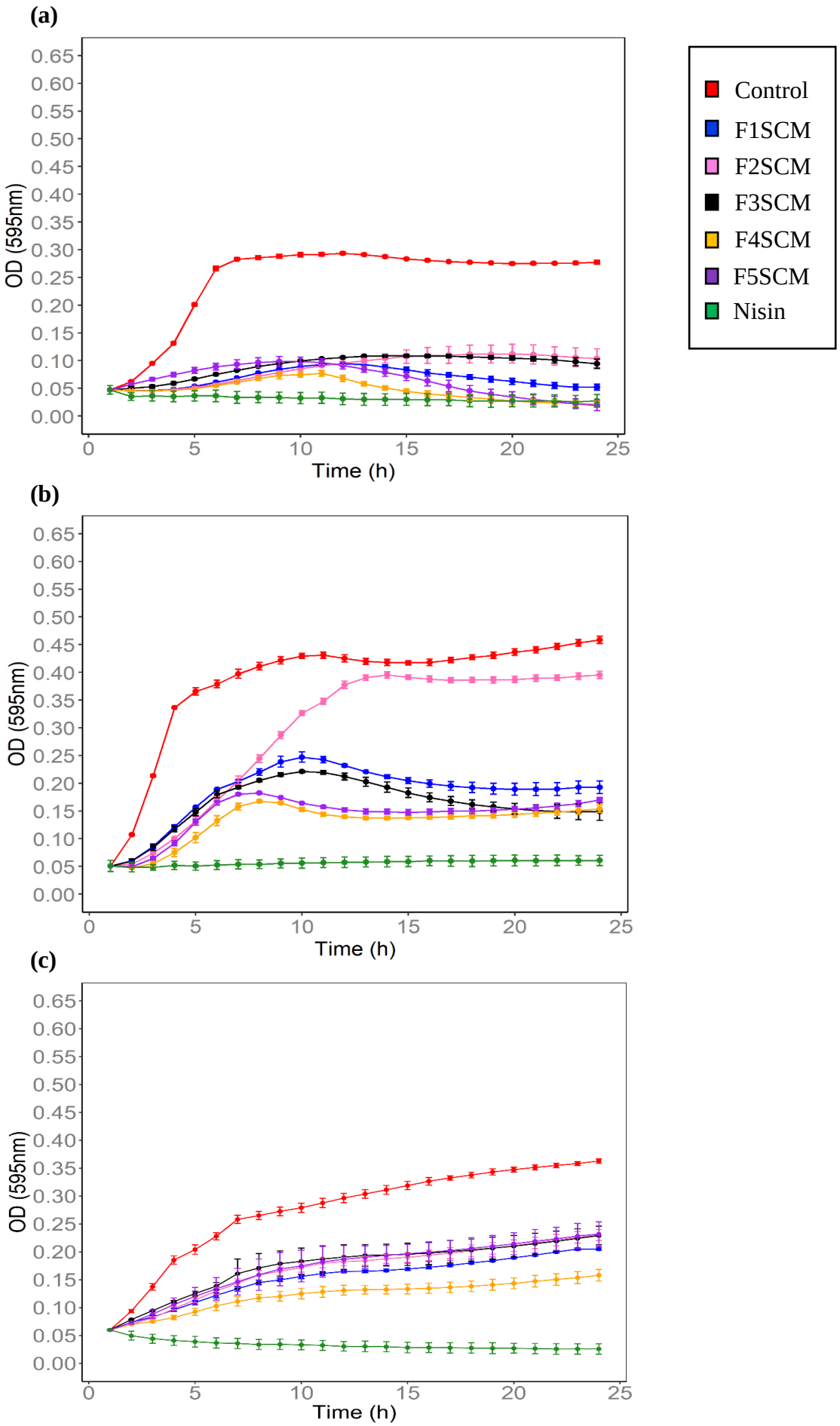
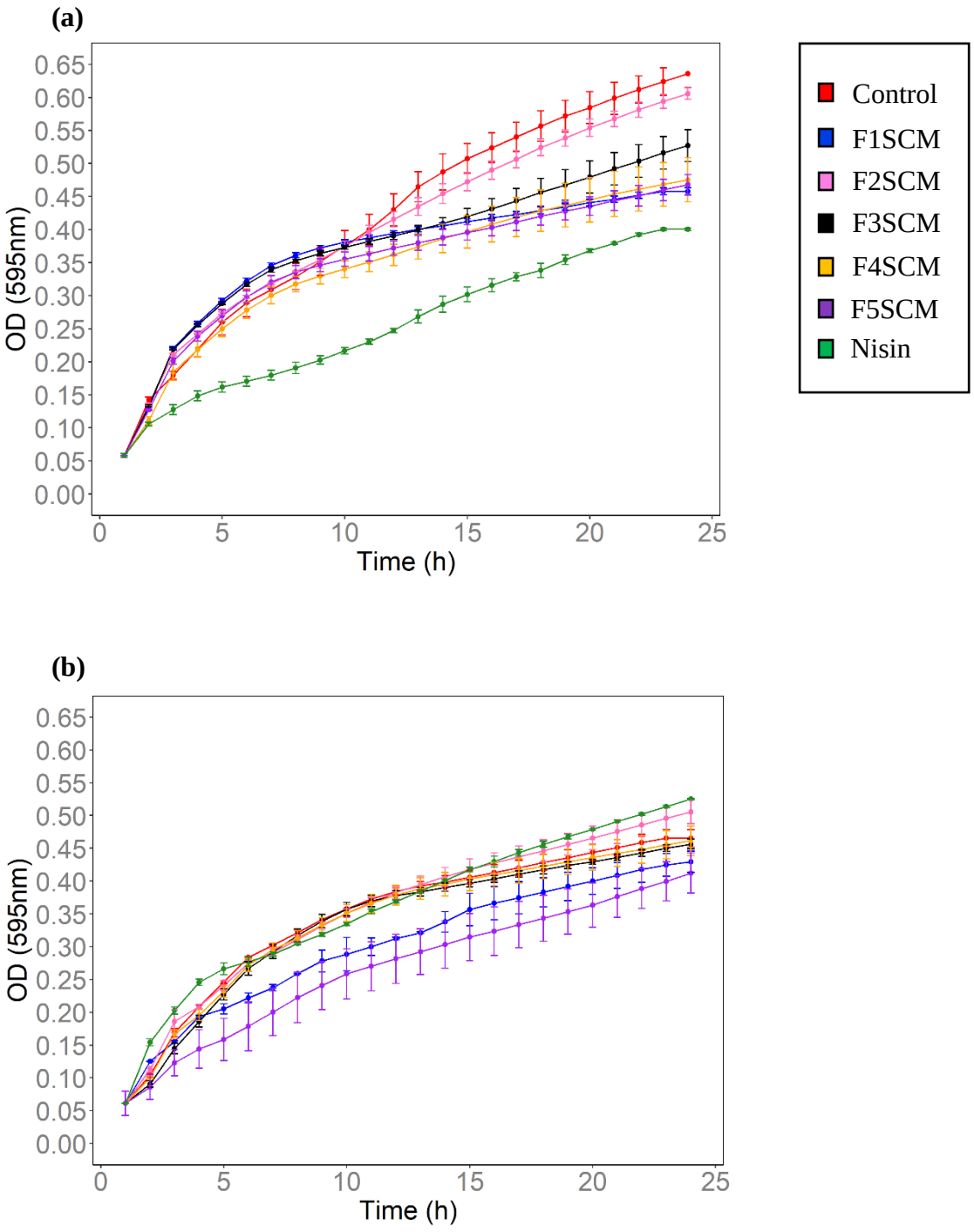

(a)
(b)
(c)
OD (595nm)
Time (h)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50
0.55
0.60
0.65
0
5
10
15
20
25
Control
F1SCM
F2SCM
F3SCM
F4SCM
F5SCM
Nisin

Figure 4.5: Effect of five soil conditioned media on the growth of Gram-positive bacteria. *B. mycoides* ATCC6462 **(a)**, *B. cereus* NZRM5 **(b)**, and *B. pumilus* ATCC14884 **(c)**. Bacteria were grown in the presence of butterfield's diluent (red), F1SCM (Farm 1 soil conditioned medium; blue), F2SCM (Farm 2 soil conditioned medium; pink), F3SCM (Farm 3 soil conditioned medium; black), F4SCM (Farm 4 soil conditioned medium; yellow), F5SCM (Farm 5 soil conditioned medium; purple) and nisin (green) in the growth media (CMGS). Nisin (45 μM) and butterfield's diluent served as positive and untreated control respectively. Each curve represents the mean growth ± S.D (n = 3).

The antimicrobial activity profiles of five soil CMs against Gram-negative bacteria were different from the Gram-positive bacteriaå. All five soil CMs were not effective in controlling the growth of *E. coli* (Figure 4.6a). There was a shift in the growth of *E. coli* compared to the untreated control after around 10 h in the presence of CMs except for F2SCM, but the bacteria showed a rapid growth throughout the incubation time. The growth of *Salmonella* Hadar was not significantly reduced by any soil CMs except F5SCM ($p < 0.05$) (Figure 4.6b). Statistically, there was a significant influence on the growth of *Salmonella* Hadar by F5SCM in comparison to untreated control ($p < 0.05$), however the bacteria showed a rapid and continuous growth throughout the incubation time (Figure 4.6b). There was no significant inhibition against *Salmonella* Typhimurium NZRM3970 by F1SCM, F2SCM, F3SCM and F5SCM. Only F4SCM significantly affected the growth of *Salmonella* Typhimurium compared to the untreated control ($p < 0.05$) (Figure 4.6c). As expected, nisin was not active against *E. coli* and the two *Salmonella enterica* serovars but was effective against Gram-positive *Bacillus* species. In general, all five soil CMs were not effective in inhibiting the growth of *E. coli* and two *Salmonella enterica* serovars. In contrast, all five soil CMs displayed strong antimicrobial activity against *P. aeruginosa* ATCC25668 by significantly reducing the growth compared to the untreated control ($p < 0.05$) (Figure 4.6d). Interestingly, the activities of soil CMs were stronger than the activity of nisin against *P. aeruginosa*, which is a human and plant pathogen having intrinsic and acquired resistance to many antibiotics [314]. Nisin is known to have relatively low activities against Gram-negative bacteria as it cannot effectively pass through the outer-membrane of Gram-negative bacteria [315]. This highlighted the significance of soil CMs for further investigations in search of underline active compounds, which could be highly effective against *P. aeruginosa*. Overall, the stage II screening identified F4SCM as the best soil CM among all five in

terms of antimicrobial profiles against the test bacteria. Therefore, it was selected for further experiments investigating its antimicrobial potential.



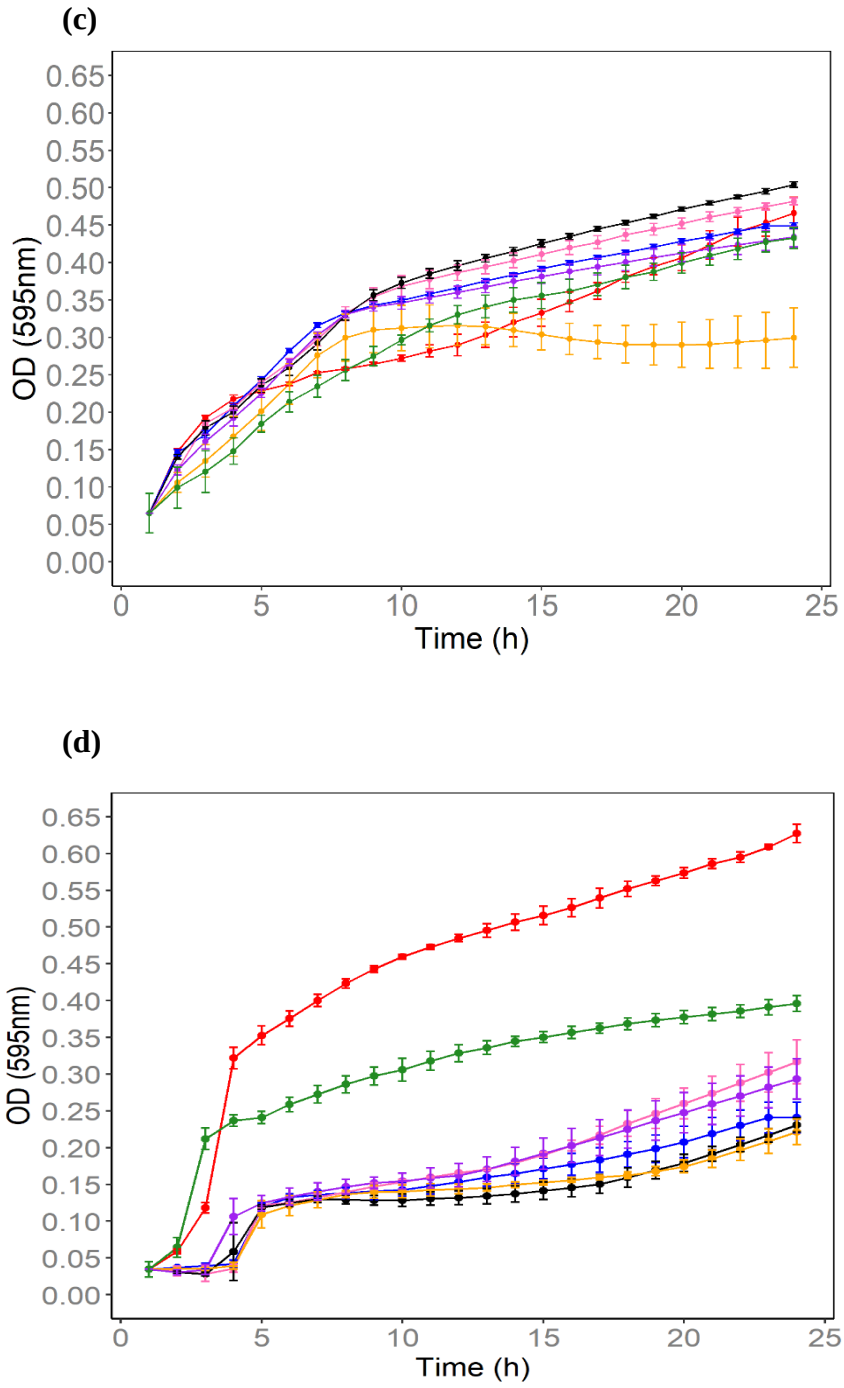


Figure 4.6: Effect of five soil conditioned media on the growth of Gram-negative bacteria. *E. coli* O157:H7 NCTC12900 **(a)**, *Salmonella* Hadar NZRM4206 **(b)**, *Salmonella* Typhimurium NZRM3970 **(c)**, and *P. aeruginosa* ATCC25668 **(d)**. Bacteria were grown in the presence of butterfield's diluent (red), F1SCM (Farm 1 soil conditioned medium; blue), F2SCM (Farm 2 soil conditioned medium; pink), F3SCM (Farm 3 soil conditioned medium; black), F4SCM (Farm 4 soil conditioned medium; yellow), F5SCM (Farm 5 soil conditioned medium; purple) and nisin (45 µM, green) in the growth media (CMGS). Each curve represents the mean growth ± S.D (n = 3).

4.2.3 Isolation and identification of anaerobic spore forming bacteria from farm soil samples

Anaerobic spore forming bacteria responsible for the antimicrobial properties of five soil conditioned media were isolated from respective soil samples using the method described in the materials and methods chapter 3.1.5. All soil isolates were identified using 16S rRNA gene amplicon sequencing to the closest taxonomically described species (See the materials and methods chapter 3.1.6).

In total, 110 bacterial isolates were recovered from spore enrichments of five soil samples, and a total of 54 representative bacterial isolates from five samples based on their morphologies were included in the 16S rRNA gene analysis. They were assigned to six *Clostridium* species, two *Paraclostridium* species (but formally belonged to *Clostridium* species) and one *Terrisporobacter* species (but formerly belonged to *Clostridium* species) based on the 16S rRNA gene sequence similarities. Different bacterial species identified from all five soil samples are shown in the Table 4.3 (16S rRNA gene sequences of one representative isolate per identified species are provided in Appendix A2.1). The bacterial species composition in all five samples was different even though some species were found in multiple samples (Figure 4.7). Bacterial isolates closely related to the species *Clostridium cadaveris* and *Terrisporobacter glycolicus* were present in all five soil samples. In contrast, the species *Clostridium botulinum*, *Clostridium amazonense*, and *Paraclostridium bifermentans* were only found in Farm 2, Farm 5, and Farm 4 respectively.

The antimicrobial potential of each soil CM should be attributed to the active metabolites in the metabolome of each CM produced by the metabolic activities of associated *Clostridium* and closely related species in each soil sample. Varied bacterial species composition in the five soil CMs partially explained the differences in the antimicrobial profiles of the five soil CMs. However, it is not as simple as bacteria interact with each other when they grow together in a mixed culture and this can affect the growth and metabolic activities of each bacteria [316]. Specific biosynthetic pathways can be triggered by the molecules produced by other bacteria [317]. Therefore, the metabolome of the mixed cultures is a result of the combined metabolic activities of all bacteria. Based on the outcome from Stage II screening, four bacterial isolates closely related to *Paraclostridium bifermentans, Clostridium cadaveris, Terrisporobacter glycolicus,* and

Clostridium senegalense, present in Farm 4 soil resulted in a CM with a relatively high antimicrobial potency (F4SCM).

Table 4.3: Closely related spore forming bacteria isolated from farm soil samples.

Farm ID	**Isolate ID**	**Closest related taxonomically described species**	**Maximum identity (%)**	**GenBank accession number**
Farm 1	PT06, PT08	*Paraclostridium benzoelyticum* (T); JC272	99.5	LN846800
	PT07, PT11, PT13, PT21	*Terrisporobacter glycolicus* (T); DSM1288	95.0	X76750
	PT8.1, PT15, PT20, PT24	*Clostridium cadaveris* (T); JCM1392	100	AB542932
Farm 2	LB02	*Clostridium botulinum* NCTC8550	100	CP010521
	LB03	*Terrisporobacter glycolicus* (T); DSM1288	98.2	X76750
	LB05, LB12	*Clostridium cadaveris* (T); JCM1392	100	AB542932
	LB09, LB10, LB11, LB15	*Clostridium perfringens* (T); ATCC13124	100	CP000246
	LB17	*Clostridium butyricum* (T); VPI3266	98.1	AJ458420
Farm 3	RT01, RT07, RT19, RT22	*Terrisporobacter glycolicus* (T); DSM1288	95.8	X76750
	RT09, RT23	*Clostridium cadaveris* (T); JCM1392	100	AB542932
	RT10	*Paraclostridium benzoelyticum* (T); JC272	99.5	LN846800
	RT24	*Clostridium butyricum* (T); VPI3266	95.5	AJ458420

	RT16, RT26	*Clostridium senegalense* (T); JC122	98.1	JF824801
Farm 4	FS01, FS2.1, FS06, FS07, FS08, FS09, FS16	*Paraclostridium bifermentans* (T); ATCC638	98.1	AB075769
	FS2.2, FS10, FS15, FS17	*Clostridium cadaveris* (T); JCM1392	100	AB542932
	FS03, FS14	*Terrisporobacter glycolicus* (T); DSM1288	95.6	X76750
	FS04, FS4.2, FS12, FS13	*Clostridium senegalense* (T); JC122	98.2	JF824801
Farm 5	PH01, PH15, PH19.1	*Clostridium amazonense* (T); NE.08	99.6	KP281434
	PH05, PH19	*Terrisporobacter glycolicus* (T); DSM1288	96.4	X76750
	PH08	*Clostridium perfringens* (T); ATCC13124	99.5	CP000246
	PH10	*Paraclostridium benzoelyticum* (T); JC272	99.2	LN846800
	PH21	*Clostridium cadaveris* (T); JCM1392	99.5	AB542932

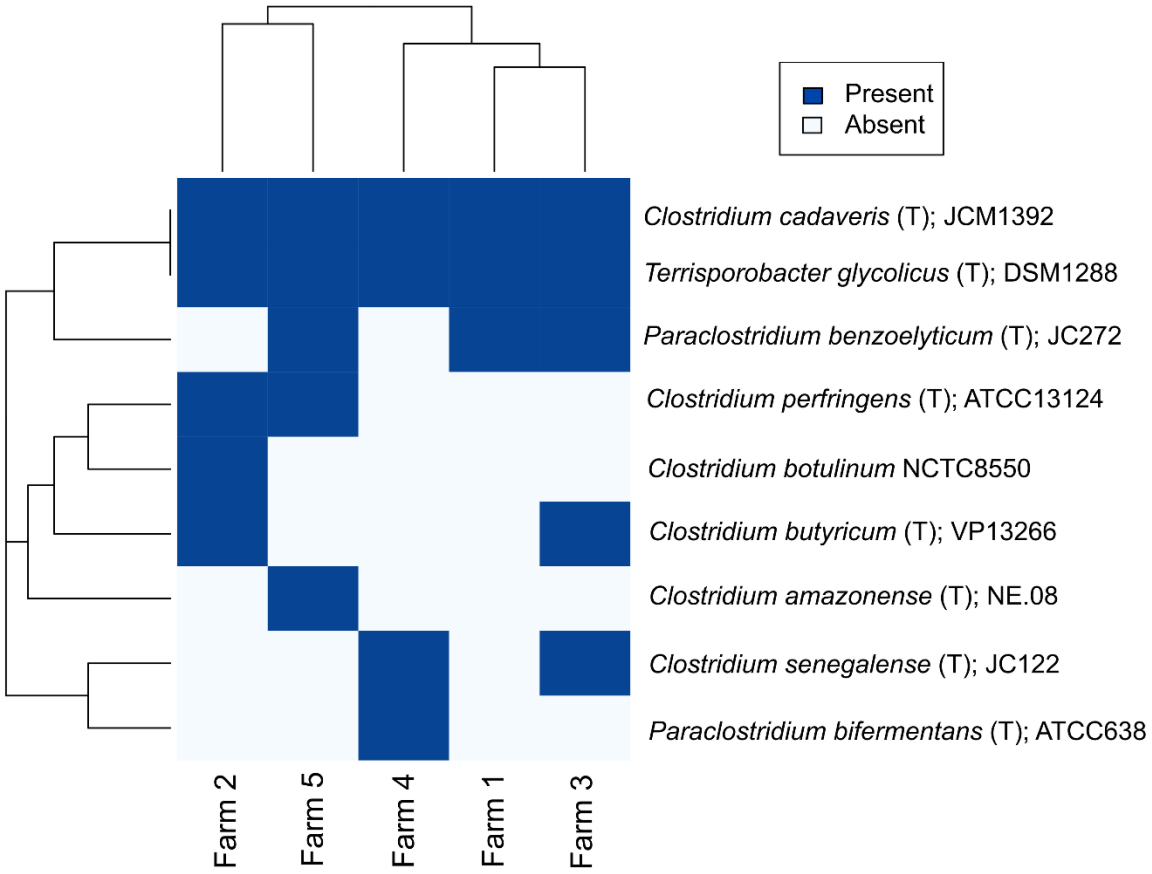


Figure 4.7: The presence of various bacterial species in five soil samples identified by 16S rRNA gene amplicon sequence analysis. The heat map with hierarchical clustering was generated to show the presence and absence of all identified anaerobic spore forming bacteria in five soil samples and relationships between samples based on the observations using R.

Soil is one of the ecosystems harbouring a large and diversified group of bacteria. Soil microorganisms have developed various strategies to survive and multiply under variable abiotic and biotic conditions in the soil [317]. Antimicrobial producing microorganisms are commonly found from soil microbial communities. It has been traditionally perceived that these microbes play a role in antagonistic species interactions by producing antimicrobial molecules giving competitive advantages to producers in competitive habitats like soil [318]. In recent years, these molecules are suggested to have roles mainly as signalling molecules that may mediate various species interactions in complex ecosystems like soil [319]. Soil *Streptomycetes* are the microbes most commonly

screened for antimicrobials and they produce more than 70% of known natural antimicrobials used in today's medicine and other applications [319]. However, soil anaerobes such as *Clostridium* spp. have been mostly ignored in antimicrobial discovery (More information can be found in the literature review chapter 2.5). This study revealed that CMs from different populations of soil *Clostridium* and closely related species possessed various degrees of antimicrobial potential. This suggests that soil *Clostridium* and closely related species may be a viable source of microorganisms to look for potent antimicrobial compounds. A previous study, which evaluated 211 published anaerobic genomes for the presence of gene clusters for the synthesis of NRPS and PKS reported that bacteria isolated from soil possessed three times higher genetic content to produce secondary metabolites than all other anaerobes from different habitats [13]. The present study highlighted the antimicrobial potential of soil anaerobes such as *Clostridium* and closely related species against bacteria associated with food quality, safety, and human health.

4.2.4 The involvement of Farm 4 soil isolates in antimicrobial activity

Among the five soil conditioned media, F4SCM was selected for further investigation in search of potent antimicrobial compounds based on its relatively stronger antimicrobial properties. First, the involvement of Farm 4 soil isolates in the antimicrobial activity of F4SCM was verified by spiking all four isolates into sterile Farm 4 soil and evaluating antimicrobial activities of conditioned media from sterile Farm 4 soil and bacteria-spiked Farm 4 soil.

Conditioned medium prepared from sterile Farm 4 soil ($F4SCM_{Sterile}$) showed no growth inhibition against *B. mycoides*, *B. cereus*, and *P. aeruginosa*. In contrast, $F4SCM_{Spiked}$ prepared from Farm 4 soil spiked with all four bacterial isolates, significantly inhibited the growth of all three test bacteria ($p < 0.05$) (Figure 4.8). These results were also very similar to the growth inhibition profiles of F4SCM against *B. mycoides*, *B. cereus*, and *P. aeruginosa*. These findings confirmed the involvement of FS01, FS2.2, FS03, and FS04 isolates in F4SCM's antimicrobial activity against test bacteria. In other words, this work highlighted the transformation of bacterial growth supporting CMGS medium into conditioned medium with antimicrobial properties by FS01, FS2.2, FS03, and FS04 isolates.

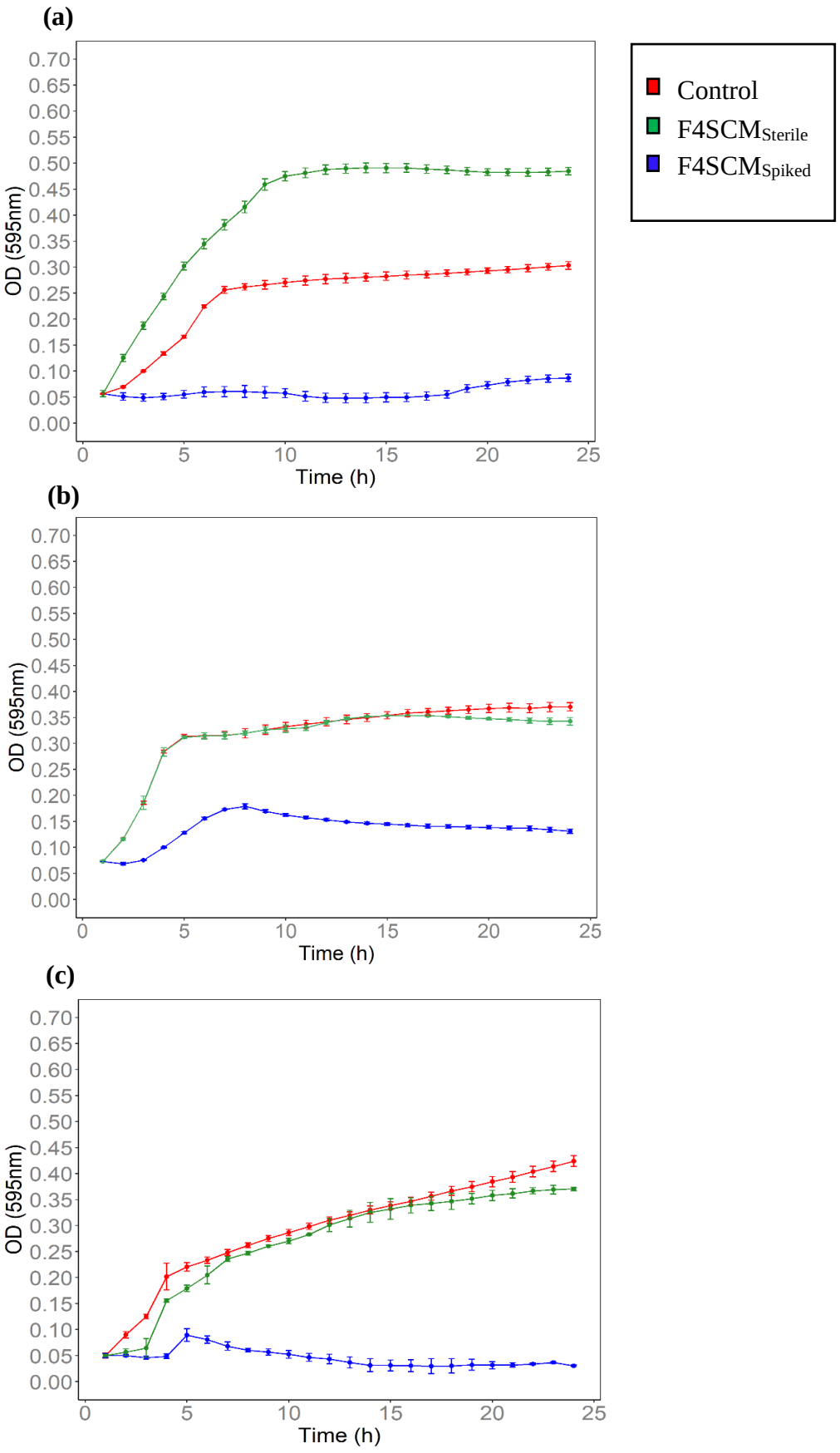

(a)
(b)
(c)
Control
F4SCMSterile
F4SCMSpiked
OD (595nm)
Time (h)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50
0.55
0.60
0.65
0.70
0
5
10
15
20
25

Figure 4.8: Involvement of *Clostridium* and closely related species isolated from Farm 4 soil in F4SCM's antimicrobial activity. CMs were prepared from sterile Farm 4 soil ($F4SCM_{Sterile}$), and Farm 4 soil spiked with all four soil isolates ($F4SCM_{Spiked}$) and evaluated their antimicrobial activities against *B. mycoides* ATCC6462 **(a)**, *B. cereus* NZRM5 **(b)**, and *P. aeruginosa* ATCC25668 **(c)**. Bacteria were grown in the presence of butterfield's diluent (red), $F4SCM_{Sterile}$ (green), and $F4SCM_{Spiked}$ (blue). Each curve represents the mean growth rate ± S.D (n = 3).

4.2.5 Preliminary characterization of Farm 4 soil conditioned medium (F4SCM)

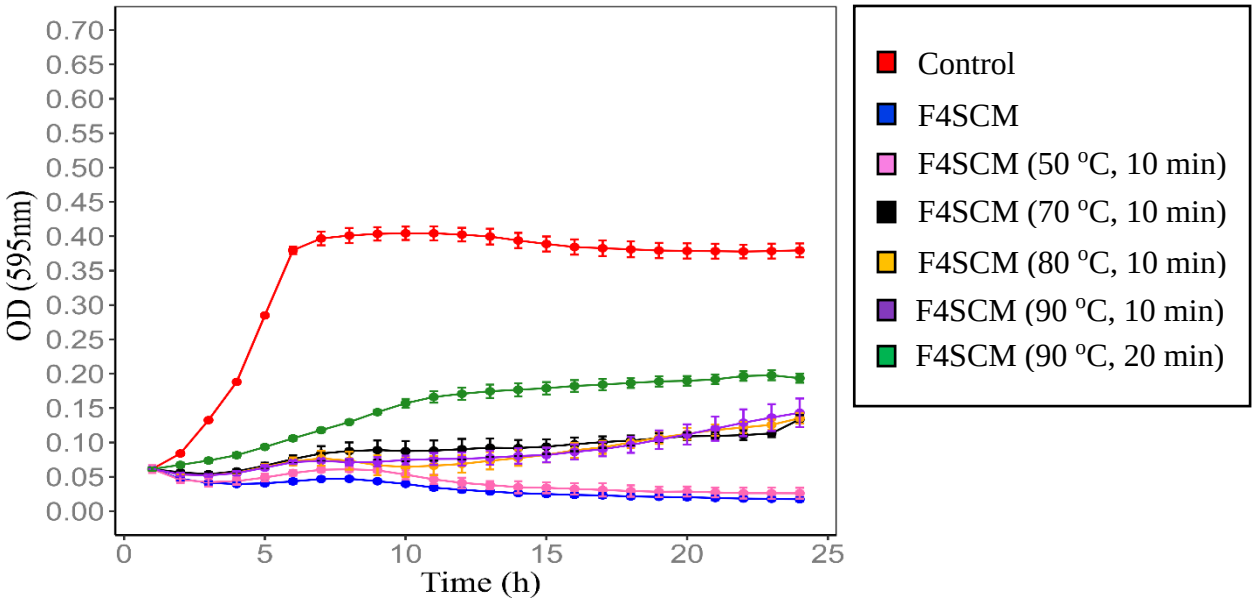
F4SCM was a crude medium potentially consisted of a wide range of molecules including metabolites and other extracellular molecules secreted by Farm 4 soil isolates. The behaviour of F4SCM's activity was evaluated after protease enzyme treatment and various temperature and pH treatments to get a basic understanding about the physiochemical properties of the antimicrobial compound/s.

4.2.5.1 Influence of heat on the antimicrobial activity of F4SCM

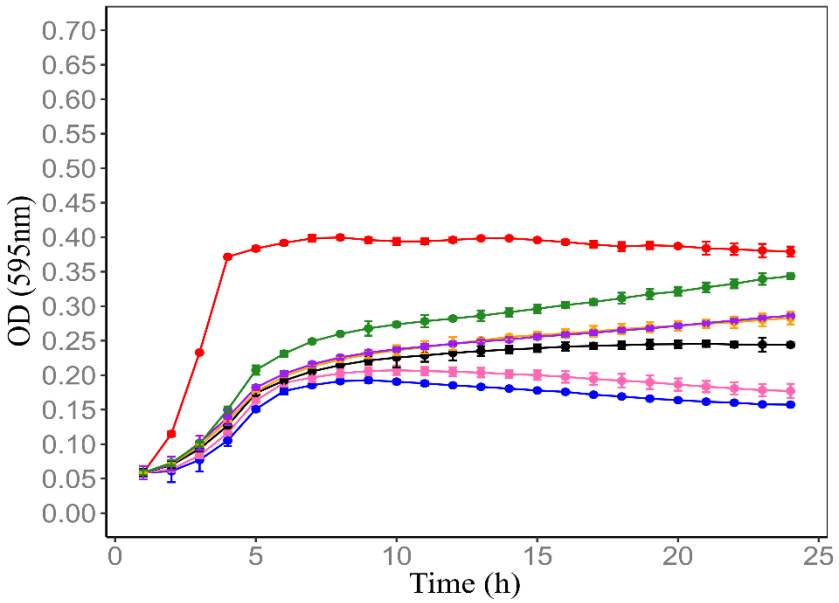
Thermal stability of the antimicrobial compounds of F4SCM was tested by subjecting F4SCM to various heat treatments and evaluating the residual antimicrobial activity against three test bacteria (See the materials and methods chapter 3.1.8). Antimicrobial capacity of F4SCM against three test bacteria was influenced to various degrees by heat treatments compared to the unheated control (Figure 4.9). Unheated F4SCM had the strongest growth inhibition against *B. mycoides* and *B. cereus* while there was a temperature-dependant reduction in the growth inhibition activity of F4SCM (Figure 4.9a and Figure 4.9b). F4SCM, heat treated at 50 ^{o}C for 10 min, showed no significant reduction of its activity against both *B. mycoides* and *B. cereus* ($p < 0.05$), although its activity was lower than the unheated control. In contrast, antimicrobial activity of F4SCM was significantly reduced following heat treatment at 70 ^{o}C or higher for 10 min ($p < 0.05$). F4SCM subjected to the most intensive heat treatment (90 ^{o}C for 20 min) in this study showed the lowest antimicrobial activity against *B. mycoides* and *B. cereus*. On the contrary, antimicrobial activity of F4SCM against *P. aeruginosa* was found to be highly stable (Figure 4.9c). Thermal treatments up to 90 ^{o}C for 20 min showed no significant change in the antimicrobial activity of F4SCM against *P. aeruginosa*

compared with the unheated control ($p < 0.05$). In general, these findings revealed that putative active compound/s in F4SCM against *P. aeruginosa* and two *Bacillus* spp. had different thermostabilities.

(a)



(b)



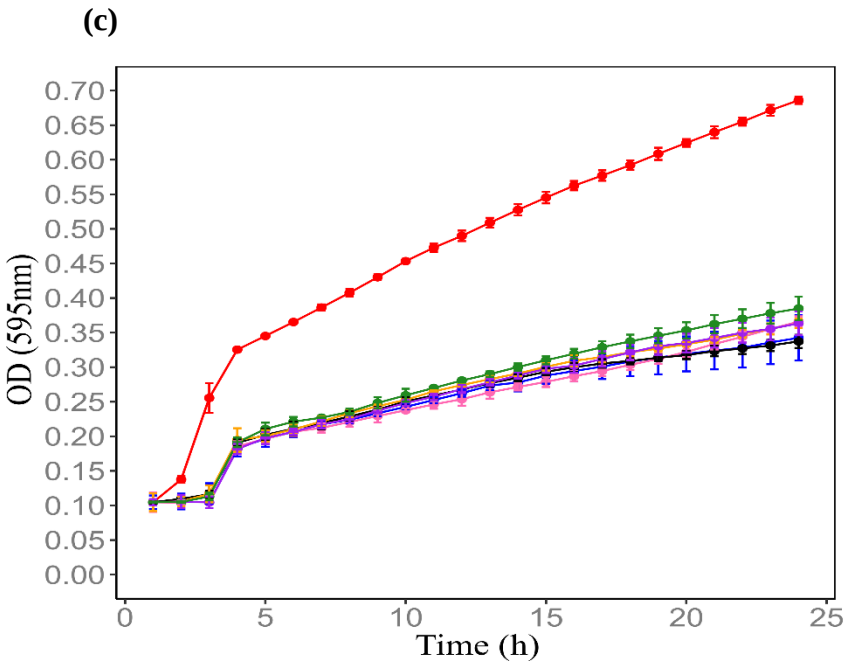


Figure 4.9: Influence of heat on the antimicrobial activity of Farm 4 soil conditioned medium (F4SCM). Growth inhibitory effect of heat treated and control F4SCM samples were tested against *B. mycoides* ATCC6462 **(a)**, *B. cereus* NZRM5 **(b)**, and *P. aeruginosa* ATCC25668 **(c)**. Bacteria were grown in the presence of butterfield's diluent (red), F4SCM (blue), F4SCM treated at 50 ^{o}C for 10 min (pink), F4SCM treated at 70 ^{o}C for 10 min (black), F4SCM treated at 80 ^{o}C for 10 min (yellow), F4SCM treated at 90 ^{o}C for 10 min (purple), and F4SCM treated at 90 ^{o}C for 20 min (green) in the growth media (CMGS). Each curve represents the mean growth rate ± S.D (n = 3).

4.2.5.2 Influence of pH on the antimicrobial activity of F4SCM

The influence of pH change on the antimicrobial property of F4SCM was evaluated by measuring the residual antimicrobial activity of F4SCM against three test bacteria after subjecting to various pH levels. F4SCM treated with near neutral pH levels (pH 6 and 8) showed no significant change in the antimicrobial activity against *B. mycoides* compared with F4SCM not subjected to any pH change ($p < 0.05$). However, significant loss of activity was observed at highly acidic (pH 2 and 4) and basic pH values (pH 10 and 12) compared with F4SCM without pH change (Figure 4.10a). In a similar fashion, exposing F4SCM to pH 2, 4, 10, 12 values significantly reduced the antimicrobial activity of F4SCM against *B. cereus* compared with F4SCM not subjected to any pH change ($p < 0.05$). There was no significant influence when subjected to pH 6 and 8 (Figure 4.10b). All pH treatments of F4SCM except pH 8 significantly reduced the antimicrobial

activity of F4SCM against *P. aeruginosa* compared with F4SCM without pH change ($p < 0.05$) (Figure 4.10c). In general, the antimicrobial capacity of F4SCM against all three test bacteria was influenced by the pH change particularly at highly acidic and basic levels.

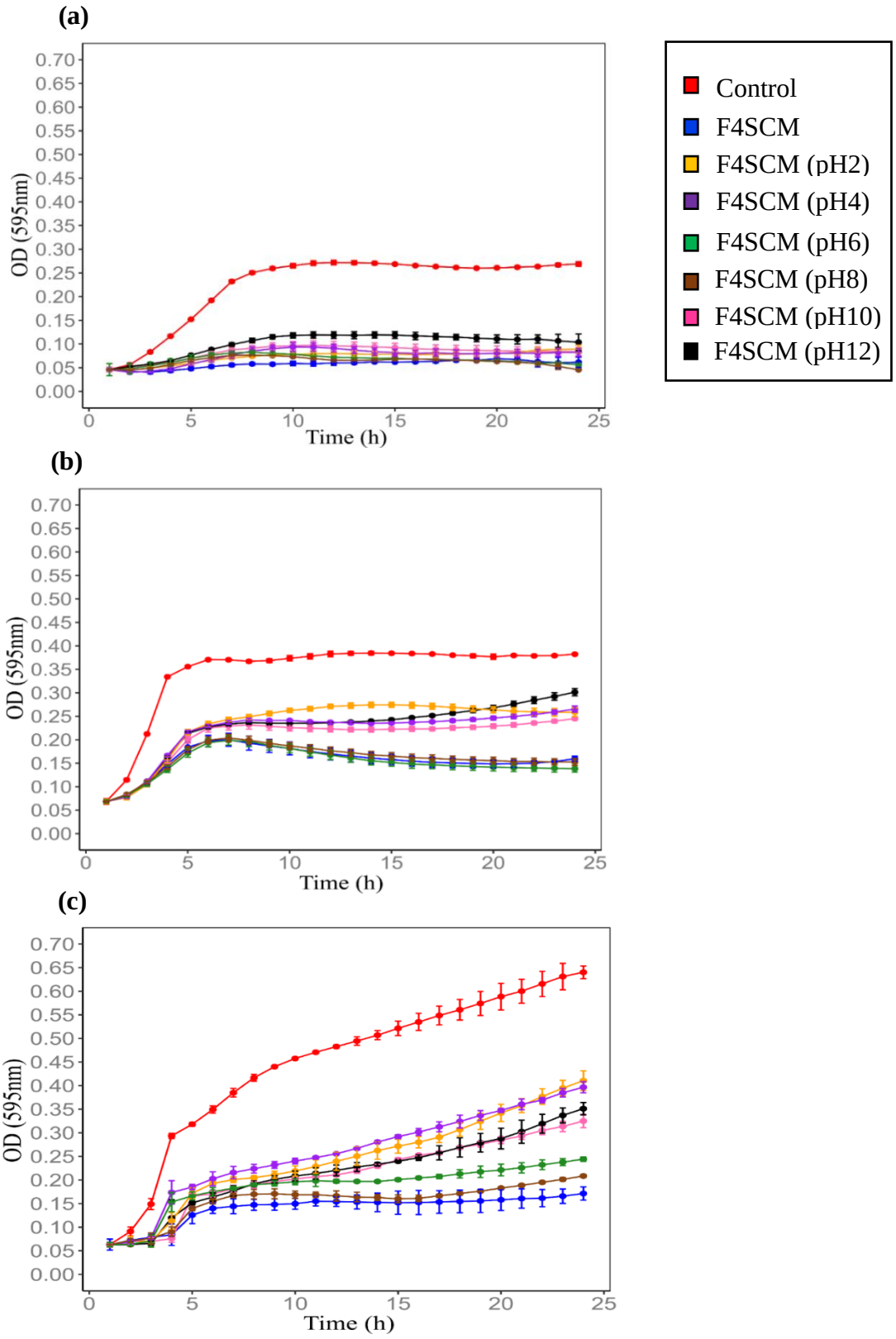


Figure 4.10: Influence of exposing Farm 4 soil conditioned medium (F4SCM) to different pH conditions on its antimicrobial activity. Growth inhibitory effect of pH treated and control F4SCM samples were tested against *B. mycoides* ATCC6462 **(a)**, *B. cereus* NZRM5 **(b)**, and *P. aeruginosa* ATCC25668 **(c)**. Bacteria were grown in the presence of butterfield's diluent (red), F4SCM (blue), F4SCM exposed to pH2 (yellow), F4SCM exposed to pH4 (purple), F4SCM exposed to pH6 (green), F4SCM exposed to pH8 (brown), F4SCM exposed to pH10 (pink), F4SCM exposed to pH12 (black). Each curve represents the mean growth rate ± S.D (n = 3).

4.2.5.3 Effect of protease on the antimicrobial activity of F4SCM

The influence of protease on the antimicrobial capacity of F4SCM was evaluated by measuring the residual activity of protease treated F4SCM against three test bacteria. Antimicrobial activity of F4SCM against *B. mycoides* and *P. aeruginosa* remained stable after 1 mg/mL protease treatment, showing similar levels of growth inhibition as F4SCM not subjected to protease treatment (Figure 4.11a and 4.11c). In contrast, protease treatment significantly reduced the antimicrobial activity of F4SCM against *B. cereus* compared with F4SCM not treated with protease ($p < 0.05$) (Figure 4.11b).

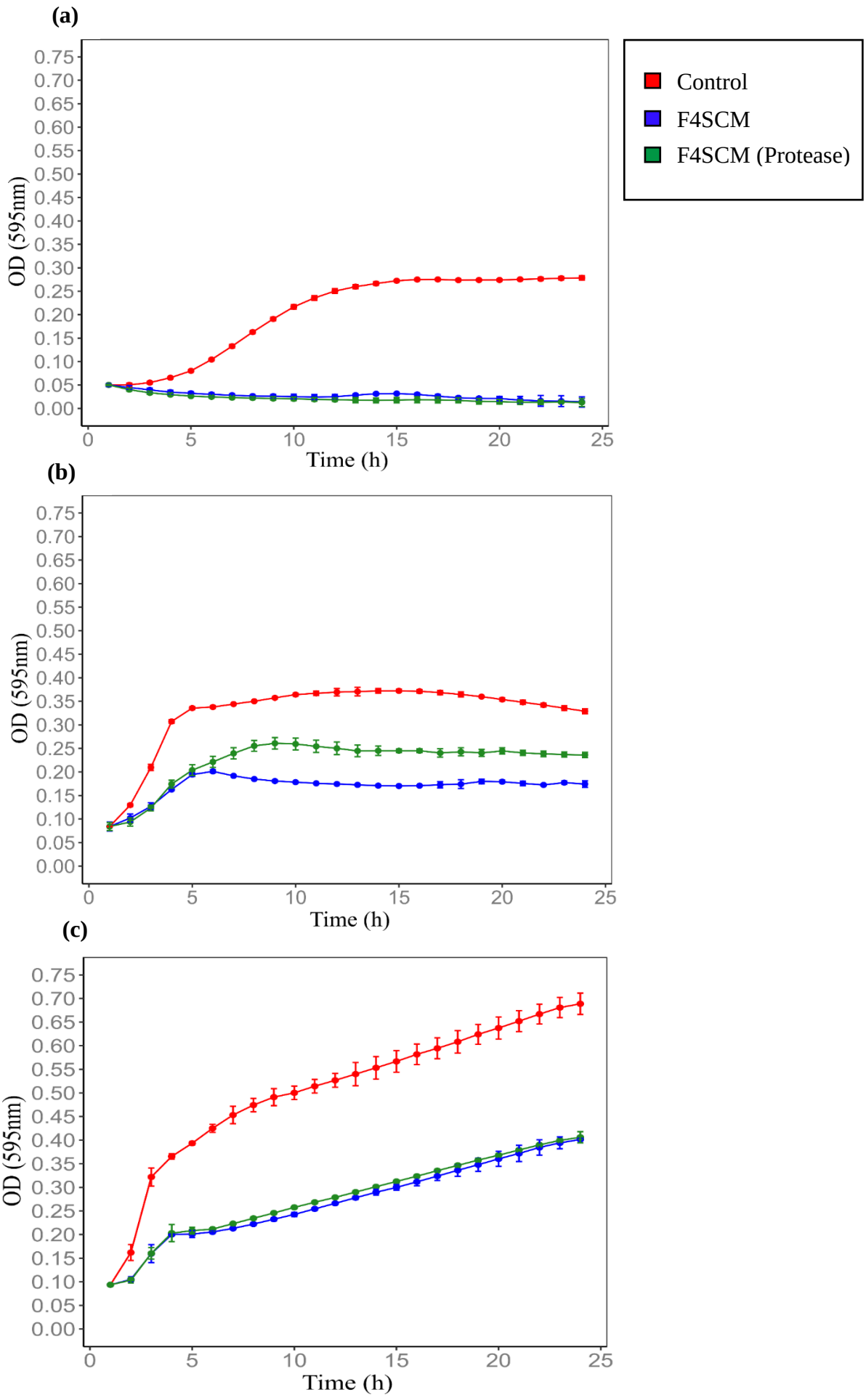

(a)
(b)
(c)
Control
F4SCM
F4SCM (Protease)
OD (595nm)
Time (h)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50
0.55
0.60
0.65
0.70
0.75
0
5
10
15
20
25

Figure 4.11: Effect of protease enzyme on the antimicrobial activity of Farm 4 soil conditioned medium (F4SCM). Growth inhibitory effect of protease treated and control F4SCM samples were tested against *B. mycoides* ATCC6462 **(a)**, *B. cereus* NZRM5 **(b)**, and *P. aeruginosa* ATCC25668 **(c)**. Bacteria were grown in the presence of butterfield's diluent (red), F4SCM (blue), and F4SCM treated with 1 mg/mL protease enzyme (green). Each curve represents the mean growth rate ± S.D (n = 3).

The putative active compound/s present in F4SCM against *P. aeruginosa* were thermostable and insensitive to protease indicating that they may not be proteinaceous in nature. On the other hand, putative antimicrobial/s in F4SCM inhibiting *B. mycoides* were heat labile at high temperatures (70 ^{o}C for 10 min or higher) but resistant to protease treatment, showing their differences in physiochemical nature from those against *P. aeruginosa*. Antimicrobial activity of F4SCM against *B. cereus* was influenced by both heat and protease, segregating those presumptively active compound/s from others. F4SCM's activity against all three tested microorganisms was partially affected by pH when deviated from its original pH (7.4 ± 0.2). These findings suggest that F4SCM may contain different active compounds having diverse physiochemical characteristics. Moreover, these putative compounds may be providing synergistic antimicrobial activity.

4.2.6 Conclusions

One of the main objectives of the work presented in this chapter was to screen conditioned media prepared from various farm samples for antimicrobial activity to select a CM with promising antimicrobial potential for further investigations. Stage I screening suggested that CMs prepared from farm soil samples possessed superior antimicrobial activities than CMs prepared from the other two farm sources. This finding led to screening more CMs prepared from various farm soil samples for antimicrobial activities against both Gram-positive and Gram-negative bacteria during the next stage of screening process (stage II). Among five soil CMs, Farm 4 soil conditioned medium (F4SCM), which displayed promising antimicrobial activities against Gram-positive bacteria (*B. mycoides, B. cereus, B. pumilus*) and Gram-negative *P. aeruginosa*, was found to be an attractive candidate for further investigation in the search for potent antimicrobials.

The isolation and identification of anaerobic spore forming bacteria from five soil samples revealed the presence of *Clostridium* and closely related species in all soil CM.

All five soil CMs contained different *Clostridium* and closely related species populations even though some identified bacterial species were present in multiple samples. Spiking sterile Farm 4 soil with all four bacterial species derived from the same soil confirmed the involvement of those *Clostridium* and closely related species in F4SCM's antimicrobial activity. The behaviour of F4SCM's antimicrobial activity against various temperature, pH, and protease treatments suggested that F4SCM might contain different active compounds having diverse physiochemical characteristics.

Chapter 5

Non-targeted metabolomics analysis of soil conditioned media

5.1 Introduction

The work presented in this chapter is associated with the third objective of the research, which was the investigation of metabolite profiles of CMs of interest with the focus on identifying putative antimicrobial compounds. As discussed in chapter 4, antimicrobial activity screening reported promising antimicrobial activities from farm soil conditioned media. F4SCM possessed the most promising antimicrobial profiles among all five soil CMs (Figure 5.1). The five soil CMs were selected to investigate their metabolite profiles and further analysis aimed to identify potential antimicrobial metabolites from the F4SCM metabolome.

The anaerobic fermentation of animal protein rich CMGS medium by *Clostridium* species and closely related species resulted CMs with various degrees of growth inhibition as reported in chapter 4. The metabolite composition of each soil CM should be a result of combined metabolic activities of all anaerobic bacterial species associated with the soil sample. These anaerobic spore-forming bacterial species associated with the five soil conditioned media were isolated and identified in the previous chapter (See the antimicrobial potential of conditioned media chapter 4.2.3).

Non-targeted metabolomics is increasingly used in antimicrobial compound discovery from various natural sources [174, 315]. This approach can provide a comprehensive analysis of all measurable analytes including chemically known and unknown compounds in a sample [320]. Therefore, non-targeted metabolomics by liquid-chromatography

coupled to mass spectrometry (LC-MS) was employed in the current study for metabolite profiling of soil CMs. Two LC conditions, Hydrophilic Interaction Liquid Chromatography (HILIC) and C18 stationary-phases with appropriate mobile-phases, were used to increase the metabolite coverage by targeting both polar and intermediate-polar compounds in the samples. Moreover, both positive and negative ionization modes were used to increase the detection coverage of metabolites. Therefore, data were obtained from four channels as C18 positive, C18 negative, HILIC positive, and HILIC negative ionization modes.

Non-targeted metabolomics data processing uses chemometrics tools to obtain chemical information and translate them into biological knowledge due to the complexity and multi-dimensionality of metabolomics data [321]. Chemometrics is a discipline that extracts information from multivariate chemical data with the help of statistical and mathematical tools. It involves a range of methods such as signal processing, basic statistics, calibration, curve fitting, detection, and pattern recognition [322]. Chemometrics has become a powerful tool for fast analysis of metabolomics data with the advancement of computational techniques. In the current study, various chemometrics analyses were carried to obtain information on the metabolomes of all samples. Multivariate analysis was used to identify directions that best explain variance in the data set, while univariate analysis identified differences between individual features in different samples leading to discriminating the conditions under study.

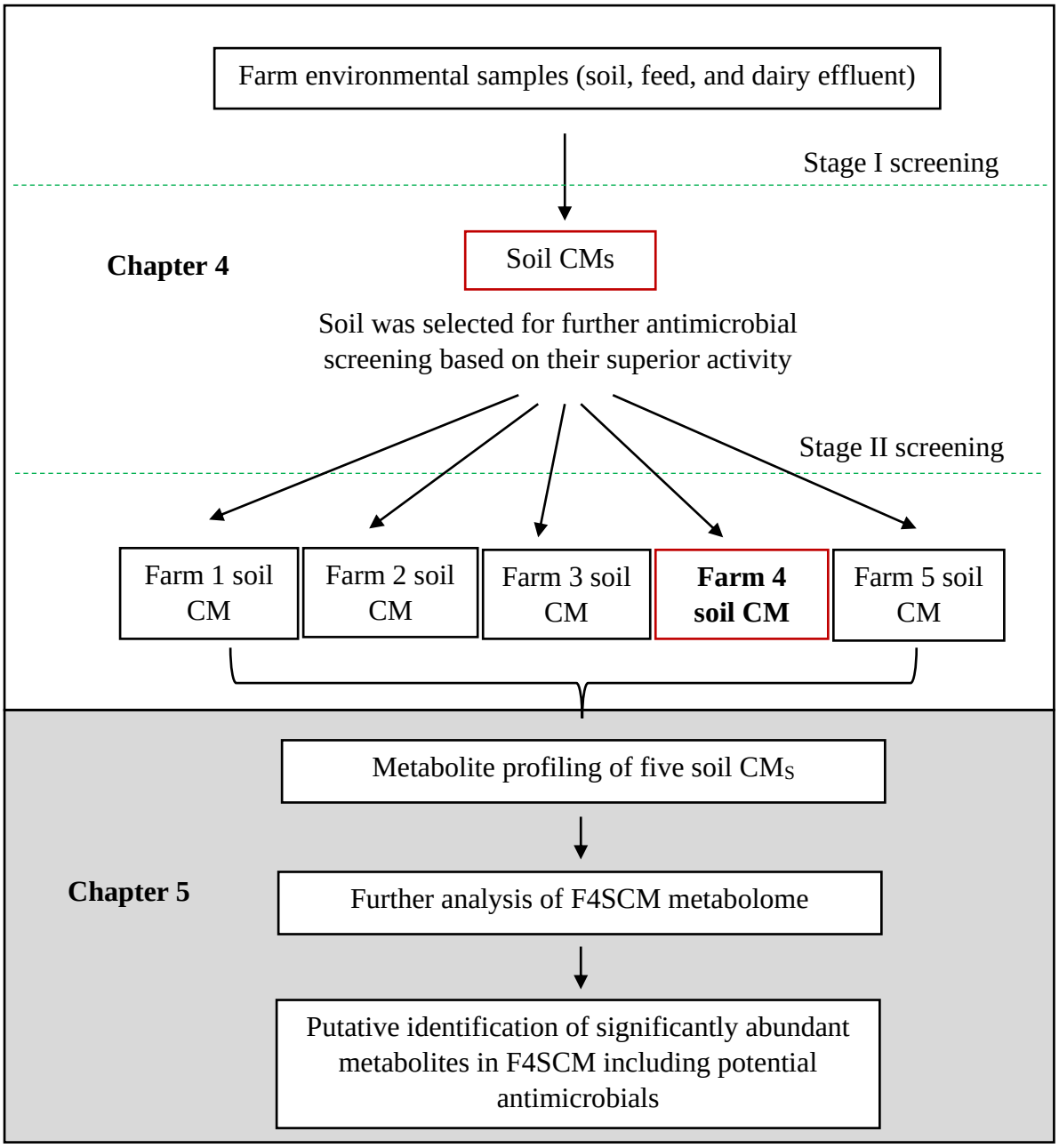


Figure 5.1: Schematic diagram showing the focus of the chapter 5 and associations to the previous works. Farm 4 soil CM (in bold) produced the most promising antimicrobial activity.

5.2 Results and discussion

5.2.1 Metabolite profiling of five soil conditioned media

Initial sample preparation consisted of liquid-liquid extraction with methanol-chloroform-water solvent system to separate polar and non-polar metabolites in all the samples (See materials and methods chapter 3.2.1). Polar and semi-polar metabolites extracted into the upper aqueous phase were used for subsequent LC-MS analysis with C18 and HILIC liquid chromatography columns. LC columns were connected to the Exactive Orbitrap™ mass spectrometer with electrospray ionization to detect ions with *m/z* values at 55-1,100 amu range. Representative total ion chromatograms (TIC) of all five samples in both positive and negative ionization modes are shown in Appendix A3.1.

Principal component analysis (PCA) is a commonly employed chemometrics technique, which is used as an unsupervised pattern recognition tool for handling multivariate data without previous knowledge of the samples under study [322]. It allows a reduction of many variables into a smaller number of composite variables (PCs) and visualization of the overall relationships between the samples based on the variance within the data sets from different samples. The first composite variable (PC1) contains the highest variant and the second composite variable (PC2) is orthogonal to the PC1 and contains the second highest variant in the original data set [323]. The variability of five soil CMs in terms of metabolite profiles was evaluated using PCA. A total of 1663 metabolite features were detected in all five soil CMs and CMGS by all four channels of LC-MS analysis (497 in the C18 negative stream, 209 in the C18 positive stream, 490 in the HILIC negative stream, and 467 in the HILIC positive streams). PCA was employed to obtain 2D-PCA score plots visualizing the spatial distribution of five soil CMs and CMGS medium along the composite variables (PC1 and PC2). The first composite variable (PC1) and the second composite variable (PC2) in C18 negative data set accounted for 43.1% and 12.2% of the overall variability. PC1 and PC2 components explained 38.5%, 13.4%; 34%, 13.7% and 37.1%, 10.8% of the total variability in C18 positive, HILIC negative, and HILIC positive data sets respectively. C18 and HILIC PCA score plots of both ionization modes showed a clear separation between CMGS and soil CMs (Figure 5.2). This revealed the distinctive changes in metabolic composition of all five soil CMs from CMGS, which indicated the combined metabolic activities of various *Clostridium* and closely related species associated with the samples in CMGS. In other words, the growth of *Clostridium* and closely related species in CMGS medium considerably changed its metabolite

composition after 48 h of incubation. Farms 1, 3, and 5 clustered together in both the C18 and HILIC PCA score plots showing less variability among samples in terms of metabolite composition. However, metabolite composition of F4SCM was found to be distinct from all other samples in both C18 and HILIC negative and positive ionization mode plots. This may partially explain the relatively strong antimicrobial activity of F4SCM compared to other soil CMs. F2SCM, which showed significant antagonistic activity, also had a distinctive metabolite composition in C18 negative, C18 positive, and HILIC positive ionization mode plots (Figure 5.2). These differences in the metabolite composition of five soil CMs were a result of the combined metabolic activities of soil sample associated different *Clostridium* and closely related species populations under given growth conditions.

In addition to PCA analysis, hierarchical cluster analysis, unsupervised clustering technique, was carried out to cluster samples into a dendrogram based on the dissimilarity and similarity of their metabolite profiles. Hierarchical clustering demonstrated descriptively similar results to those of the 2D-PCA plots. There was a clear distance between F4SCM and rest of the samples in all four ionization modes (Figure 5.3). These two multivariate analyses revealed the substantial and different chemical composition of F4SCM from other soil CMs consistent with its promising antimicrobial activity.

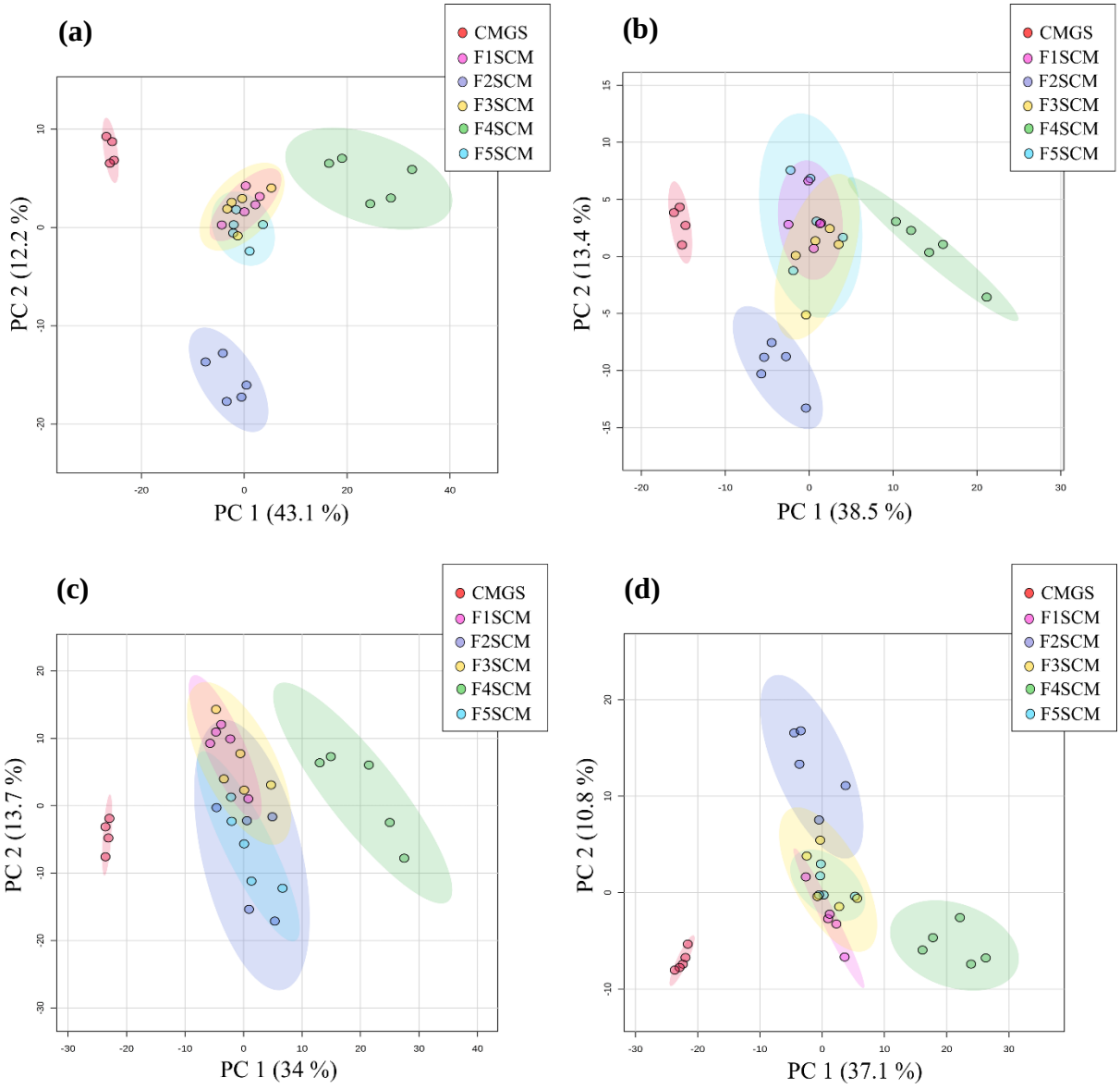
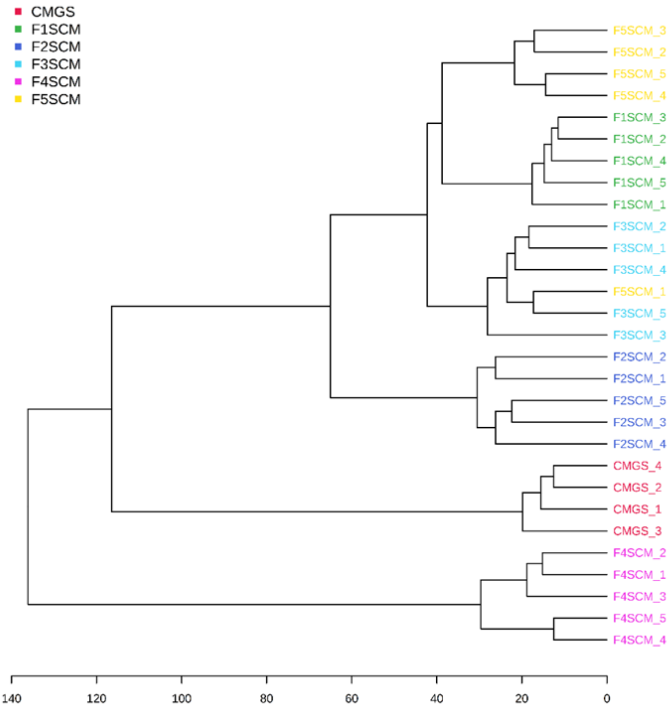
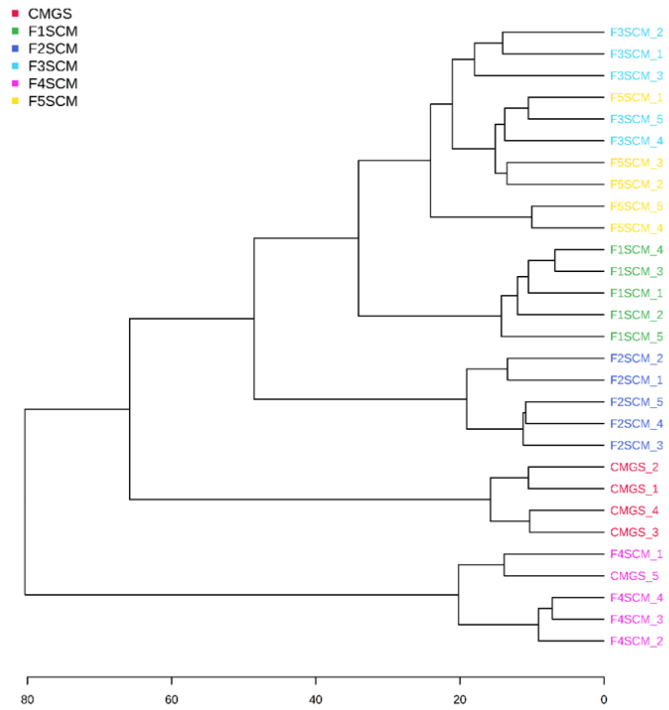


Figure 5.2: Principal component analysis (PCA) score plots of five soil conditioned media. 2D- PCA score plots of C18 negative **(a)**, C18 positive **(b)**, HILIC negative **(c)**, and HILIC positive **(d)** ionization mode data, showing differences in metabolite profiles of CMGS and Farm 1-5 soil conditioned media. CMGS (Cooked meat glucose starch media supplemented with 0.0005% yeast extract, 0.1% hemin and 1% vitamin K) was used as negative control (n = 5). Samples are colour coded as indicated in the figure legend.

(a)


CMGS
F1SCM
F2SCM
F3SCM
F4SCM
F5SCM
F5SCM_3
F5SCM_2
F5SCM_5
F5SCM_4
F1SCM_3
F1SCM_2
F1SCM_4
F1SCM_5
F1SCM_1
F3SCM_2
F3SCM_1
F3SCM_4
F5SCM_1
F3SCM_5
F3SCM_3
F2SCM_2
F2SCM_1
F2SCM_5
F2SCM_3
F2SCM_4
CMGS_4
CMGS_2
CMGS_1
CMGS_3
F4SCM_2
F4SCM_1
F4SCM_3
F4SCM_5
F4SCM_4
140
120
100
80
60
40
20
0

(b)


CMGS
F1SCM
F2SCM
F3SCM
F4SCM
F5SCM
F3SCM_2
F3SCM_1
F3SCM_3
F5SCM_1
F3SCM_5
F3SCM_4
F5SCM_3
F5SCM_2
F5SCM_5
F5SCM_4
F1SCM_4
F1SCM_3
F1SCM_1
F1SCM_2
F1SCM_5
F2SCM_2
F2SCM_1
F2SCM_5
F2SCM_4
F2SCM_3
CMGS_2
CMGS_1
CMGS_4
CMGS_3
F4SCM_1
CMGS_5
F4SCM_4
F4SCM_3
F4SCM_2
80
60
40
20
0

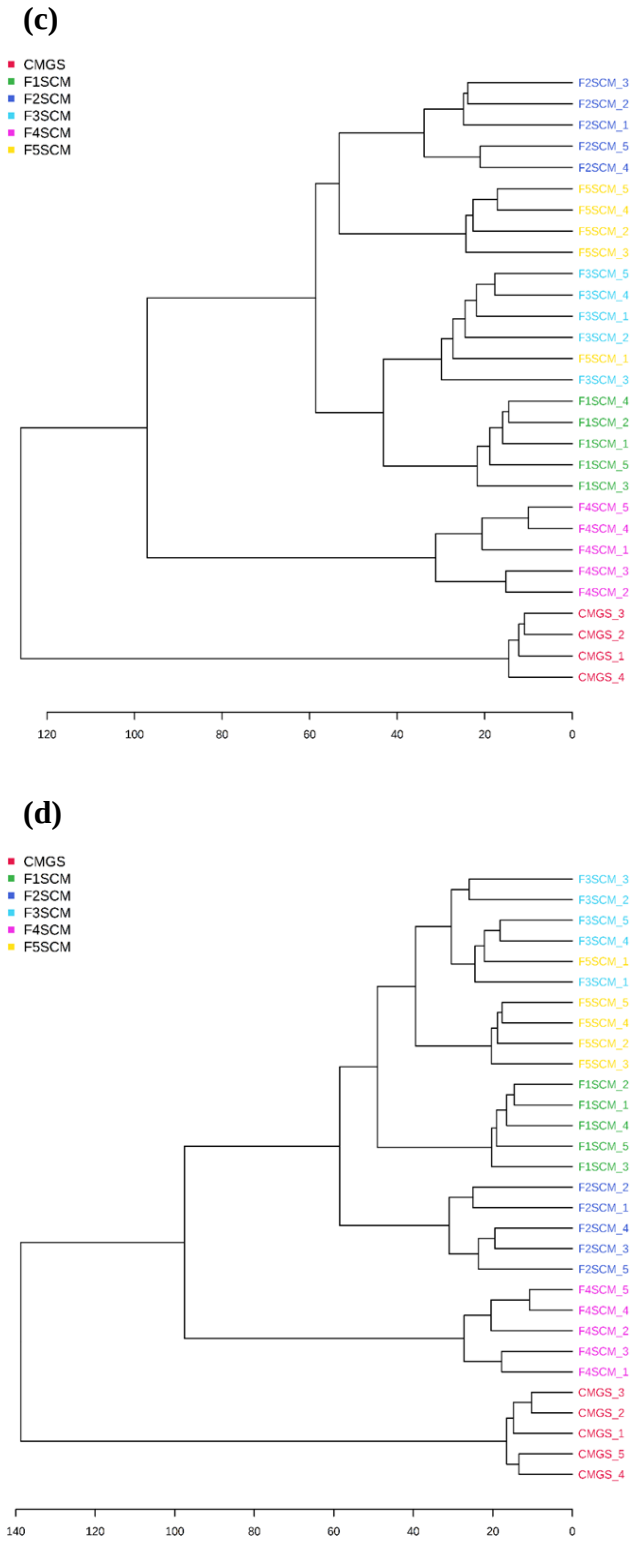


Figure 5.3: Dendrograms showing the relationship between five soil CMs and CMGS medium. Samples were clustered based on their C18 negative **(a)**, C18 positive **(b)**, HILIC negative **(c)**, and HILIC positive **(d)** metabolite profiles. Dendrogram was constructed by ward clustering on the closest Euclidean distances between samples. Samples are colour coded as indicated in the figure legend. Numbers represent sample IDs.

5.2.2 Evaluation of differentiating metabolites in Farm 4 soil conditioned medium (F4SCM) from growth medium (CMGS)

F4SCM demonstrated the highest relative antimicrobial activity and significantly different metabolite profiles from all other soil CMs. Therefore, F4SCM metabolite profiles were further evaluated to identify metabolite features that differentiate them from growth medium (CMGS). These significantly discriminating metabolite features of F4SCM belong to the metabolites produced by Farm 4 soil associated *Clostridium* and closely related species and they are most likely responsible for the antimicrobial properties of F4SCM. As discussed in chapter 4, four different bacterial species closely related to *Paraclostridium bifermentans*, *Clostridium cadaveris*, *Terrisporobacter glycolicus*, and *Clostridium senegalense*, are involved in the production of those metabolites during their growth in CMGS.

All metabolite features detected in F4SCM and CMGS medium (497 from C18 negative, 209 from C18 positive, 490 from HILIC negative, and 467 from HILIC positive) were used to prepare volcano plots showing significantly changed metabolites between F4SCM and CMGS (Figure 5.4). A volcano plot is a scatter plot of the statistical significance (*p*-value) against a magnitude of change (FC) displaying differential expression of features in two comparative groups [324]. In the volcano plot, metabolite features with a fold change (FC) > 2 and *p*-value < 0.05 were considered significantly different. A total of 539 metabolite features (210 from C18 negative, 84 from C18 positive, 100 from HILIC negative, and 135 from HILIC positive) were found to be significantly higher in the F4SCM group compared to CMGS. Significantly higher metabolite features in the F4SCM group, which are likely to be associated with its antimicrobial activity are presented on the right square shown by red coloured dots in volcano plots (Figure 5.4).

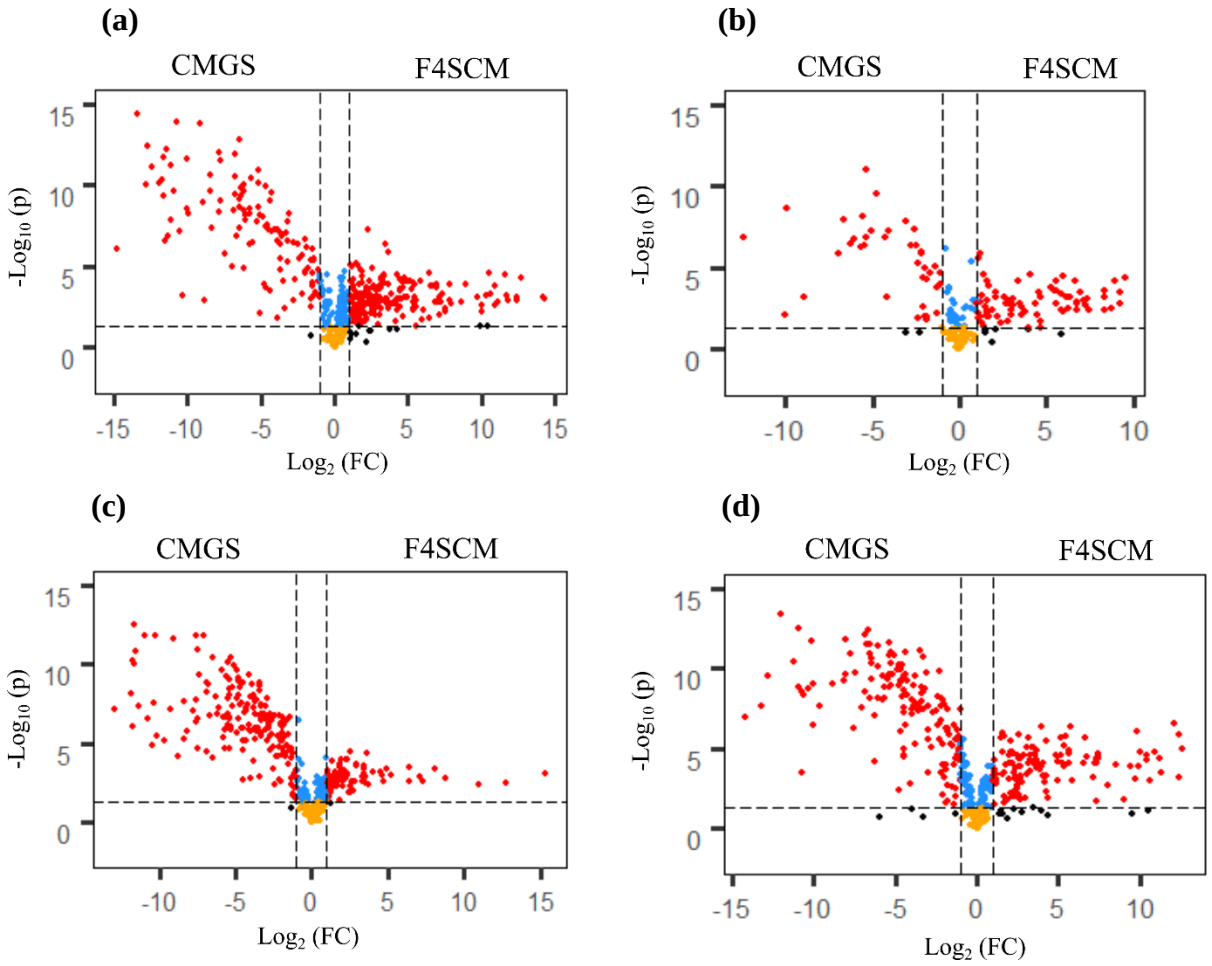


Figure 5.4: Volcano plots show the relative abundance of metabolites in CMGS and F4SCM groups. They were constructed by combining the statistical t-test [-log10(*p*-value)] and the magnitude of the change (log_2 [FC]) in C18 negative **(a)**, C18 positive **(b)**, HILIC negative **(c)**, and HILIC positive **(d)** ionization mode datasets. Red dots represent the metabolites with *p*-value < 0.05 and FC > 2. Blue points represent the metabolites with *p*-value < 0.05 and FC < 2. Yellow points represent the metabolites with *p*-value > 0.05 and FC < 2. Black dots represent the metabolites with *p*-value > 0.05 and FC > 2. CMGS (Cooked meat glucose starch media supplemented with 0.0005% yeast extract, 0.1% hemin, and 1% vitamin K), F4SCM (Farm 4 soil conditioned medium).

Significantly high metabolite features in F4SCM were ranked using the *p*-values (lowest to highest) resulting in a prioritized list of tentative candidates based on the signal abundance and it was used for metabolite identification. Table 5.1 shows a list of the top 50 significantly different metabolite features in F4SCM compared with CMGS from all four streams.

Table 5.1: Significantly high metabolite features in F4SCM group (top 50 features ranked based on the *p*-value).

	C18 negative ionization				C18 positive ionization				HILIC negative ionization				HILIC positive ionization			
	m/z	RT	*p*-value	FC	*m/z*	RT	*p*-value	FC	*m/z*	RT	*p*-value	FC	*m/z*	RT	*p*-value	FC
01	172.0974	282.38	$2.39X10^{-10}$	5.4	180.9041	37.57	$4.52X10^{-9}$	2.3	374.1563	691.87	$1.75X10^{-7}$	6.0	161.107	622.03	$8.20X10^{-10}$	7972.2
02	362.9675	344.83	$3.34X10^{-10}$	11.9	142.9482	37.5	$1.33X10^{-8}$	2.2	336.1193	697.84	$2.00X10^{-7}$	13.0	72.0811	629.24	$1.41X10^{-9}$	60.7
03	131.0344	238.75	$2.15X10^{-9}$	18.9	126.0524	40.93	$1.06X10^{-7}$	15.3	261.0724	694.38	$8.29X10^{-7}$	4.5	274.1657	622.65	$1.77X10^{-9}$	13.8
04	61.9878	380.41	$5.35X10^{-8}$	2.2	164.9301	37.27	$1.29X10^{-7}$	2.8	245.125	869.8	$8.67X10^{-7}$	5.0	141.0653	632.7	$3.80X10^{-9}$	850.6
05	227.1399	267.28	$5.91X10^{-8}$	3.1	89.1075	36.04	$1.63X10^{-7}$	77.5	302.1352	690.07	$9.53X10^{-7}$	3.7	143.0686	632.55	$5.99X10^{-9}$	3.0
06	374.1566	247.69	$6.22X10^{-8}$	3.4	141.0656	55.86	$2.10X10^{-7}$	763.2	261.0877	681.74	$1.36X10^{-6}$	2.7	92.0708	861.87	$6.53X10^{-9}$	6.2
07	298.1517	209.11	$6.42X10^{-8}$	2.2	104.0706	42.41	$3.64X10^{-7}$	11.8	188.1033	781.06	$2.66X10^{-6}$	8.4	123.0553	632.58	$7.29X10^{-9}$	5452.1
08	390.1158	60.69	$9.61X10^{-8}$	3.8	177.0741	343.31	$3.93X10^{-7}$	343.31	242.1138	886.45	$3.08X10^{-6}$	6.5	167.0812	657.18	$8.25X10^{-9}$	3.3
09	250.0574	362.26	$1.10X10^{-7}$	103.2	176.0707	343.59	$4.13X10^{-7}$	111.2	258.0724	742.65	$3.28X10^{-6}$	11.3	81.0449	632.74	$1.02X10^{-8}$	9.3
10	256.0646	278.02	$1.15X10^{-7}$	1493.9	188.1758	39.64	$4.41X10^{-7}$	50.2	189.0509	695.32	$3.49X10^{-6}$	3.4	100.0758	651.25	$1.18X10^{-8}$	112.2
11	380.1825	290.49	$1.29X10^{-7}$	3.1	148.0343	41.03	$6.92X10^{-7}$	3.5	166.0172	591.4	$4.13X10^{-6}$	3.7	144.0805	622.42	$1.20X10^{-8}$	37.1
12	243.1348	240.69	$1.35X10^{-7}$	11.3	202.144	343.19	$1.68X10^{-6}$	42.7	61.9873	633.62	$4.55X10^{-6}$	3.5	131.1178	655.11	$1.27X10^{-8}$	2.8
13	107.0496	295.05	$1.36X10^{-7}$	4.9	137.0267	61.99	$2.09X10^{-6}$	3.0	204.0619	750.87	$4.58X10^{-6}$	12.4	59.0493	650.66	$2.01X10^{-8}$	20.0
14	131.0708	284.2	$1.65X10^{-7}$	19.3	208.1335	314.05	$2.85X10^{-6}$	314.8	232.0819	692.87	$4.60X10^{-6}$	40.1	181.1043	550.47	$4.02X10^{-8}$	4.0
15	102.0553	214.63	$1.66X10^{-7}$	3106.1	184.1334	343.01	$3.26X10^{-6}$	46.0	141.0537	634.1	$4.71X10^{-6}$	88.8	87.0443	692.59	$7.12X10^{-8}$	11.4
16	181.0362	207.4	$2.39X10^{-7}$	13.7	199.144	214.91	$3.31X10^{-6}$	48.4	139.0501	634.1	$5.47X10^{-6}$	313.8	275.123	384.19	$7.63X10^{-8}$	8.3
17	408.1411	259.96	$2.64X10^{-7}$	9.5	131.1177	45.81	$3.83X10^{-6}$	4.7	102.0186	324.23	$8.06X10^{-6}$	2.0	86.0603	692.66	$8.38X10^{-8}$	10.7
18	131.0344	225.55	$2.72X10^{-7}$	29.2	264.1595	416.39	$4.38X10^{-6}$	115.7	227.1143	875.22	$8.55X10^{-6}$	3.9	180.1014	550.23	$1.12X10^{-7}$	5289.9
19	101.0601	313.44	$2.93X10^{-7}$	6818.2	115.0504	93.81	$4.62X10^{-6}$	21.3	175.024	502.39	$9.42X10^{-6}$	4.2	68.0497	692.68	$1.26X10^{-7}$	57.8
20	213.0878	256.96	$3.93X10^{-7}$	2.5	144.0806	262.44	$4.65X10^{-6}$	798.8	167.9963	854.34	$1.16X10^{-5}$	3.6	245.1492	591.97	$1.48X10^{-7}$	13.1
21	85.0655	283.37	$4.14X10^{-7}$	9.3	177.1027	231.18	$5.81X10^{-6}$	59.2	152.0567	647.54	$1.20X10^{-5}$	17.9	82.0652	650.63	$1.64X10^{-7}$	1228.0
22	132.0742	282.84	$4.32X10^{-7}$	19.8	133.0972	35.41	$7.56X10^{-6}$	2.6	187.108	745.98	$1.24X10^{-5}$	3.6	118.086	649.96	$1.72X10^{-7}$	4.4
23	193.9946	268.55	$4.57X10^{-7}$	28.7	105.0739	42.5	$8.56X10^{-6}$	4.6	155.0817	650.65	$1.30X10^{-5}$	20.8	61.0844	597.12	$1.85X10^{-7}$	2892.2
24	346.198	273.22	$5.30X10^{-7}$	90.1	100.0756	214.9	$1.07X10^{-5}$	30.7	96.9686	747.63	$1.31X10^{-5}$	2.2	119.0895	650.13	$1.93X10^{-7}$	4.6
25	345.1499	242.3	$5.62X10^{-7}$	5.3	229.1551	203.66	$1.09X10^{-5}$	6.8	95.0604	634.1	$1.43X10^{-5}$	41043.0	148.0964	690.6	$2.40X10^{-7}$	4.8
26	279.0984	251.61	$5.78X10^{-7}$	2.4	101.079	214.83	$1.11X10^{-5}$	73.6	299.1145	651.04	$1.46X10^{-5}$	11.9	245.1491	558.09	$2.65X10^{-7}$	12.5
27	175.0243	116.1	$6.38X10^{-7}$	37.8	161.1071	262.45	$1.14X10^{-5}$	427.0	132.0293	692.36	$1.50X10^{-5}$	2.8	101.0597	650.39	$2.74X10^{-7}$	191.5
28	180.9993	312.17	$6.43X10^{-7}$	2.3	121.0648	122.53	$1.23X10^{-5}$	46.2	233.0232	739.66	$1.57X10^{-5}$	3.5	74.0715	654.79	$2.75X10^{-7}$	3.4
29	327.167	231.58	$6.65X10^{-7}$	4.4	170.0926	38.67	$1.63X10^{-5}$	8.6	257.1251	893.41	$1.72X10^{-5}$	5.0	105.074	692.58	$2.94X10^{-7}$	12.0
30	310.1406	272.94	$6.71X10^{-7}$	30.9	138.0913	122.62	$1.66X10^{-5}$	180.6	171.0403	693.86	$1.77X10^{-5}$	3.8	134.0957	550.08	$3.00X10^{-7}$	40.3
31	144.066	214.23	$8.88X10^{-7}$	23.1	376.1723	246.56	$1.68X10^{-5}$	3.4	431.1775	696.4	$2.00X10^{-5}$	29.4	56.0497	650.38	$3.23X10^{-7}$	32.7
32	231.0982	204.07	$8.94X10^{-7}$	10.8	102.0801	214.58	$2.07X10^{-5}$	7.8	121.0397	634.1	$2.05X10^{-5}$	26.3	93.0701	636.27	$3.48X10^{-7}$	1274.4
33	130.0867	344.67	$8.97X10^{-7}$	4.2	100.0488	214.72	$2.21X10^{-5}$	145.1	144.0657	650.53	$2.27X10^{-5}$	2.2	103.0504	741.82	$3.61X10^{-7}$	36.9
34	238.021	112.49	$9.10X10^{-7}$	32.0	247.1292	235.16	$2.36X10^{-5}$	2.4	129.0184	455.53	$2.39X10^{-5}$	2.9	104.0706	692.68	$3.64X10^{-7}$	10.1

	C18 negative ionization				C18 positive ionization				HILIC negative ionization				HILIC positive ionization			
	m/z	RT	*p*-value	FC	*m/z*	RT	*p*-value	FC	*m/z*	RT	*p*-value	FC	*m/z*	RT	*p*-value	FC
35	151.0395	295.3	$9.61X10^{-7}$	3.8	132.102	343.31	$2.80X10^{-5}$	2.4	271.0568	785.47	$2.60X10^{-5}$	79.9	170.0808	581.53	$3.67X10^{-7}$	4.0
36	186.113	310.03	$1.03X10^{-6}$	145.6	174.1129	270.86	$2.89X10^{-5}$	120.4	206.9961	597.53	$2.62X10^{-5}$	12.0	188.1279	583.53	$3.70X10^{-7}$	5.4
37	179.0709	345.72	$1.16X10^{-6}$	289.0	154.0972	55.62	$3.43X10^{-5}$	455.6	214.035	695.33	$2.64X10^{-5}$	4.8	376.1706	691.48	$3.89X10^{-7}$	7.2
38	133.075	284.16	$1.22X10^{-6}$	3.4	230.1752	411.4	$3.55X10^{-5}$	52.7	146.0449	694.73	$2.68X10^{-5}$	3.0	289.0834	648.77	$3.91X10^{-7}$	478.0
39	165.0551	326.7	$1.25X10^{-6}$	44.7	360.1925	288.38	$3.60X10^{-5}$	2.2	164.0014	648.7	$2.70X10^{-5}$	2.4	69.0338	692.48	$4.14X10^{-7}$	32.6
40	180.0741	345.69	$1.25X10^{-6}$	777.4	220.1006	304.23	$3.74X10^{-5}$	6.9	228.0983	652.23	$2.83X10^{-5}$	4.5	105.066	437.15	$4.33X10^{-7}$	6.5
41	382.1729	224.02	$1.28X10^{-6}$	11.7	194.1181	285.91	$3.76X10^{-5}$	23.4	207.0879	655.51	$3.11X10^{-5}$	10.5	139.0942	637.23	$4.76X10^{-7}$	112.1
42	336.12	254.66	$1.42X10^{-6}$	6.9	177.102	216.42	$3.81X10^{-5}$	11.7	227.1031	674.94	$3.16X10^{-5}$	3.9	121.0648	636.81	$4.94X10^{-7}$	188.5
43	262.1446	417.53	$1.58X10^{-6}$	106.0	188.1284	308.67	$4.48X10^{-5}$	109.4	130.0501	748.19	$3.42X10^{-5}$	4.9	76.076	664.05	$5.41X10^{-7}$	190.1
44	263.1481	417.51	$1.61X10^{-6}$	951.3	192.598	222.94	$4.94X10^{-5}$	9.1	147.0288	455.34	$3.69X10^{-5}$	3.0	55.0545	649.88	$6.01X10^{-7}$	6.7
45	149.0603	380.88	$1.73X10^{-6}$	107.6	190.0864	376.55	$5.10X10^{-5}$	142.0	171.0403	844.2	$3.71X10^{-5}$	3.3	138.0909	637.15	$7.04X10^{-7}$	181.6
46	344.1461	242.32	$1.77X10^{-6}$	101.4	360.2131	298.49	$5.15X10^{-5}$	8.4	279.1094	641.69	$3.72X10^{-5}$	2.4	405.0904	515.91	$7.13X10^{-7}$	3234.7
47	245.114	236.91	$1.88X10^{-6}$	2.4	141.0655	78.9	$6.44X10^{-5}$	12.7	403.0761	516.58	$3.84X10^{-5}$	338.7	229.1541	511.18	$8.04X10^{-7}$	13.3
48	288.1198	200.13	$2.05X10^{-6}$	6.3	155.0792	36.58	$7.34X10^{-5}$	3.0	127.039	650.04	$4.34X10^{-5}$	3.7	212.1025	529.08	$8.14X10^{-7}$	5.6
49	383.158	223.78	$2.06X10^{-6}$	7.7	318.1668	243.86	$1.13X10^{-4}$	2.0	215.0667	833.28	$4.69X10^{-5}$	2.6	145.133	639.18	$8.28X10^{-7}$	10.5
50	188.0347	253.01	$2.19X10^{-6}$	7.3	216.1596	382.5	$1.17X10^{-4}$	93.7	214.0827	689.38	$4.81X10^{-5}$	3.0	123.0998	601.36	$9.35X10^{-7}$	1553.7

m/z – mass to charge ratio

RT – retention time (s)

p-value - *t*-test between F4SCM and CMGS groups

FC – fold change value (F4SCM/CMGS)

5.2.3 Putative identification of significantly abundant molecules in F4SCM

In the present study, metabolite identification was carried out with level 2 metabolite identification confidence according to the Chemical Analysis Working Group (CAWG) guidelines, which was a comparison of experimental mass measurements (MS1 or MS/MS) with corresponding matches in public spectral libraries, METLIN [199], and MassBank [200]. More information on the metabolite identification has been discussed in the literature review chapter 2.4.2.4. All the selected metabolite features for identification showed statistically significant high intensity in F4SCM compared to CMGS (FC > 2 and *p*-value <0.05). Five metabolites were putatively identified with level two confidence from all four channels of data, they were 2-hydroxyisocaproic acid, 3-hydroxyphenylacetic acid, γ-aminobutyric acid, tryptamine, and creatine (Table 5.2). γ-aminobutyric acid and tryptamine were identified from both C18 and HILIC positive ionization mode data. Appendix A3.2 shows extracted ion chromatograms for parent masses and co-eluting diagnostic fragments of putatively identified compounds. As shown in Figure 5.5, all five putatively identified compounds were detected at very high levels in F4SCM compared to CMGS.

The presence of tryptamine in high abundance in F4SCM was revealed by both C18 and HILIC ionization mode data. Tryptamines are a group of monoamine alkaloids consisting of indole ring structures and derivatives of amino acid tryptophane. They can be found in natural sources including a variety of plants, animals, fungi, and microbes [325]. Tryptamines include a broad range of compounds including neurotransmitters present in the human brain; serotonin and melatonin, psilocybin and bufotenine [326]. Gut microorganisms such as *Clostridium* spp. and *Ruminococcus* spp. have been reported to synthesize tryptamine from tryptone [327]. This group of compounds were reported to have antimicrobial activities against some bacteria, fungi, and yeast. Tryptamine and its derivative, 6-bromo-8,10-dihydro-isoplysin A, extracted from a marine sponge *Fascaplysinopsis reticulata*, demonstrated promising antibacterial activity against *Vibrio carchariae* and *Vibrio natrigens*, respectively [328]. Tryptamine inhibited the growth of yeast species; *Saccharomyces cerevisiae*, *Candida krusei*, and *Candida tropicalis* when grown with ethanol and glucose as the carbon source [329]. The presence of tryptamine in F4SCM may contribute to its antimicrobial property. As tryptamines

have mainly hallucinogenic effects interacting with the serotonin neurotransmitter system in humans, their use as an antimicrobial agent could be highly limited [326].

Another significantly abundant molecule putatively identified from F4SCM using both C18 and HILIC ionization mode data was γ-aminobutyric acid (GABA). GABA is a non-protein amino acid broadly distributed in natural sources including animals, plants, and microorganisms [330]. Bacteria, particularly, lactic acid bacteria (LAB) isolated from fermented food products such as kimchi, paocai, black raspberry juice, yoghurt, cheese, and fermented fish were found to produce high levels of GABA [330, 331]. No information could be found in the literature on GABA production by *Clostridium* species. The major functional property of GABA is acting as an inhibitory neurotransmitter in the mammalian central nervous system affecting personality and stress management [332]. GABA was reported to possess hypotensive, diuretic, tranquilizing, and antidiabetic effects [330, 333]. Alizadeh Behbahani, Jooyandeh [334] demonstrated the production of GABA using *Lactobacillus brevis* A3 in culture medium containing whey protein and monosodium glutamate, and the antimicrobial activity of fermented extract against several food spoilage and pathogenic bacteria. Similarly, the current study reported the presence of GABA in *Clostridium* and closely related species grown conditioned medium with antimicrobial properties. No information could be found on the antimicrobial activity of pure GABA in the literature. Therefore, GABA requires further studies to understand its antimicrobial potential. Considering GABA's other health benefits and its good biosafety profile, it would be worth investigating its antimicrobial potential.

C18 negative ionization mode data revealed the presence of 2-hydroxyisocaproic acid (HICA) in F4SCM in high abundance. HICA is a product of leucine metabolism and a typical constituent of human plasma [254]. Fermentative bacteria including *Clostridium* spp. have been reported to produce HICA [256]. HICA has been reported to possess antimicrobial activity against several bacteria and fungi. More information on the biochemistry, distribution, and reported antimicrobial activity of HICA can be found in the literature review chapter 2.6.2. Since it is naturally found in the human body and food products, it could be a novel, safe, and natural antimicrobial compound to control food spoilage and pathogenic bacteria.

Table 5.2: Putatively identified metabolite features in Farm 4 soil conditioned medium (F4SCM).

Compound name	**m/z**	**RT (sec)**	**Molecular formula**	**Stream**	**Adduct**	***p*-value**	**FDR**	**FC**	**Level of identification**
2-hydroxyisocaproic acid	131.0708	284.2	$C_6H_{12}O_3$	C18_Neg	[M-H]-	1.65×10^{-7}	9.83×10^{-7}	19.3	2
3-hydroxyphenylacetic acid	151.0395	295.3	$C_8H_8O_3$	C18_Neg	[M-H]-	9.61×10^{-7}	4.08×10^{-6}	3.8	2
γ-aminobutyric acid	104.0706	42.41	$C_4H_9NO_2$	C18_Pos	[M+H]+	3.64×10^{-7}	2.54×10^{-6}	11.8	2
Tryptamine	161.1071	262.45	$C_{10}H_{12}N_2$	C18_Pos	[M+H]+	1.14×10^{-5}	4.42×10^{-5}	427.0	2
Creatine	130.0612	723.24	$C_4H_9N_3O_2$	HILIC_Neg	[M-H]-	7.95×10^{-5}	1.66×10^{-4}	2.1	2
Tryptamine	161.107	622.03	$C_{10}H_{12}N_2$	HILIC_Pos	[M+H]+	8.20×10^{-10}	6.84×10^{-9}	7972.2	2
γ-aminobutyric acid	104.0706	692.68	$C_4H_9NO_2$	HILIC_Pos	[M+H]+	3.64×10^{-7}	1.12×10^{-6}	10.1	2

m/z – mass to charge ratio, RT–retention time, *p*-value- t-test between F4SCM and CMGS groups, FDR – false discovery rate, FC-fold change value (F4SCM/CMGS)

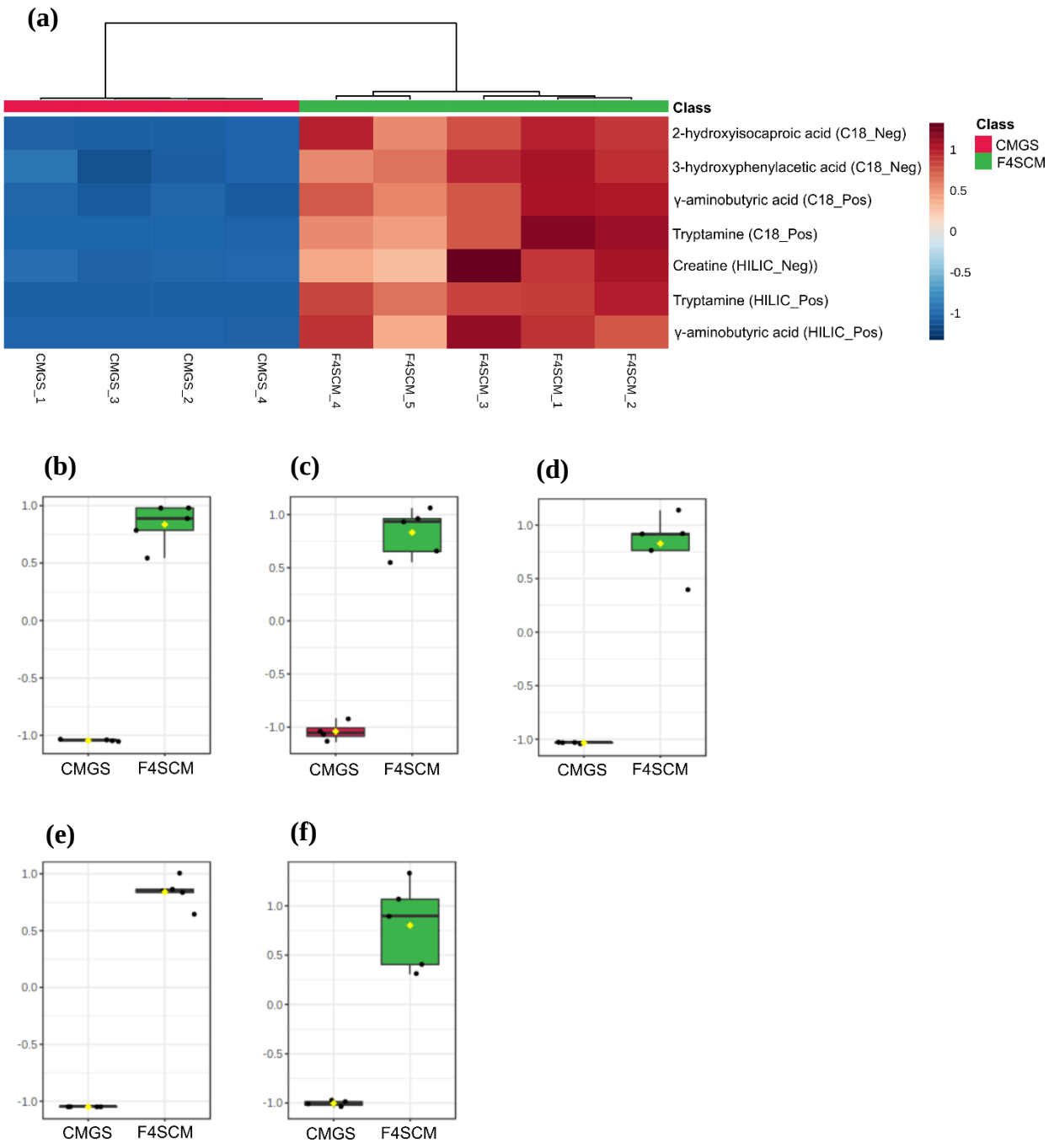


Figure 5.5: Intensity of putatively identified metabolites. Heatmap of seven metabolites identified as significantly high in F4SCM **(a)**. Rows represent putatively identified metabolites, whereas columns represent replicates. Metabolite intensity levels are shown using a pseudocolour scale (-1.0 to 1.0) with red representing high intensity levels and blue representing low intensity levels. Box plots represent the relative abundance of putatively identified 2-hydroxyisocaproic acid in C18 negative **(b)**, 3-hydroxyphenylacetic acid in C18 negative **(c)**, γ-aminobutyric acid in HILIC positive **(d)**, tryptamine in HILIC positive **(e)**, and creatine in HILIC negative **(f)** datasets. Data are presented as normalized values ± S.D (n = 5). CMGS (Cooked meat glucose starch medium supplemented with 0.0005% yeast extract, 0.1% hemin and 1% vitamin K), F4SCM- Farm 4 soil conditioned medium.

The compound, 3-hydroxyphenylacetic acid (3-HPAA) is a monocarboxylic acid and a member of phenols [335]. It has a simple structure with only one hydroxy group connected to its benzine ring. 3-HPAA has been detected in olive oil wastewater [336]. 3-HPAA is found to be associated with phenylalanine metabolism by overgrown *Clostridium* spp. in the gut of children with autism spectrum disorders [337]. The current study revealed the production of 3-HPAA in CMGS by *Clostridium* and closely related species. A recent study showed that 3-HPAA possessed antimicrobial activity against *P. aeruginosa* by affecting multiple bacterial processes including DNA replication and repair, RNA modification, proteins, cell envelope, nutrient availability, and oxidative stress [338]. Therefore, the presence of 3-HPAA in F4SCM may contribute to the antimicrobial property of F4SCM. However, more work needs to be done to understand its antimicrobial efficacy against a wide range of bacteria.

Creatine is an amino acid derivative consisting of methyl and amidino groups connected to the nitrogen [339]. It is naturally produced by vertebrate animals in the liver, kidney, and pancreas using the amino acids arginine, glycine, and methionine [340]. Various bacteria including *Pseudomonas*, *Alcaligenes*, *Flavobacterium*, *Micrococcus*, and *Clostridium* species have been reported to be capable of degrading creatinine to creatine and further metabolizing to urea and sarcosine [340, 341]. The present work demonstrated the presence of creatine in conditioned medium, resulted by the growth of *Clostridium* and closely related species in animal protein rich CMGS. Creatine has been reported to possess neuroprotective effects against a variety of neurological conditions such as Parkinson's disease, Huntington's disease, and traumatic brain injury [342-344]. However, there is no information available on the antimicrobial potential of creatine in the literature.

Among putatively identified compounds, HICA, 3-hydroxyphenylacetic acid, and tryptamine have previously been reported to show antimicrobial activities against some bacteria. Nevertheless, none of the compounds has been considered or assessed to control food spoilage and pathogenic bacteria. Considering the current knowledge on the biocompatibility and safety profiles of identified compounds, HICA was selected for further studies to assess its antimicrobial efficacy against bacterial associated with food spoilage, safety, and human health and its possible antimicrobial mechanism.

All annotated metabolites significantly abundant in F4SCM were associated with the protein/amino acid metabolic pathways signifying the activation of protein metabolism by *Clostridium* and closely related species in CMGS. Putatively characterized compounds based on the exact mass and formula matches to compounds in the spectral libraries (Level 3 identification confidence) are given in Appendix A3.3. These data also confirmed the synthesis of compounds associated with protein metabolism by the growth of *Clostridium* and closely related species in CMGS. This is not surprising because CMGS is an animal protein rich growth medium and *Clostridium* spp. are known as protein and amino acid fermentative microorganisms [345]. Conceivably, the proteolytic activity of *Clostridium* and closely related spp. might enable the degradation of proteins to peptides. Low molecular weight peptides produced by microbial fermentation of protein have been reported to possess various bioactivities including antimicrobial activity [346]. Therefore, there could be peptides contributing to the antimicrobial property of F4SCM. However, in the present study, the focus was only on the secondary metabolites produced by the metabolic activities of *Clostridium* and closely related species in CMGS and their prospective contribution to the antimicrobial activity of F4SCM.

The identification of metabolites is the greatest bottleneck so far in non-targeted metabolomics approach [347]. The limitation of this study includes incomplete annotation of significantly abundant metabolites in F4SCM due to limited mass spectral information in the currently available databases. This could be due to the lack of experimental data of pure compounds and the novelty of detected metabolites [347]. Unidentified metabolites significantly abundant in F4SCM might also contribute to the antimicrobial property of F4SCM. Therefore, further metabolomics studies are required to identify unknown metabolites in the F4SCM metabolome.

5.2.4 Conclusions

Multivariate analyses (PCA analysis and hierarchical cluster analysis) revealed that the metabolite composition of CMGS changed significantly after the growth of *Clostridium* and closely related species in CMGS. Among five soil CMs, F4SCM possessed distinctive polar and intermediate-polar metabolite profiles from all other soil CMs indicating the presence of some characteristic metabolites.

The volcano plot comparison of F4SCM and CMGS discriminated metabolites produced by Farm 4 soil associated *Clostridium* and closely related species in CMGS. Metabolite identification at level two identification confidence revealed the presence of 2-hydroxyisocaproic acid, 3-hydroxyphenylacetic acid, γ-aminobutyric acid, tryptamine, and creatine in F4SCM in significant amounts compared to CMGS. 2-hydroxyisocaproic acid, 3-hydroxyphenylacetic acid, and tryptamine, which have been previously reported to have antimicrobial activity against certain bacteria, may contribute to the antimicrobial property of F4SCM. Considering the biocompatibility and safety profiles of identified compounds, 2-hydroxyisocaproic acid was selected for further studies to assess its antimicrobial efficacy and possible antimicrobial mechanism of action.

Chapter 6

Antimicrobial potential of *Clostridium* and closely related species isolated from Farm 4 soil

6.1 Introduction

Conventional antimicrobial studies involve the testing of bacteria grown under standard laboratory conditions for antimicrobial activity as used in the current study. In recent years, the development of culture-independent approaches such as genome mining have permitted additional understanding into the antimicrobial potential of various microorganisms from different environments. Genome mining for the existence of biosynthetic pathways that allow microorganisms to synthesise putative natural products including antimicrobials has become an important approach to antimicrobial discovery. More information on genome mining for natural compound discovery can be found in the literature review chapter 2.4.1.

This chapter focuses on the antimicrobial potential of four different bacterial species isolated from Farm 4 soil, which is the fourth objective of this study. Farm 4 soil isolates were selected for further antimicrobial studies based on the promising antimicrobial activity of their product, F4SCM. Part of the objective five, which is the investigation of 2-hydroxyisocaproic acid (HICA) production by F4SCM associated bacterial isolates, is also included in this section. Chapter 4 described the promising antimicrobial potential of F4SCM, associated with the growth of these four bacterial isolates. In this section, the antimicrobial potential of these four individual Farm 4 soil isolates (FS01, FS2.2, FS03,

and FS04) was investigated using both culture-based and genome-based methods. FS01, FS2.2, FS03, and FS04 were previously identified as *Clostridium* and closely related spp. by 16S rRNA gene sequence analysis (Chapter 4 section 4.2.3). Culture-based methods used in this study involved the preparation of condition media from individual isolates and assessing their antimicrobial activities. The genome-based approach consisted of whole genome sequencing of four isolates and mining their genomes for secondary metabolite biosynthetic gene clusters. The association between HICA, one of the abundant metabolites present in F4SCM, and Farm 4 soil isolates was also investigated using genomics and metabolomics approaches. Additionally, phylogenetic analysis using both whole genome sequence identity and extracted 16S rRNA gene sequence identity was performed to further confirm the taxonomic assignment of Farm 4 soil isolates (Figure 6.1).

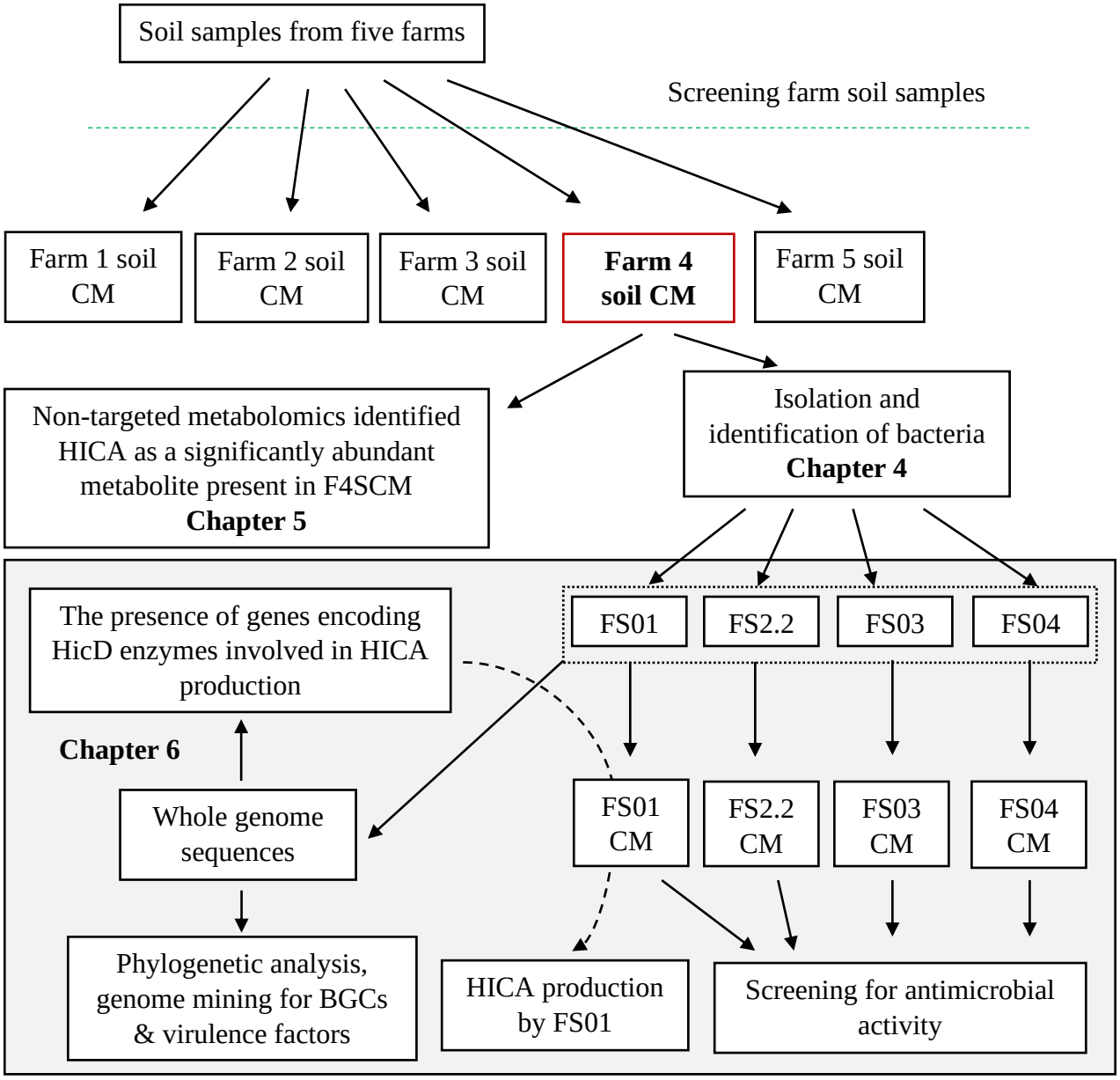


Figure 6.1: Schematic diagram showing the focus of the chapter 6 and associations to the previous works.

6.2 Results and discussion

6.2.1 Screening of Farm 4 soil isolates for antimicrobial activities using culture-based method

The antimicrobial potential of Farm 4 soil isolates was investigated by preparing conditioned media from all four bacterial isolates after growing them in CMGS as monocultures and testing them for growth inhibitory activities against *Bacillus mycoides* ATCC6462, *Bacillus cereus* NZRM5, *Pseudomonas aeruginosa* ATCC25668, *E. coli* O157:H7 NCTC12900, and *Salmonella* Typhimurium NZRM3970.

All CMs (FS01CM, FS2.2CM and FS03CM) except FS04CM significantly inhibited the growth of *B. mycoides* compared to the untreated controls, with FS03CM showing the strongest activity ($p < 0.05$). The growth of *B. cereus* was significantly reduced by FS03CM ($p < 0.05$) only. All four CMs were active against *P. aeruginosa* by significantly inhibiting the growth in comparison to the untreated control ($p < 0.05$). In contrast, all four CMs displayed no antimicrobial activity against *E. coli*, and *Salmonella* Typhimurium (Figure 6.2). Among all four isolates, the strongest and broadest antimicrobial activity was reported from FS03CM showing strong growth inhibition activity against *B. mycoides*, *B. cereus*, and *P. aeruginosa*. FS04CM demonstrated the least antimicrobial activity by inhibiting only the growth of *P. aeruginosa*. Nisin showed strong antimicrobial activity against Gram-positive *Bacillus* spp., but not against Gram-negative *P. aeruginosa, E. coli*, and *Salmonella* Typhimurium consistent with previous reports [315, 348]. Interestingly, all four CMs from the four bacterial isolates produced stronger activity against *P. aeruginosa* than the widely used commercial food preservative, nisin. Moreover, the antimicrobial activity results from Farm 4 soil isolates in this section were very similar to the activity of F4SCM, which demonstrated promising antimicrobial potential against *B. mycoides*, *B. cereus*, and *P. aeruginosa*, but not against *E. coli* and *Salmonella* Typhimurium (See the antimicrobial potential of conditioned media chapter 4.2.2). This was not surprising because F4SCM was a result of Farm 4 soil isolates, and these outcomes indirectly showed their involvement in the antimicrobial property of F4SCM.

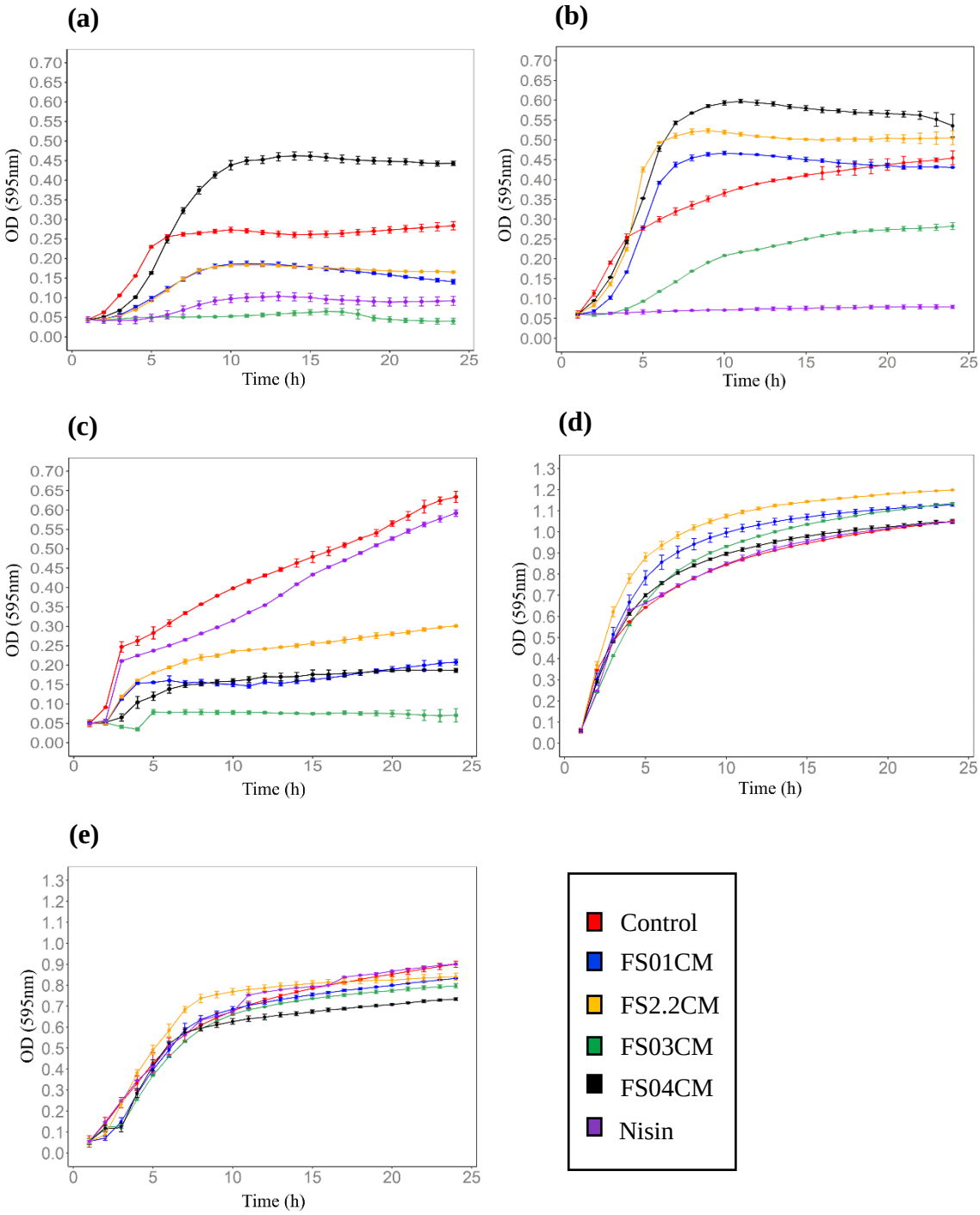


Figure 6.2: Effect of conditioned media derived from four soil isolates on the growth of Gram-positive and Gram-negative bacteria. *B. mycoides* ATCC6462 **(a)**, *B. cereus* NZRM5 **(b)**, *P. aeruginosa* ATCC25668 **(c)**, *E. coli* O157:H7 NCTC12900 **(d)**, and *Salmonella* Typhimurium NZRM3970 **(e)**. Bacteria were grown in the presence of butterfield's diluent (red), FS01CM (FS01 conditioned medium; blue), FS2.2CM (FS2.2 conditioned medium; yellow), FS03CM (FS03 conditioned medium; green), FS04CM (FS04 conditioned medium; black), and nisin (purple) in the growth media (CMGS). Nisin (45 µM) and butterfield's diluent served as positive and untreated controls respectively. Each curve represents the mean growth ± S.D (n = 3).

However, as only a single growth condition was used to prepare conditioned media from bacterial isolates, this may not demonstrate their full antimicrobial potential as activation of some metabolic pathways may require specific abiotic and/or biotic environmental cues [66]. For instance, the production of closthioamide, the first antibiotic identified from *Clostridium cellulolíticum*, required the addition of aqueous soil extract to the growth medium [14]. Therefore, variations in the growth conditions need to be tested to explore any additional antimicrobial potential. Alternatively, genome mining may be able to identify additional antimicrobials in a culture-independent manner.

6.2.2 Effect of FS03CM on the *B. cereus* spore germination and outgrowth

As FS03CM showed superior antimicrobial activity among four CMs prepared from four Farm 4 isolates, it was selected for further investigation including its activity against *B. cereus* spore germination and outgrowth. *B. cereus* NZRM5 spores prepared at two spore densities as 1×10^3 CFU/mL and 1×10^4 CFU/mL were treated with FS03CM and evaluated for spore germination and subsequent vegetative growth by measuring the OD595 of the spore cultures (See the materials and methods chapter 3.3.3). Chloramphenicol is a well-studied antibiotic known to inhibit of *B. cereus* spores and its mechanisms has also been identified. Therefore, chloramphenicol was used as the positive control in this study. FS03CM inhibited the germination and vegetative growth of *B. cereus* NZRM5 spores compared to untreated control (Figure 6.3). These results demonstrated the effectiveness of FS03CM against spore forming bacteria such as *B. cereus*.

This assay demonstrated the inhibitory action of FS03CM on *B. cereus* spore germination by assessing the spore outgrowth and subsequent vegetative growth through the measurement of OD of spore cultures. Therefore, this assay shows the overall effect of FS03CM on the spore germination but provides no information on at what stage/s of spore germination process, FS03CM exerts its inhibitory effect.

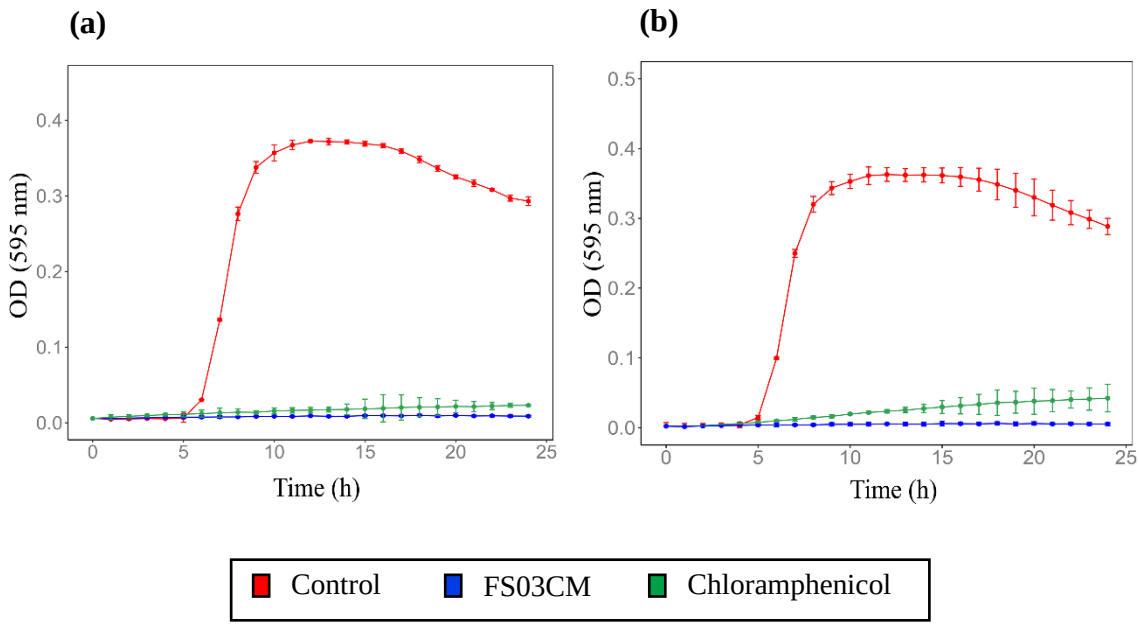


Figure 6.3: Effect of FS03CM on the spore germination of *B. cereus* NZRM5. Untreated spores (red line), spores treated with FS03CM (blue line), and spores treated with 0.1 mg/ml chloramphenicol (green line). Two initial spore densities were evaluated, 1×10^3 CFU/mL **(a)** and 1×10^4 CFU/mL **(b)**. Each curve represents the mean growth ± S.D (n = 3).

6.2.3 Genome sequencing

The genomes of four Farm 4 soil isolates were sequenced using Illumina MiSeq version 3 sequencing platform. The size of draft assembled genomes of the four bacterial isolates ranged from 3.5 to 3.9 Mb in line with the size of previously reported clostridial genomes [349]. Their Glycine-Cytosine content (mol %) ranged from 27 to 31% falling within the typical range of GC content found in *Clostridium* genomes [350, 351] and several other genomic features are summarized in Table 6.1. Genome data of FS01, FS2.2, FS03 and FS04 isolates were submitted to NCBI under the accession number PRJNA705805, PRJNA642910, PRJNA706025, and PRJNA605262 respectively.

Table 6.1: General features of Farm 4 soil bacterial genomes.

	Isolate ID			
	FS01	**FS2.2**	**FS03**	**FS04**
Genome size (bp)	3529942	3620293	3953295	3984260
Completeness (%)	98	97	83	98
G+C (mol%)	28	31	28	27
Genes (total)	3541	3614	3900	3640
CDSs (total)	3375	3489	3752	3483
Genes (coding)	3363	3428	3719	3430
rRNAs (5s, 16s, 23s)	16, 25, 23	9, 16, 13	13 ,19, 18	11, 19, 16
tRNAs	98	83	94	107
Number of contigs	44	88	32	96
Contig N50	1993941	106458	730680	91086

6.2.4 Taxonomic characterization of isolates

A preliminary identification of Farm 4 soil isolates was performed using PCR based 16S rRNA gene sequence analysis as outlined in the materials and methods chapter 3.1.6. According to 16S rRNA gene sequence results, FS01, FS2.2, FS03, and FS04 were taxonomically closely related to *Paraclostridium bifermentans* (98.1%), *Clostridium cadaveris* (100%), *Terrisporobacter glycolicus* (95.6%), and *Clostridium senegalense* (98.2%) respectively (Chapter 4 section 4.2.3). In this section, whole genome sequences were used to further confirm the taxonomic assignment and the functional relationships between isolates and closely related microorganisms.

6.2.4.1 Phylogenetic analysis using Type (Strain) Genome Server (TYGS)

Phylogenetic analysis considering both whole genome sequence identity as well as 16S rRNA gene sequence similarity was performed using TYGS webserver. TYGS is a free, publicly available automated high-throughput platform, which provides an integrated approach for genome-based bacterial taxonomy. It uses a comprehensive genome database of type strains (species and sub-species) and automatic detection of closest

relatives of query genomes [289]. TYGS workflow involves several important data processing and analysis steps as described in the materials and methods chapter 3.3.6.

A phylogenetic tree was constructed using extracted 16S rRNA gene sequences from whole genome sequences and it consisted of four soil isolates and 65 closely related species. The 16S rRNA gene sequence based phylogenetic tree indicated that FS01 clustered together with *Paraclostridium bifermentans* and *Paraclostridium benzolyticum*; FS2.2 was closely related to *Clostridium cadaveris*; FS03 was closely related to *Terrisporobacter glycolicus* and FS04 was closely related to *Clostridium senegalence* (Figure 6.4). These phylogenetic neighbours were similar to those previously reported by PCR based 16S rRNA gene sequence results for all four isolates. *C. cadaveris* genomes were manually added to the TYGS analysis due to the absence of any draft genome sequence of its type strains in the TYGS database. A phylogenetic tree based on the whole genome sequences was also constructed and it indicated that four soil isolates clustered with the same closest relatives as in the 16S rRNA based phylogenetic tree (Figure 6.5).

Genome-based methods including in-silico digital DNA-DNA hybridization (dDDH) and average nucleotide identity (ANI) have been identified as important criteria in microbial species delineation [295, 352]. These values have been coined as the overall genome related index (OGRI) [353]. Digital DNA:DNA hybridization values for each genome of bacterial isolate and their closest neighbours were calculated, and results are shown in Table 6.2. ANI values, which can be used as a measure of genetic relatedness between genomes, were also calculated for the same genome pairs using an online ANI tool (http://enve-omics.ce.gatech.edu/ani/) [295]. dDDH values and ANI values obtained for genome pairs of FS01 and *Paraclostridium bifermentans* (formerly known as *Clostridium bifermentans*), FS2.2 and *Clostridium cadaveris*, FS04 and *Clostridium senegalense* were over recommended species boundary cut-off values of 70% dDDH and 95% ANI [295, 354]. These results indicate that these three isolates belong to the same species they have been compared to (Table 6.2). In contrast, dDDH values (66.8%) and ANI values (92.48% and 92.52%) found between FS03 and *Terrisporobacter glycolicus* ATCC638, and FS03 and *Terrisporobacter glycolicus* DSM1288 were below species cut-off values. Therefore, FS03 does not belong to the species *T. glycolicus* (formerly known as *Clostridium glycolicum*) even though it is the closest relative according to 16S rRNA based and whole genome-based phylogenetic analysis. These results indicate that FS03 may represent a novel species of the genus *Terrisporobacter*.

Table 6.2: Intergenomic digital DNA-DNA hybridization and average nucleotide identity values between Farm 4 bacterial isolates and their closest phylogenetic neighbours.

Isolate	**Subject strain**	**dDDH (CI) in %**	**Two-way ANI (%)**
FS01	*Paraclostridium bifermentans* ATCC 638	83.2 (79.9-86.1)	96.24
FS2.2	*Clostridium cadaveris* AGR2141	90.2 (87-92.7)	99.61
FS03	*Terrisporobacter glycolicus* ATCC14880	66.8 (63.4-70.0)	92.48
	Terrisporobacter glycolicus DSM1288	66.8 (63.4-70.1)	92.52
FS04	*Clostridium senegalense* JC122	86.6 (83.5-89.2)	95.38

CI - confidence intervals dDDH – Digital DNA-DNA hybridization
ANI – average nucleotide identity

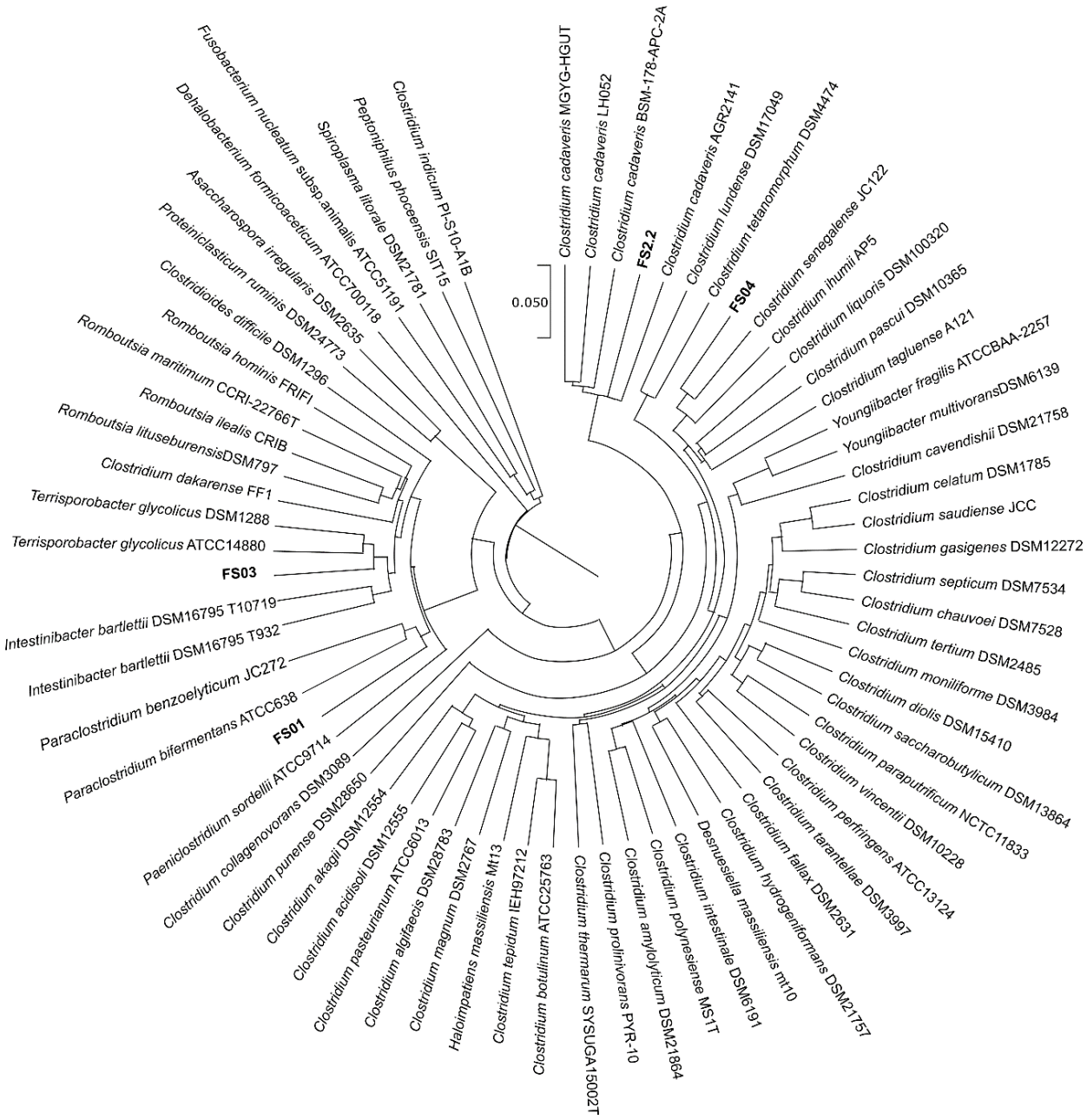


Figure 6.4: 16S rRNA-based phylogenetic tree inferred with FastME 2.1.6.1 from genome blast distance phylogeny (GBDP) distances calculated from 16S rRNA gene sequences. The branch lengths are scaled in terms of GBDP distance formula d_5. Genomes in bold represent isolates sequenced in this study.

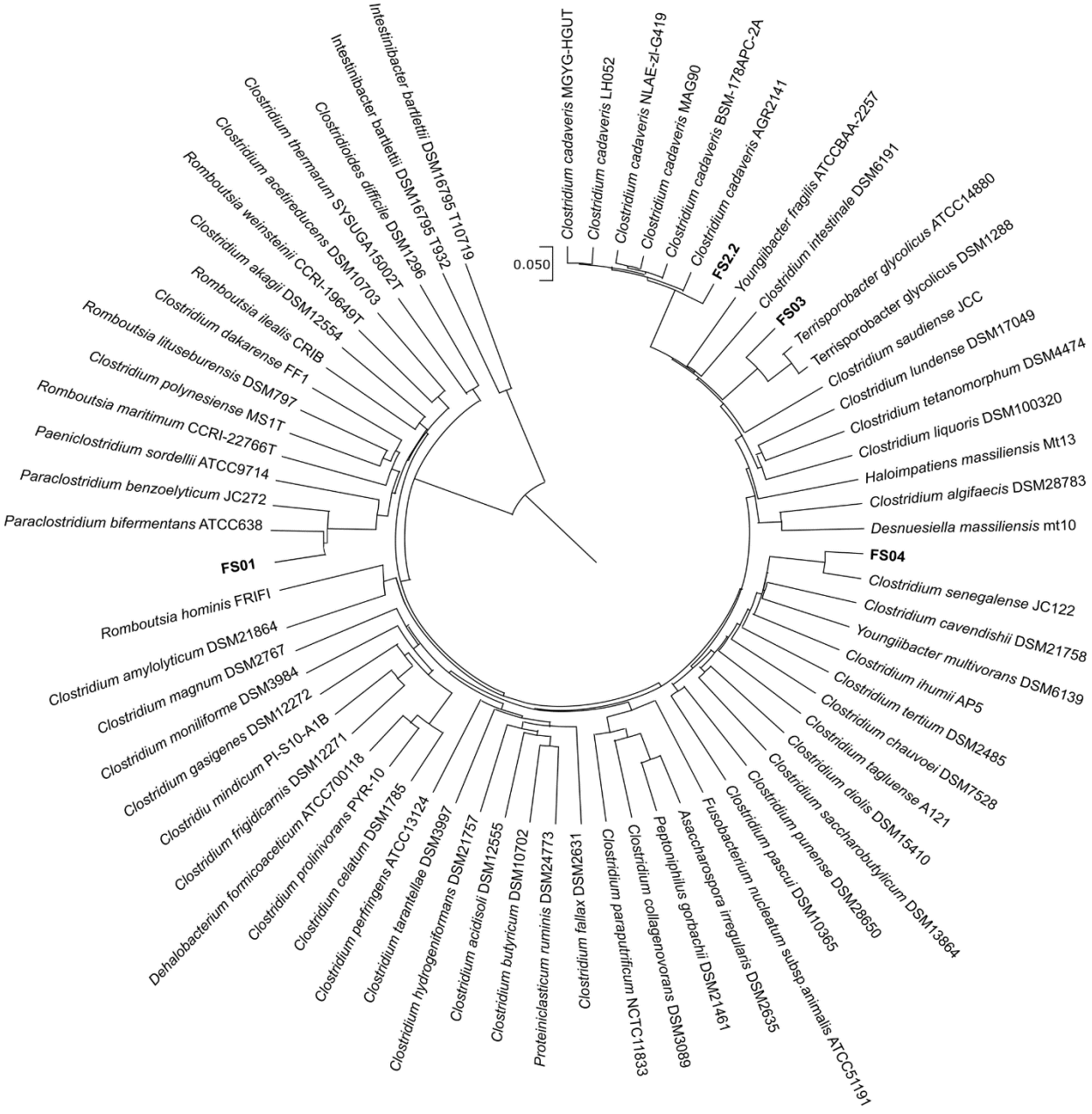


Figure 6.5: Whole genome based-phylogenetic tree inferred with FastME 2.1.6.1 from genome blast distance phylogeny (GBDP) distances calculated from genome sequences. The branch lengths are scaled in terms of GBDP distance formula d_5. Genomes in bold represent isolates sequenced in this study.

6.2.4.2 Functional genome distribution (FGD) analysis

A comparative whole genome analysis of four soil isolates and their closely related bacterial species was conducted by computing functional genome distribution (FGD). FGD analysis computes the overall levels of microbial genome similarities by the comparisons between amino acid sequences predicted from each bacterial open read frames (ORFeomes) [296]. Therefore, the FGD comparative genomics approach carries out genome to genome comparisons highlighting functional associations rather than evolutionary relationships. Based on this analysis, it was confirmed that FS01, FS2.2 and FS04 isolates are functionally closely related to their previously assigned species, *Paraclostridium bifermentans*, *Clostridium cadaveris*, and *Clostridium senegalense* respectively (Figure 6.6). FS03 showed a close functional relationship to *Terrisporobacter glycolicus* similar to its PCR based 16S rRNA analysis and TYGS phylogenetic analysis results. However, overall genome related index (ANI and dDDH values) suggested it as a novel species different from *Terrisporobacter glycolicus* (Table 6.2).

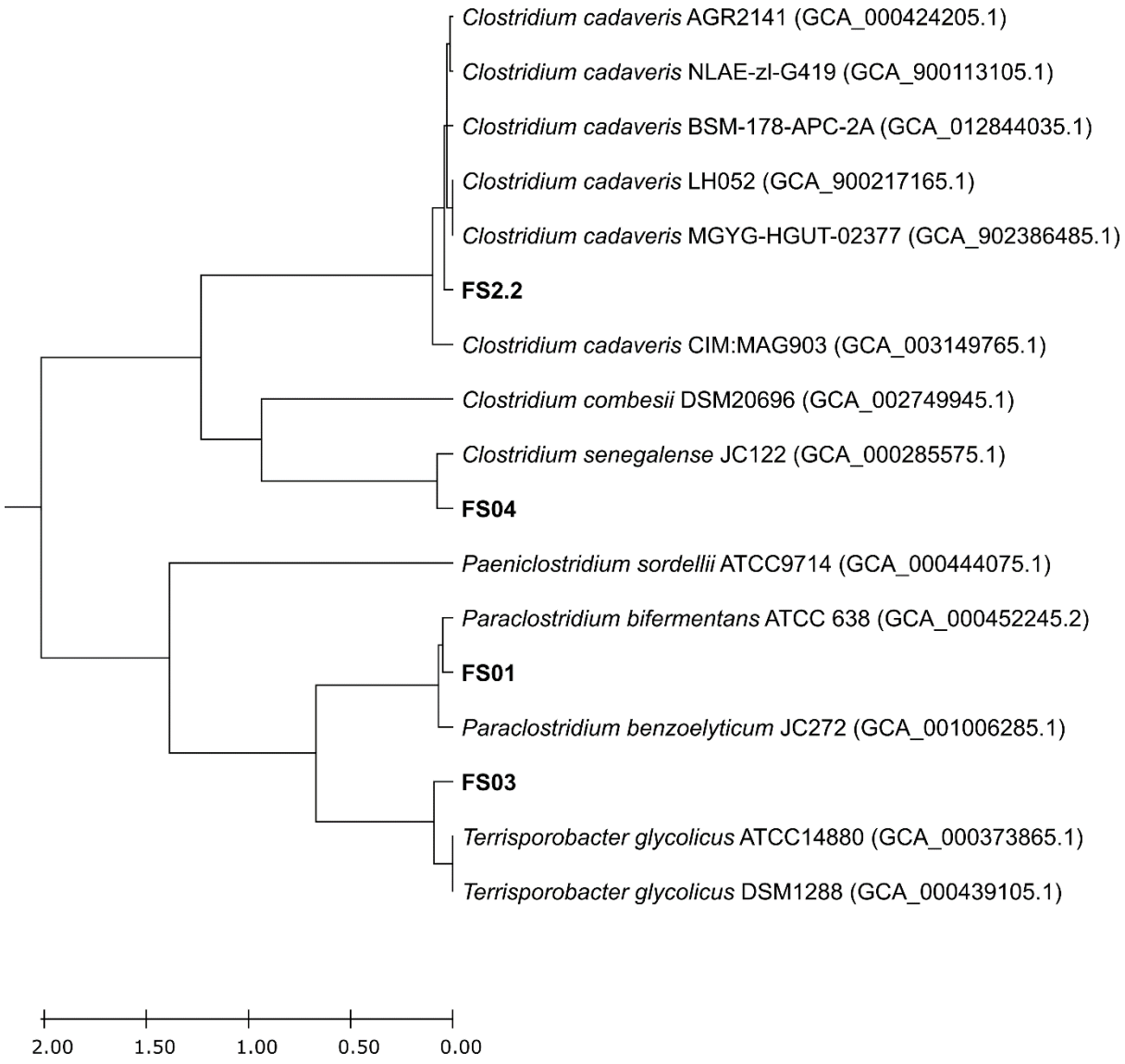


Figure 6.6: Inferred phylogenetic tree based on the functional genome distribution (FGD) analysis. All four genomes were subjected to FGD analysis and the resulted distance values were imported into MEGA X version 10.2.2. and visualized using unweighted pair group method with arithmetic mean (UPGMA). Genomes in bold represent isolates sequenced in this study. The tree is drawn to scale with the branch lengths with the same units of the functional distances used to infer the distribution tree.

6.2.5 Detection of putative virulence factors (VFs)

Bacteria exhibiting antagonistic activity against other harmful microorganisms are often considered for developing probiotics, however their pathogenicity needs to be considered. An array of animal and microbiological assays is available to demonstrate the safety of potential probiotic strains. Nowadays, the evaluation of genome sequences has become a vital part of a thorough safety evaluation [355, 356].

The genus *Clostridium* includes important human and animal pathogens and some of the members produce exotoxins responsible for diseases such as those causing botulism, tetanus, and gas gangrene. *Clostridium perfringens* and *Clostridium botulinum* are two main toxin producers causing foodborne illnesses [357]. In the present work, soil *Clostridium* species and closely related species shown to possess antimicrobial properties were evaluated for virulence factors using their whole genome sequences.

The presence of putative virulence genes in all four genomes was investigated using VFanalyzer. VFanalyzer is a virulence factor data base (VFDB) integrated automatic pipeline, which can identify known or potential virulence factors in complete or draft genomes of bacteria [298]. The VFanalyzer predicted three putative *Clostridium* toxin related genes in the FS01 genome and one in the FS2.2 genome (Table 6.3). FS03 and FS04 were not found to harbour putative genes for the main *Clostridium* toxins including alpha-clostripain (*cloSI*), alpha-toxin (*pIc*), beta2 toxin (*cpb2*), *Botulinum* neurotoxin (*atx*), *C. novyi* alpha-toxin (*tcnA*), *C. perfringens* enterotoxin (*cpe*), *Clostridium difficile* toxin (*cdtA*, *cdtB*), enterotoxin (*entA*, *entB*, *entC*, *entD*), kappa-toxin (*colA*), mu-toxin (*nagH*, *nagI*, *nagJ*, *nagK*, *nagL*), perfringolysin O (*pfoA*), sialidase (*nanH*, *nanI*, *nanJ*), tetanus toxin (*tetX*), toxin A (*toxA*), and toxin B (*toxB*). Based on the results, FS03 and FS04 isolates could be more suitable for developing probiotics as genes for virulence factors were absent. However, the expression of *plc*, *colA* and *pfoA* toxin genes detected in FS01 genome are usually regulated by the VirS/VirR two component regulatory system [358] and these two component regulatory genes, *VirS* and *VirR* were not detected in the genome of FS01. Therefore, even though FS01 carries these three toxin genes, they may not be expressed to produce extracellular toxins. Further assessments are required to confirm their safety in humans.

Table 6.3: Putative virulence factors detected.

Isolate ID	Virulence factor	Related genes
FS01	Alpha-toxin	*plc*
	Kappa-toxin	*colA*
	Perfringolysin O	*pfoA*
FS2.2	Mu-toxin	*nagH*

6.2.6 Identification of biosynthetic gene clusters (BGCs)

In recent years, strict anaerobes such as *Clostridium* spp. have been considered as potent antimicrobial producers with the advent of computational genomics [66]. However, to date comparatively limited genomic information is available on the metabolic and biosynthetic capabilities of *Clostridium* and closely related species compared to other bacteria. In this context, the four soil bacteria belonging to *Clostridium* and closely related species shown to display antimicrobial activity against significant food and human associated bacteria were investigated for their genetic potential to produce various secondary metabolites belonging to known antimicrobial compound groups, using their assembled whole genome sequences and the antiSMASH 5.0 genome mining pipeline.

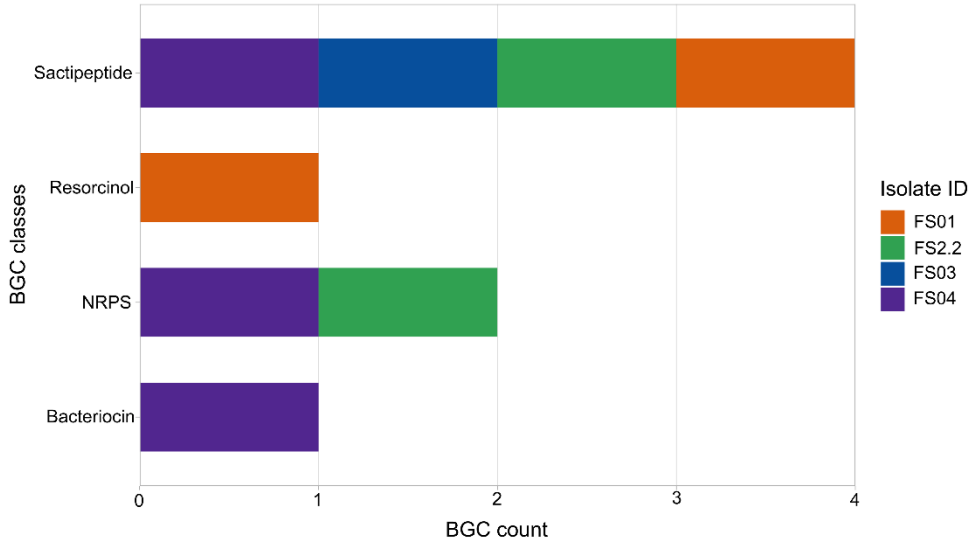


Figure 6.7: Total number of different BGCs (BGC count) predicted in all four bacterial isolates. Different colours depict in which isolate they were detected.

antiSMASH analysis predicted a total of 8 potential secondary metabolite biosynthetic gene clusters in four Farm 4 soil isolates (Figure 6.7). FS04 showed the highest BGC count detecting one sactipeptide, one NRPS, and one bacteriocin gene clusters in its genome. FS03 harboured only one BGC, which encoded for a sactipeptide, while other two genomes, FS01 and FS2.2 were found to feature two BGCs each (Figure 6.7). None of the detected clusters could be assigned to a known compound (Table 6.4). Therefore, it is likely that all the metabolites encoded by the detected BGCs have not been fully characterized.

Table 6.4: Details of predicted BGCs in all four bacterial isolates by antiSMASH 5.0.

Isolate	**BGC type**	**Location (nt)**		**Most similar known cluster**
		From	**To**	
FS01	Resorcinol	157,419	198,534	NA
	Sactipeptide	149,074	169,217	NA
FS2.2	NRPS	45,623	88,681	NA
	Sactipeptide	4,427	24,633	NA
FS03	Sactipeptide	143,920	164,063	NA
FS04	Sactipeptide	63,762	83,899	NA
	Bacteriocin	1	10,155	NA
	NRPS	41,090	83,957	NA

6.2.6.1 RiPPs gene clusters (sactipeptides and other bacteriocins)

The most abundant BGC type annotated in the genomes of four bacterial isolates was sactipeptide (Figure 6.7). Sactipeptides (Sulfur-to-alpha carbon thioether crosslinked peptides) are a class of ribosomally synthesized post-translationally modified peptides (RiPPs). The unique feature of compounds in this group is assembling of precursor peptides from encoded genes followed by maturation to the relevant core peptide by introducing at least a single intramolecular thioether bridge, connecting cysteine sulphurs with an unreactive α-carbon of the partnering amino acid [359, 360]. The first reported sactipeptide was subtilosin A from *Bacillus subtilis* [361]. Since then, a few more

members of this class have been identified from *Bacillus* species. In recent years, genome mining of various bacterial genomes has revealed the presence of putative sactipeptide biosynthesis gene clusters in other microorganisms including *Clostridium* species [245]. It is postulated that during the maturation process, thioether bond formation is catalysed by a radical *S*-adenosylmethionine (SAM) enzyme encoded by the relevant biosynthetic gene cluster [362]. antiSMASH detected the core biosynthetic genes of all four predicted sactipeptide clusters using the TIGR03973 HMM profile. Members of the TIGR03973 peptides are designated "Six Cysteines in Forty-Five residues" (SCIFF) and predicted ribosomal natural product precursors linked with an uncharacterized radical SAM protein [363]. The antiSMASH analysis showed that predicted core peptides of all detected sactipeptides are SCIFF belong to the genus *Clostridium*. They were not associated with any known sactipeptides.

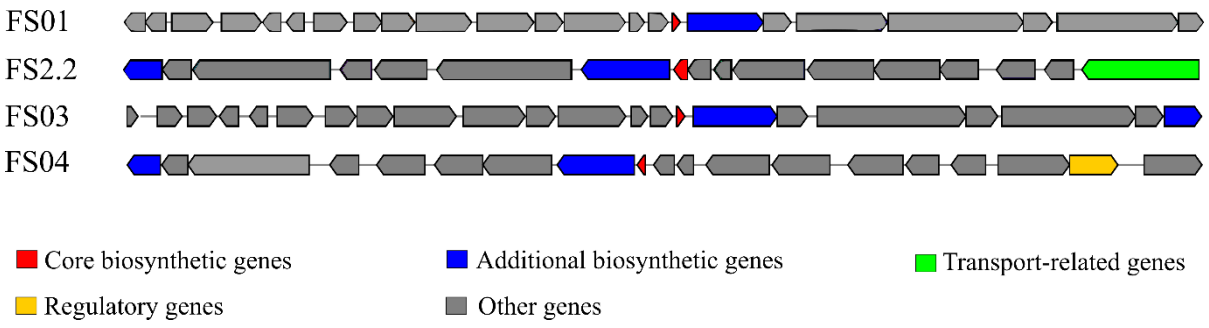
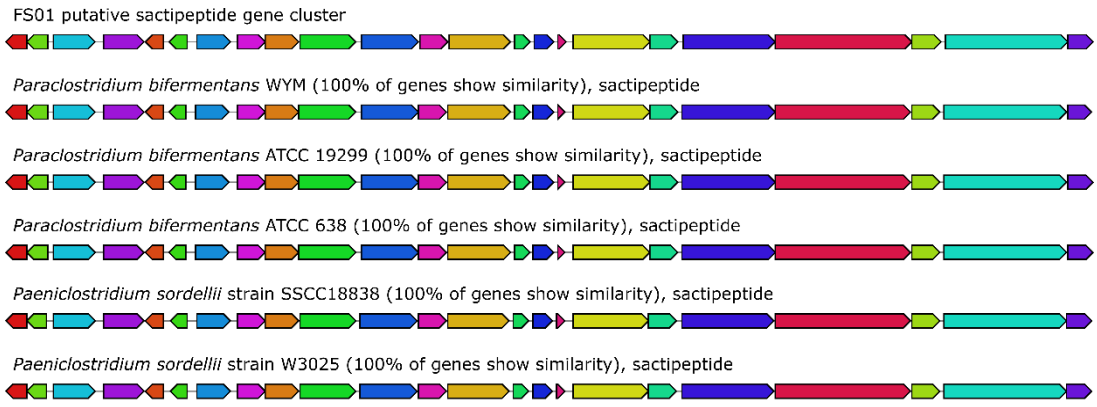


Figure 6.8: Detected putative sactipeptide gene clusters in four soil isolates. Gene clusters are colour coded with respect to the predicted function of the genes.

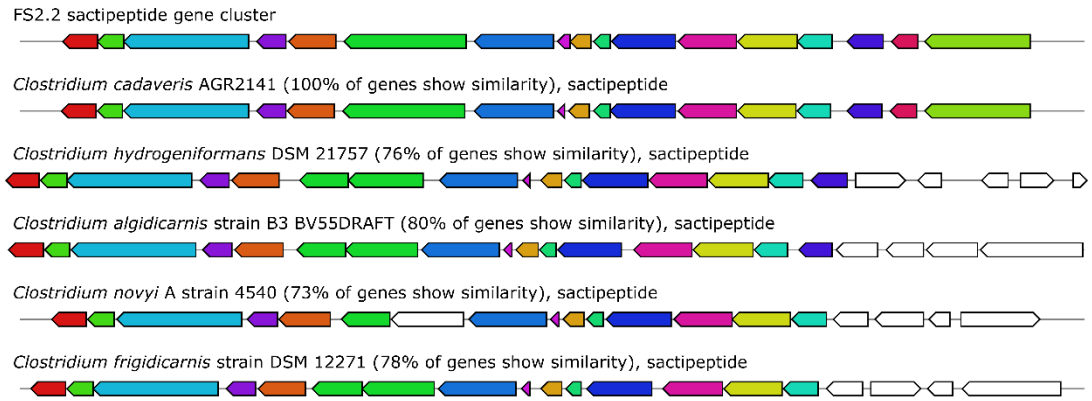
The antiSMASH 'ClusterBlast' tool compares the similarity of individual genes and their arrangement in the query cluster with a comprehensive database of predicted BGCs of publicly available genomes. This comparison identifies other microorganisms harbouring similar BGCs as the query cluster [164]. Furthermore, the 'KnownClasterBlast' module compares any predicted BGCs with the known/characterized BGCs in the Minimum Information about a Biosynthetic Gene cluster (MIBiG) database to identify closest compound/product [167]. ClusterBlast results indicated that several other *Paraclostridium bifermentans* strains and *Paeniclostridium sordellii* strains harbour 100% identical gene clusters to the predicted FS01 sactipeptide gene cluster in this study (Figure 6.9a). AntiSMASH annotated sactipeptide clusters in FS2.2 and FS03 genomes

were also found to have 100% similar gene clusters in *Clostridium cadaveris* and *Terrisporobacter glycolicus* strains respectively (Figure 6.9b and Figure 6.9c). No other bacteria were found to have 100% identical gene clusters to the FS04 sactipeptide cluster. The best match was 94% gene cluster similarity with *Clostridium senegalense*. However, only a single gene is potentially different between two gene clusters (Figure 6.9d). Since the non-identical gene is located at the margin of the gene cluster and annotated as 'other' gene, it may not be involved in the sactipeptide biosynthesis. Accordingly, two clusters would be 100% identical in terms of functionality. Sactipeptide clusters predicted in all four bacterial genomes showed no similarity to a BGC cording for a known sactipeptide. This is not surprising as only one sactipeptide cluster, thuricin CD from *Bacillus thuringiensis*, has been characterized and included in the MIBiG database so far [167]. To date, no sactipeptide has been chemically identified and isolated from *Clostridium* species [66]. Therefore, these results encourage further studies aiming at identifying and characterizing sactipeptides from *Clostridium* and closely related species.

(a)



(b)



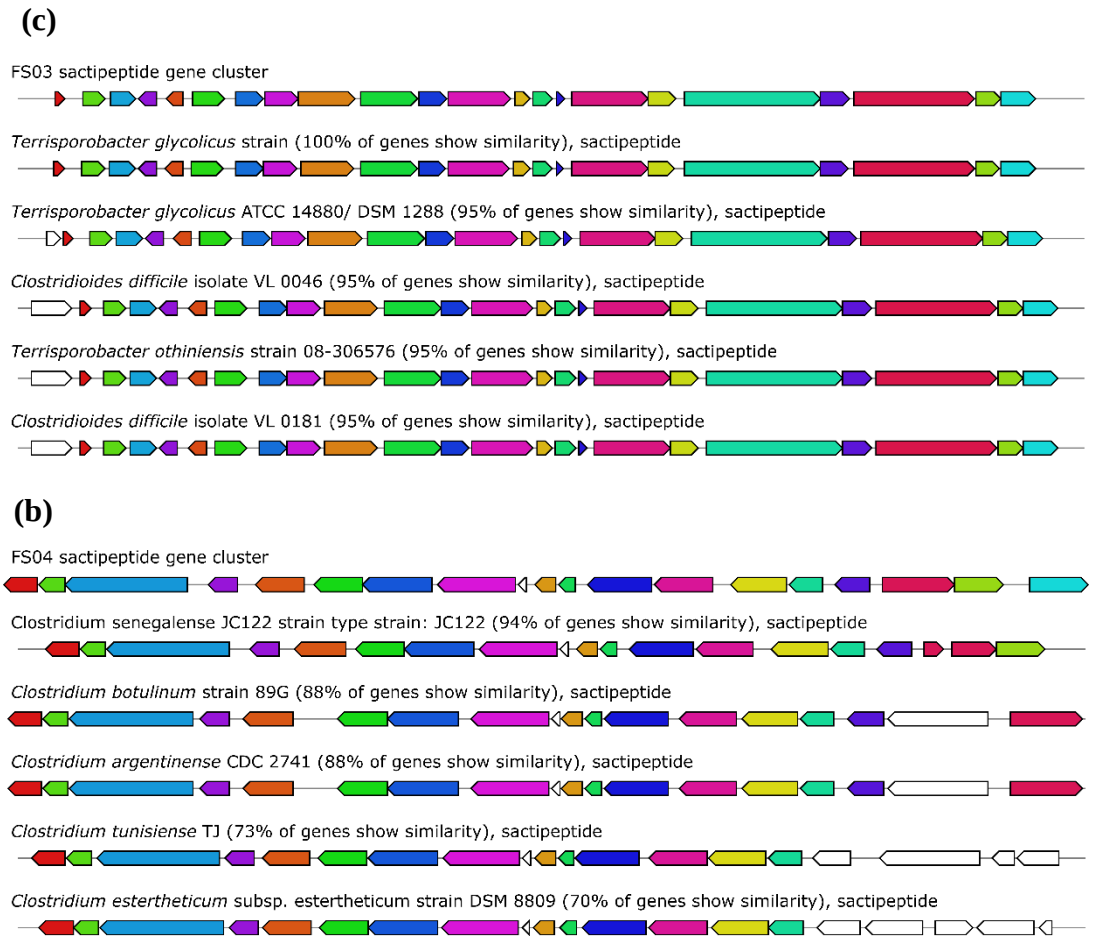


Figure 6.9: Homologous gene clusters of predicted sactipetide gene clusters in four soil isolates. FS01 **(a)**, FS2.2 **(b)**, FS03 **(c)**, and FS04 **(d)**. Genes with the same colour are putative homologs based on significant Blast hits. Top five matches from antiSMASH database are shown with the bacteria name and the similarity percentage.

Another bacteriocin/unspecified RiPP gene cluster was detected in the FS04 genome (Figure 6.10). This gene cluster contains a core gene encoding a protein harbouring a DUF692 domain. This domain has been frequently found in bacteriocin gene clusters. An additional biosynthetic gene encoding for a putative peptidase was also annotated in the same gene cluster. There was no other regulatory, transport-related or resistance related gene annotated in the cluster.

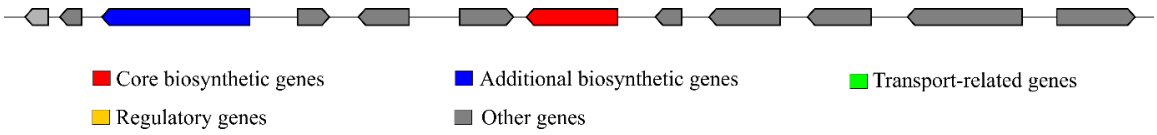


Figure 6.10: Detected bacteriocin gene cluster in FS04 genome. Gene clusters are colour coded with respect to the predicted function of the genes.

Cluster blast analysis of the bacteriocin gene cluster found no 100% identical gene cluster in any other bacteria in the antiSMASH database. The bacteriocin cluster showed no relatedness to any known compounds and the most similar cluster was found in the *Clostridium senegalense* JC122 strain, which showed only 75% similarity to the FS04 bacteriocin cluster (Figure 6.11).

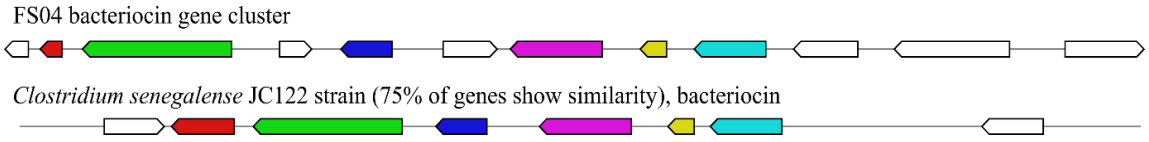


Figure 6.11: Homologous gene cluster of FS04 bacteriocin gene cluster. Genes with the same colour are putative homologs based on significant Blast hits. The best match cluster from antiSMASH database is shown with the bacteria name and the similarity percentage.

We cannot further elaborate on the putative functions of these RiPPs as they showed no relatedness to known compounds. This could be due to the lack of information with only three RiPP clusters from *Clostridium* spp. lodged in the MIBiG database [167]. However, these results expand the knowledge regarding the presence/abundance of potentially novel RiPP gene clusters among *Clostridium* and closely related species. Further studies are required to understand their products and functions.

6.2.6.2 NRPS gene clusters

Microbial natural products of non-ribosomal peptide (NRP) origin have been reported as bioactive compounds primarily as antimicrobials followed by antifungals, anti-tumour compounds, and immunosuppressants [364]. NRPs are synthesized by dedicated non-ribosomal peptide synthetases (NRPSs), which are modular multi-domain enzyme complexes, which serve as templates and biosynthetic machinery for non-ribosomal peptide synthesis [70, 90, 91]. NRPSs assemble non-ribosomal peptides through a series of repeating steps carried out by three catalytic domains: adenylation domain (A), peptidyl carrier protein domain (PCP) or thiolation domain, and condensation domain (C) [365]. These catalytic domains are organized into modules, which select and in-cooperate one define monomer into the final peptide product. Above three catalytic domains consist of a minimal NRPS module. A fourth domain, thioesterase catalyses the release of the peptide from NRPS [70].

In the present study, antiSMASH analysis of four bacterial isolates predicted the presence of two putative NRPS gene clusters in the genomes of FS2.2 and FS04. These two gene clusters and their domain organization were different to each other (Figure 6.12). Notably, both clusters shared no similarity with those of previously characterized known antimicrobial compounds in the MIBiG database. Furthermore, they appear to be unique NRPS clusters due to the absence of identical clusters in closely related microorganisms. According to ClusterBlast results, the FS2.2 cluster found the best gene cluster match in the *Clostridium cadaveris* AGR2141 genome with 64% cluster similarity. The NRPS cluster detected in FS04 showed the highest similarity of 55% to a NRPS cluster in the genome of *Clostridium senegalense* JC122. These results demonstrate that detected clusters may encode producer specific novel NRPS compounds.

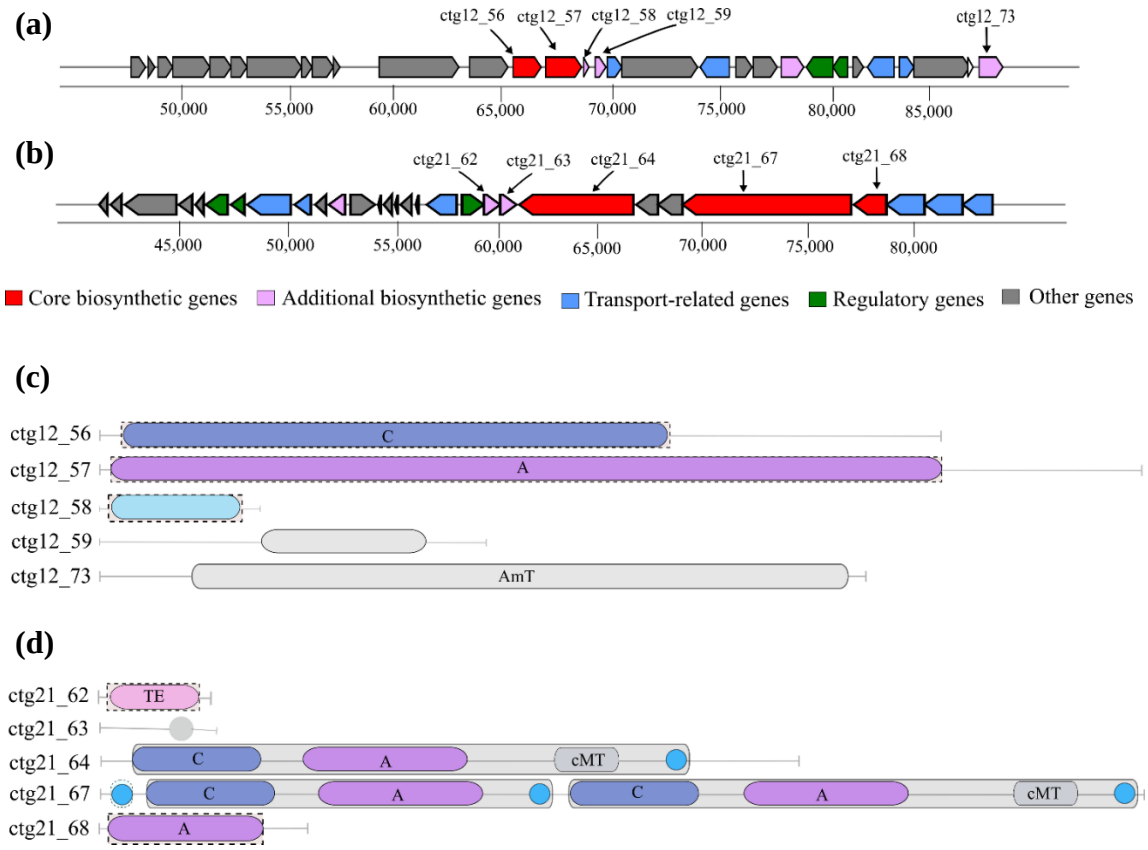


Figure 6.12: The predicted NRPS gene clusters. NRPS gene cluster detected in FS2.2 genome **(a)**. NRPS gene cluster detected in FS04 genome **(b)**. Module and domain annotation of FS2.2 NRPS gene cluster **(c)**. Module and domain annotation of FS04 NRPS gene cluster **(d)**. Gene locus tags are given on the left of the domain architecture and in the corresponding genes of the gene cluster.

Clostridium spp. have not been thoroughly explored for their secondary metabolite or bioactive compound production. In recent years, several studies have predicted the secondary metabolite synthesis potential of anaerobic bacteria including some *Clostridium* species [13, 245, 248]. To date, only a few antimicrobial secondary metabolites have been identified and characterised from *Clostridium* species (See the literature review chapter 2.5.1 for more information). Therefore, there is limited information on the biosynthetic gene clusters associated with known clostridial antimicrobials and this limits the prediction capabilities. In agreement with the antagonistic activities, all four Farm 4 soil isolates were predicted to have at least one or more biosynthetic gene clusters in their genomes. All putative gene clusters identified in the present study were not associated with any currently known clostridial or other

secondary metabolites in the MiBiG database indicating that they might encode for novel compounds. Furthermore, some of the putative clusters were unique to the bacterial isolate used in this study indicating their respective novelty to produce putative secondary metabolites.

However, it should be taken into consideration that neither the genome-based nor the culture-based approach alone can show the true antimicrobial potential of microorganisms due to their own limitations. Genome mining techniques including antiSMASH are not able to exactly predict the full capacity of a bacterium to produce bioactive secondary metabolites, including antimicrobials. Bioinformatic tools such as antiSMASH use BGC prediction algorithms which are based on identification of known biosynthetic pathways to some extend and it can be speculated that truly novel biosynthetic pathways may not be detected. Most of the genome mining tools use conserved domains of biosynthetic enzymes or pHMMs (profile Hidden Markov Models) signature genes for identification of core genes of a biosynthetic pathway. This implies a limitation of these tools not being able to identify novel pathways and enzymes. To improve the BGC detection, antiSMASH has implemented 'ClusterFinder' algorithm to identify BGCs that are not detected by the rule-based genome mining. But this technique still has some bias towards currently known pathways as BGC detection has been trained using source data of known pathways [164]. The assembly quality of the query genomes is also important for getting reliable results from antiSMASH as its genome analyses rely on gene finding from the coding regions [164]. On the other hand, the prediction of the presence of a certain biosynthetic pathway does not fully ensure the production of associated secondary metabolite. Biosynthetic gene clusters could be either silent or active under specific growth conditions. Activation of gene clusters may require specific external factors such as presence/density of other microbes or (lack of) nutrients [366]. However, the detection of silent metabolic pathways (not activated under standard laboratory conditions) by genome mining is overcoming a limitation of conventional culture-based methods. Furthermore, anaerobic bacteria such as *Clostridium* spp. can utilize a wide range of substrates for their anaerobic fermentation and result in various metabolites based on the substrates used [367]. Some of these small metabolites produced from their metabolic pathways other than metabolites synthesized as a direct regulation of gene clusters such as NRPS or RiPP may also possess antimicrobial activities. For instance, *Clostridium difficile* was reported to produce para-cresol through the

fermentation of tyrosine, which showed antagonistic activity against some bacteria including *Bacteroides thetaiotaomicron*, *Escherichia coli*, *Klebsiella oxytoca* and *Proteus mirabilis* [125]. Despite the above limitations, genome mining has been shown to assist the antimicrobial discovery by providing valuable insight into the genomic potential of bacteria for bioactive secondary metabolite biosynthesis.

6.2.7 2-hydroxyisocaproic acid production by Farm 4 soil isolates

2-hydroxyisocaproic acid (HICA) was one of the compounds putatively identified from F4SCM and detected at very high levels compared to CMGS (See the non-targeted metabolomics analysis of soil conditioned media chapter 5.2.3). Among all metabolites putatively identified from F4SCM, HICA was investigated in the current study to learn more about its antimicrobial potential considering its reported biocompatibility and safety profile. As F4SCM was a result of FS01, FS2.2, FS03, and FS04 isolates, HICA production in F4SCM should be associated with the metabolic activities of these bacterial isolates or at least one of them.

HICA is a by-product of the leucine degradation pathway. In this pathway, the reduction of 2-ketoisocaproic acid (KICA) into HICA is catalysed by hydroxyisocaproic/ hydroxyisocaproate acid dehydrogenase (HicD) enzyme (See the literature review chapter section 2.6.1 for more information). Therefore, HicD is an important enzyme involved in the production of HICA through the leucin degradation pathway. HicDs belong to a subgroup of lactate dehydrogenases (LDH) in the family of 2-hydroxycarboxylate dehydrogenases [368]. The HicD enzyme activity was found to vary between bacterial species and strains [369]. In this section, Farm 4 soil isolates were screened for predicted enzyme function of HicD as an indication of their capability to produce HICA through the leucine degradation pathway.

6.2.7.1 Analysis of hydroxyisocaproate dehydrogenases (HicDs)

Hydroxyisocaproate acid dehydrogenase (HicD) enzyme function in all four bacterial isolates was predicted using the HicD enzyme sequence searched against the translated nucleotide sequence of the whole genomes of FS01, FS2.2, FS03, and FS04 through TBLASTN. Six clostridial HicD enzyme sequences derived from the UniProt database (Table 6.5) were used to assess the availability of homologous HicD enzyme-coding

regions in the draft whole genomes of all four bacterial isolates. To date, there were six clostridial HicD proteins available in the UniProt database, five belonging to *Clostridium difficile* and one annotated in *Clostridium sporogenes* (Table 6.5). All six HicD enzyme amino acid sequences found 61-66 % amino acid identities in the FS01 isolate. Alignment characteristics of all six amino acid sequences such as the positive percentage (79-81%) and E value ($2e^{-136}$ or less) showed high quality hits for homology matches in the translated nucleotide sequence of FS01 (Appendix A4.1). These results suggested the presence of homologous HicD enzyme-coding region in the draft whole genome of FS01. FS2.2, FS03 and FS04 isolates were found to have best significant alignments to six query amino acid sequences at a range of 30-36 % similarity only indicating less likelihood to produce HicD enzymes (Table 6.6). These outcomes suggested that the production of HICA might be associated with the isolate FS01, which was identified as a *Paraclostridium bifermentans* strain.

Table 6.5: HicD enzymes used in sequence similarity search.

Protein entry in UniProt	**Associated gene name**	**Organism**	**Length**
A0A125V2B4	ldhA, CDIF1296T_00590	*Clostridioides difficile* ATCC 9689, DSM 1296	332
D5PZU0	ldhA, HMPREF0220_0172	*Clostridioides difficile* NAP08	332
Q5U922	ldhA	*Clostridioides difficile (Peptoclostridium difficile)*	331
A0A0H3MZ08	ldhA, CD196_0379	*Clostridioides difficile* (strain CD196) *(Peptoclostridium difficile)*	332
C9YIH1	ldhA, CDR20291_0365	*Clostridioides difficile* (strain R20291) *(Peptoclostridium difficile)*	332
J7TF96	ldhA, CLOSPO_00317	*Clostridium sporogenes* (strain ATCC 15579)	334

Table 6.6: Sequence homology percentages of best significant alignment in all four Farm 4 soil isolates.

Gene names of query protein sequences	**Identities %**			
	FS01	**FS2.2**	**FS03**	**FS04**
ldhA, CDIF1296T_00590 (A0A125V2B4)	61	36	33	30
ldhA, CD196_0379 (A0A0H3MZ08)	61	36	33	30
ldhA, CDR20291_0365 (C9YIH1)	61	36	33	30
ldhA, CLOSPO_00317 (J7TF96)	66	38	32	30
ldhA, HMPREF0220_0172 (D5PZU0)	61	36	33	31
ldhA (Q5U922)	61	36	33	30

6.2.7.2 HICA production by FS01

Previous genome analysis predicted that FS01 might have the capacity to produce HICA through the leucine degradation pathway. In this section, HICA production by FS01 was confirmed by quantifying the HICA content in conditioned medium prepared from FS01 (FS01CM). An additional sample ($FS01CM_{Leu+}$) was prepared by culturing FS01 in CMGS medium enriched with extraneous 0.2% leucine to evaluate the effect of leucine in the growth medium on HICA production by FS01. Conditioned media were analysed using an UHPLC system coupled to a Q-TOF mass spectrometer with an electrospray ionization source (See the materials and methods chapter 3.3.11). Quantification of HICA was done by comparing the peak area of relevant peaks from extracted ion chromatograms (HICA, *m/z* 131.0700) with a calibration curve (Appendix A4.2). HICA content in the CMGS growth medium was very low, 0.155 µg/mL. The growth of FS01 in CMGS medium significantly increased the concentration of HICA ($p < 0.05$), which was indicated by detecting 3.638 µg/mL HICA in FS01CM. These findings confirmed that FS01 can synthesize HICA during its growth in CMGS medium under the given growth conditions. The addition of 0.2% extraneous leucine to the CMGS growth medium significantly increased the production of HICA (7.363 µg/mL) by FS01 (Figure 6.13).

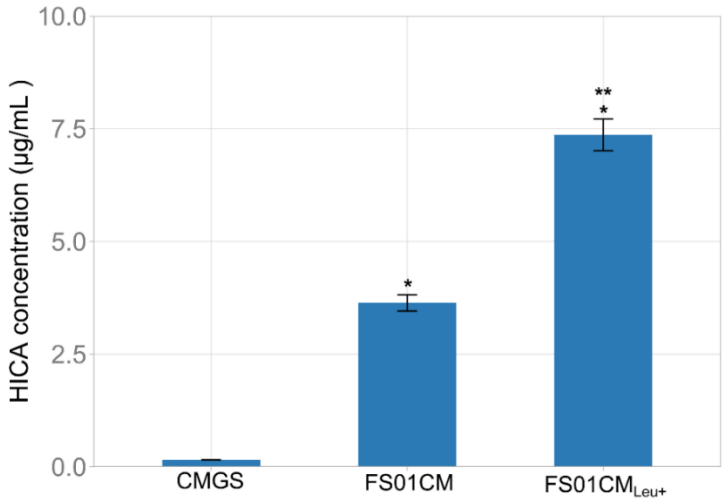


Figure 6.13: HICA concentrations detected in cooked meat glucose starch (CMGS) medium, FS01 conditioned medium (FS01CM), and FS01 conditioned medium enriched with 0.2% leucine ($FS01CM_{Leu+}$). Data are presented as mean ± S.D HICA concentration measured in µg/mL (n = 5). $^{*}p < 0.05$ compared with the CMGS. $^{**}p < 0.05$ compared with the FS01CM.

This is the first study reporting the production HICA by *Cl. bifermentans* and the second report of HICA production by *Clostridium* species. Previously, Brooks, Nunez-Montiel [256] described the increased production of HICA by *Clostridium difficile* in trypticase yeast salt broth enriched with leucine. HICA production was mostly reported from lactic acid bacteria. A study conducted by Park, Hwang [261], investigated the HICA production by several lactic acid bacteria isolated from kimchi. They reported the production of HICA by *Lactococcus lactis*, *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Leuconostoc lactis* at the concentration range of 153.1 – 526 µg/mL, with lower production reported from *Lactobacillus sakei* (21.4 µg/mL) and *Pediococcus pentosaceus* (26.1 µg/mL). HICA production by *Cl. bifermentans* (FS01) reported in the current study was smaller compared to the HICA production by lactic acid bacteria in the previous study. Nevertheless, it was shown that the HICA production by FS01 can be increased by adding leucine to the growth medium. This is not surprising as HICA is a side product of the leucine degradation, and the presence of readily available leucine

possibly accelerates its degradation by the metabolic activity of FS01 producing more HICA.

6.3 Conclusions

FS01, FS2.2, and FS04 belong to the species *Paraclostridium bifermentans*, *Clostridium cadaveris*, and *Clostridium senegalense* respectively, while FS03 may represent a novel species of the genus *Terrisporobacter* as evident by phylogenetic analysis together with DNA-DNA hybridization (dDDH), average nucleotide identity (ANI), and functional genome distribution (FGD) analyses.

All four bacterial isolates derived from Farm 4 soil possess varying levels of antimicrobial activity as indicated by culture-based study using conditioned media prepared from each individual bacterial isolate. BGCs encoding uncharacterized RiPP and/or NRPS were identified from all four Farm 4 soil isolates, indicating their genetic potential to produce novel secondary metabolites belonging to antimicrobial compound groups.

The FS01 isolate, identified as *Clostridium bifermentans* was predicted to have enzyme function to produce HICA through the leucine degradation pathway as evident by the high-quality hits for homology matches for hydroxyisocaproate acid dehydrogenase (HicD) enzyme sequences in translated nucleotide sequence of FS01. A metabolomics study, which quantified the HICA content in FS01CM confirmed the capacity of FS01 to produce HICA in CMGS medium. Moreover, the addition of leucine to CMGS growth medium increases the HICA production by FS01.

Overall, this work reveals the antimicrobial potential of Farm 4 soil isolates, which belong to *Clostridium* and closely related species by showing their genetic potential to produce putative secondary metabolites as a direct regulation of gene clusters such as NRPS or RiPP, and their metabolic capability to produce putative antimicrobial metabolites such as HICA through their metabolic pathways. This knowledge certainly serves as a basis for future investigations aimed to identify and characterize potent antimicrobials from *Clostridium* and closely related species and expands the current knowledge base emphasizing the bioactive compound production potential of the genus *Clostridium* and closely related species.

Chapter 7

Antimicrobial potential of 2-hydroxyisocaproic acid (HICA)

7.1 Introduction

The work presented in this chapter relates to the fifth objective of the study, which was to investigate the antimicrobial efficacy of 2-hydroxyisocaproic acid (HICA) and provide some insights into its antimicrobial mechanism. HICA was one of the putatively identified compounds from Farm 4 soil conditioned medium (F4SCM) which exhibited promising antimicrobial activities against *B. mycoides*, *B. cereus*, and *P. aeruginosa.* HICA was highly abundant in F4SCM, after the growth of *Clostridium* and closely related species associated with Farm 4 soil in CMGS and was postulated to be associated with F4SCM's antimicrobial activity (Figure 7.1). Genomic studies predicted that the FS0l (*Clostridium bifermentans* strain) might produce HICA through the leucine degradation pathway, and this was confirmed by detecting HICA in FS01CM using metabolomics analysis.

As discussed in the literature review chapter 2.1.2, the rapid emergence of antimicrobial resistant bacteria and the consumer demand for natural food preservatives instead of synthetic chemicals have driven the search for novel natural antimicrobial compounds, that can be safely applied in food products. HICA is a by-product of the leucine degradation pathway in humans and certain bacteria [251]. This compound has been reported to have functional properties such as antimicrobial, antifungal, and anti-inflammatory activities [252]. It has also been reported to improve the muscle mass and speed up the muscle recovery after exercise, therefore it is now commercially available

as a supplement [253, 268]. More information on its biochemistry, distribution, and functional properties can be found in the literature review chapter 2.6.

In the present study, it is suggested that HICA could be a novel and safe antimicrobial, which may have applications in food preservation and human health as it is a natural compound in food products and is metabolized by the human body. Yet, no study has investigated its potential application in food preservation. Therefore, the aim of this section was to evaluate the antimicrobial efficacy of HICA against several significant bacteria associated with food quality, safety, and human health and provide some insights into its possible antimicrobial mechanism against bacteria (Figure 7.1).

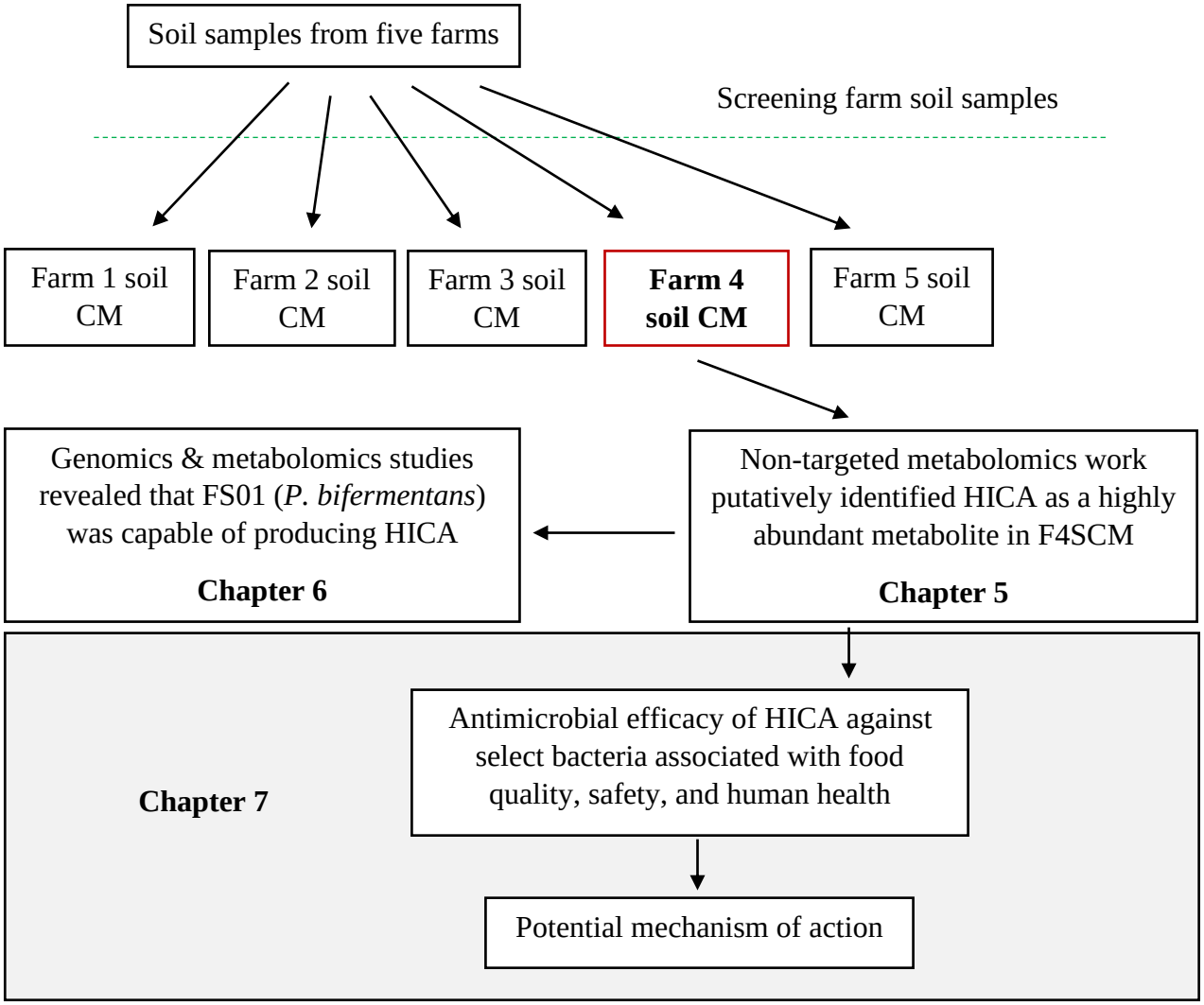


Figure 7.1: Schematic diagram showing the focus of chapter 7 and its association to the previous studies.

7.2 Results and discussion

7.2.1 Antibacterial efficacy of HICA

Antimicrobial efficacy of HICA against a range of Gram-positive and Gram-negative bacteria associated with food quality, safety, and human health was investigated using the broth dilution method (See the materials and methods chapter 3.4.1). Bacterial susceptibility to HICA was determined in terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). According to the Clinical and Laboratory Standards Institute (CLSI) guidelines, MIC is the lowest concentration of an antimicrobial compound, that completely inhibits the visible growth of test bacteria. MBC is defined as the lowest concentration of an antimicrobial required to kill at least 99.9% of the final inoculum after 24 h incubation [370]. In the present study, bacterial growth was monitored by measuring the optical density (OD) of the culture and the MIC was the lowest concentration of HICA, which resulted no OD change in the bacterial culture after 24 h incubation. MBC was the minimum concentration, that resulted no colonies on SBA plates after 24 h incubation.

Susceptibility testing of fourteen bacteria including some reference strains and environmental, meat, and milk isolates belonging to eleven species was performed to investigate the antimicrobial efficacy of HICA (Table 7.1). These bacterial species were selected based on their association with food quality, food safety, and human health. *B. cereus, E.coli*, and *S. aureus* are three major foodborne pathogens [371]. *Shewanella putrefaciens* and *Serratia proteamaculans* are known food spoilage bacteria associated with vacuum-packed meat, chicken, and fish [372, 373]. *Paenibacillus odorifer, Bacillus subtilis,* and *Bacillus mycoides* are involved with the spoilage of various types of food products [374-376]. *Pseudomonas* spp. are a major group of food spoilage bacteria; *Pseudomonas lundensis* and *Pseudomonas fragi* are involved with the spoilage of milk, meat, and fish at chilled temperatures [377]. *P. aeruginosa* is a significant opportunistic pathogen causing infections in patients with immunocompromising conditions such as those hospitalized in intensive care units or with cystic fibrosis [378] and it has also been reported as a spoilage bacterium in dairy products [312].

HICA inhibited the growth of all test bacteria in a dose-dependent manner. The antimicrobial efficacy of HICA against both Gram-positive and Gram-negative bacteria used in this study was similar indicating a MIC of 1 mg/mL except for *Shewanella*

putrefaciens SM26, which was the most susceptible bacterium to HICA with only 0.5 mg/mL MIC. Both *Pseudomonas aeruginosa* ATCC25668 (clinical isolate) and multi-drug resistant *Pseudomonas aeruginosa* NZRM4034 (Ceftazidime/piperacillin resistant) showed the same level of susceptibility to HICA (MIC = 1 mg/mL). According to MBC comparison, *B. cereus* strains were the most resistant bacteria demonstrating the highest MBC value (32 mg/mL). This high MBC value could be attributed to the resistant *B. cereus* spores present in the growth medium. *B. cereus* spores have been reported to survive under adverse environments such as extreme pH, high temperatures, and antimicrobial compounds [379]. The MBC for all other test bacteria ranged from 1 to 4 mg/mL as shown in Table 7.1. The *Bacillus mycoides* reference strain and *Shewanella putrefaciens* meat isolate were the most susceptible to HICA with MBC values as low as 1 mg/mL. A previous study evaluated the cytotoxicity and genotoxicity of HICA and reported that it was safe at concentrations < 10 mg/mL [263]. All the MIC values obtained in this study were less than the reported cytotoxicity and genotoxicity levels showing some indications of its safe use in food products.

Table 7.1: Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of HICA.

Microorganism	**MIC (mg/mL)**	**MBC (mg/mL)**
Bacillus mycoides ATCC6462	1.0	1.0
Bacillus cereus NZRM5	1.0	32.0
Bacillus cereus M4 (milk isolate)	1.0	32.0
Pseudomonas aeruginosa ATCC25668	1.0	2.0
Pseudomonas aeruginosa NZRM4034	1.0	2.0
Escherichia coli O157:H7 NCTC12900	1.0	2.0
Escherichia coli AGR3789 (soil isolate)	1.0	2.0
Staphylococcus aureus NZRM917	1.0	2.0
Shewanella putrefaciens SM26 (meat isolate)	0.5	1.0
Serratia proteamaculans ENT68 (meat isolate)	1.0	2.0
Bacillus subtilis F2MCUH1 (environmental isolate)	1.0	4.0
Paenibacillus odorifer F1OSP28 (environmental isolate)	1.0	4.0
Pseudomonas lundensis F2MCUH2 (environmental isolate)	1.0	2.0
Pseudomonas fragi F1NBUH38 (environmental isolate)	1.0	2.0

7.2.2 Effect of HICA on the cell viability of Gram-positive and Gram-negative bacteria

Bacterial cell viability testing of seven bacteria including reference strains and environmental isolates belonging to both Gram-positive and Gram-negative bacteria was performed using a luciferase bioluminescence-based method (See the materials and methods chapter 3.4.4). The luminescent signal obtained in this assay is proportional to the amount of ATP present in the bacterial culture, which is directly proportional to the number of metabolically active viable cells present in the culture [380].

The cell viability of bacteria after treatment with 4 mg/mL HICA was evaluated over a period of 180 min at different time intervals and the cell viability was expressed as a percentage of the untreated control. HICA treatment reduced the cell viability of all test bacteria in a time dependant manner despite the differences in the percentage of viable cells in each bacterial group at different treatment times. Except *P. aeruginosa* strains, all other test bacteria displayed a significant reduction in their cell viability after 15 min of HICA treatment compared to their respective controls ($p < 0.05$). Gram-negative bacteria (*P. aeruginosa* and *E. coli*) had relatively higher viable cell percentages than Gram-positive bacteria (*B.* cereus and *S. aureus)* during the first 30 min of HICA treatment. *P. aeruginosa* strains were the most resistant to HICA showing significant reduction of their viability after only 30 min ($p < 0.05$) and maintaining relatively higher percentages of cell viability compared to other test bacteria at all treatment times. Overall, HICA was shown to be effective in reducing the cell viability of both Gram-positive and Gram-negative bacteria (Figure 7.2).

These results reveal the future application of HICA as an antimicrobial agent that can control the growth of both Gram-positive and Gram-negative bacteria, which compromise food quality and safety. Additionally, HICA may have medical applications as a potential antimicrobial agent against *P. aeruginosa*.

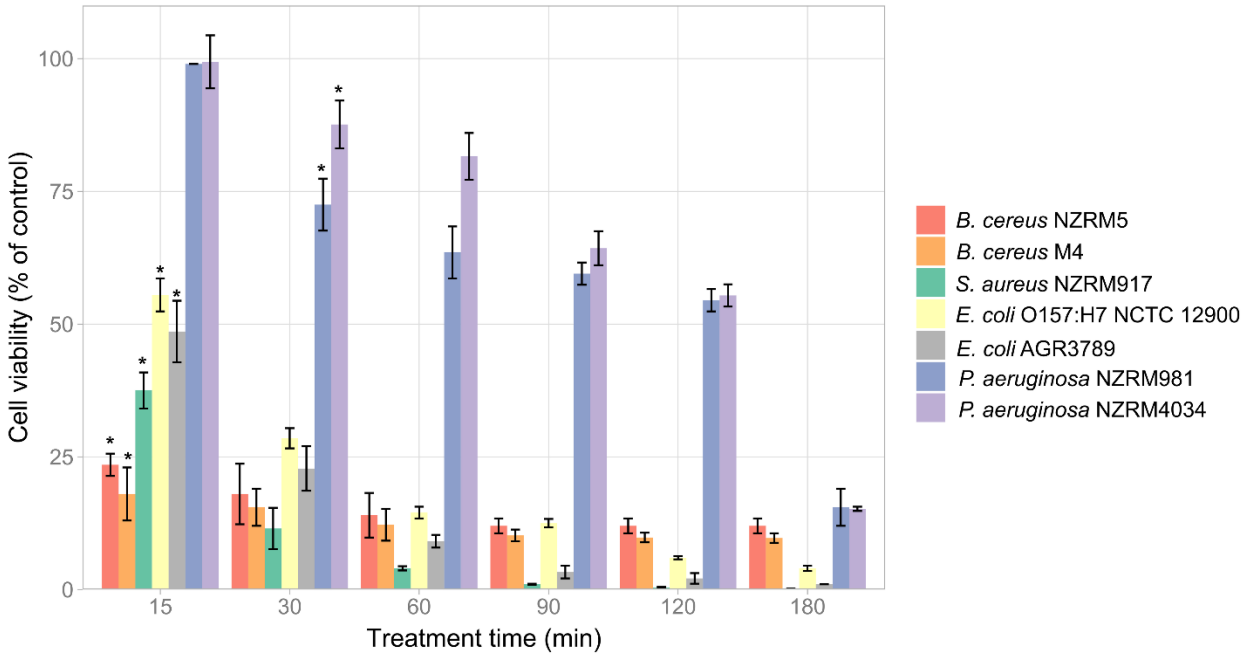


Figure 7.2: Antimicrobial effect of HICA on selected Gram-positive and Gram-negative bacteria. Data are presented as mean ± S.D of cell viability expressed as a percentage of control (n = 3) *significant reduction **(***p* < 0.05) compared with control (only the starting points of significant reduction in each bacterium are marked in the graph).

7.2.3 Effect of HICA on the *B. cereus* spore germination

B. cereus is a spore forming bacterium, which is associated with food-borne illnesses. Their spores remain a challenge to the food industry as they have the capacity to resist heat, radiation, and chemical treatments [379]. Therefore, there is a need for more effective and less intense preservation techniques to control *B. cereus* spores in food and/or food processing environments. In the present study, HICA was assessed for its effect on *B. cereus* spore germination.

7.2.3.1 Germination of *B. cereus* spores at four concentrations of HICA

To understand the antimicrobial effect of HICA on *B. cereus* spores, the spores were prepared at two densities (1×10^3 CFU/mL and 1×10^4 CFU/mL), treated with four HICA concentrations (0.5, 1, 2, and 4 mg/mL), and evaluated for spore germination and subsequent vegetative growth by measuring the OD_{595} of the spore cultures (See the materials and methods chapter 3.4.3). HICA completely inhibited the germination of *B.*

cereus spores at ≥ 1 mg/mL regardless of the initial spore densities used in this study. Similarly, the positive control, 0.1 mg/mL chloramphenicol showed no germination and subsequent vegetative growth of *B. cereus* spores. In contrast, untreated spores and the spores treated with 0.5 mg/mL HICA showed exponential vegetative growth after similar lag time (exponential growth started after ≈ 4h of incubation). However, the spores treated with 0.5 mg/mL HICA indicated different growth kinetics from the untreated spores by showing a slow exponential growth rate (Figure 7.3). This could be due to HICA inhibiting *B. cereus* cells entering the vegetative growth phase.

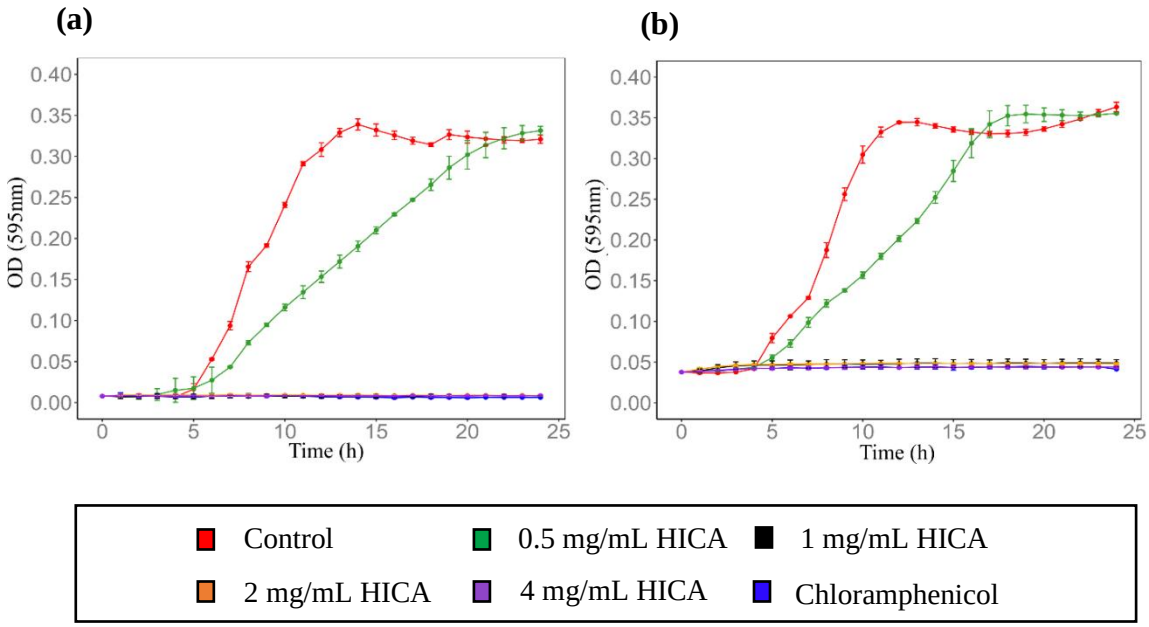


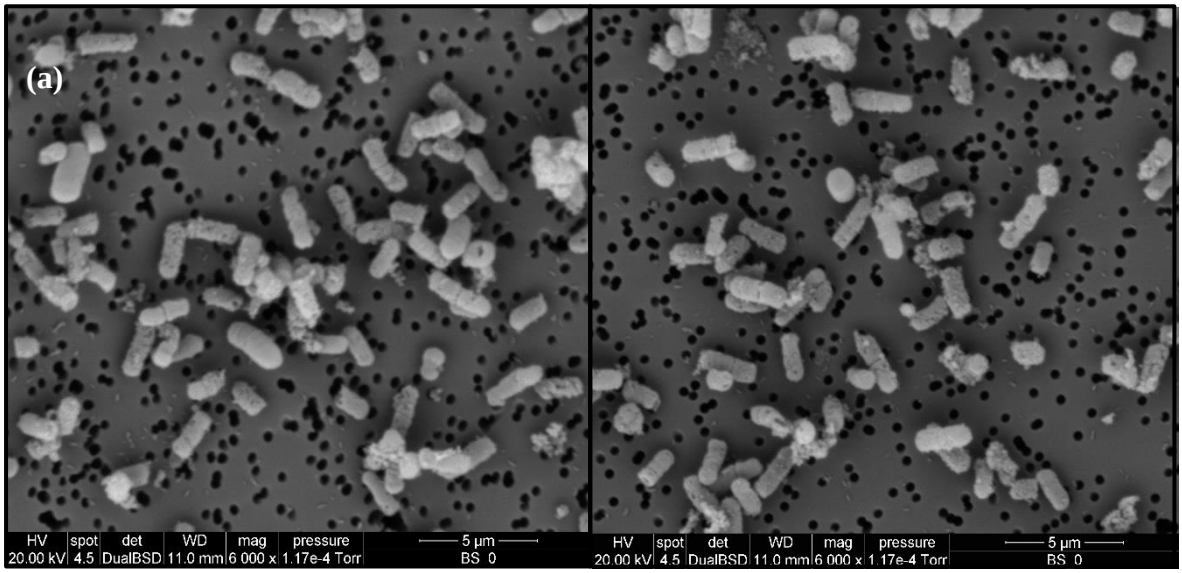
Figure 7.3: Effect of HICA treatment on the spore germination of *B. cereus* NZRM5. Untreated spores (red line), spores treated with 0.5 mg/mL HICA (green line), spores treated with 1 mg/mL HICA (black line), spores treated with 2 mg/mL HICA (orange line), spores treated at 4 mg/mL HICA (purple line), and spores treated at 0.1 mg/ml chloramphenicol (blue line). Two initial spore densities were evaluated, 1×10^3 CFU/mL **(a)**, and 1×10^4 CFU/mL **(b)**. Each curve represents the mean growth ± S.D (n = 3).

These results indicate that HICA may have a future application as a controlling agent for the spore-forming bacteria such as *B. cereus*. For instance, acid resistance spore-forming bacteria are a concern in acid dairy products stored at ambient temperatures and HICA may be a potential candidate that can inhibit the germination of those bacterial spores. This activity could be due to the interference of HICA at any stage/s of the spore germination and outgrowth process preventing the spores becoming vegetative cells. Further studies are required to understand more details of HICA's interference on *B.*

cereus spore germination and outgrowth, as well as its effect on the germination of other bacterial spores produced by food spoilage and pathogenic bacteria.

7.2.3.2 Observation of the inhibitory effect of HICA on the germination of *B. cereus* spores using Scanning Electron Microscopy (SEM)

HICA (≥ 1 mg/mL) was capable of inhibiting the germination of *B. cereus* NZRM5 spores as described in the previous section (7.2.3.1). In this section, additional evidence was provided by observing the morphological characteristics of *B. cereus* NZRM5 spores after HICA treatment using scanning electron microscopy. Spores were prepared with multiple washing steps to minimize cellular debris, but it was not practical to eliminate the debris as seen in all SEM micrographs. Figure 7.4a shows SEM images of starting *B. cereus* spores (HICA treatment for 0 h). They were cylindrical in shape with rough surfaces. As shown in Figure 7.4b, HICA treatment (4 mg/mL for 24 h) inhibited the germination and subsequent vegetative growth of *B. cereus* spores, whereas the untreated spores had become vegetative cells (Figure 7.4d). Chloramphenicol (0.1 mg/mL) also stopped the germination of *B. cereus* spores very similar to HICA treated spores (Figure 7.4c). Chloramphenicol was found to inhibit the germination of *B. cereus* spores at the swelling stage by inhibiting the protein synthesis [381]. These SEM micrographs clearly showed that HICA treatment could inhibit the germination and subsequent vegetative growth of *B. cereus* spores (Figure 7.4). However, it was not clear at what stage/s of spore germination process, HICA exerts its effect. Therefore, further studies are required to understand more about its inhibitory effect on spore germination.



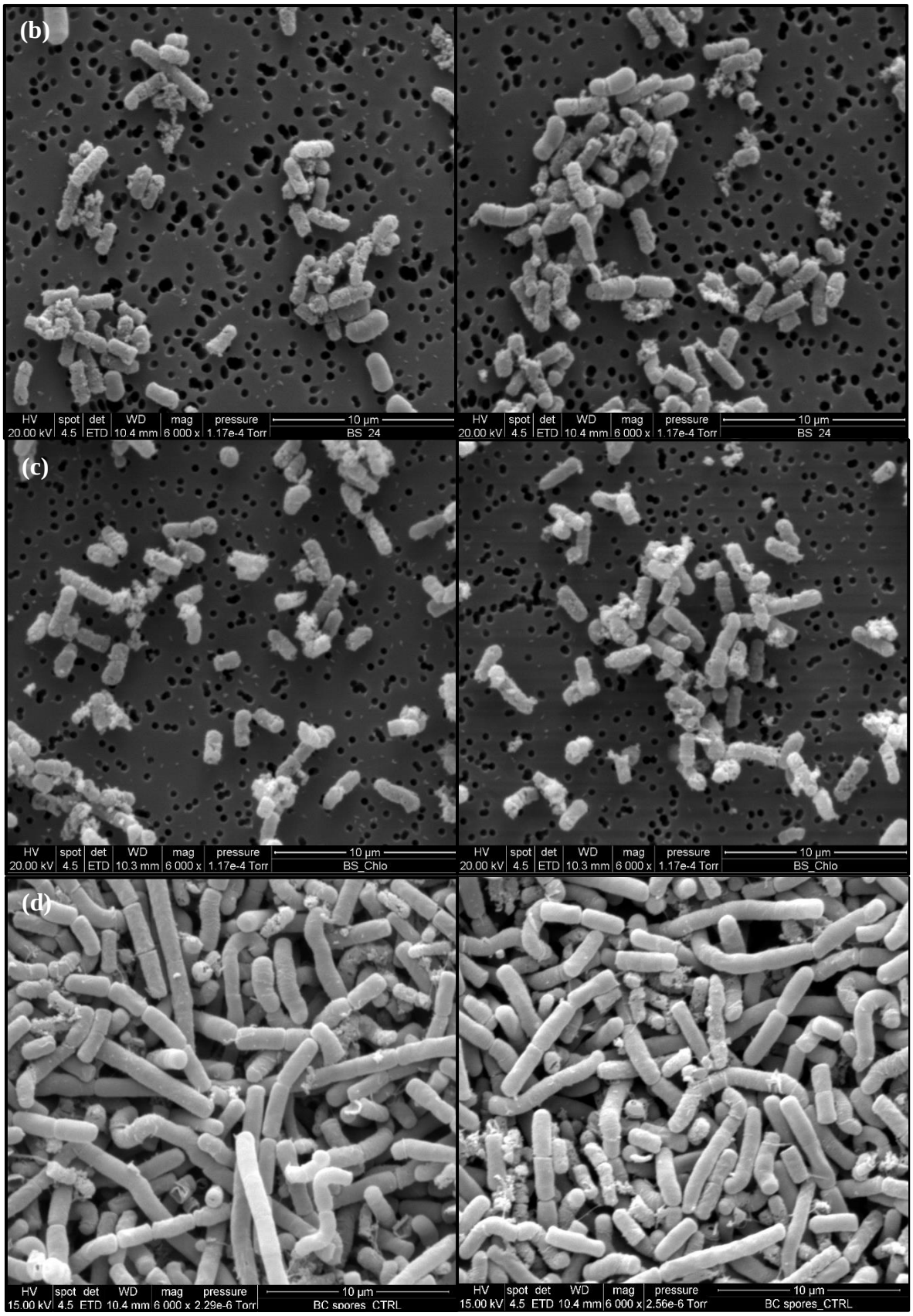


Figure 7.4: Scanning electron microscopic micrographs depicting the inhibitory effect of HICA on *B. cereus* NZRM5 spore germination and subsequent vegetative growth. The spores were treated with 4 mg/mL HICA for 0 h **(a)**, 4 mg/mL HICA for 24 h **(b)**, 0.1 mg/mL Chloramphenicol for 24 h **(c),** and water (untreated control) for 24 h **(d)**.

7.2.4 Effect of HICA on bacterial cell membrane integrity

The bacterial cell membrane is vitally important for many cell functions including transportation of molecules, osmoregulation, respiration, and synthesis of lipids. Therefore, cell membrane integrity is critically important for cell viability and its interruption can lead to metabolic dysfunction and ultimately cell death [382]. The disruption of cell membrane integrity has been recognized as a potential mechanism for cell death. As discussed in the literature review chapter 2.3.2, some antimicrobial compounds cause cell toxicity by disrupting the bacterial cell membrane integrity.

The effect of HICA on the bacterial cell membrane integrity was assessed using CellTox™ green cytotoxicity assay kit (See the materials and methods chapter 3.4.5). This assay uses a fluorescence DNA binding dye, which can bind with DNA from bacterial cells upon losing their membrane integrity. Since the dye is non-permeable to viable cells, the fluorescence occurs only with cells that have lost their membrane integrity and correlates with cell death. In the present study, the fluorescence intensities of bacterial cultures treated with 4 mg/mL HICA were monitored at various treatment times. In the absence of HICA, fluorescence intensities of all bacteria were very low indicating their intact/undamaged cell membranes. After the treatment with HICA, there was a significant increase in the fluorescence intensity of *B. cereus* NZRM5, *B. cereus* M4, and *S. aureus* NZRM917 compared to untreated controls ($p < 0.05$) indicating the loss of cell membrane integrity (Figure 7.5). Similarly, Gram negative bacteria (*E. coli* O157:H7 NCTC12900, *E. coli* AGR3789, *P. aeruginosa* ATCC25668, and *P. aeruginosa* NZRM4034) treated with HICA lost their cell membrane integrity, indicated by a significant increase ($p < 0.05$) in fluorescence over the treatment time compared to the relevant controls (Figure 7.6). Nevertheless, the maximum fluorescence intensity level (Relative Fluorescence Units) of each test bacterium was different to each other. Gram-positive bacteria demonstrated a more rapid loss of cell membrane integrity than Gram-negative bacteria achieving the maximum florescence values within 30 min of treatment. These results are consistent with the bacterial cell viability assay, which showed a relatively faster reduction in the cell viability of Gram-positive bacteria compared to Gram-negative bacteria after HICA treatment. This could be attributed to the additional outer membrane of Gram-negative bacteria providing an extra barrier for the access of HICA to the cytoplasmic membrane and to the inner cell structures. It has been reported that outer membrane of Gram-negative bacteria provides an extra layer of protection

against antimicrobials by lowering/not allowing to access inner cellular targets such as cytoplasmic membrane and other intracellular structures [383]. Overall, HICA disrupted the cell membrane integrity of both Gram-positive and Gram-negative bacteria causing the cell death.

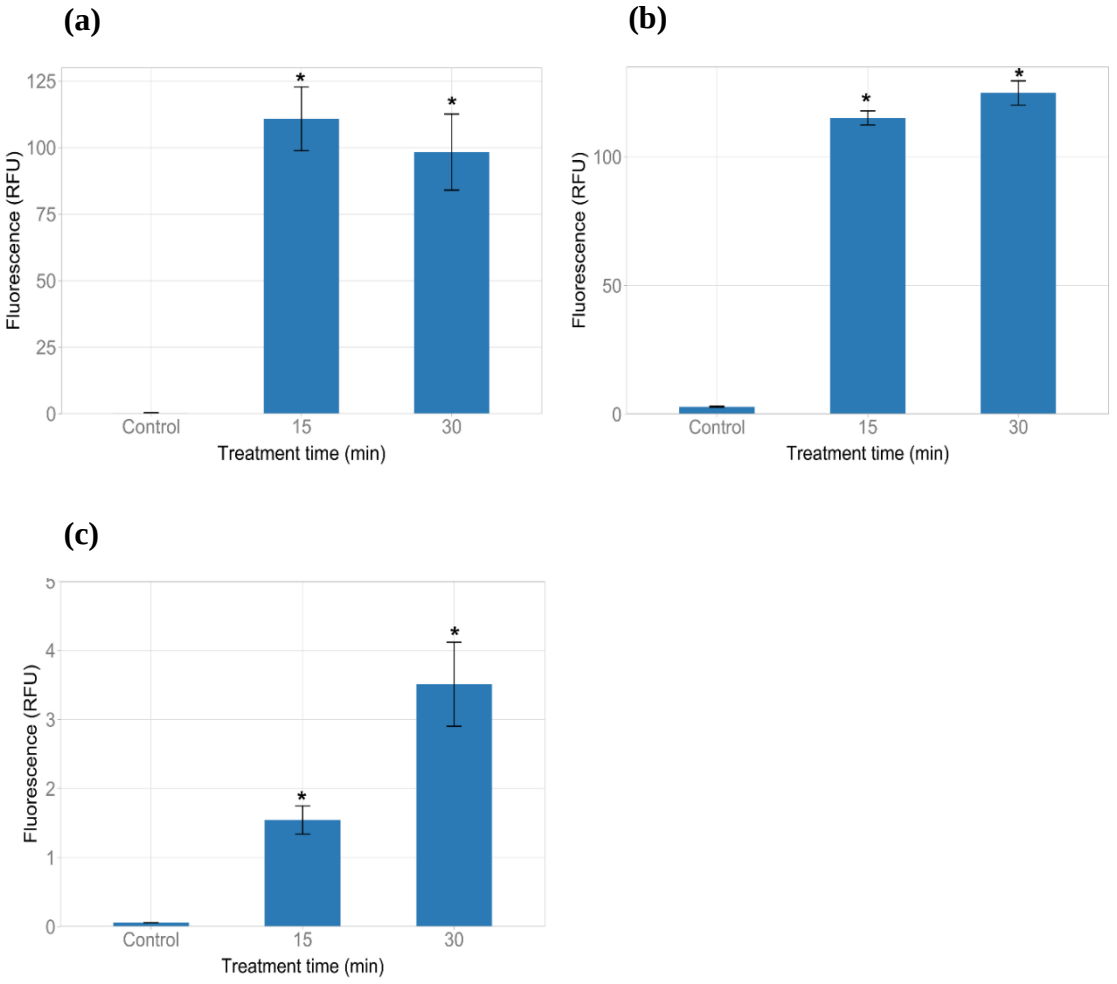


Figure 7.5: Effect of HICA on the cell membrane integrity of Gram-positive bacteria. *B. cereus* NZRM5 **(a)**, *B. cereus* M4 **(b)**, and *S. aureus* NZRM917 **(c)**, were treated with 4 mg/mL HICA and the loss of cell membrane integrity was assessed by measuring the fluorescence intensity at various treatment times. Untreated cells were used as controls. Data are presented as mean ± S.D of relative fluorescence units (RFU) (n = 3) $^{*}p < 0.05$ compared with the control.

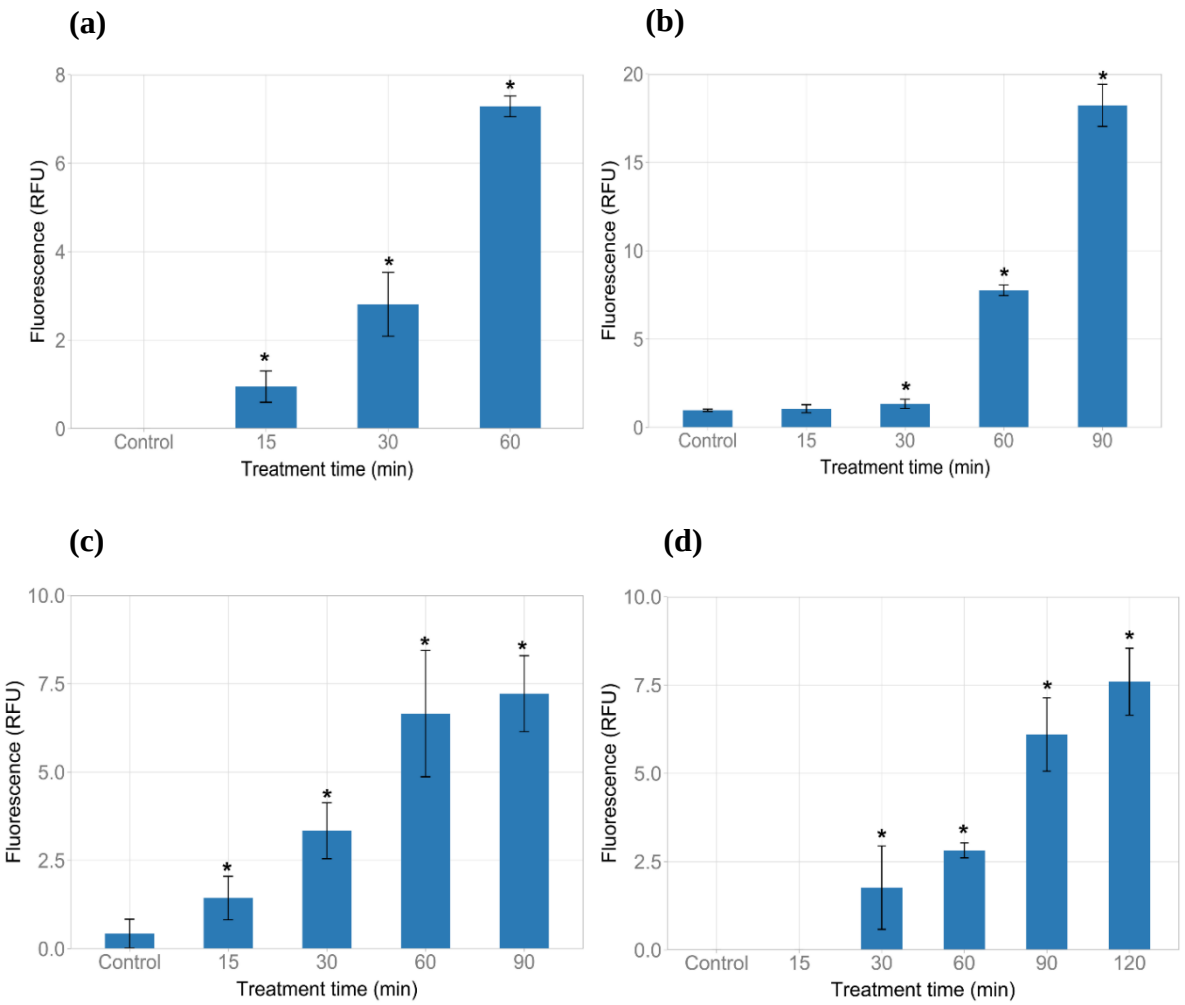


Figure 7.6: Effect of HICA on the cell membrane integrity of Gram-negative bacteria. *Escherichia coli* O157:H7 NCTC12900 **(a)**, *Escherichia coli* AGR3789 **(b)**, *P. aeruginosa* ATCC25668 **(c)**, and *Pseudomonas aeruginosa* NZRM4034 **(d)** were treated with 4 mg/mL HICA and the loss of cell membrane integrity was assessed by measuring the fluorescence intensity at various treatment times. Untreated cells were used as controls. Data are presented as mean ± S.D of relative fluorescence units (RFU) (n = 3) $^{*}p < 0.05$ compared with the control.

7.2.5 Effect of HICA on the outer membrane permeability of Gram-negative bacteria

Gram-negative bacteria consist of an extra membrane layer, the outer membrane (OM). The outer membrane in Gram-negative bacteria plays a vital function as a selective permeation barrier. It usually prevents the entry of harmful compounds and allows the entry of nutrient molecules [384]. The discovery of antimicrobial compounds against Gram-negative bacteria has been a major challlenge mainly due to the poor activity against Gram-negative bacteria during the screening of various compounds. The main reasons for the poor activity were the low permeability of many compounds across two membrane envelopes of the Gram-negative bacteria, thus preventing many antimicrobial compounds from reaching their intracellular targets [385]. Increasing the outer membrane permeability of Gram-negative bacteria by potent antimicrobial compounds is vital for their activities.

The previous section (7.2.4) suggested that HICA disrupted the cell membrane integrity of both Gram-positive and Gram-negative bacteria. In this section, the impact of HICA on the outer membrane permeability of Gram-negative bacteria was investigated. The fluorescence probe 1-*N*-phenylnapthylamine (NPN) was used to assess if the HICA caused the outer membranes of *E. coli* and *Pseudomonas aeruginosa* strains to be more permeable. NPN is a hydrophobic fluorophore, which cannot effectively penetrate through the outer membranes of Gram-negative bacteria. It gives a weak fluorescence signal in aqueous solution and a strong one when it binds to a phospholipid layer [386]. This characteristic of NPN is used to examine the permeability of the outer membrane of Gram-negative bacteria [302]. Cells having intact outer membranes show a weak fluorescence as NPN cannot effectively cross through the outer membrane, but cells with damaged/compromised outer membranes show stronger fluorescence as NPN can penetrate through the outer membrane accessing to the periplasmic space and bind with the phospholipids of the inner and outer membranes.

HICA permeabilized the outer membranes of *E. coli* O157:H7 NCTC12900, *E. coli* AGR3789, *P. aeruginosa* ATCC25668, and *P. aeruginosa* NZRM4034 in a dose dependant manner as observed by an increase in NPN fluorescence (Figure 7.7). Even, 0.5 mg/mL HICA (sub-MIC) showed a significant increase in the permeability of outer membrane compared to relevant untreated controls ($p < 0.05$). HICA increased the outer

membrane permeability of *P. aeruginosa* NZRM4034 (multi-drug resistant strain) and *P. aeruginosa* ATCC25668 (clinical isolate) in a similar way.

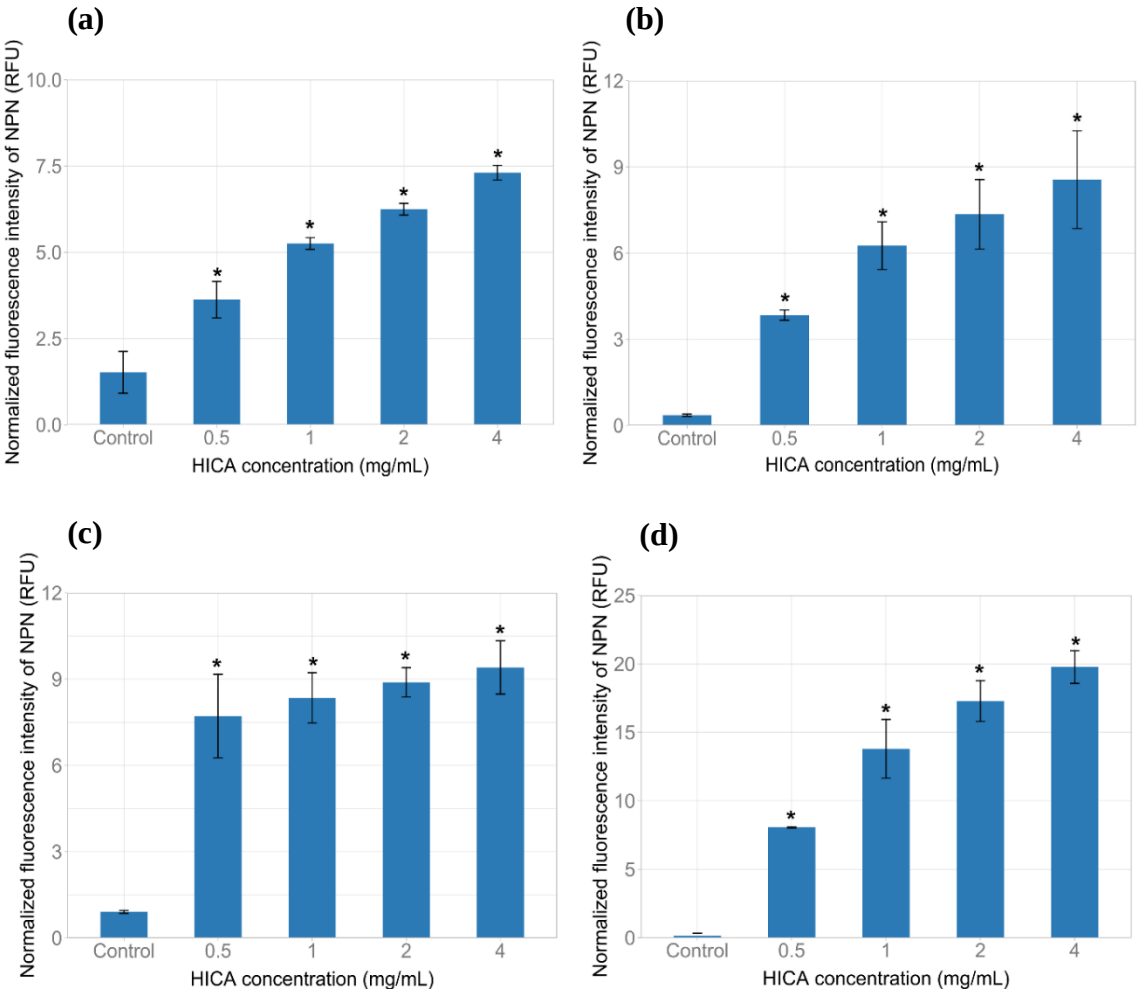


Figure 7.7: Effect of HICA on the outer membrane permeability of Gram-negative bacteria. *E. coli* O157:H7 NCTC12900 **(a)**, *E. coli* AGR3789 **(b)**, *P. aeruginosa* ATCC25668 **(c)**, and *P. aeruginosa* NZRM4034 **(d)** were treated with various concentrations (0.5 - 4 mg/mL) of HICA and the outer membrane permeability was assessed by measuring the fluorescence intensity. Untreated cells were used as controls. Data are presented as mean ± S.D of normalized fluorescence intensity of 1-*N*-phenylnapthylamine (NPN) measured in relative fluorescence units (RFU) (n = 3) $^{*}p < 0.05$ compared with the control.

These results demonstrate that HICA disrupts the integrity of the outer membrane resulting in a loss of barrier function in *E. coli* and *P. aeruginosa* strains. This provides the access of HICA to inner cellular targets including the cytoplasmic membrane of Gram-negative bacteria. The ability of HICA to permeabilize the outer membrane even

at concentrations lower than the MIC makes it a permeabilizer lacking inherent toxicity at the particular concentration but can sensitize bacteria to other antimicrobial agents when it is used together with other antimicrobial interventions.

7.2.6 Effect of HICA on cytoplasmic membrane potential

The cytoplasmic membrane potential plays a vital role in the chemical and mechanical integrity of bacterial cells and electrical potential [387]. The electric potential across membrane is important for bacterial cell division and ATP production [387, 388]. The dissipation of the membrane potential of bacteria by antimicrobial compounds may disrupt the indispensable barrier function and other essential cell functions of bacteria [303]. The disruption of the membrane potential of bacterial cells could be either the whole antimicrobial mechanism or contribute to the effectiveness of the compound by allowing access to additional inner molecular targets.

HICA disrupted the cell membrane integrity of both Gram-positive and Gram-negative bacteria as discussed in section 7.2.4. Furthermore, HICA permeabilized the outer membranes of Gram-negative bacteria gaining access to the cytoplasmic membrane (section 7.2.5). Therefore, further studies were conducted to assess the effect of HICA on the cytoplasmic membrane potential of both Gram-positive and Gram-negative bacteria using the membrane potential-sensitive dye 3,3'-Dipropylthiadicarbocyanine iodide [$DiSC_3(5)$]. The cationic and hydrophobic nature of $DiSC_3(5)$ allows it to penetrate through the lipid bilayers and to function as a potentiometric probe. When the dye is added to the cell suspension, it accumulates in energized/polarized cells until a Nernstian equilibrium is archived. This movement of the dye from the media to cells results in quenching of the overall fluorescence in the cell suspension. When the cytoplasmic membrane is depolarized, the dye will be rapidly released back into the medium (dequenching), that can be measured fluorometrically as a rapid increase in fluorescence [303].

HICA addition induced a rapid depolarization of the cytoplasmic membranes of both Gram-positive and Gram-negative bacteria used in this study as evident by rapid increase in fluorescence intensities (Figures 7.8 and 7.9). In contrast, the addition of water (untreated control) showed no alteration in the cytoplasmic membrane potential of all test bacteria measured by no change in fluorescence intensities. These results demonstrate

that HICA can alter the cytoplasmic membrane potential of both Gram-positive and Gram-negative bacteria. Cell membrane depolarization caused by HICA may lead to changes in the barrier properties of the cells and other cell functions leading to cell death. Studies have suggested that cytoplasmic membrane depolarization could result either through the formation of ion-conducting membrane pores, increasing membrane ion permeability or acting as an ion carrier [389, 390].

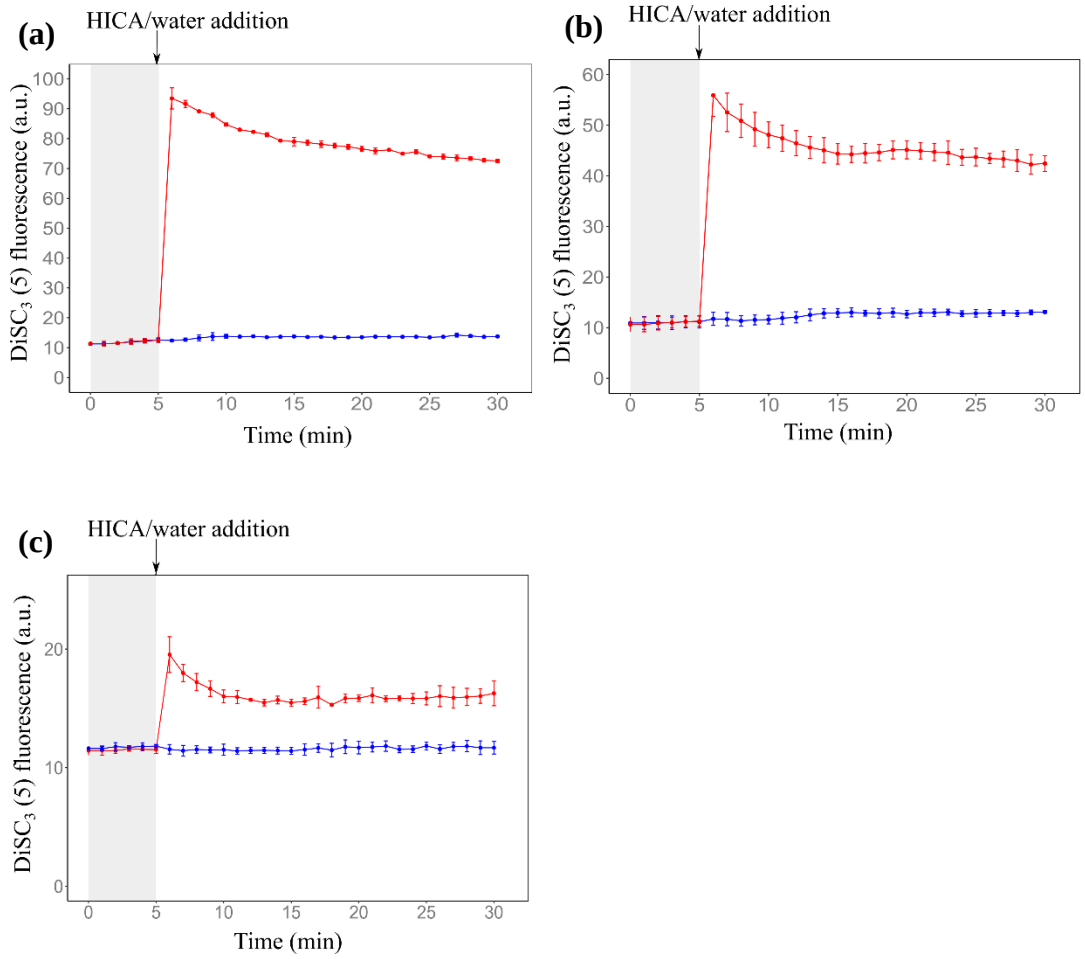


Figure 7.8: Cytoplasmic membrane depolarization of Gram-positive bacteria by HICA. Membrane potential levels of *B. cereus* NZRM5 **(a)**, *B. cereus* M4 **(b)**, and *S. aureus* NZRM917 **(c)** upon addition of 4 mg/mL HICA (red line) or water (untreated control, blue line) were assessed by the release of the membrane potential-sensitive dye $DiSC_3(5)$ measured spectroscopically at 610 nm excitation and 660 nm emission wavelengths. The time point of HICA/water addition is highlighted with arrows. Data are presented as mean ± S.D of $DiSC_3(5)$ fluorescence (n = 3).

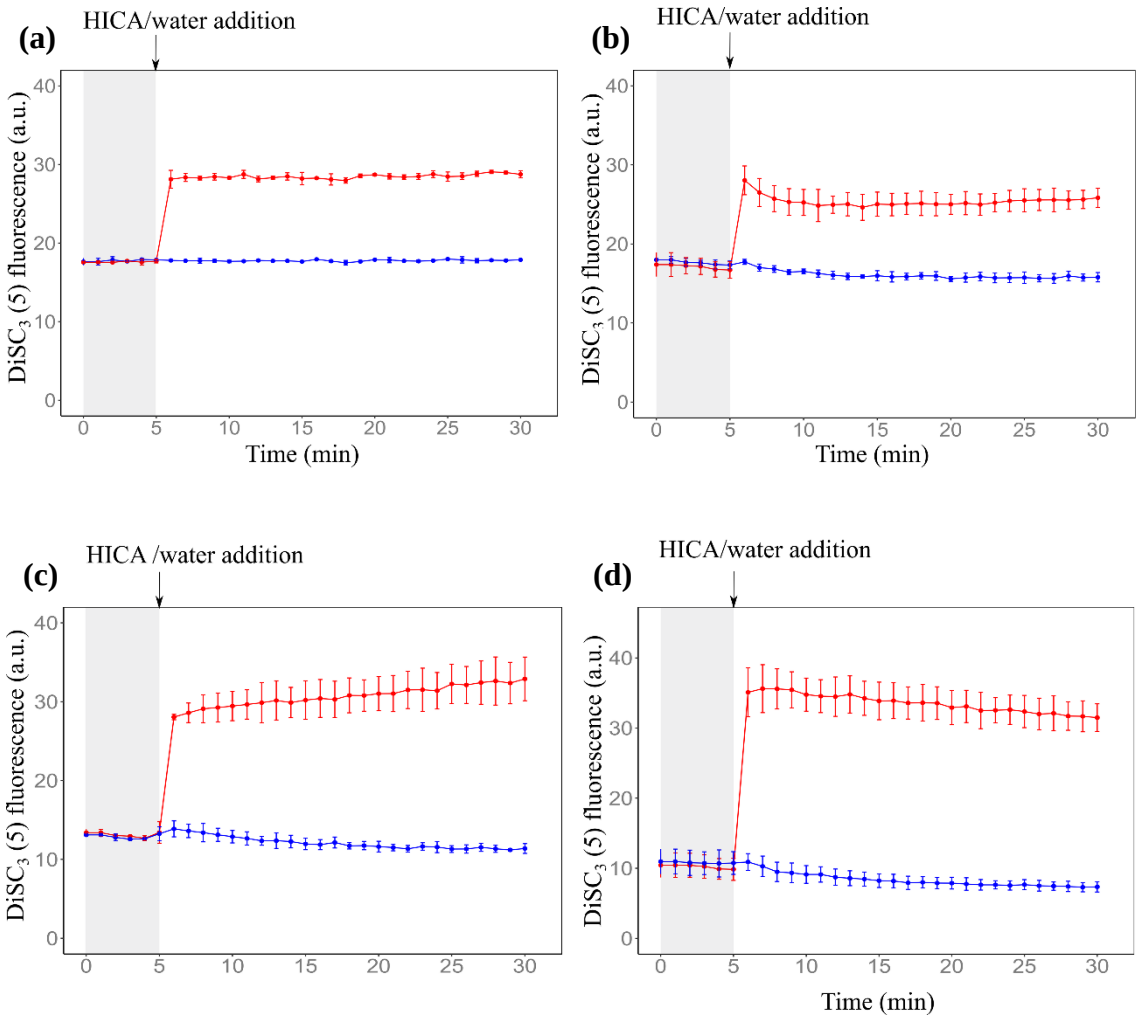


Figure 7.9: Cytoplasmic membrane depolarization of Gram-negative bacteria by HICA. Membrane potential levels of *E. coli* O157:H7 NCTC12900 **(a)**, *E. coli* AGR3789 **(b)**, *P. aeruginosa* ATCC25668 **(c)**, and *P. aeruginosa* NZRM4034 **(d)** upon addition of 4 mg/mL HICA (red line) or water (untreated control, blue line) were assessed by the release of the membrane potential-sensitive dye $DiSC_3(5)$ measured spectroscopically at 610 nm excitation and 660 nm emission wavelengths. The time point of HICA addition is highlighted with arrows. Data are presented as mean ± S.D of $DiSC_3(5)$ fluorescence (n = 3).

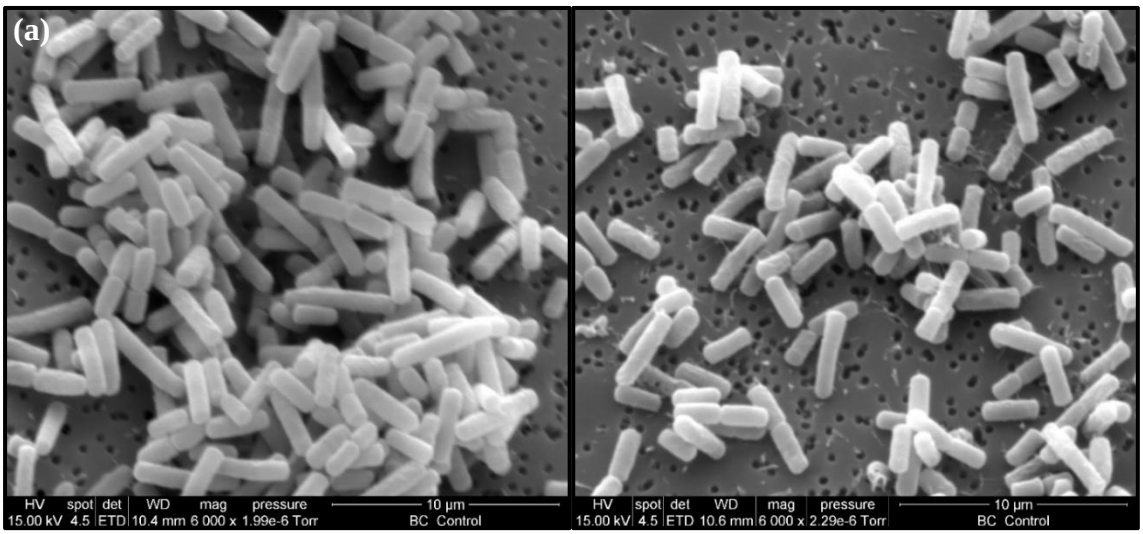
7.2.7 Morphological changes after HICA treatment

The examination of bacteria treated with antimicrobial agent for alterations to their morphology and ultrastructure is commonly used to investigate the potential antimicrobial mechanism. It is used as both an early investigative step and confirmatory step for a suspected mechanism [391]. In this section, *B. cereus* and *P. aeruginosa* were selected as test microorganisms to investigate the morphological changes in Gram-positive and Gram-negative bacteria after HICA treatment. Morphological and ultrastructural alterations were visualized and examined using scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

7.2.7.1 Scanning electron microscopy (SEM)

7.2.7.1.1 The effect of HICA on the cell morphology of B. cereus

The morphological characteristics of *B. cereus* cells treated with 4 mg/mL HICA for various time periods were observed by SEM. The untreated *B. cereus* cells were approximately 3 - 4 µm long and had smooth and intact surfaces (Figure 7.10a). The surfaces of some cells treated with HICA for 30 min appeared corrugated and there were ruptures in these cells, however other cells looked like untreated cells (Figure 7.10b). However, the incubation of cells with HICA for 60 min or more (120 min) caused excessive leakage of the cellular content (Figure 7.10c and 7.10d) and there were ruptures in the cell surfaces. SEM images demonstrated the distinct signs of damage to the cell envelope including roughening, rupturing, and the leak of the cellular content after the HICA treatment in a time dependant manner.



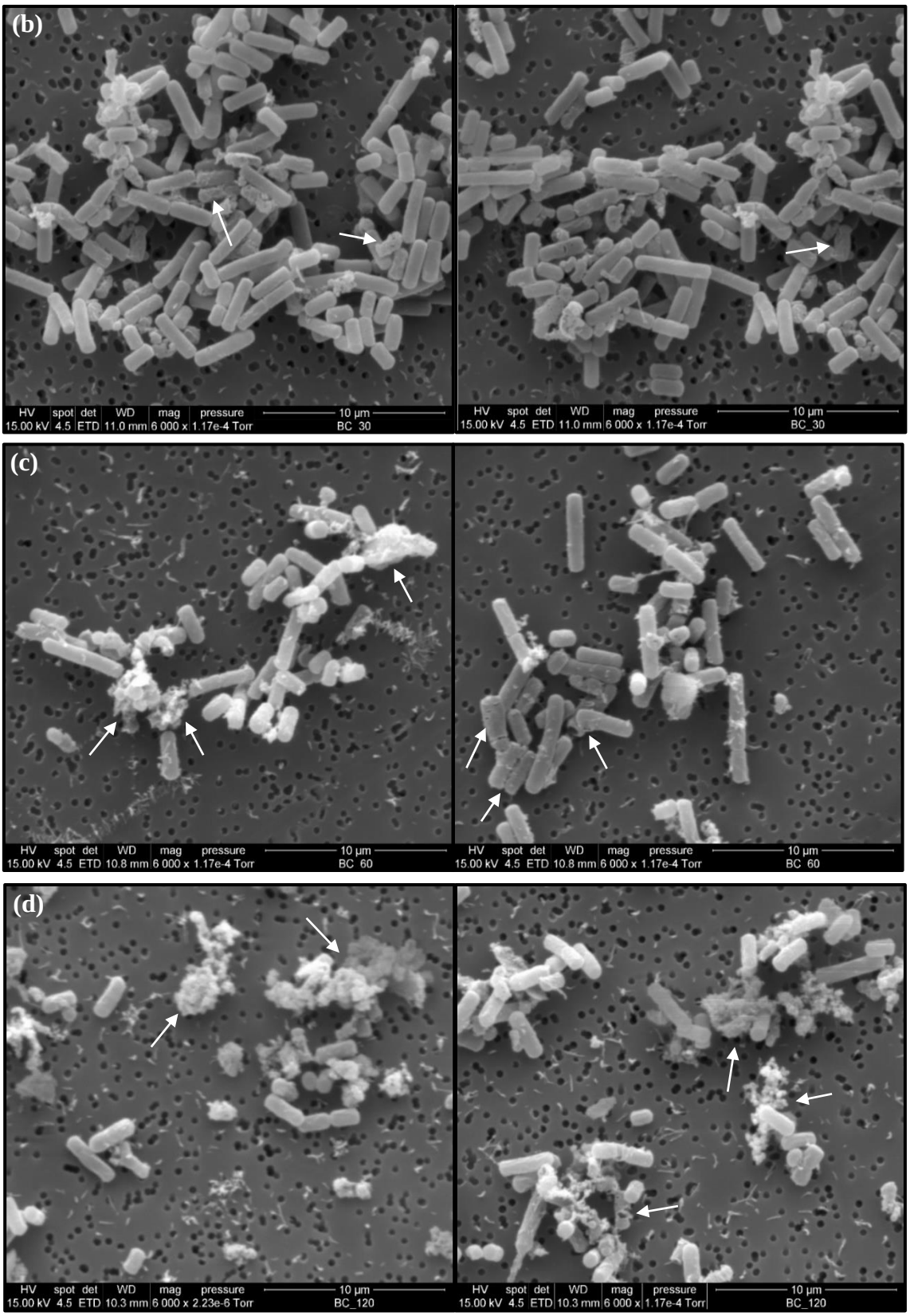
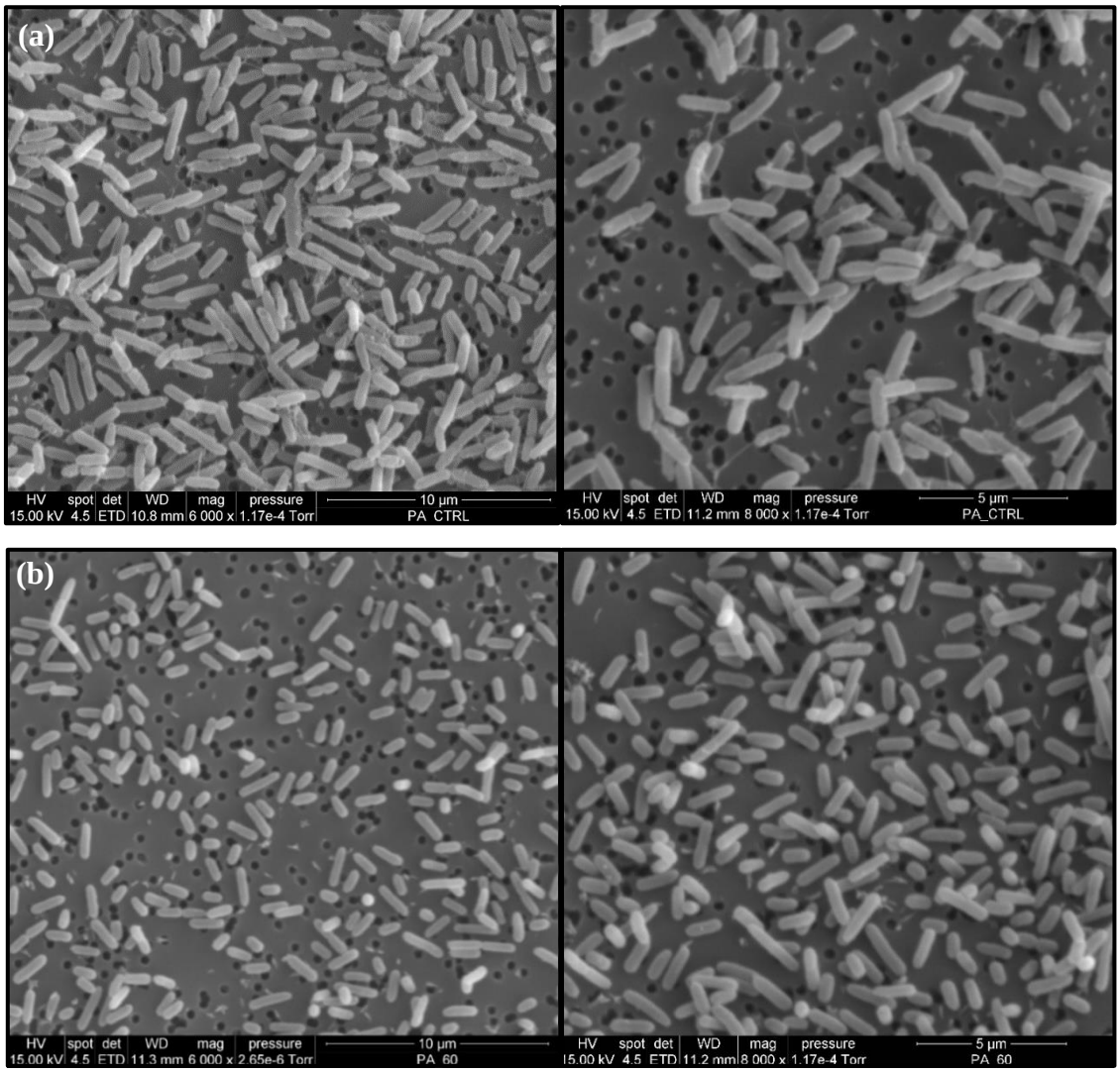


Figure 7.10: Scanning electron microscopic micrographs depicting the effect of HICA on the cell morphology of *B. cereus* NZRM5. The cells were treated with water (untreated control) **(a)** or with 4 mg/mL HICA for 30 min **(b)**, 60 min **(c)**, and 120 min **(d)**. Arrows show the locations of the ruptures of cell envelope and released cellular content.

7.2.7.1.2 The effect of HICA on the cell morphology of P. aeruginosa

In the control sample of *P. aeruginosa*, the cells were around 2 - 2.5 µm long, rod-shaped, and showed a smooth and undamaged cell structure. Additionally, there were extracellular polymeric-like substances (exopolysaccharide) [392] on the surfaces of untreated cells (Figure 7.11a). The HICA treatment for 60 min or more, reduced the size of the cells to as little as approximately 1 µm long and lost extracellular polymeric like substances on their cell surfaces. As the treatment time increases, cells had become shorter (Figure 7.11). However, there was no visible damage to the cell envelope and no cellular content was observed outside of HICA treated cells.



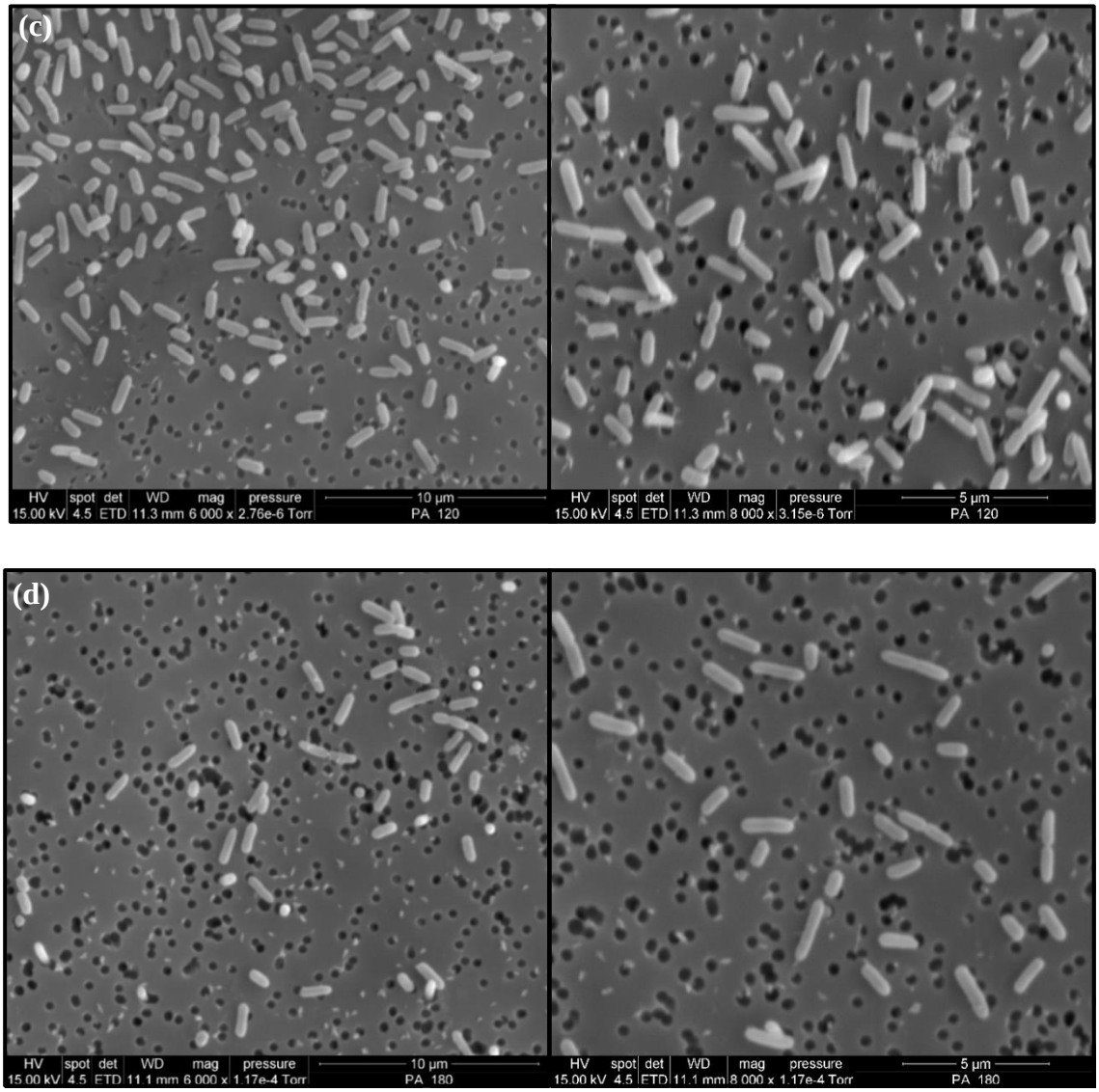
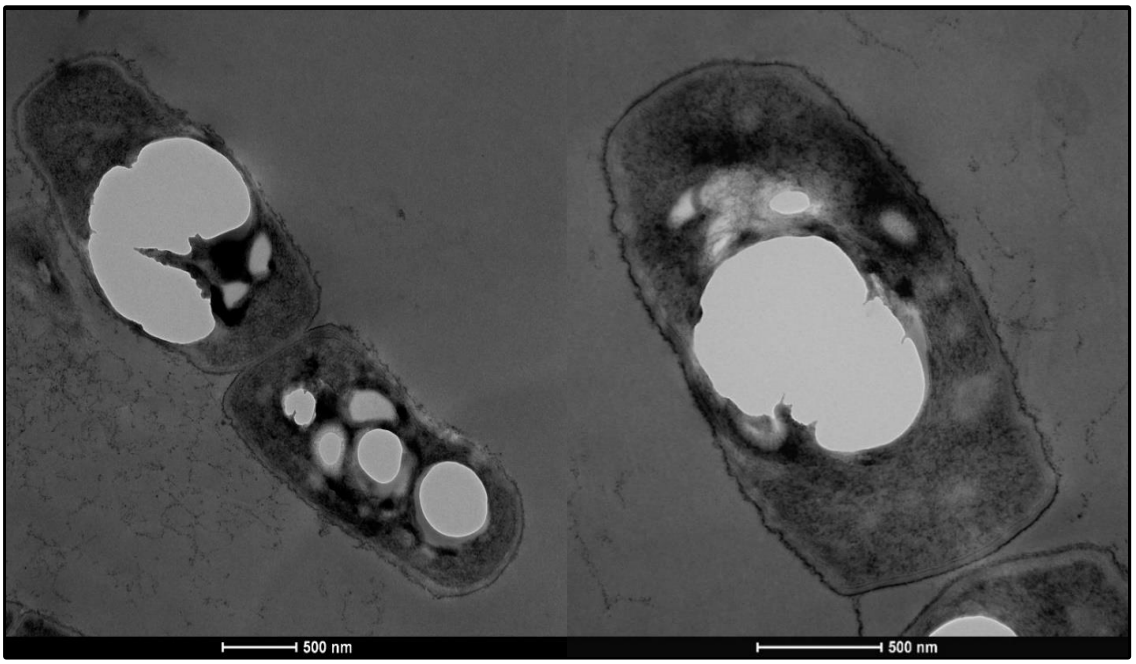


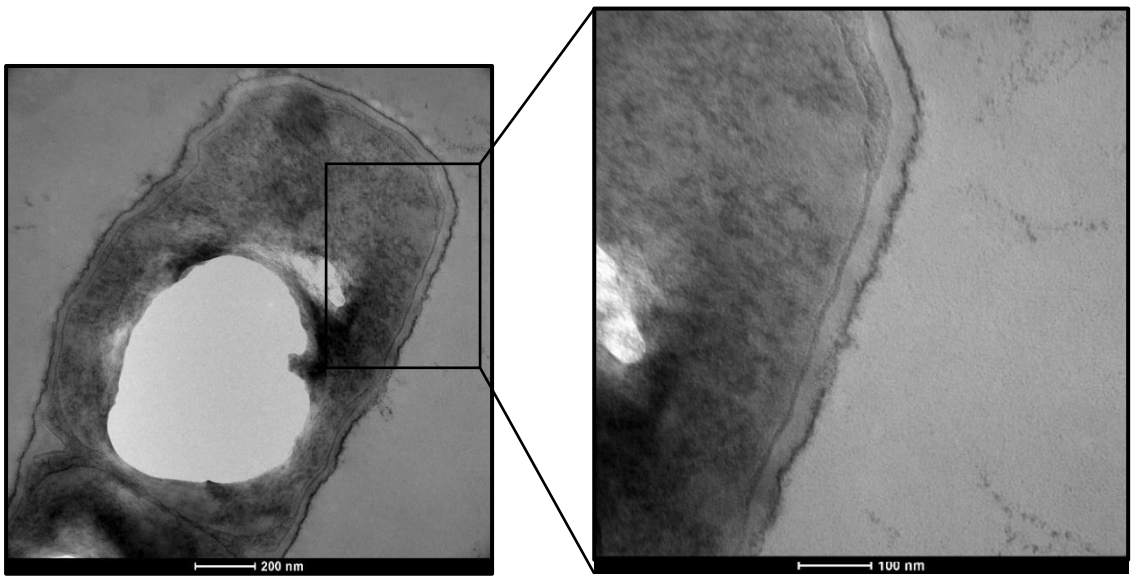
Figure 7.11: Scanning electron microscopic micrographs depicting the effect of HICA on the cell morphology of *P. aeruginosa* ATCC25668. The cells were treated with water (untreated control) **(a)** or with 4 mg/mL HICA for 30 min **(b)**, 60 min **(c)**, and 120 min **(d)**.

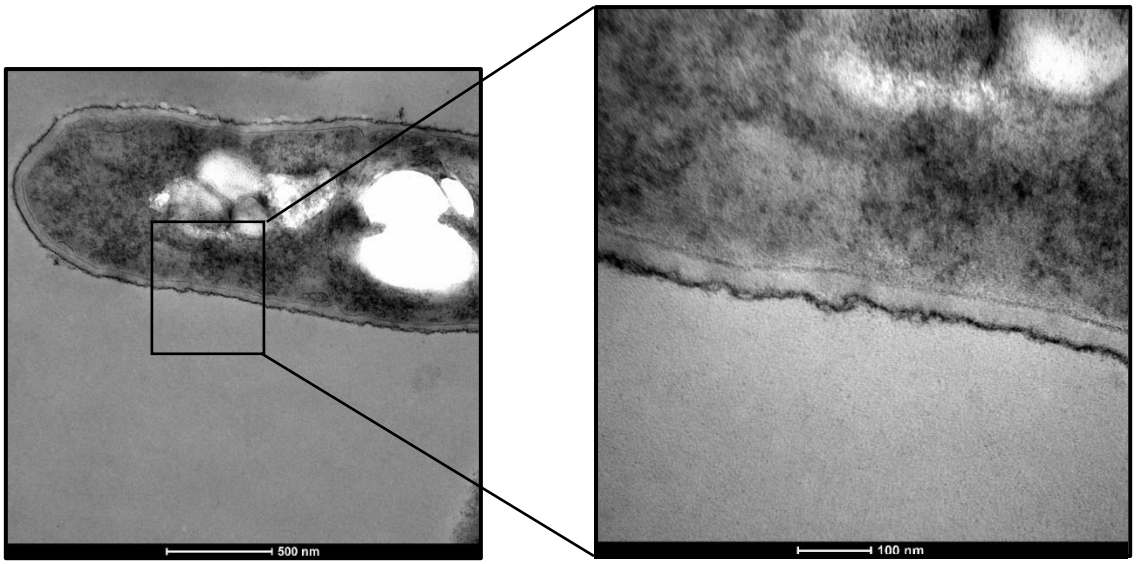
7.2.7.2 Transmission electron microscopy (TEM)

Ultrastructural alterations in *B. cereus* NZRM5 and *P. aeruginosa* ATCC25668 cells upon 4 mg/mL HICA treatment were investigated using transmission electron microscopy (TEM). Control/untreated *B. cereus* cells were rod shaped, intact, and presented the complete cell membrane and the cell wall (Figure 7.12a). After HICA treatment (4 mg/mL HICA for 60 min), several perimortem changes were observed in *B. cereus* cells as shown in Figure 7.12b. The breakage of the cell wall and cytoplasmic membrane followed by the leakage of cell contents were observed. Lysed cells ('ghost cells") devoid of cytoplasm were also observed.

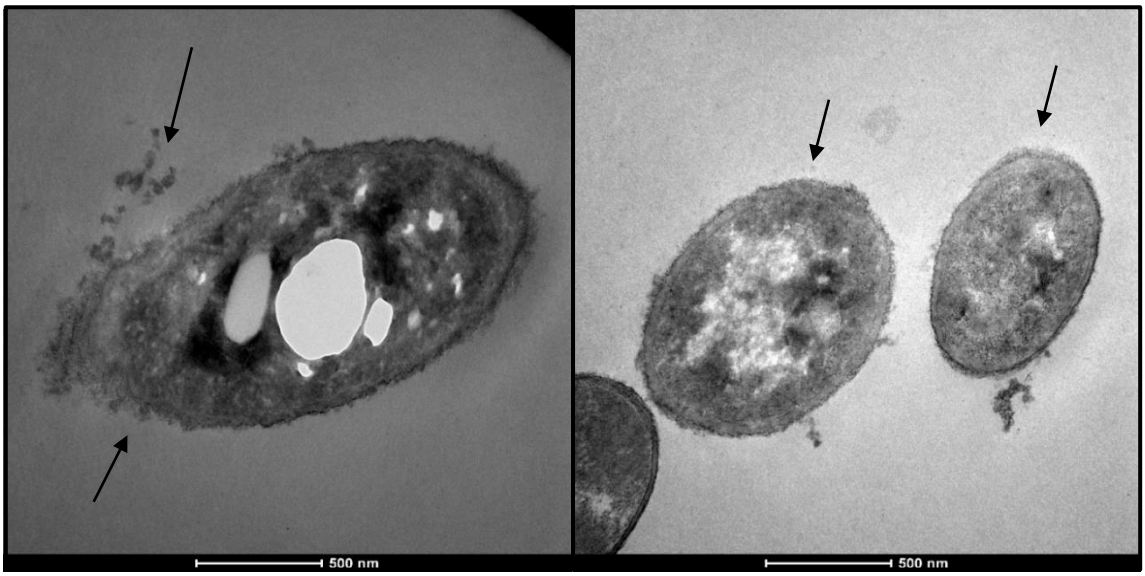
(a)

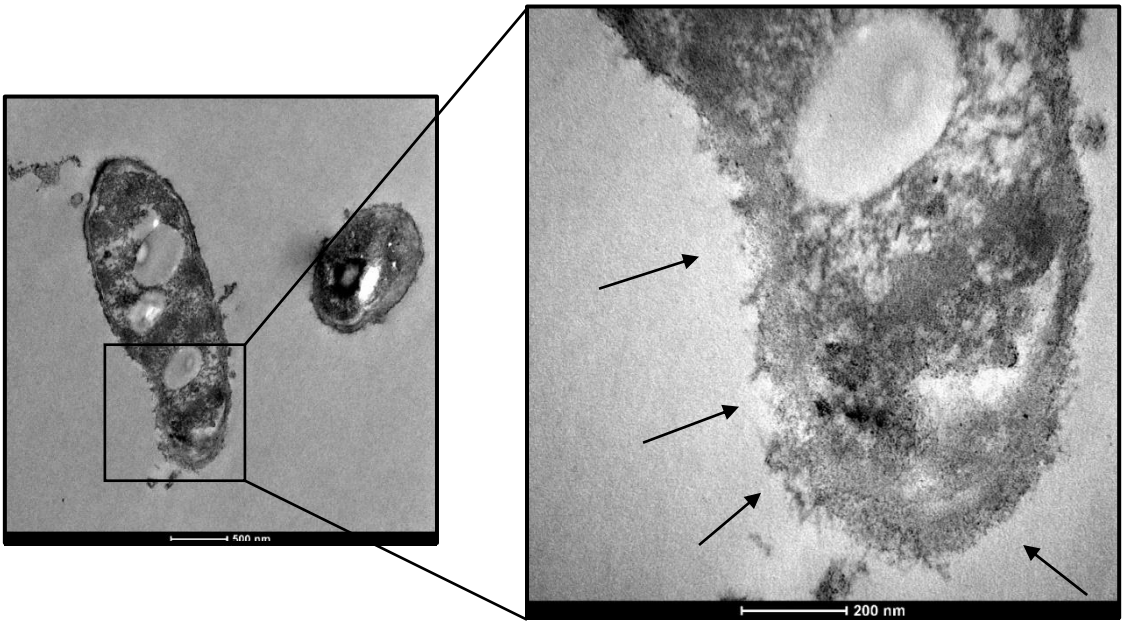






(b)





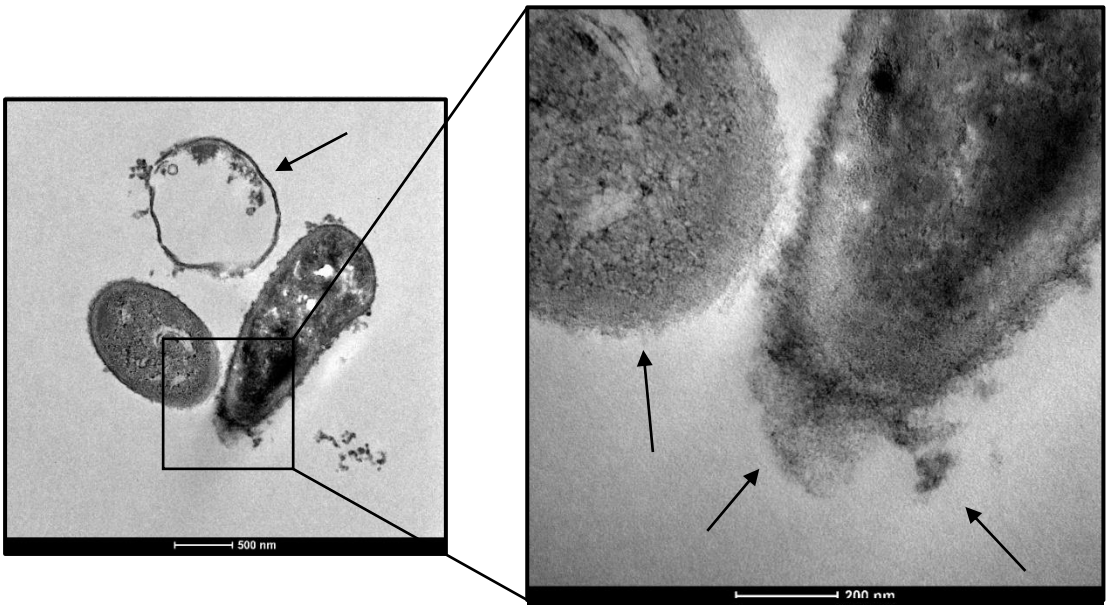
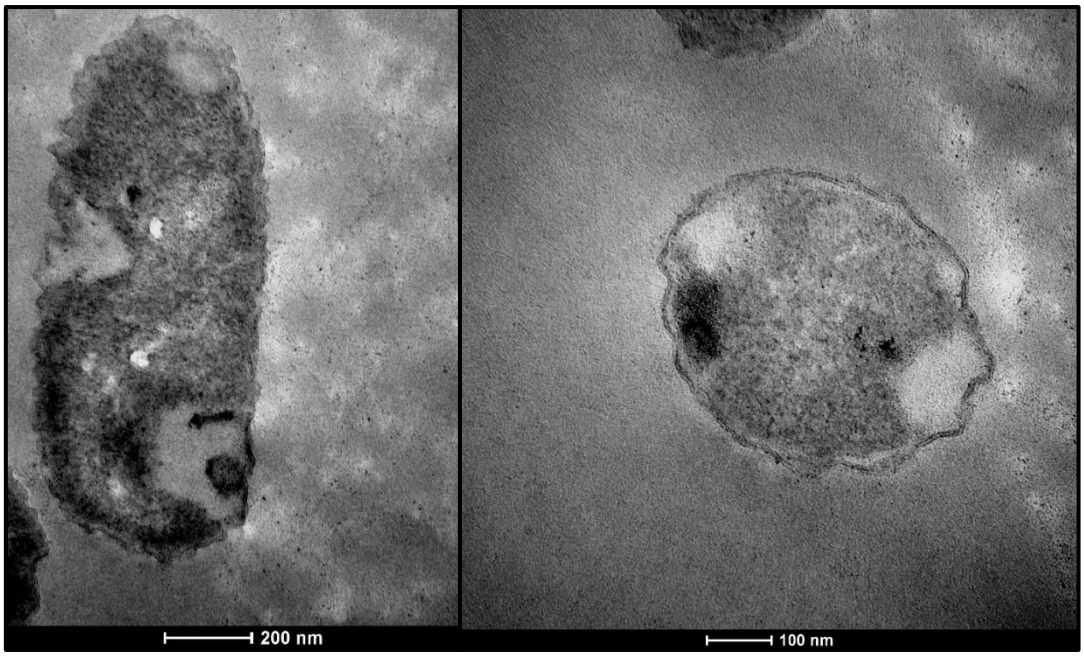


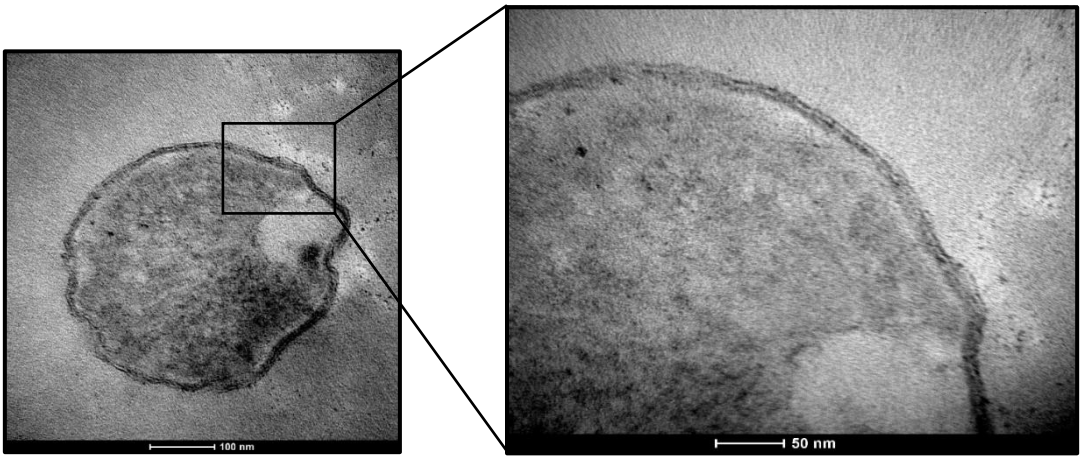
Figure 7.12: Transmission electron microscopic micrographs depicting the effect of HICA on the cell ultrastructure of *B. cereus* NZRM5. The cells were treated with water (untreated control) **(a)** or with 4 mg/mL HICA for 60 min **(b)**. Arrows show the locations of the breakage of cell wall and cytoplasmic membrane, release of cellular content or lysed cells.

Both SEM and TEM images of *B. cereus* demonstrated significant morphological alterations including signs of damage to the cell envelope (roughening and rupturing) and the leak of the cellular content after the HICA treatment.

Significant perimortem changes were also observed in *P. aeruginosa* cells following 120 min incubation with HICA (4 mg/mL). Untreated cells presented an undamaged wavy cell membrane structure (Figure 7.13a). Compared to untreated cells, the rupture of the cell envelope, and subsequent release of cellular content were observed in HICA treated cells (Figure 7.13b). The cellular damage caused by HICA in *P. aeruginosa* cells was not very clear in SEM images, whereas TEM images clearly showed the loss of cell membrane integrity and the leakage of cellular content in *P. aeruginosa* cells after HICA treatment.

(a)





(b)

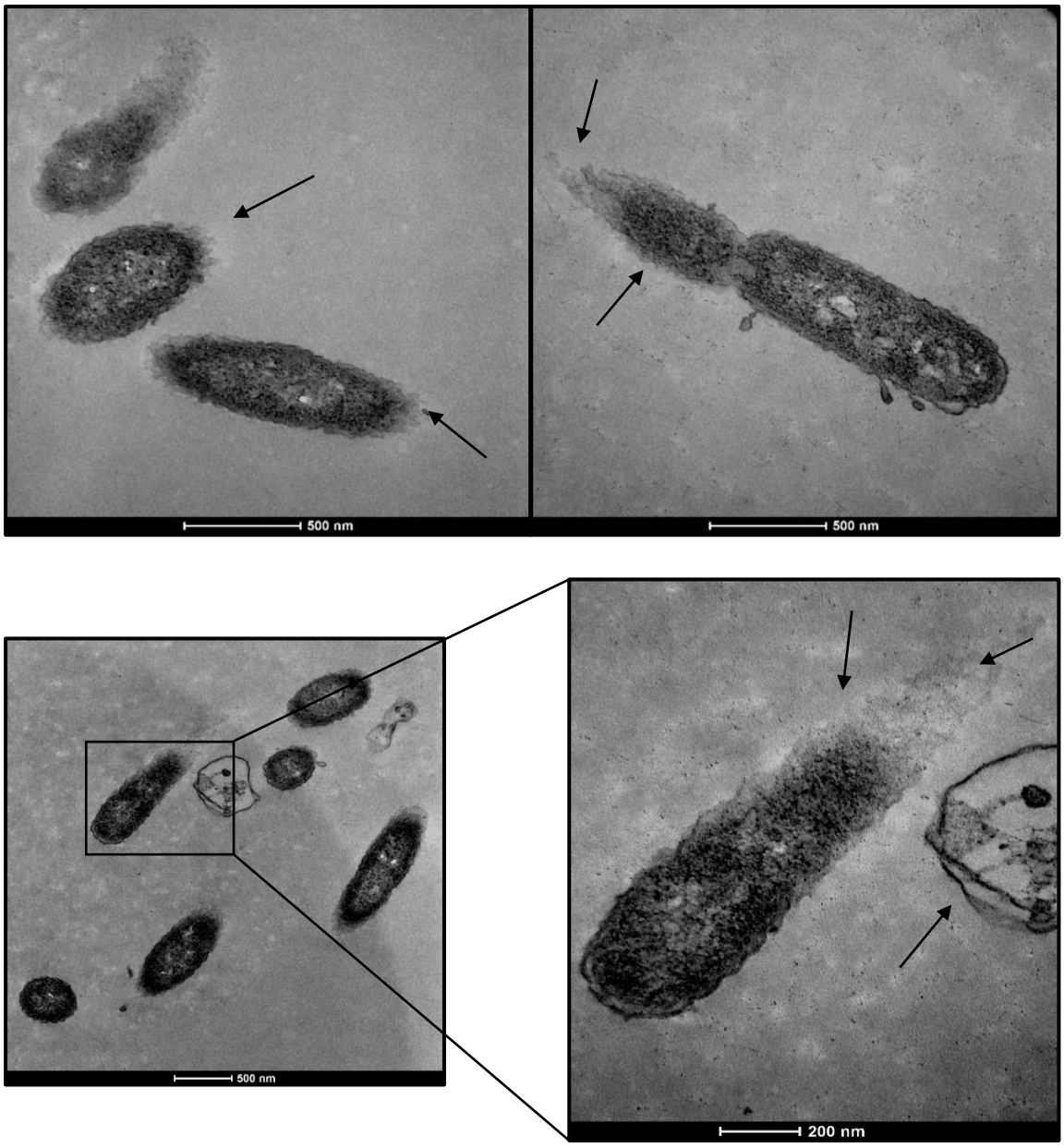


Figure 7.13: Transmission electron microscopic micrographs depicting the effect of HICA on the cell ultrastructure of *P. aeruginosa* ATCC25668. The cells were treated with water (untreated control) **(a)** or with 4 mg/mL HICA for 120 min **(b)**. Arrows show the locations of the breakage of cell envelope, release of cellular content or lysed cells.

Clarification of the antimicrobial mechanism of action is vital in the development of any putative antimicrobial agent. This information is important to identify problems relating to the potential applications, bacterial resistance, and the optimization of the antimicrobial compound. Also, it allows the selection of a combination of interventions targeting multiple cellular targets optimizing the control and lowing the risk of bacterial resistance [391].

The evidence provided in this chapter indicates that the cell membrane (permeability of outer membrane, depolarization of cytoplasmic membrane and loss of cell membrane integrity) represents the primary target of HICA. HICA might target other cellular structures or processes and its activity could be attributed to more than one mechanism. In this respect, further studies are required to understand other cellular interference of HICA, that may lead to the cell death. A significant feature of HICA, which will benefit its potential application, is its activity against both Gram-positive and Gram-negative bacteria showing similar MIC values. The low permeability of the outer membrane of Gram-negative bacteria has been reported to prevent the effect of some antimicrobial compounds, which are active against Gram-positive bacteria [393]. For instance, the outer membrane of Gram-negative bacteria prevents nisin, a widely used natural food preservative, from reaching the inner membrane, where it exerts its effect [315]. This study demonstrated that HICA was capable of disrupting the integrity of the outer membrane of Gram-negative bacteria resulting in the loss of barrier function. This is interesting to note, as HICA is a hydrophobic compound and Gram-negative bacteria have been reported to be more resistant to hydrophobic antimicrobial compounds compared to Gram-positive bacteria [394].

The disruption of the bacterial cell envelope by HICA could be partially or largely associated with its ability to reduce the pH of the cell environment. Antimicrobial activity of lactic acid has been attributed to its ability to reduce intracellular pH, disrupt cell membranes and affect other cellular processes and structures including enzyme activities, protein, and DNA structure [395, 396]. Therefore, further studies are required to understand how HICA permeabilizes the outer membrane of Gram-negative bacteria and depolarizes the cytoplasmic membrane of both Gram-positive and Gram-negative bacteria leading to the loss of cell membrane integrity. Nevertheless, the current study revealed HICA's ability to cause bacterial cell death via depolarization, permeabilization, rupture of bacterial cell membranes, and subsequent leakage of cellular content.

Hydroxy acids such as HICA are not major flavour compounds in fermented food products but known as precursors of flavour compounds [397]. Therefore, one of the practical limitations of using HICA in food preservation could be the influence of HICA on the flavour profile of certain food products if added directly as a food additive. However, as it is naturally present in fermented food products, it might not contribute to undesirable flavour profiles when added to fermented foods. Another approach to use HICA in food preservation is using it as a surface decontamination agent to reduce the spoilage and pathogenic bacteria on food surfaces such as meat, fruits, and vegetables. It could be used as a part of the hurdle technology applied to maintain the quality and safety of food products.

7.3 Conclusions

This study explored the antimicrobial efficacy of HICA against a range of Gram-positive and Gram-negative bacteria associated with food spoilage, food safety, and human health. The results reveal that HICA is effective in controlling the growth of both Gram-positive and Gram-negative test bacteria including a multi-drug resistant *P. aeruginosa* strain. Additionally, HICA inhibited the germination and subsequent vegetative growth of *B. cereus* spores.

Further studies were conducted to provide some insights into the possible antimicrobial mechanism of HICA. It was found that the antimicrobial activity of HICA was achieved via disruption of the cytoplasmic membrane of both Gram-positive and Gram-negative bacteria with significant membrane depolarization, which lead to loss of cell membrane integrity resulting in cell death. The permeabilization of the outer membrane of Gram-negative bacteria by HICA allowed it to reach to the inner cellular target, the cytoplasmic membrane. Taken together, HICA exhibited promising antimicrobial potential via penetration of the bacterial cell membranes and causing depolarization, permeabilization, rupture of membranes, and subsequent leakage of cellular contents leading to cell death. Antimicrobial activity combined with its previously reported safety profile suggests that HICA could be considered as a promising antimicrobial agent against food spoilage and pathogenic bacteria.

Chapter 8

Chemical isotope labelling (CIL) liquid chromatography mass spectrometry (LC-MS) analysis of FS03CM

8.1 Introduction

The work presented in this chapter is associated with the third objective of the project, which was to evaluate the metabolite profile of FS03CM and identify potent antimicrobials from its metabolome. The CM prepared from FS03 (FS03CM), was found to possess the strongest antimicrobial activity among all four CMs prepared from Farm 4 soil isolates (Figure 8.1). FS03 was found to be closely related to *Terrisporobacter glycolicus* (See antimicrobial potential of conditioned media chapter 4.2.3). Its metabolome was investigated to identify putative secondary metabolites with potential antimicrobial properties using chemical isotope labelling (CIL) liquid chromatography mass spectrometry (LC-MS) analysis. A non-targeted approach was used in this study to provide a comprehensive analysis of all measurable analytes in FS03CM.

Chapter 5 described the non-targeted metabolomics analysis of five soil conditioned media using several analytical tools with various metabolite detectability. Reversed phase (RP) liquid chromatography with two different separation columns were used to analyse compounds with a wider polarity range (polar and intermediate polar metabolites). Positive and negative ionization modes of MS detection were separately used to ionize as many metabolites as possible in the samples. This chapter describes the metabolite profiling of FS03CM using an alternative strategy of non-targeted metabolomics, which is chemical isotope labelling (CIL) liquid chromatography mass spectrometry (LC-MS).

This technique is based on classifying metabolites into different subgroups depending on the availability of common functional moieties and carrying out in-depth analysis of individual chemical groups [196]. The combination of data obtained from submetabolomes provides information on the whole metabolome with a high coverage [398]. More information on the CIL LC-MS can be found in the literature review chapter 2.4.2.3.

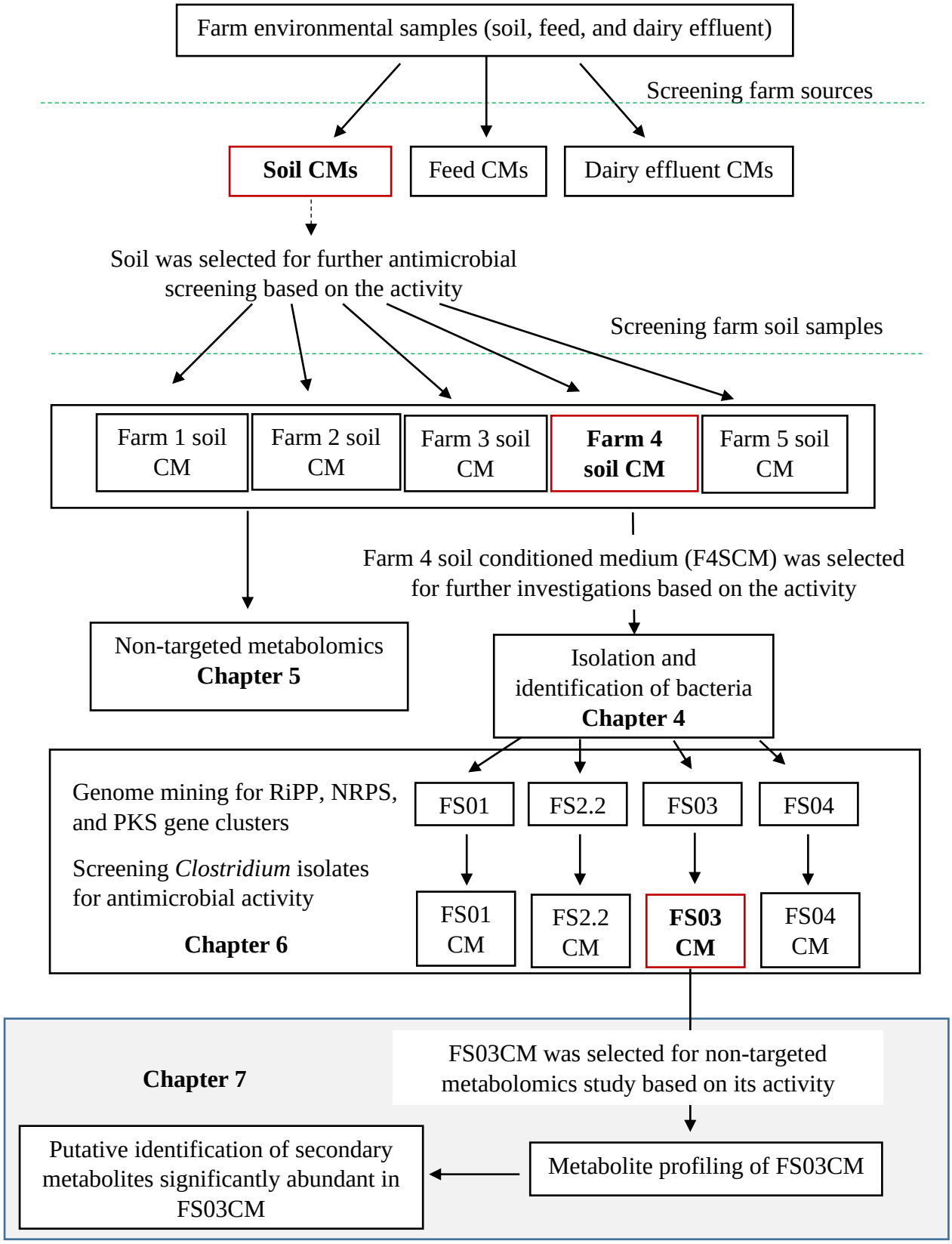


Figure 8.1: Schematic diagram showing the focus of chapter 7 and association to the previous work.

In the current study, two isotope labelling techniques, ^{12}C-/^{13}C-dansyl chlorite labelling and ^{12}C-/^{13}C-dimethylaminophenacyl (DmPA) bromide labelling, were carried out for profiling different submetabolomes in FS03CM and CMGS (Figure 8.2). FS03 transformed the starting growth medium, CMGS into FS03CM with antimicrobial properties through its metabolic activities during anaerobic growth. Therefore, CMGS was included in the study for metabolite profile comparison purpose and to identify compounds generated during the growth of FS03. An isotope reagent ^{12}C-/^{13}C-dansyl chlorite was used to label metabolites containing primary amines, secondary amines, or phenolic hydroxyl group(s) in the samples. Previously, the amine/phenol submetabolome had been analysed with high coverage using dansylation LC-MS [196]. The ^{12}C-/^{13}C-dimethylaminophenacyl (DmPA) bromide labelling was used to label carboxylic acid containing metabolites in the samples. This isotope labelling was effective in both quantification and identification of carboxylic acid containing metabolites [195]. In CIL-LC-MS technique, the differential isotope labelling (^{12}C-/^{13}C) is used for accurate relative quantification [194]. The two isotope labelling reactions employed in this study are described in the literature review Figure 2.1 and Figure 2.2.

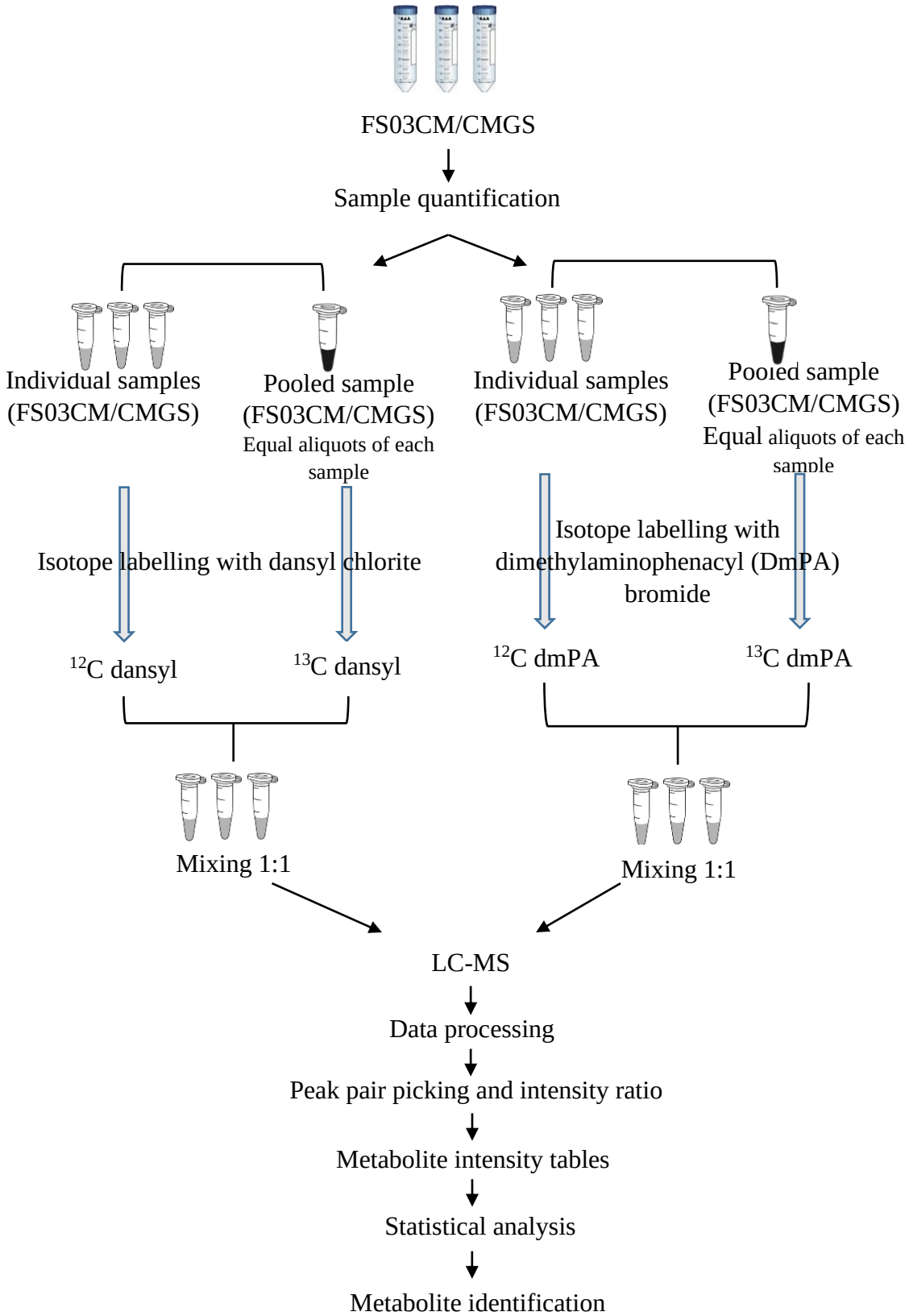


Figure 8.2: Schematic diagram showing the chemical isotope labelling liquid chromatography mass spectrometry (CIL LC-MS) workflow used in this study.

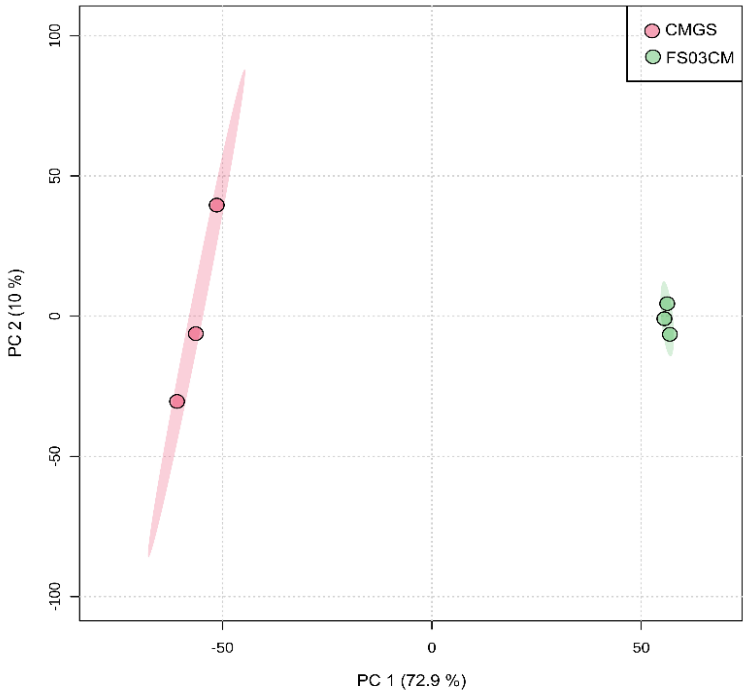
8.2 Results and discussion

8.2.1 Metabolite profiling of FS03CM and CMGS

Two-channel (amine/phenol channel and carboxyl channel) CIL LC-MS analysis was carried out to obtain the information on the metabolomes of FS03CM and CMGS. LC-MS data obtained in two labelling channels were processed and combined to get a single file with all detected peak pairs for each sample. After filtering for peak pairs that presented in at least 80% of samples in any group, 4783 ± 23 average peak pairs (n = 3) in the CMGS group and 4697 ± 9 average peak pairs (n = 3) in the FS03CM group were taken for further analyses. Less commonly detected peak pairs were removed to maintain the data quality. There could be biological and technical reasons for not detecting all the metabolites in every sample. The biological reason could be due to lower concentrations of missing metabolites below the detection threshold in some samples. The technical reason could be due to ion suppression by other compounds or matrix effects on the detection of metabolites [399].

Multivariate analyses, principal component analysis (PCA) and hierarchical cluster analysis were carried out to determine the variability of samples with regards to metabolite profiles. In the PCA analysis, the first composite variable (PC1) and the second composite variable (PC2) accounted for 72.9% and 10% of the overall variability (Figure 8.3a) in the dataset. The FS03CM group was clearly separated from the CMGS group. This indicates the characteristic changes occurred in the metabolite composition of CMGS during the growth of FS03. Similar results were obtained through hierarchical cluster analysis, which demonstrated a clear distance between CMGS and FS03CM (Figure 8.3b).

(a)



(b)

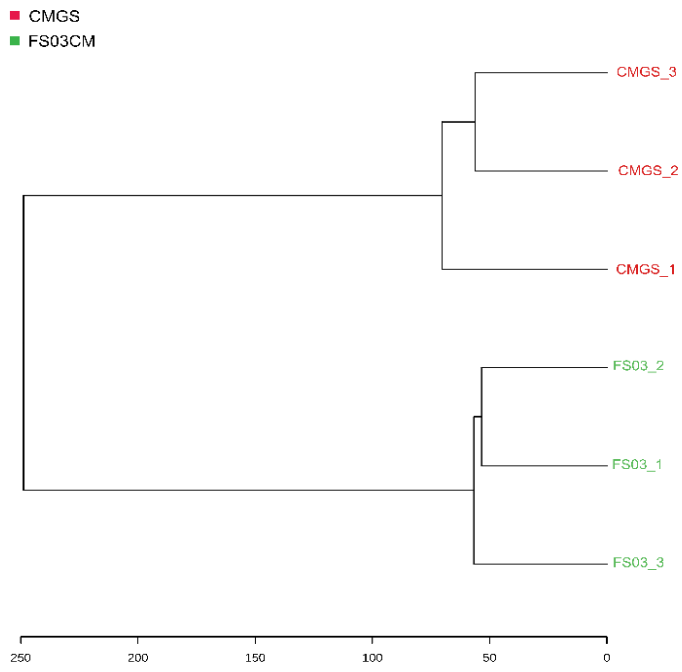


Figure 8.3: 2D-PCA score plot and hierarchical cluster analysis dendrogram of CIL LC-MS data obtained for FS03CM and CMGS. PCA score plot shows the differences in metabolite profiles of FS03CM and CMGS **(a)**. Dendrogram shows the relationship between FS03CM and CMGS medium based on their metabolite profiles **(b)**. Dendrogram was constructed by ward clustering on the closest Euclidean distances between samples. Samples are colour coded as indicated in the figure legend. Numbers represent sample IDs.

8.2.2 Evaluating differentiating metabolites of FS03CM from growth medium (CMGS)

All metabolites detected in CMGS and FS03CM groups were used to compute a volcano plot showing significantly discriminating metabolites in both groups (Figure 8.4). The x-axis represents the $\log_2$ of magnitude of the change/fold change (FC) between FS03CM and CMGS, and the y-axis represents the negative $\log_{10}$ of the *p* value (t-test of significance) between FS03CM and CMGS. The metabolites having a FC > 2 and *p*-value < 0.05 were considered significantly different and denoted in red dots in the volcano plot. In the present study, the focus was only on statistically significant metabolites detected in FS03CM compared to CMGS as they were most likely to be associated with FS03CM's antimicrobial activity. A total of 683 metabolites were found to be significantly abundant in FS03CM in comparison to CMGS (shown in right upper quadrant of the volcano plot using red dots). They were primarily produced because of the growth of FS03 in CMGS and possibly associated with its antimicrobial activity.

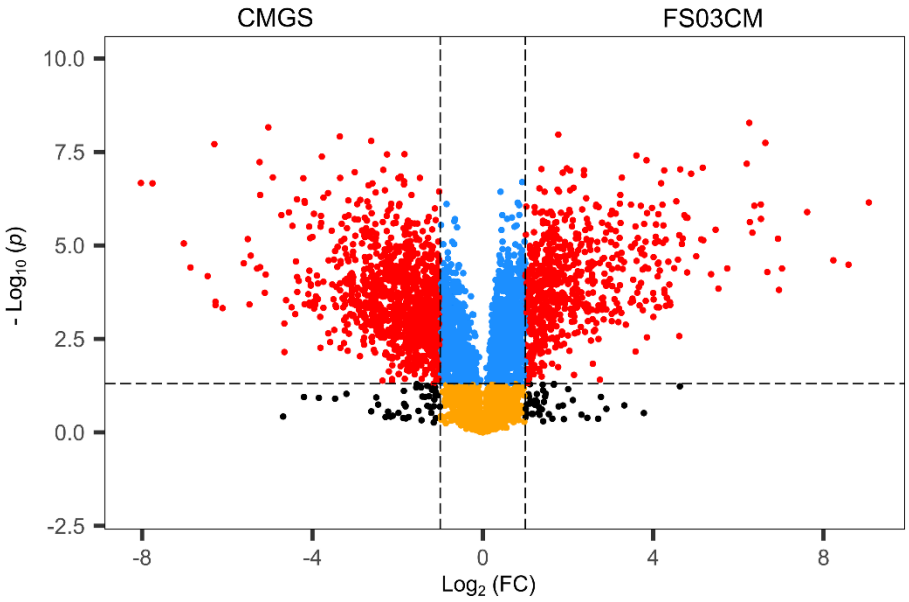


Figure 8.4: Volcano plot shows the relative abundance of metabolites in CMGS and FS03CM groups. It was constructed by combining the statistical t-test [$-\log_{10}$(*p*-value)] and the magnitude of the change [$\log_2$(FC)]. Red dots represent the metabolites with *p*-value < 0.05 and FC > 2. Blue points represent the metabolites with $p < 0.05$ and FC < 2. Yellow points represent the metabolites with *p*-value > 0.05 and FC < 2. Black dots represent the metabolites with $p > 0.05$ and FC > 2. CMGS (Cooked meat glucose starch media supplemented with 0.0005% yeast extract, 0.1% hemin, and 1% vitamin K), FS03CM (Conditioned medium prepared from FS03).

8.2.3 Metabolite identification

Metabolite identification used in the current study consisted of three levels as described in the Table 8.1. The number of significantly discriminating peak pairs identified from FS03CM using a three-tier identification are given in Table 8.2.

Table 8.1: The "Three tier" metabolite identification used in this study [398].

Level of identification	Description
Tier 1 positive identification	Metabolites are positively identified with the highest confidence based on their accurate mass and retention time matches with those of labelled standard compounds in chemical isotope labelling library (CIL library).
Tier 2 high confidence putative identification	Metabolites are putatively identified with high confidence based on accurate mass and predicted retention time matches against a linked identity library (LI library)
Tier 3 putative identification	Metabolites are putatively identified using accurate mass matches against the MycompoundID (MCID) library (zero-reaction) and their predicted metabolite products from one reaction (one-reaction library) and two metabolic reactions (two reaction library)

All peak pairs, which were significantly abundant in FS03CM were searched against the library containing labelled standard compounds (CIL library) [398] and 48 peak pairs were positively identified based on their accurate mass and retention time matches (Table 8.2 and 8.3). As shown in Figure 8.5, all positively identified metabolites were detected at high levels in FS03CM in comparison to CMGS. A total of 45 peak pairs in FS03CM were putatively identified with tier 2 identification confidence, which was based on the

accurate mass and predicted retention time matches against a linked identity library. As tier 3 putative identification used only accurate mass, the exact structure of the metabolite was hard to determine. Nevertheless, it provided some indications about the peak pair identity.

Table 8.2: Number of significantly discriminating peak pairs identified at different identification confidence levels from FS03CM.

	Number of peak pairs
Total number of significantly discriminating peak pairs in FS03CM (FC $\geq$ 2 and $p \leq 0.05$).	683
Number of peak pairs identified at various identification levels	
Tier 1	48
Tier 2	45
Tier 3 (Zero-reaction)	64
Tier 3 (One-reaction)	178
Tier 3 (Two-reaction)	106

The metabolites, which were identified at the highest identification confidence (tier 1) were used for a literature research to find out any previously reported information on their antimicrobial potential. The outcome of the literature research showed that 4-hydroxyphenyllactate, 3-hydroxyphenylacetic acid, acetic acid, isobutyric acid, valeric acid, and tryptamine, positively identified from FS03CM had previously been reported to possess antimicrobial activities against some microorganisms. The relative intensities of these metabolites in FS03CM and CMGS suggested that they were produced by the metabolic activities of FS03 during the growth in CMGS (Figure 8.6). However, positively identified metabolites with no previous information on antimicrobial activity cannot be neglected for their antimicrobial potential as they have not been assessed for antimicrobial potential yet.

Table 8.3: Positively identified significantly abundant metabolites in FS03CM.

External identifier	Compound name	Neutral mass (Da)	Normalized RT (s)	Fold change	*p*-value	Level of identification
C02043	Indolelactate	205.0722	320.7	73.34	6.63×10^{-8}	1
C05629	Hydrocinnamic Acid / 3-Methylphenylacetic Acid	150.0686	426.5	24.83	2.54×10^{-7}	1
C05145	3-Aminoisobutanoic Acid	103.0625	208.7	11.99	1.14×10^{-5}	1
C05984	2-Hydroxyglutaric Acid	148.0361	340.7	5.18	1.43×10^{-5}	1
C03672	Hydroxyphenyllactic Acid	182.058	239.6	4.91	1.52×10^{-5}	1
C01620	Threonic Acid	136.0387	130	3.32	3.42×10^{-5}	1
C00327	Citrulline	175.0963	222.1	4.33	3.45×10^{-5}	1
C00954	Indoleacetic Acid	175.0631	364.6	5.65	3.72×10^{-5}	1
HMDB0029005	Phenylalanyl-Threonine	266.1259	530.6	2.38	6.65×10^{-5}	1
C00398	Tryptamine	160.1009	1070.7	27.79	1.75×10^{-4}	1
C00346	O-Phosphoethanolamine	141.0197	121.4	18.21	1.83×10^{-4}	1
C00632	3-Hydroxyanthranilic acid	153.0422	1070.4	3.31	2.55×10^{-4}	1
C00334	Gamma-Aminobutyric Acid	103.0622	176.2	4.58	2.98×10^{-4}	1
C00803	Valeric Acid	102.0689	404.9	80.42	3.51×10^{-4}	1
C05852	3-Hydroxyphenylacetic acid	152.0483	990.4	92.25	3.65×10^{-4}	1
C00431	5-Aminopentanoic acid	117.0803	513.2	196.10	4.61×10^{-4}	1
C05852	Ortho-Hydroxyphenylacetic acid	152.0471	980.7	16.75	4.99×10^{-4}	1
C00099	Beta-Alanine	89.0481	430.3	18.88	6.28×10^{-4}	1
C02632	Isobutyric Acid	88.053	352.6	44.18	7.42×10^{-4}	1
C00232	Succinic Semialdehyde	102.0319	226.9	2.32	7.50×10^{-4}	1
HMDB0000479	3-Methylhistidine	169.0846	121.9	6.45	7.57×10^{-4}	1
C00020	Adenosine monophosphate	347.0643	110.2	5.83	7.85×10^{-4}	1
C00064	Glutamine	146.0689	216.8	3.80	1.01×10^{-3}	1
C00262	Hypoxanthine	136.0392	545.6	3.33	1.03×10^{-3}	1
C00033	Acetic Acid	60.021	247.6	19.62	1.03×10^{-3}	1
C03672	Hydroxyphenyllactici acid	182.0573	837.3	25.53	1.10×10^{-3}	1
C00666	Diaminopimelic acid	190.094	740	6.45	1.16×10^{-3}	1
HMDB0000678	N-Isovaleroylglycine	159.0887	260.5	2.12	1.56×10^{-3}	1
C02372	4-Aminophenol	109.0511	1473.3	2.36	1.62×10^{-3}	1
HMDB0028991	Phenylalanyl-Aspartate	280.1052	493.6	2.07	1.92×10^{-3}	1

External identifier	Compound name	Neutral mass (Da)	Normalized RT (s)	Fold change	*P*-value	Level of identification
C01732	Mesaconic Acid	130.0261	441.4	6.71	1.94×10^{-3}	1
C00642	Parahydroxyphenylacetic Acid	152.0478	291.7	300.33	2.04×10^{-3}	1
HMDB0000866	N-Acetyl-Tyrosine	223.083	227.3	2.00	2.05×10^{-3}	1
C01161	3,4-Dihydroxybenzeneacetic acid	168.0427	1422.5	6.41	8.98×10^{-3}	1
C2136	2-Hydroxybutyric Acid	104.0472	243	5.54	9.31×10^{-3}	1

External identifier - KEGG/HMDB entry of the identified compound; Neutral mass (Da) - The neutral monoisotope mass of the metabolite (i.e., labelled mass - the mass of the labelling group); Normalized RT (s) - The corrected retention time of the peak pair with universal RT Calibrant data; Fold change - The ratio of the average peak ratio values (FS03CM/CMGS); *p*-value - From student's t-test (FS03CM Vs CMGS)

Hydroxyphenyllactic acid or 4-hydroxyphenyllactate is a tyrosine metabolite found to be produced by bacteria. It is found in individuals with unusual gut microflora [400] and is a microbial metabolite detected in culture medium of a wide range of bacteria including *Clostridium* species [401]. Hydroxyphenyllactic acid has shown antifungal and antimycotoxin properties [402, 403]. To date, no study could be found in the literature describing antibacterial properties of hydroxyphenyllactic acid. Therefore, future studies are required to understand the antibacterial potential of hydroxyphenyllactic acid. Nevertheless, this compound was found to possess carcinogenic properties by inducing leukemic changes and hepatomas in mice [404, 405].

Another metabolite produced by FS03 using CMGS as the growth medium was 3-hydroxyphenylacetic acid (3-HPAA). 3-HPAA is a monocarboxylic acid belonging to the hydroxycinnamic acid group and the structure consists of a one hydroxyl group attached to its benzene ring [335]. This compound has been reported to have antimicrobial activity against *Pseudomonas aeruginosa* by affecting multiple bacterial processes [338]. Therefore, it may play a role in the antimicrobial activity of FS03CM against *P. aeruginosa*. As there is no information on its antimicrobial efficacy against other bacteria, it will be worth investigating its antimicrobial potential against a wide range of bacteria with the intention of identifying its potential antimicrobial applications in food quality, safety, and human health.

Acetic acid is a monocarboxylic acid and has found applications in medicine and food industry [406]. *Clostridium* spp. have been reported to produce acetic acid through anaerobic fermentation of glucose and cellulose [406]. *Paraclostridium bifermentans* NCTC506 has previously been found to produce acetic acid in fastidious anaerobe broth (FAB) containing glucose [407]. The current study reported the production of acetic acid by anaerobic bacterium FS03, closely related to *Terrisporobacter glycolicus* derived from soil, in glucose containing CMGS medium. Acetic acid has a long history of usage as an effective anti-septic agent. It has successfully been used to treat plague, infections (chest, ear, and urinary tract), and wounds during the World War I [408, 409]. Acetic acid is used in the food industry as a natural preservative and acidulant and has been recognized as a GRAS (generally recognized as safe) additive [410]. Acetic acid has been tested against food spoilage and pathogenic bacteria in various food matrixes and considered in hurdle

technology for preservation [411, 412]. The presence of acetic acid in FS03CM may contribute to its antimicrobial property.

Isobutyric acid/isobutanoic acid is a carboxylic acid and a branched short chain fatty acid. It is produced by the fermentation of branched amino acids resulted from undigested proteins in the colon by gut microbiota in humans [413]. *Clostridium uticellarii* has been reported to produce isobutyric acid from methanol [414]. The current study reported the production of isobutyric acid by anaerobic bacterium FS03, closely related to *Terrisporobacter glycolicus* in animal protein rich CMGS medium. This compound has found applications in the food industry as a flavouring agent and is permitted for direct addition to food for human consumption by U.S. Food and Drug Administration (FDA) [415]. A previous study investigated the antibacterial activity of short chain fatty acids (SCFAs), including isobutyric acid, against several oral microorganisms and demonstrated that isobutyric acid was active against all test bacteria at concentration ranging from 1.4 mg/mL to over 2.5 mg/mL [416]. It was also found to inhibit the biofilm formation of *Staphylococcus epidermidis* 1457 [417]. The presence of isobutyric acid in FS03CM may be associated with its antimicrobial activity. Nevertheless, further studies are required to understand the antimicrobial potential of isobutyric acid against food spoilage and pathogenic bacteria.

Valeric acid/pentanoic acid is an alkyl carboxylic acid and it is found in plants and animals [418]. This compound is detected in human faeces and known to be produced by gut microbiota including *Clostridium* species [419]. The current study suggested the production of valeric acid by anaerobic bacterium FS03, closely related to *Terrisporobacter glycolicus* in CMGS medium. Kovanda, Zhang [420] investigated the antimicrobial activity of seven organic acids and their derivatives including valeric acid and the results demonstrated that valeric acid was active against both Gram-positive and Gram-negative test bacteria with minimum inhibitory concentration (MIC) ranging between 0.5 – 3.2 mg/mL. As valeric acid was detected in a significant amount in FS03CM compared to CMGS, it might contribute to the antimicrobial property of FS03CM.

This work also detected the production of tryptamine by FS03, closely related to *Terrisporobacter glycolicus* in CMGS medium. Tryptamines are a group of monoamine alkaloids and found in a variety of natural sources including plants, animals, and

microorganisms [325]. This group of compounds possess mainly hallucinogenic effects interacting with the neurotransmitter system in humans [326]. Tryptamine and its derivatives have been reported to have antimicrobial activities against some bacteria and yeast [328, 329]. More information on tryptamine can be found in the non-targeted metabolomics analysis of soil conditioned media chapter 5.2.3.

Three metabolites positively identified from FS03CM; 3-hydroxyphenylacetic acid, γ-aminobutyric acid, and tryptamine were also detected in the previous work from F4SCM (Chapter 5). This is not surprising as the metabolome of F4SCM was a result of the metabolic activities of FS03 and three other *Clostridium* and closely related species in CMGS medium. It is postulated that 4-hydroxyphenyllactate, 3-hydroxyphenylacetic acid, acetic acid, isobutyric acid, valeric acid, and tryptamine may play a role in the antimicrobial property of FS03CM.

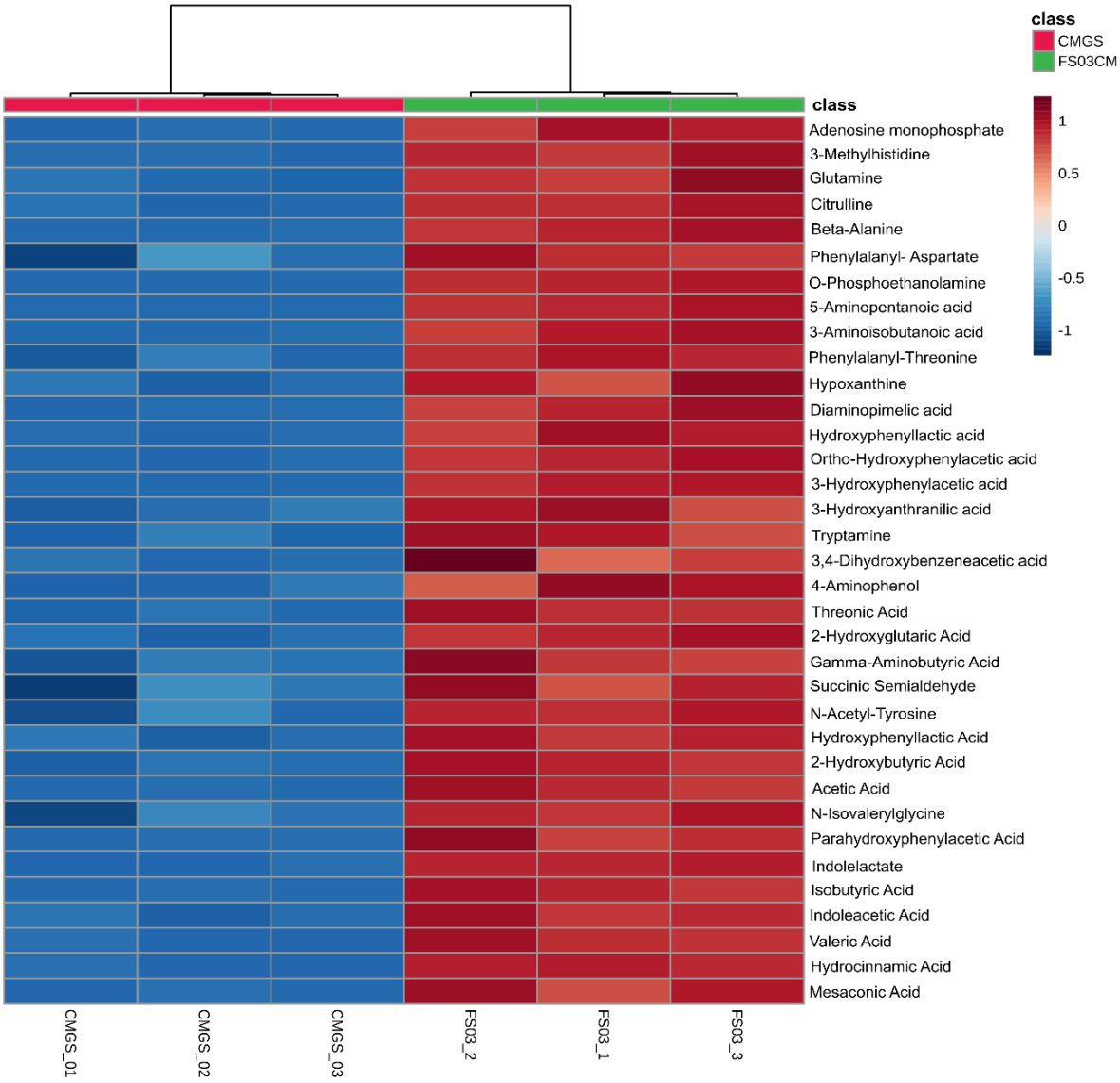


Figure 8.5: Intensity of positively identified metabolites (with tier 1 identification confidence). Heatmap shows positively identified metabolites with their abundance in FS03CM and CMGS groups. Rows represent positively identified metabolites, whereas columns represent replicates (n = 3). Metabolite intensity levels are shown using a pseudocolour scale (-1.0 to 1.0) with red representing high intensity levels and blue representing low intensity levels. CMGS (Cooked meat glucose starch medium supplemented with 0.0005% yeast extract, 0.1% hemin, and 1% vitamin K), FS03CM-conditioned medium prepared from FS03.

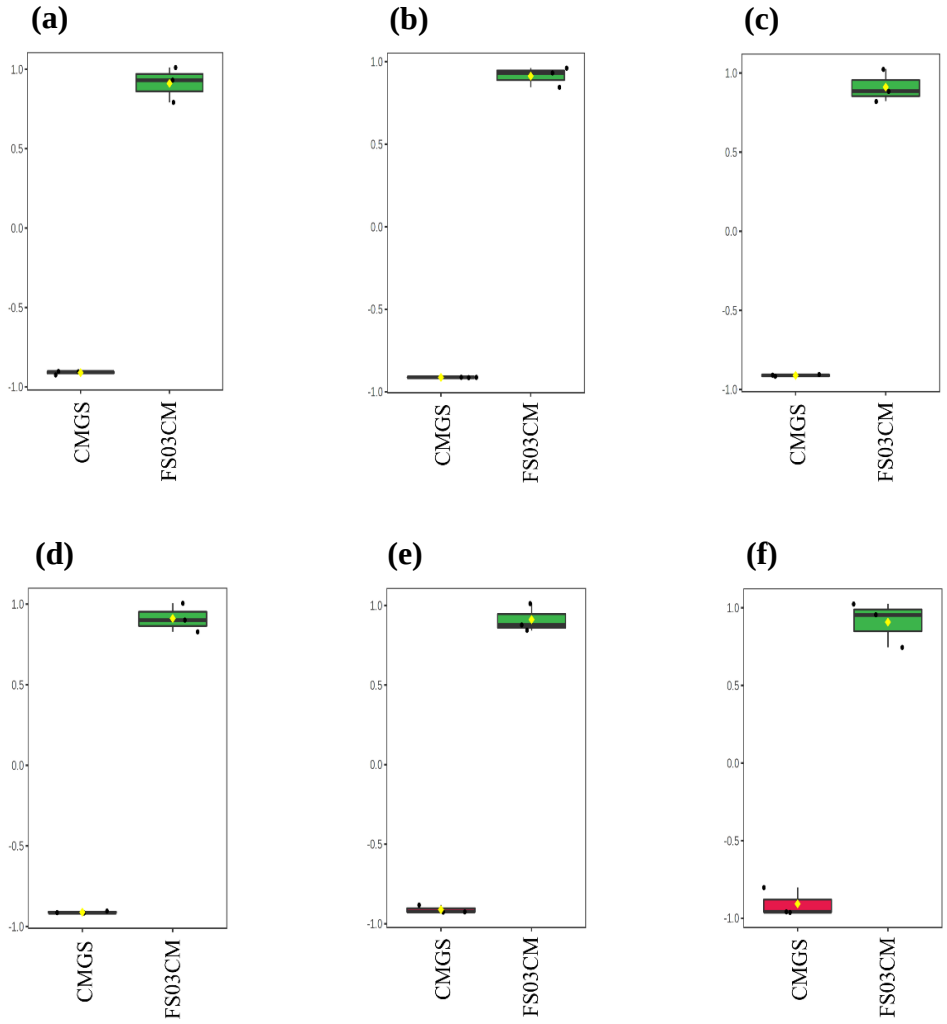


Figure 8.6: Box plots represent the relative abundance of positively identified metabolites from FS03CM, which have been reported to have antimicrobial activities. Relative abundance of 4-hydroxyphenyllactate **(a)**, 3-hydroxyphenylacetic acid **(b)**, Acetic acid **(c)**, Isobutyric acid **(d)**, Valeric acid **(e)**, and Tryptamine **(f)** in CMGS and FS03CM. Data are presented as normalized values ± S.D (n = 3). CMGS (Cooked meat glucose starch medium supplemented with 0.0005% yeast extract, 0.1% hemin, and 1% vitamin K), FS03CM- conditioned medium prepared from FS03.

This work found that anaerobic bacterium FS03 closely related to *Terrisporobacter glycolicus* produced several short chain fatty acids including acetic acid, isobutyric acid, and valeric acid as a result of its metabolic activities during the growth in cooked meat glucose starch medium. These SCFAs are some of the end products of the fermentation of dietary proteins and fibre by anaerobic intestinal microbiota in the human colon [421]. The SCFAs in the colon are believed to play a vital role in human health including the

inhibition of harmful bacteria, and regulation of inflammation, intestinal barrier function, and oxidative stress [422]. Moreover, probiotic bacteria such as *Lactobacilli* spp. and *Bifidobacterium* spp. have been reported to produce SCFAs [423, 424]. It is proposed that SCFAs together with other compounds such as hydrogen peroxide, bacteriocins, bacteriocin-like inhibitory substances (BLIS) produced by probiotic bacteria are responsible for their protective effect against harmful bacteria [425]. The metabolome of FS03CM together with its promising antimicrobial potential suggests that FS03 possesses characteristics of a potential probiotic bacterium.

Positively identified metabolites (tier 1) were only considered for identifying potential antimicrobial compounds present in the FS03CM metabolome in order to maintain the high quality of results. However, the metabolites putatively identified from FS03CM based on accurate mass and predicted retention time matches (tier 2), also showed the synthesis of compounds associated with protein and carbohydrate metabolism resulting from the growth of FS03 in CMGS. The putatively identified metabolites (tier 2) are given in Appendix 5.1 and their relative intensities in FS03CM and CMGS groups are shown in Figure 8.7.

This study focused only on identifying metabolites with potential antimicrobial activities from FS03CM. However, there could be compounds other than metabolites having antimicrobial activities in FS03CM such as peptides. The proteolytic activities of FS03 during its growth in animal protein rich CMGS medium may result in short peptides with antimicrobial properties. Low molecular weight peptides produced by microbial fermentation of protein have been reported to show various bioactivities including antimicrobial activity [346]. Therefore, it would be worth conducting future proteomics work focusing on antimicrobial peptides from FS03CM.

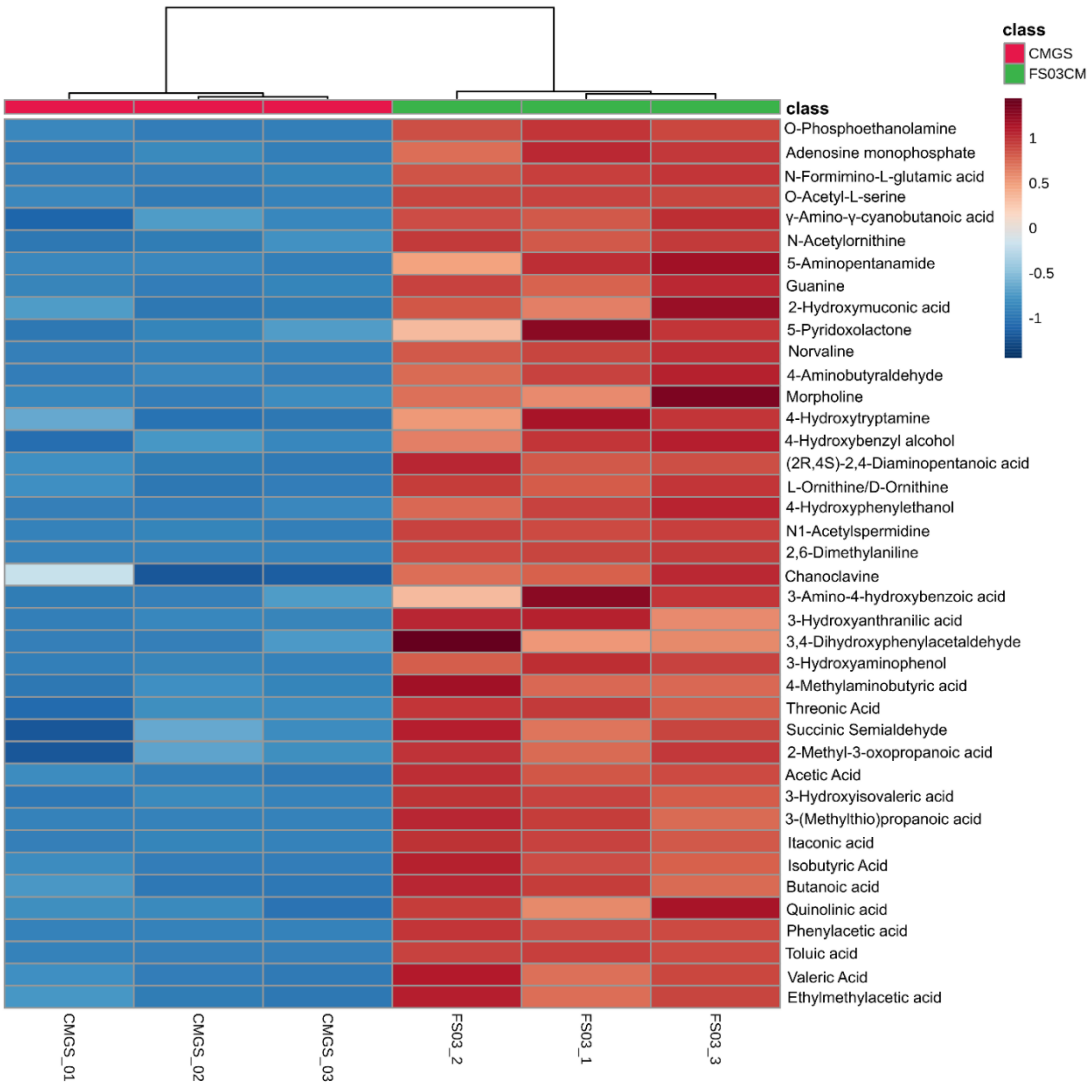


Figure 8.7: Intensity of putatively identified metabolites (with tier 2 identification confidence). Heatmap shows putatively identified metabolites with their abundance in FS03CM and CMGS groups. Rows represent putatively identified metabolites, whereas columns represent biological replicates (n = 3). Metabolite intensity levels are shown using a pseudocolour scale (-1.0 to 1.0) with red representing high intensity levels and blue representing low intensity levels. CMGS (Cooked meat glucose starch medium supplemented with 0.0005% yeast extract, 0.1% hemin, and 1% vitamin K), FS03CM-conditioned medium prepared from FS03.

CIL-LC-MS is a powerful metabolomics technique, which can be used for in-depth metabolome analysis with good quantification accuracy [398]. The present work included two isotope labelling techniques, ^{12}C-/^{13}C-dansyl chlorite labelling and ^{12}C-/^{13}C-dimethylaminophenacyl (DmPA) bromide labelling targeting metabolites containing amine, phenol, and carboxyl groups. The use of two channels certainly increased the

metabolome coverage of FS03CM. However, one of the limitations of this study was not targeting metabolites with other functional groups such as hydroxyls and carbonyls. Therefore, this study might have missed the detection of some metabolites with those functional groups.

The present work focused on known unknown metabolites (previously characterized metabolites, but unknown in the sample) in FS03CM as metabolite identification was carried out using metabolite libraries containing previously characterized metabolites. Therefore, another limitation of this study includes incomplete annotation of significantly abundant metabolites in FS03CM. Out of 683 significantly abundant peak pairs detected in FS03CM, only 48 peak pairs were identified with tier 1 metabolite identification confidence. This could be due to the lack of experimental data of labelled standard compounds in the library and the novelty of detected metabolites. These unidentified metabolites produced by FS03 may also contribute to the antimicrobial activity of FS03CM.

8.2.4 Conclusions

The present study showed significant changes in the metabolite composition of CMGS after the growth of FS03 closely related to *Terrisporobacter glycolicus*, using multivariate data analysis (PCA analysis and hierarchical cluster analysis). The volcano plot analysis of FS03CM and CMGS peak pairs discriminated metabolites produced by the metabolic activities of FS03 in CMGS. Metabolite identification with the highest confidence level (tier 1) confirmed 35 metabolites produced by FS03 in CMGS medium including several putative antimicrobial metabolites; 4-hydroxyphenyllactate, 3-hydroxyphenylacetic acid, acetic acid, isobutyric acid, valeric acid and tryptamine, which were previously reported to have antimicrobial activity against certain bacteria.

Taken together, this study revealed that FS03 could produce several metabolites with potential antimicrobial activities and many uncharacterized metabolites during its growth in CMGS medium. Further research should focus on assessing the antimicrobial potential of identified metabolites both previously known and unknown for antimicrobial activity against food spoilage and pathogenic bacteria to understand future applications of these metabolites in food preservation.

Chapter 9

General discussion and future directions

9.1 General discussion

The studies presented in this thesis focused on investigating the antimicrobial potential of *Clostridium* and closely related bacterial species. The overall objective was to understand the antimicrobial potential of *Clostridium* and closely related species and to identify secondary metabolites with potential to control the growth of bacteria associated with food spoilage, food safety, and human health. Only a few bacterial groups have been extensively studied for antimicrobial producing potential. The genus *Clostridium* and closely related bacterial species have been overlooked in bioactive compound discovery efforts such as antimicrobials although they are suggested to play important roles in the human gut and natural environments [345, 426]. In this context, the understanding acquired in the current study will help to consider the genus *Clostridium* and closely related species as a viable source of potential antimicrobial compounds and to extend the knowledge of antimicrobial potential of strict anaerobes.

This project originated from a previous observation, which showed antagonistic activities of *Clostridium* and closely related spp. against facultative anaerobes such as *Bacillus* species (Figure 1.1). This led to the hypothesis that anaerobic spore-forming bacteria (*Clostridium* and closely related species) derived from farm environments produce compounds with antimicrobial activities against other bacteria such as *Bacillus* species during their growth in CMGS medium. The initial experiments screened CMs produced by various *Clostridium* and closely related bacterial spp. derived from farm samples for

antimicrobial activity. It was suggested that metabolite composition of CMs produced from various farm samples could be different depending on the bacterial population in each farm sample leading to various degrees of antimicrobial activity. The results obtained in the stage I screening showed that there was a superior antimicrobial potential in CMs prepared from soil than feed and dairy effluents (Figure 4.1 - 4.3). Soil is a complex ecosystem harbouring a large and diversified group of bacteria. Soil bacteria, actinomycetes, have been extensively screened for antimicrobials and they produce more than 80% of known antimicrobials used in today's medicine and other applications [427]. However, soil anaerobes such as *Clostridium* species, have not been thoroughly investigated for their antimicrobial production [11].

The antimicrobial activity-based screening of five soil CMs (stage II) suggested that CM prepared from Farm 4 soil (F4SCM) was an attractive candidate for further investigation in search of potential antimicrobial compounds. Nevertheless, there was a variability in the susceptibility of test bacteria against the five soil CMs including F4SCM, where it was found that CMs were active against all test Gram-positive bacteria (*Bacillus mycoides*, *Bacillus cereus*, and *Bacillus pumilus*), but not active against Gram-negative bacteria (*Escherichia coli* and two *Salmonella enterica* serovars) except *Pseudomonas aeruginosa* (Figure 4.4 and 4.5). However, the growth inhibition activity of soil CMs against *P. aeruginosa* suggests that soil CMs may have selective activity against some Gram-negative bacteria.

The isolation and identification of bacteria confirmed the presence of *Clostridium* and closely related bacterial species in the five soil CMs. Six *Clostridium* species, two *Paraclostridium* species (formally belonging to *Clostridium* species) and one *Terrisporobacter* species (formally belonging to *Clostridium* species) were identified across the five soil CMs using 16S rRNA gene sequence analysis (Figure 4.6). The varied bacterial species composition in all five soil CMs partially explained the variations in their antimicrobial profiles. F4SCM, which demonstrated the strongest antimicrobial activity among the five soil CMs, was a result of four different bacterial species closely related to *Paraclostridium bifermentans*, *Clostridium cadaveris*, *Clostridium glycolicum/Terrisporobacter glycolicus*, and *Clostridium senegalense*. The spiking study further confirmed the involvement of these four *Clostridium* and closely related species in the antimicrobial property of F4SCM (Figure 4.7). The impact of various temperature,

pH, and protease treatments on the activity of F4SCM suggested that there might be multiple active compounds with various physiochemical characteristics in the F4SCM (Figure 4.8 – 4.10). It is commonly reported that multiple active compounds in a crude solution like CM act in a synergistic manner contributing to the overall antimicrobial activity of the crude medium [428, 429].

Antimicrobial studies have benefited from the advancement of omics platforms such as metabolomics and genomics. These next-generation approaches have increased the chances of finding antimicrobial compounds from a wide range of microorganisms including poorly investigated bacterial and fungal species [430]. CM is conceivably a complex mixture of metabolites of the extracted *Clostridium* and closely related spp., whose compounds consist of a wide range of polarity and molecular mass. Therefore, the non-targeted metabolomics was used for a comprehensive analysis of all the measurable metabolites in the CM of interest looking for putative antimicrobial compounds (Chapter 5 and Chapter 8). The first non-targeted metabolomics study was performed to understand the metabolite composition of five soil CMs using LC-MS. The purpose of using two LC conditions and both positive and negative ionization modes of MS detection was to improve the metabolome coverage. As expected, the growth of *Clostridium* and closely related spp. in CMGS significantly changed the growth medium's metabolite composition (Figure 5.2, and Figure 5.3). This partially explains the transformation of CMGS growth medium into CMs with antimicrobial properties. There were some variabilities among the five soil CMs in terms of their metabolite profiles and these probably resulted from the combined metabolic activities of different bacterial populations. Distinctive metabolite profiles of F4SCM may explain its relatively strong antimicrobial activity. Therefore, the metabolite composition of F4SCM was further analysed to identify putative antimicrobial metabolites. The comparison between F4SCM and CMGS metabolite profiles using the t-test and fold change analyses identified metabolites produced by *Clostridium* and closely related species and those were the compounds associated with F4SCM's antimicrobial activity (Figure 5.4). The putative identification of significantly abundant metabolite features in F4SCM with level 2 identification confidence confirmed the presence of three putative antimicrobial metabolites: 2-hydroxyisocaproic acid, 3-hydroxyphenylacetic acid, and tryptamine (Table 5.2). The analysis of microbial exacts has always been inherently challenging and no single study has been able to detect or annotate its entire metabolome as in the current study [431]. There were many

significantly abundant metabolite features in F4SCM resulting from the growth of *Clostridium* and closely related species, which were not identified at level 2 identification confidence. Therefore, arguably some of the unknown metabolites and other compounds such as peptides, which have not been detected by the current study, may also contribute to the antimicrobial activity of F4SCM.

Four different bacterial isolates belonging to *Clostridium* and closely related species (FS01, FS2.2, FS03, and FS04) derived from F4SCM, were investigated for their antimicrobial potential using culture and genome-based methods. As expected, the culture-based studies revealed that all four isolates possessed various degrees of antimicrobial activity against Gram-positive and Gram-negative bacteria (Figure 6.2). Their overall antimicrobial activity profile was similar to the antimicrobial activity profile of F4SCM indicating their relationship. However, one set of laboratory growth conditions cannot be expected to show the full antimicrobial potential of these bacteria as the activation of some genes may require specific environmental signal molecules. Alternatively, genome mining for BGCs may uncover the hidden metabolic potential of these isolates in producing antimicrobial secondary metabolites in a culture-independent manner. The genome-based approach consisted of whole-genome sequencing of four bacterial isolates and mining their genomes for the presence of secondary metabolite biosynthetic gene clusters. Whole genome-based phylogenetic analysis confirmed that FS01, FS2.2, and FS04 belong to the species *Paraclostridium bifermentans*, *Clostridium cadaveris*, and *Clostridium senegalense* respectively, while FS03 may represent a novel species (closely related to *Terrisporobacter glycolicus*) (Table 6.2). antiSMASH predicted the presence of BGCs encoding for uncharacterized RiPPs and NRPs in the genomes of four *Clostridium* and closely related species indicating their potential to produce antimicrobial secondary metabolites (Figure 6.7).

HICA was given much attention in the current study among the putative antimicrobials identified from F4SCM because of its good biosafety profile. As F4SCM was a product of the combined metabolic activities of FS01, FS2.2, FS03, and FS04, the ability of these bacterial isolates to produce HICA was predicted using their whole-genome sequences. Hydroxyisocaproate dehydrogenase (HicD) is an important enzyme necessary to produce HICA through leucine degradation pathway. Therefore, the presence of HicD enzyme-coding regions in the draft whole genomes of all four bacterial isolates was used as an

indication of their potential to produce HICA. The results predicted that FS01 might be able to produce HICA through leucine degradation pathway (Table 6.6). This was confirmed by quantifying HICA in its CM (FS01CM) using LC-MS analysis. The addition of extraneous leucine to the CMGS increased the production of HICA by FS01 indicating the activation of the leucine degradation pathway. These results confirm that FS01 can produce HICA in CMGS medium (Figure 6.13).

The only application of HICA, that has been studied so far is its use as an interappointment medication in the treatment of root canal infections [266]. The natural distribution of HICA in the human body and some food products suggests its potential use as a natural food preservative. However, there was no study focusing on evaluating the antimicrobial potential of HICA against food spoilage and pathogenic bacteria. Therefore, the current study investigated the antimicrobial effectiveness of HICA against several food spoilage and pathogenic bacteria and its possible antimicrobial mechanism against Gram-positive and Gram-negative bacteria. HICA was a potent antimicrobial compound against both Gram-positive and Gram-negative bacteria as evident by MIC values (Table 7.1). The time dependant reduction of the cell viability of both Gram-positive and Gram-negative bacteria after HICA treatment confirmed the bactericidal effect of HICA. Nevertheless, the Gram-positive bacteria tended to lose their cell viability faster than Gram-negative bacteria after the HICA addition (Figure 7.2). This might be because of barrier properties of the outer membrane of Gram-negative bacteria slowing down the access of HICA to inner cell target/s. The inhibition of *B. cereus* spore germination and subsequent vegetative growth further indicated the potential of HICA as a food bio-preservative (Figure 7.3).

Many antimicrobial agents exert their antimicrobial action by initiating their interactions with the cell envelope [432]. Several fluorescence-based assays were conducted in the current study to assess the effect of HICA on the cell envelope of Gram-positive and Gram-negative bacteria. HICA induced the loss of cell membrane integrity of both Gram positive and Gram-negative bacteria indicating cell membrane damage (Figures 7.5 and 7.6). These results indicate that the barrier properties of the outer membrane of Gram-negative bacteria should have been compromised by HICA treatment. As expected, NPN fluorescence assay confirmed HICA's ability to permeabilize the outer membrane allowing its access to the cytoplasmic membrane and inner structures (Figure 7.7). HICA

was able to disrupt the cytoplasmic membrane potential of both Gram-positive and Gram-negative bacteria (Figure 7.8 and 7.9). HICA may interfere with other cellular functions by the depolarization of cytoplasmic membrane of the bacteria [433]. SEM and TEM images further confirmed the loss of cell membrane integrity and the release of cellular content (Figure 7.10, 7.11, 7.12, and 7.13). These results indicate that the cell cytoplasmic membrane represents the primary target of HICA.

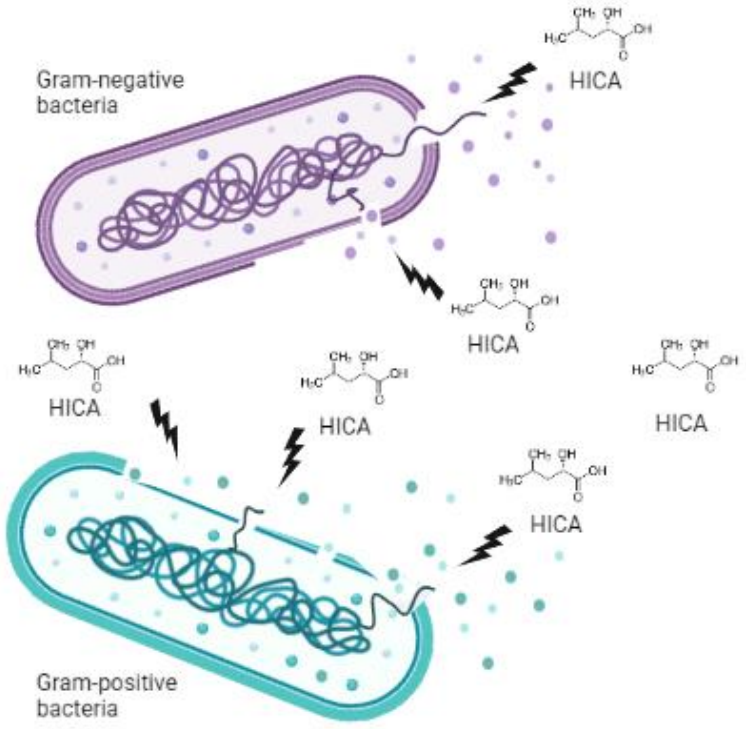


Figure 9.1: Illustration showing the potential antimicrobial mechanism of HICA against Gram-positive and Gram-negative bacteria.

The second non-targeted metabolomics study was carried out to investigate the metabolite profile of FS03CM and to identify putative antimicrobial metabolites from its metabolome. FS03CM was selected among four CMs prepared from F4SCM isolates based on its promising antimicrobial activity (Figure 6.2). The methods involved carrying out two-channel isotope labelling, mass detection by LC-MS, and data processing to get the total metabolome profile. As expected, the metabolite composition of CMGS was significantly altered by the growth of FS01 as evident by PCA and hierarchical cluster

analysis (Figure 8.3). The peak pairs belonging to metabolites produced by FS01 were discriminated by comparing the metabolite profile of FS03CM with CMGS (Figure 8.4). Metabolite identification at the tier 1 positive identification level annotated 35 different metabolites produced by FS01 including 4-hydroxyphenyllactate, 3-hydroxyphenylacetic acid, acetic acid, isobutyric acid, valeric acid, and tryptamine, which had previously been reported to possess antimicrobial activities against some microorganisms (Table 8.3 and Figure 8.5). Most of the positively identified metabolites had no reported information on their antimicrobial potential against bacteria as evident by the literature review. Therefore, further studies need to be conducted to assess their antimicrobial potential against significant bacteria associated with food spoilage, pathogenicity, and human health.

9.2 Limitations and opportunities for future studies

Even though the work presented in this thesis produced useful information regarding the antimicrobial potential of *Clostridium* and closely related species, the findings uncovered other unanswered questions and there were limitations in the methods used. The findings, unanswered questions, and limitations present opportunities for future work.

1) Culture-based and culture-independent methods were used in the current study to evaluate the antimicrobial potential of farm 4 soil isolates. The culture-based screening studies used only a single laboratory growth condition to assess the antimicrobial potential of *Clostridium* and closely related spp. isolated from Farm 4 soil. However, due to the requirement of specific abiotic and/or biotic environmental cues for the activation of some metabolic pathways in bacteria, variations in the growth conditions need to be tested to explore the possibility of any additional antimicrobials. Therefore, further studies can be carried out by mimicking the natural physiochemical conditions of bacterial isolates under laboratory setting to uncover any hidden antimicrobial compound synthesis (e.g., co-cultivation of two or more different bacteria and the introduction of soil extract to growth medium).

2) Another limitation of this study was not being able to characterize all identified BGCs belonging to the RiPP and NRPS groups as they had no associations to any currently known clostridial or other secondary metabolites. Therefore, possible extension of this work is to uncover the associated products of uncharacterized RiPPs and NRPS biosynthetic gene clusters identified from four *Clostridium* and closely related isolates. Heterologous expression of predicted BGCs is one of the approaches that can be used to identify associated products [434].

3) The non-targeted metabolomics work carried out in the current study only focused on identifying known/previously characterized metabolites in the metabolome. Therefore, one of the limitations was incomplete annotation of metabolites of interest. The unknown/previously uncharacterized metabolites in the metabolome were not identified in this study. Therefore, other metabolomics techniques need

to be considered targeting novel active metabolites in the CM of interest. The use of liquid chromatography coupled to nuclear magnetic resonance spectrometry (LC-NMR) will provide the possibility of obtaining structural information of unknown active metabolites in CM [435].

4) Several metabolites putatively identified from F4SCM and FS03CM had been reported previously to possess antimicrobial activities against some microorganisms and they were considered as putative antimicrobials in this study. However, most of the identified metabolites from the two CMs had no information on their antimicrobial potential against some key food spoilage and pathogenic bacteria. The current study investigated only one metabolite (HICA) identified from F4SCM for further information on its antimicrobial property. As these identified metabolites are natural compounds produced through the metabolic activities of *Clostridium* and closely related species, it is worth evaluating their antimicrobial potential with the intention of potential applications in food bio-preservation.

5) This study provided no information on the active peptides, which might be present in the CM and a major group of antimicrobial compounds. The proteolytic activity of *Clostridium* and closely related bacterial spp. during their growth in animal protein rich CMGS growth medium would most likely result in various protein fragments in the CM. Future proteomics studies can be performed to purify and identify any antimicrobial peptides present in the CM of interest.

6) HICA increased the permeability of the outer membrane of Gram-negative bacteria and disrupted the cytoplasmic membrane potential of both Gram-positive and Gram-negative bacteria suggesting its possible antimicrobial target. Future studies are required to understand what chemical properties of HICA are responsible for its activity and how it interrupts the bacterial membranes.

7) This study employed only a few bacterial strains from each target bacterial species for the assessment of antimicrobial activity of CMs and HICA. It is well established that there could be strain variations in the same bacterial species to the susceptibility of the same antimicrobial compound. Therefore, antimicrobials need to be tested against a reasonably large number of strains from each target species to provide solid evidence on their activity against each target bacterial species.

9.3 Conclusions

This research study has revealed that *Clostridium* and closely related species derived from farm environments can produce metabolites with antimicrobial properties. The genome-based studies have disclosed the genetic potential of select *Clostridium* and closely related spp. for producing secondary metabolites belonging to antimicrobial compound groups including RiPPs and NRPs. Non-targeted metabolomics together with culture-based methods have shown the metabolic capability of *Clostridium* and closely related spp. to produce putative antimicrobial metabolites such as HICA. HICA showed a promising potential for use in food preservation and medical purposes with its broad-spectrum antimicrobial activity. HICA exerts its antimicrobial activity by primarily targeting the bacterial cytoplasmic membrane. The knowledge obtained in this study will help future investigations to identify and characterize potent antimicrobials from *Clostridium* and closely related species and expands the current understanding of the potential for the production of bioactive compounds from anaerobic bacteria, *Clostridium* and closely related species.

Reference List

[1] Burnett-Boothroyd SC, McCarthy BJ. Antimicrobial treatments of textiles for hygiene and infection control applications: an industrial perspective. In: McCarthy BJ, editor. Textiles for Hygiene and Infection Control: *Woodhead Publishing*; 2011. p. 196-209.

[2] Tufail M, Hussain S, Malik F, Mirza T, Parveen G, Shafaat S, et al. Isolation and evaluation of antibacterial activity of bacteriocin produced by *Lactobacillus bulgaricus* from yogurt. *African Journal of Microbiology Research*. 2011;5:3842-7.

[3] Dyke JW, Angones D, Bhcikta D, Tenjarla G, Kumar A. Antimicrobial activity of new antibiotics against bacterial isolates from a community hospital. *Chemotherapy*. 1993;39:315-21.

[4] Wang XB, Ren ZJ, Mei YD, Liu MH, Chen M, Si WJ, et al. Design, synthesis, and antifungal activity of 3-(thiophen-2-yl)-1,5-dihydro-2H-pyrrol-2-one derivatives bearing a carbonic ester group. *Journal of Heterocyclic Chemistry*. 2019;56:165-71.

[5] Angulo FJ, Baker NL, Olsen SJ, Anderson A, Barrett TJ. Antimicrobial use in agriculture: controlling the transfer of antimicrobial resistance to humans. *Seminars in Pediatric Infectious Diseases*. 2004;15:78-85.

[6] Quinto EJ, Caro I, Villalobos-Delgado LH, Mateo J, De-Mateo-Silleras B, Redondo-Del-Río MP. Food safety through natural antimicrobials. *Antibiotics*. 2019;8:208.

[7] Mohr KI. History of antibiotics research. In: Stadler M, Dersch P, editors. How to Overcome The Antibiotic Crisis : Facts, Challenges, Technologies and Future Perspectives. Cham: *Springer International Publishing*; 2016. p. 237-72.

[8] National research council committee on new directions in the study of antimicrobial therapeutics: New classes of a, national research council committee on new directions in the study of antimicrobial therapeutics i. The national academies collection: reports funded by national institutes of health. Treating Infectious Diseases in a Microbial World: Report of Two Workshops on Novel Antimicrobial Therapeutics. Washington (DC): *National Academies Press (US)*; 2006.

[9] Stubbendieck RM, Vargas-Bautista C, Straight PD. Bacterial communities: interactions to scale. *Frontiers in Microbiology*. 2016;7.

[10] Watve MG, Tickoo R, Jog MM, Bhole BD. How many antibiotics are produced by the genus *Streptomyces*? *Archives of Microbiology*. 2001;176:386-90.

[11] Schmitz R, Daniel R, Deppenmeier U, Gottschalk G. The anaerobic way of life. The Prokaryotes2006. p. 86-101.

[12] Behnken S, Hertweck C. Anaerobic bacteria as producers of antibiotics. *Applied Microbiology and Biotechnology*. 2012;96:61-7.

[13] Letzel A, Pidot SJ, Hertweck C. A genomic approach to the cryptic secondary metabolome of the anaerobic world. *Natural Product Reports*. 2013;30:392-428.

[14] Lincke T, Behnken S, Ishida K, Roth M, Hertweck C. Closthioamide: an unprecedented polythioamide antibiotic from the strictly anaerobic bacterium *Clostridium cellulolyticum*. *Angewandte Chemie International Edition*. 2010;49:2011-3.

[15] Pidot S, Ishida K, Cyrulies M, Hertweck C. Discovery of clostrubin, an exceptional polyphenolic polyketide antibiotic from a strictly anaerobic bacterium. *Angewandte Chemie International Edition*. 2014;53:7856-9.
[16] Schieferdecker S, Shabuer G, Knuepfer U, Hertweck C. Clostrindolin is an antimycobacterial pyrone alkaloid from *Clostridium beijerinckii*. *Organic & Biomolecular Chemistry*. 2019;17:6119-21.
[17] Davies J, Ryan KS. Introducing the parvome: bioactive compounds in the microbial world. *ACS Chemical Biology*. 2012;7:252-9.
[18] Sinha RP, Häder D-P. Chapter 1 - Introduction. In: Sinha Rp, Häder D-P, editors. Natural Bioactive Compounds: *Academic Press*; 2021. p. 1-17.
[19] Fraser K, Lane GA, Otter DE, Hemar Y, Quek S, Harrison SJ, et al. Analysis of metabolic markers of tea origin by UHPLC and high resolution mass spectrometry. *Food Research International*. 2013;2013 v.53 no.2:pp. 827-35.
[20] Demain AL, Sanchez S. Microbial drug discovery: 80 years of progress. *The Journal of Antibiotics*. 2009;62:5-16.
[21] Wright PM, Seiple IB, Myers AG. The evolving role of chemical synthesis in antibacterial drug discovery. *Angewandte Chemie (International ed in English)*. 2014;53:8840-69.
[22] Lahlou M. Screening of natural products for drug discovery. *Expert Opinion on Drug Discovery*. 2007;2:697-705.
[23] CDC-National Centre for Health Statistics. Life Expectancy.
[24] Barber M, Waterworth PM. Antibacterial activity of the penicillins. *British Medical Journal*. 1962;1:1159-64.
[25] Nathwani D, Wood MJ. Penicillins. *Drugs*. 1993;45:866-94.
[26] Cabello FC. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environmental Microbiology*. 2006;8:1137-44.
[27] Vidaver AK. Uses of antimicrobials in plant agriculture. *Clinical Infectious Diseases*. 2002;34:S107-S10.
[28] Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. *Pharmacy and Therapeutics*. 2015;40:277-83.
[29] World Health Organization. WHO guidelines on use of medically important antimicrobials in food-producing animals. Geneva, Switzerland2017.
[30] World Health Organization. WHO estimates of the global burden of foodborne diseases. Geneva, Switzerland2015.
[31] Yang S, Lin C, Sung CT, Fang J. Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. *Frontiers in Microbiology*. 2014;5.
[32] Kraszewska J, Beckett MC, James TC, Bond U. Comparative analysis of the antimicrobial activities of plant defensin-like and ultrashort peptides against food-spoiling bacteria. *Applied and Environmental Microbiology*. 2016;82:4288-98.
[33] Cammack R, Joannou CL, Cui X, Torres Martinez C, Maraj SR, Hughes MN. Nitrite and nitrosyl compounds in food preservation. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*. 1999;1411:475-88.
[34] Juneja VK, Sofos JN. Control of foodborne microorganisms: *Boca Raton, Fla. : CRC Press, 2001*; 2001.
[35] Hintz T, Matthews KK, Di R. The use of plant antimicrobial compounds for food preservation. *BioMed Research International*. 2015;2015:1-12.
[36] Fan AM, Steinberg VE. Health implications of nitrate and nitrite in drinking water: an update on methemoglobinemia occurrence and reproductive and

developmental toxicity. *Regulatory Toxicology and Pharmacology*. 1996;23:35-43.
[37] Kennedy N, Smith CP, McWhinney P. Faulty sausage production causing methaemoglobinaemia. *Archives of Disease in Childhood*. 1997;76:367-8.
[38] Reis JA, Paula AT, Casarotti SN, Penna ALB. Lactic acid bacteria antimicrobial compounds: characteristics and applications. *Food Engineering Reviews*. 2012;4:124-40.
[39] Delves-Broughton J, Blackburn P, Evans RJ, Hugenholtz J. Applications of the bacteriocin, nisin. *Antonie Van Leeuwenhoek*. 1996;69:193-202.
[40] de Arauz LJ, Jozala AF, Mazzola PG, Vessoni Penna TC. Nisin biotechnological production and application: a review. *Trends in Food Science & Technology*. 2009;20:146-54.
[41] Hancock REW. The end of an era? *Nature Reviews Drug Discovery*. 2007;6:28.
[42] CDC. Antibiotic resistance threats in the United States. Atlanta, GA: *U.S. Department of Health and Human Services, CDC*; 2019.
[43] Davies D. Understanding biofilm resistance to antibacterial agents. *Nature Reviews Drug Discovery*. 2003;2:114-22.
[44] Iglewski BH. *Pseudomonas*. In: Baron S, editor. Medical Microbiology. Galveston (TX): University of Texas Medical Branch at Galveston1996.
[45] Food Standards Agency. Approved additives and E numbers. UK2018.
[46] Verheul A, Russell NJ, Van'T Hof R, Rombouts FM, Abee T. Modifications of membrane phospholipid composition in nisin-resistant *Listeria monocytogenes* Scott A. *Applied and Environmental Microbiology*. 1997;63:3451-7.
[47] Ming X, Daeschel MA. Nisin resistance of foodborne bacteria and the specific resistance responses of *Listeria monocytogenes* Scott A. *Journal of Food Protection*. 1993;56:944-8.
[48] Rolain JM, Baquero F. The refusal of the society to accept antibiotic toxicity: missing opportunities for therapy of severe infections. *Clinical Microbiology and Infection*. 2016;22:423-7.
[49] Hibbing ME, Fuqua C, Parsek MR, Peterson SB. Bacterial competition: surviving and thriving in the microbial jungle. *Nature Reviews Microbiology*. 2010;8:15-25.
[50] Spiteller P. Chemical ecology of fungi. *Natural Product Reports*. 2015;32:971-93.
[51] Zain ME, Awaad AS, Al-Othman MR, Alafeefy AM, El-Meligy RM. Biological activity of fungal secondary metabolites. In: Dekker M, editor. Handbook of Industrial Mycology. NY2002.
[52] Ruiz B, Chávez A, Forero A, García-Huante Y, Romero A, Sánchez M, et al. Production of microbial secondary metabolites: regulation by the carbon source. *Critical Reviews in Microbiology*. 2010;36:146-67.
[53] Yim G, Wang HH, Davies J. Antibiotics as signalling molecules. *Philosophical Transactions of the Royal Society of London Series B, Biological Sciences*. 2007;362:1195-200.
[54] Nicolaou KC, Rigol S. A brief history of antibiotics and select advances in their synthesis. *Journal of Antibiotics*. 2018;71:153-84.
[55] Fleming A. On the antibacterial action of cultures of a *Penicillium*, with special reference to their use in the isolation of *B. influenzæ*. *British Journal of Experimental Pathology*. 1929;10:226-36.
[56] Künzler M. How fungi defend themselves against microbial competitors and animal predators. *PLOS Pathogens*. 2018;14:e1007184.
[57] Wenzel SC, Müller R. Myxobacteria—'microbial factories' for the production of bioactive secondary metabolites. *Molecular BioSystems*. 2009;5:567-74.

[58] Welker M, Dittmann E, von Döhren H. Chapter Two - Cyanobacteria as a source of natural products. In: Hopwood DA, editor. Methods in Enzymology: *Academic Press*; 2012. p. 23-46.
[59] Fickers P. Antibiotic compounds from *Bacillus*: why are they so amazing? *American Journal of Biochemistry and Biotechnology*. 2012;8:38-43.
[60] Gross H, Loper JE. Genomics of secondary metabolite production by *Pseudomonas* spp. *Natural Product Reports*. 2009;26:1408-46.
[61] Chen H, Hoover DG. Bacteriocins and their food applications. *Comprehensive Reviews in Food Science and Food Safety*. 2003;2:82-100.
[62] Pidot SJ, Coyne S, Kloss F, Hertweck C. Antibiotics from neglected bacterial sources. *International Journal of Medical Microbiology*. 2014;304:14-22.
[63] Sammer UF, Völksch B, Möllmann U, Schmidtke M, Spiteller P, Spiteller M, et al. 2-amino-3-(Oxirane-2,3-dicarboxamido)-propanoyl-valine, an effective peptide antibiotic from the epiphyte *Pantoea agglomerans* 48b/90. *Applied and Environmental Microbiology*. 2009;75:7710-7.
[64] Bode HB. Entomopathogenic bacteria as a source of secondary metabolites. *Current Opinion in Chemical Biology*. 2009;13:224-30.
[65] Stincone P, Brandelli A. Marine bacteria as source of antimicrobial compounds. *Critical Reviews in Biotechnology*. 2020;40:306-19.
[66] Pahalagedara ASNW, Flint S, Palmer J, Brightwell G, Gupta TB. Antimicrobial production by strictly anaerobic *Clostridium* spp. *International Journal of Antimicrobial Agents*. 2020:105910.
[67] Juturu V, Wu JC. Microbial production of bacteriocins: latest research development and applications. *Biotechnology Advances*. 2018;36:2187-200.
[68] Zou J, Jiang H, Cheng H, Fang J, Huang G. Strategies for screening, purification and characterization of bacteriocins. *International Journal of Biological Macromolecules*. 2018;117:781-9.
[69] Fontaine A, Kucherov G, Pupin M, Jacques P, Leclère V, Caboche S. NORINE: a database of nonribosomal peptides. *Nucleic Acids Research*. 2007;36:D326-D31.
[70] Felnagle EA, Jackson EE, Chan YA, Podevels AM, Berti AD, McMahon MD, et al. Nonribosomal peptide synthetases involved in the production of medically relevant natural products. *Molecular Pharmaceutics*. 2008;5:191-211.
[71] Hammami R, Zouhir A, Le Lay C, Ben Hamida J, Fliss I. Bactibase. 2017.
[72] Todorov SD, Wachsman MB, Knoetze H, Meincken M, Dicks LMT. An antibacterial and antiviral peptide produced by *Enterococcus mundtii* ST4V isolated from soya beans. *International Journal of Antimicrobial Agents*. 2005;25:508-13.
[73] Belguesmia Y, Choiset Y, Rabesona H, Baudy-Floc'h M, Le Blay G, Haertlé T, et al. Antifungal properties of durancins isolated from *Enterococcus* durans A5-11 and of its synthetic fragments. *Letters in Applied Microbiology*. 2013;56:237-44.
[74] Sharma G, Dang S, Gupta S, Gabrani R. Antibacterial activity, cytotoxicity, and the mechanism of action of bacteriocin from *Bacillus subtilis* GAS101. *Medical Principles and Practice*. 2018;27:186-92.
[75] Wiedemann I, Breukink E, van Kraaij C, Kuipers OP, Bierbaum G, de Kruijff B, et al. Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. *Journal of Biological Chemistry*. 2001;276:1772-9.
[76] Gratia A. Sur un remarquable exemple d'antagonisme entre deux souches de colibacille. *Comptes Rendus Biologies (Paris)*. 1925;93:1040-1.

[77] Whitehead HR. A substance inhibiting bacterial growth, produced by certain strains of lactic streptococci. *The Biochemical Journal*. 1933;27:1793-800.
[78] Mattick ATR, Hirsch A, Berridge NJ. Further observations on an inhibitory substance (nisin) from lactic streptococci. *The Lancet*. 1947;250:5-8.
[79] Eluned J, Victoria S, Gary WW. Nisin and the market for commercial bacterions. TAMRC Consumer and Product Research Report. Texas, US2005.
[80] Zhang H, Liu L, Hao Y, Zhong S, Liu H, Han T, et al. Isolation and partial characterization of a bacteriocin produced by *Lactobacillus plantarum* BM-1 isolated from a traditionally fermented Chinese meat product. *Microbiology and Immunology*. 2013;57:746-55.
[81] Abdel-Haliem M, Tartour E, Enan G. Characterization, production and partial purification of a bacteriocin produced by *Lactobacillus plantarum* LPS10 isolated from pickled olives2016.
[82] Lakshminarayanan B, Guinane CM, O'Connor PM, Coakley M, Hill C, Stanton C, et al. Isolation and characterization of bacteriocin-producing bacteria from the intestinal microbiota of elderly Irish subjects. *Journal of Applied Microbiology*. 2013;114:886-98.
[83] An J, Zhu W, Liu Y, Zhang X, Sun L, Hong P, et al. Purification and characterization of a novel bacteriocin CAMT2 produced by *Bacillus amyloliquefaciens* isolated from marine fish *Epinephelus areolatus*. *Food Control*. 2015;51:278-82.
[84] Smitha S, Bhat SG. Thermostable bacteriocin BL8 from *Bacillus licheniformis* isolated from marine sediment. *Journal of Applied Microbiology*. 2013;114:688-94.
[85] Babasaki K, Takao T, Shimonishi Y, Kurahashi K. Subtilosin A, a new antibiotic peptide produced by *Bacillus subtilis* 168: isolation, structural analysis, and biogenesis. *The Journal of Biochemistry*. 1985;98:585-603.
[86] Gebhart D, Williams SR, Bishop-Lilly KA, Govoni GR, Willner KM, Butani A, et al. Novel high-molecular-weight, R-type bacteriocins of *Clostridium difficile*. *Journal of Bacteriology*. 2012;194:6240-7.
[87] Ros-Chumillas M, Esteban MD, Huertas JP, Palop A. Effect of nisin and thermal treatments on the heat resistance of *Clostridium sporogenes* spores. *Journal of Food Protection*. 2015;78:2019-23.
[88] Chung KT, Dickson JS, Crouse JD. Effects of nisin on growth of bacteria attached to meat. *Applied and Environmental Microbiology*. 1989;55:1329-33.
[89] Negash AW, Tsehai BA. Current applications of bacteriocin. *International Journal of Microbiology*. 2020;2020:1-7.
[90] Lipmann F. Nonribosomal polypeptide synthesis on polyenzyme templates. *Accounts of Chemical Research*. 1973;6:361-7.
[91] Laland SG, Zimmer TL. The protein thiotemplate mechanism of synthesis for the peptide antibiotics produced by *Bacillus brevis*. *Essays in Biochemistry*. 1973;9:31-57.
[92] Agrawal S, Acharya D, Adholeya A, Barrow CJ, Deshmukh SK. Nonribosomal peptides from marine microbes and their antimicrobial and anticancer potential. *Frontiers in Pharmacology*. 2017;8:828-.
[93] Mootz HD, Schwarzer D, Marahiel MA. Ways of assembling complex natural products on modular nonribosomal peptide synthetases. *ChemBioChem*. 2002;3:490-504.
[94] Wang H, Fewer DP, Holm L, Rouhiainen L, Sivonen K. Atlas of nonribosomal peptide and polyketide biosynthetic pathways reveals common occurrence of

nonmodular enzymes. *Proceedings of the National Academy of Sciences of the United States of America*. 2014;111:9259-64.

[95] Horwood PF, Burgess GW, Jane Oakey H. Evidence for non-ribosomal peptide synthetase production of cereulide (the emetic toxin) in *Bacillus cereus*. *FEMS Microbiology Letters*. 2004;236:319-24.

[96] Di Lorenzo M, Poppelaars S, Stork M, Nagasawa M, Tolmasky ME, Crosa JH. A nonribosomal peptide synthetase with a novel domain organization is essential for siderophore biosynthesis in *Vibrio anguillarum*. *Journal of Bacteriology*. 2004;186:7327-36.

[97] Martínez-Núñez M, López V. Nonribosomal peptides synthetases and their applications in industry. *Sustainable Chemical Processes*. 2016;4:13.

[98] Michelsen CF, Watrous J, Glaring MA, Kersten R, Koyama N, Dorrestein PC, et al. Nonribosomal peptides, key biocontrol components for *Pseudomonas fluorescens* In5, isolated from a Greenlandic suppressive soil. *mBio*. 2015;6:e00079-e.

[99] Desai JD, Banat IM. Microbial production of surfactants and their commercial potential. *Microbiology and Molecular Biology Reviews*. 1997;61:47-64.

[100] Gaudelli NM, Long DH, Townsend CA. β-Lactam formation by a non-ribosomal peptide synthetase during antibiotic biosynthesis. *Nature*. 2015;520:383.

[101] Robbel L, Marahiel MA. Daptomycin, a bacterial lipopeptide synthesized by a nonribosomal machinery. *The Journal of Biological Chemistry*. 2010;285:27501-8.

[102] Dittmann J, Wenger RM, Kleinkauf H, Lawen A. Mechanism of cyclosporin A biosynthesis. Evidence for synthesis via a single linear undecapeptide precursor. *The Journal of Biological Chemistry*. 1994;269:2841-6.

[103] John DB. The discovery and development of modified penicillin- and cephalosporin- derived β lactamase inhibitors. *Current Medicinal Chemistry*. 2004;11:1951-64.

[104] Baerson SR, Rimando AM. A plethora of polyketides: structures, biological activities, and enzymes. Polyketides: *American Chemical Society*; 2007. p. 2-14.

[105] Pfeifer BA, Khosla C. Biosynthesis of polyketides in heterologous hosts. *Microbiology and Molecular Biology Reviews*. 2001;65:106-18.

[106] Weissman KJ. Chapter 1 Introduction to polyketide biosynthesis. Methods in Enzymology: *Academic Press*; 2009. p. 3-16.

[107] David AH, David HS. Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. *Annual Review of Genetics*. 1990;24:37-62.

[108] Onwueme KC, Vos CJ, Zurita J, Ferreras JA, Quadri LEN. The dimycocerosate ester polyketide virulence factors of mycobacteria. *Progress in Lipid Research*. 2005;44:259-302.

[109] Tam EWT, Tsang C, Lau SKP, Woo PCY. Polyketides, toxins and pigments in *Penicillium marneffei*. *Toxins*. 2015;7:4421-36.

[110] Park SR, Yoo YJ, Ban Y, Yoon Y. Biosynthesis of rapamycin and its regulation: past achievements and recent progress. *The Journal of Antibiotics*. 2010;63:434.

[111] Campbell CD, Vederas JC. Biosynthesis of lovastatin and related metabolites formed by fungal iterative PKS enzymes. *Biopolymers*. 2010;93:755-63.

[112] Grimm A, Madduri K, Ali A, Hutchinson CR. Characterization of the *Streptomyces peucetius* ATCC 29050 genes encoding doxorubicin polyketide synthase. *Gene*. 1994;151:1-10.

[113] Cane DE. Programming of erythromycin biosynthesis by a modular polyketide synthase. *The Journal of Biological Chemistry*. 2010;285:27517-23.

[114] Lule V, Garg S, Gosewade S, Khedkar C. Natamycin. 2015. p. 56–62.

[115] Wang H, He X, Sun C, Gao J, Liu X, Liu H. Enhanced natamycin production by co-expression of *Vitreoscilla* hemoglobin and antibiotic positive regulators in *Streptomyces gilvosporeus*. *Biotechnology & Biotechnological Equipment*. 2018;32:470-6.
[116] Welscher YMt, Napel HHt, Balagué MM, Souza CM, Riezman H, de Kruijff B, et al. Natamycin blocks fungal growth by binding specifically to ergosterol without permeabilizing the membrane. *Journal of Biological Chemistry*. 2008;283:6393-401.
[117] Howell C, Beier R, Stipanovic R. Production of ammonia by *Enterobacter cloacae* and its possible role in the biological control of *Pythium* preemergence damping-off by the bacterium. *Phytopathology*. 1988;78:1075-8.
[118] Cherif-Silini H, Silini A, Yahiaoui B, Ouzari I, Boudabous A. Phylogenetic and plant-growth-promoting characteristics of *Bacillus* isolated from the wheat rhizosphere. *Annals of Microbiology*. 2016;66:1087-97.
[119] Fu L-H, Hu K-D, Hu L-Y, Li Y-H, Hu L-B, Yan H, et al. An antifungal role of hydrogen sulfide on the postharvest pathogens *Aspergillus niger* and *Penicillium italicum*. *PLOS ONE*. 2014;9:104206.
[120] Korpi A, Järnberg J, Pasanen A-L. Microbial volatile organic compounds. *Critical Reviews in Toxicology*. 2009;39:139-93.
[121] Lemfack MC, Gohlke B-O, Toguem Serge M T, Preissner S, Piechulla B, Preissner R. mVOC 2.0: a database of microbial volatiles. *Nucleic Acids Research*. 2017;46:D1261-D5.
[122] Tahir HAS, Gu Q, Wu H, Niu Y, Huo R, Gao X. *Bacillus* volatiles adversely affect the physiology and ultra-structure of *Ralstonia solanacearum* and induce systemic resistance in tobacco against bacterial wilt. *Scientific Reports*. 2017;7:40481.
[123] Gu Y-Q, Mo M-H, Zhou J-P, Zou C-S, Zhang K-Q. Evaluation and identification of potential organic nematicidal volatiles from soil bacteria. *Soil Biology and Biochemistry*. 2007;39:2567-75.
[124] Chen H, Xiao X, Wang J, Wu L, Zheng Z, Yu Z. Antagonistic effects of volatiles generated by *Bacillus subtilis* on spore germination and hyphal growth of the plant pathogen, *Botrytis cinerea*. *Biotechnology Letters*. 2008;30:919-23.
[125] Passmore IJ, Letertre MPM, Preston MD, Bianconi I, Harrison MA, Nasher F, et al. *Para*-cresol production by *Clostridium difficile* affects microbial diversity and membrane integrity of Gram-negative bacteria. *PLOS Pathogens*. 2018;14:e1007191.
[126] Gürtler H, Pedersen R, Anthoni U, Christophersen C, Nielsen PH, Wellington EM, et al. Albaflavenone, a sesquiterpene ketone with a zizaene skeleton produced by a streptomycete with a new rope morphology. *The Journal of Antibiotics*. 1994;47:434-9.
[127] Trombetta D, Castelli F, Sarpietro MG, Venuti V, Cristani M, Daniele C, et al. Mechanisms of antibacterial action of three monoterpenes. *Antimicrobial Agents and Chemotherapy*. 2005;49:2474-8.
[128] Yacoub T, Rima M, Karam M, Sabatier J-M, Fajloun Z. Antimicrobials from venomous animals: An overview. *Molecules*. 2020;25:2402.
[129] Khameneh B, Iranshahy M, Soheili V, Fazly Bazzaz BS. Review on plant antimicrobials: a mechanistic viewpoint. *Antimicrobial Resistance & Infection Control*. 2019;8:118.
[130] Hooper DC. Mechanisms of action of antimicrobials: focus on fluoroquinolones. *Clinical Infectious Diseases*. 2001;32:S9-S15.

[131] Vollmer W, Blanot D, De Pedro MA. Peptidoglycan structure and architecture. *FEMS Microbiology Reviews*. 2008;32:149-67.
[132] Mc Dermott PF, Walker RD, White DG. Antimicrobials: modes of action and mechanisms of resistance. *International Journal of Toxicology*. 2003;22:135-43.
[133] Liu Y, Breukink E. The membrane steps of bacterial cell wall synthesis as antibiotic targets. *Antibiotics* 2016;5.
[134] Scheffers D-J, Pinho MG. Bacterial cell wall synthesis: new insights from localization studies. *Microbiology and Molecular Biology Reviews*. 2005;69:585-607.
[135] Batson S, de Chiara C, Majce V, Lloyd AJ, Gobec S, Rea D, et al. Inhibition of D-Ala:D-Ala ligase through a phosphorylated form of the antibiotic D-cycloserine. *Nature Communications*. 2017;8:1939.
[136] Olesen SH, Ingles DJ, Yang Y, Schönbrunn E. Differential antibacterial properties of the MurA inhibitors terreic acid and fosfomycin. *Journal of Basic Microbiology*. 2014;54:322-6.
[137] Sarkar P, Yarlagadda V, Ghosh C, Haldar J. A review on cell wall synthesis inhibitors with an emphasis on glycopeptide antibiotics. *MedChemComm*. 2017;8:516-33.
[138] Lam NH, Ma Z, Ha B-Y. Electrostatic modification of the lipopolysaccharide layer: competing effects of divalent cations and polycationic or polyanionic molecules. *Soft Matter*. 2014;10:7528-44.
[139] Epand RM, Walker C, Epand RF, Magarvey NA. Molecular mechanisms of membrane targeting antibiotics. *Biochimica et Biophysica Acta (BBA) - Biomembranes*. 2016;1858:980-7.
[140] Kwa A, Kasiakou SK, Tam VH, Falagas ME. Polymyxin B: similarities to and differences from colistin (polymyxin E). *Expert Review of Anti-infective Therapy*. 2007;5:811-21.
[141] Clausell A, Garcia-Subirats M, Pujol M, Busquets MA, Rabanal F, Cajal Y. Gram-negative outer and inner membrane models: insertion of cyclic cationic lipopeptides. *The Journal of Physical Chemistry B*. 2007;111:551-63.
[142] Falagas ME, Rafailidis PI, Matthaiou DK. Resistance to polymyxins: mechanisms, frequency and treatment options. *Drug Resistance Updates*. 2010;13:132-8.
[143] Epand RM, Epand RF. Bacterial membrane lipids in the action of antimicrobial agents. *Journal of Peptide Science*. 2011;17:298-305.
[144] Koller D, Lohner K. The role of spontaneous lipid curvature in the interaction of interfacially active peptides with membranes. *Biochimica et Biophysica Acta (BBA) - Biomembranes*. 2014;1838:2250-9.
[145] Perrin BS, Sodt AJ, Cotten ML, Pastor RW. The curvature induction of surface-bound antimicrobial peptides piscidin 1 and piscidin 3 varies with lipid chain length. *The Journal of Membrane Biology*. 2015;248:455-67.
[146] Pogliano J, Pogliano N, Silverman JA. Daptomycin-mediated reorganization of membrane architecture causes mislocalization of essential cell division proteins. *Journal of Bacteriology*. 2012;194:4494-504.
[147] Franklin TJ, Snow GA. Inhibitors of nucleic acid synthesis. In: Franklin TJ, Snow GA, editors. Biochemistry and Molecular Biology of Antimicrobial Drug Action. Dordrecht: *Springer Netherlands*; 1998. p. 61-76.
[148] Bhattacharjee MK. Antibiotics that inhibit protein synthesis. Chemistry of Antibiotics and Related Drugs. Cham: *Springer International Publishing*; 2016. p. 129-51.

[149] Kotra LP, Haddad J, Mobashery S. Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance. *Antimicrobial Agents and Chemotherapy*. 2000;44:3249-56.
[150] Speer BS, Shoemaker NB, Salyers AA. Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. *Clinical Microbiology Reviews*. 1992;5:387-99.
[151] Davis JL. Chapter 2 - Pharmacologic principles. In: Reed SM, Bayly WM, Sellon DC, editors. Equine Internal Medicine Forth ed: *W.B. Saunders*; 2018. p. 79-137.
[152] Parsons JB, Rock CO. Is bacterial fatty acid synthesis a valid target for antibacterial drug discovery? *Current Opinion in Microbiology*. 2011;14:544-9.
[153] Conly J, Johnston B. Where are all the new antibiotics? The new antibiotic paradox. *The Canadian Journal of Infectious Diseases & Medical Microbiology*. 2005;16:159-60.
[154] Lee N, Hwang S, Kim J, Cho S, Palsson B, Cho B-K. Mini review: genome mining approaches for the identification of secondary metabolite biosynthetic gene clusters in *Streptomyces*. *Computational and Structural Biotechnology Journal*. 2020;18:1548-56.
[155] Blin K, Shaw S, Steinke K, Villebro R, Ziemert N, Lee SY, et al. antiSMASH 5.0: updates to the secondary metabolite genome mining pipeline. *Nucleic Acids Research*. 2019;47:W81-W7.
[156] Alanjary M, Kronmiller B, Adamek M, Blin K, Weber T, Huson D, et al. The antibiotic resistant target seeker (ARTS), an exploration engine for antibiotic cluster prioritization and novel drug target discovery. *Nucleic Acids Research*. 2017;45:W42-W8.
[157] van Heel AJ, de Jong A, Montalbán-López M, Kok J, Kuipers OP. BAGEL3: automated identification of genes encoding bacteriocins and (non-)bactericidal posttranslationally modified peptides. *Nucleic Acids Research*. 2013;41:W448-W53.
[158] Li MHT, Ung PMU, Zajkowski J, Garneau-Tsodikova S, Sherman DH. Automated genome mining for natural products. *BMC Bioinformatics*. 2009;10:185.
[159] Tietz JI, Schwalen CJ, Patel PS, Maxson T, Blair PM, Tai H-C, et al. A new genome-mining tool redefines the lasso peptide biosynthetic landscape. *Nature Chemical Biology*. 2017;13:470-8.
[160] Khaldi N, Seifuddin FT, Turner G, Haft D, Nierman WC, Wolfe KH, et al. SMURF: genomic mapping of fungal secondary metabolite clusters. *Fungal Genetics and Biology*. 2010;47:736-41.
[161] Weber T, Kim HU. The secondary metabolite bioinformatics portal: computational tools to facilitate synthetic biology of secondary metabolite production. *Synthetic and Systems Biotechnology*. 2016;1:69-79.
[162] Cimermancic P, Medema Marnix H, Claesen J, Kurita K, Wieland Brown Laura C, Mavrommatis K, et al. Insights into secondary metabolism from a global analysis of prokaryotic biosynthetic gene clusters. *Cell*. 2014;158:412-21.
[163] Blin K, Pascal Andreu V, de los Santos ELC, Del Carratore F, Lee SY, Medema MH, et al. The antiSMASH database version 2: a comprehensive resource on secondary metabolite biosynthetic gene clusters. *Nucleic Acids Research*. 2018;47:D625-D30.
[164] Blin K, Kim HU, Medema MH, Weber T. Recent development of antiSMASH and other computational approaches to mine secondary metabolite biosynthetic gene clusters. *Briefings in Bioinformatics*. 2017;20:1103-13.

[165] Hyatt D, Chen G-L, LoCascio PF, Land ML, Larimer FW, Hauser LJ. Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics*. 2010;11:119.
[166] Blin K, Kazempour D, Wohlleben W, Weber T. Improved lanthipeptide detection and prediction for antiSMASH. *PLOS ONE*. 2014;9:e89420.
[167] Kautsar SA, Blin K, Shaw S, Navarro-Muñoz JC, Terlouw BR, van der Hooft JJJ, et al. MIBiG 2.0: a repository for biosynthetic gene clusters of known function. *Nucleic Acids Research*. 2019;48:D454-D8.
[168] Navarro-Muñoz JC, Selem-Mojica N, Mullowney MW, Kautsar S, Tryon JH, Parkinson EI, et al. A computational framework for systematic exploration of biosynthetic diversity from large-scale genomic data. *bioRxiv*. 2018:445270.
[169] Stuart KA, Welsh K, Walker MC, Edrada-Ebel R. Metabolomic tools used in marine natural product drug discovery. *Expert Opinion on Drug Discovery*. 2020;15:499-522.
[170] Peric-Concha N, Long PF. Mining the microbial metabolome: a new frontier for natural product lead discovery. *Drug Discovery Today*. 2003;8:1078-84.
[171] Wang X-J, Ren J-L, Zhang A-H, Sun H, Yan G-L, Han Y, et al. Novel applications of mass spectrometry-based metabolomics in herbal medicines and its active ingredients: current evidence. *Mass Spectrometry Reviews*. 2019;38:380-402.
[172] Emwas AH, Roy R, McKay RT, Tenori L, Saccenti E, Gowda GAN, et al. NMR spectroscopy for metabolomics research. *Metabolites*. 2019;9.
[173] Nagana Gowda GA, Raftery D. Overview of NMR spectroscopy-based metabolomics: opportunities and challenges. In: Gowda GAN, Raftery D, editors. NMR-based Metabolomics: Methods and Protocols. New York, NY: *Springer New York*; 2019. p. 3-14.
[174] Tang L, Shang J, Song C, Yang R, Shang X, Mao W, et al. Untargeted metabolite profiling of antimicrobial compounds in the brown film of *Lentinula edodes* mycelium via LC–MS/MS analysis. *ACS Omega*. 2020;5:7567-75.
[175] Ramirez T, Daneshian M, Kamp H, Bois FY, Clench MR, Coen M, et al. Metabolomics in toxicology and preclinical research. *ALTEX*. 2013;30:209-25.
[176] Zampieri M, Szappanos B, Buchieri MV, Trauner A, Piazza I, Picotti P, et al. High-throughput metabolomic analysis predicts mode of action of uncharacterized antimicrobial compounds. *Science Translational Medicine*. 2018;10:eaal3973.
[177] Verpoorte R, Choi YH, Kim HK. NMR-based metabolomics at work in phytochemistry. *Phytochemistry Reviews*. 2007;6:3-14.
[178] Kim HK, Choi YH, Verpoorte R. NMR-based plant metabolomics: where do we stand, where do we go? *Trends in Biotechnology*. 2011;29:267-75.
[179] Watrous J, Roach P, Alexandrov T, Heath BS, Yang JY, Kersten RD, et al. Mass spectral molecular networking of living microbial colonies. *Proceedings of the National Academy of Sciences*. 2012;109:E1743-E52.
[180] Wu C, Kim HK, van Wezel GP, Choi YH. Metabolomics in the natural products field – a gateway to novel antibiotics. *Drug Discovery Today: Technologies*. 2015;13:11-7.
[181] Roberts LD, Souza AL, Gerszten RE, Clish CB. Targeted metabolomics. *Current Protocols in Molecular Biology*. 2012;Chapter 30:Unit30.2-.2.24.
[182] Schrimpe-Rutledge AC, Codreanu SG, Sherrod SD, McLean JA. Untargeted metabolomics strategies-challenges and emerging directions. *Journal of the American Society for Mass Spectrometry*. 2016;27:1897-905.

[183] Martín-Blázquez A, Díaz C, González-Flores E, Franco-Rivas D, Jiménez-Luna C, Melguizo C, et al. Untargeted LC-HRMS-based metabolomics to identify novel biomarkers of metastatic colorectal cancer. *Scientific Reports*. 2019;9:20198.
[184] Koistinen VM, da Silva AB, Abrankó L, Low D, Villalba RG, Barberán FT, et al. Interlaboratory coverage test on plant food bioactive compounds and their metabolites by mass spectrometry-based untargeted metabolomics. *Metabolites*. 2018;8:46.
[185] Vincent IM, Ehmann DE, Mills SD, Perros M, Barrett MP. Untargeted metabolomics to ascertain antibiotic modes of action. *Antimicrobial Agents and Chemotherapy*. 2016;60:2281-91.
[186] Bijlsma L, Emke E, Hernández F, de Voogt P. Investigation of drugs of abuse and relevant metabolites in Dutch sewage water by liquid chromatography coupled to high resolution mass spectrometry. *Chemosphere*. 2012;89:1399-406.
[187] Mullen W, Larcombe S, Arnold K, Welchman H, Crozier A. Use of accurate mass full scan mass spectrometry for the analysis of anthocyanins in berries and berry-fed tissues. *Journal of Agricultural and Food Chemistry*. 2010;58:3910-5.
[188] Kaddurah-Daouk R, Kristal BS, Weinshilboum RM. Metabolomics: a global biochemical approach to drug response and disease. *Annual Review of Pharmacology and Toxicology*. 2008;48:653-83.
[189] Dunn WB, Broadhurst DI, Atherton HJ, Goodacre R, Griffin JL. Systems level studies of mammalian metabolomes: the roles of mass spectrometry and nuclear magnetic resonance spectroscopy. *Chemical Society Reviews*. 2011;40:387-426.
[190] Patti GJ. Separation strategies for untargeted metabolomics. *Journal of Separation Science*. 2011;34:3460-9.
[191] Hemström P, Irgum K. Hydrophilic interaction chromatography. *Journal of Separation Science*. 2006;29:1784-821.
[192] Subbaraj AK, Huege J, Fraser K, Cao M, Rasmussen S, Faville M, et al. A large-scale metabolomics study to harness chemical diversity and explore biochemical mechanisms in ryegrass. *Communications Biology*. 2019;2:87.
[193] Pitt JJ. Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. *The Clinical Biochemist Reviews*. 2009;30:19-34.
[194] Zhao S, Luo X, Li L. Chemical isotope labeling LC-MS for high coverage and quantitative profiling of the hydroxyl submetabolome in metabolomics. *Analytical Chemistry*. 2016;88:10617-23.
[195] Guo K, Li L. High-performance isotope labeling for profiling carboxylic acid-containing metabolites in biofluids by mass spectrometry. *Analytical Chemistry*. 2010;82:8789-93.
[196] Guo K, Li L. Differential 12C-/13C-isotope dansylation labeling and fast liquid chromatography/mass spectrometry for absolute and relative quantification of the metabolome. *Analytical Chemistry*. 2009;81:3919-32.
[197] Zhao S, Dawe M, Guo K, Li L. Development of high-performance chemical isotope labeling LC–MS for profiling the carbonyl submetabolome. *Analytical Chemistry*. 2017;89:6758-65.
[198] Sumner LW, Amberg A, Barrett D, Beale MH, Beger R, Daykin CA, et al. Proposed minimum reporting standards for chemical analysis chemical analysis working group (CAWG) metabolomics standards initiative (MSI). *Metabolomics : Official Journal of the Metabolomic Society*. 2007;3:211-21.
[199] Smith CA, Maille GO, Want EJ, Qin C, Trauger SA, Brandon TR, et al. METLIN: a metabolite mass spectral database. *Therapeutic Drug Monitoring*. 2005;27:747-51.

[200] Horai H, Arita M, Kanaya S, Nihei Y, Ikeda T, Suwa K, et al. MassBank: a public repository for sharing mass spectral data for life sciences. *Journal of Mass Spectrometry*. 2010;45:703-14.
[201] Wishart DS, Jewison T, Guo AC, Wilson M, Knox C, Liu Y, et al. HMDB 3.0—The human metabolome database in 2013. *Nucleic Acids Research*. 2012;41:D801-D7.
[202] Berdy J. Bioactive microbial metabolites. A personal view *Journal of Antibiotics*. 2005;58:C1-C.
[203] Spellberg B, Powers JH, Brass EP, Miller LG, Edwards JE, Jr. Trends in antimicrobial drug development: implications for the future. *Clinical Infectious Diseases*. 2004;38:1279-86.
[204] Wilson MC, Mori T, Rückert C, Uria AR, Helf MJ, Takada K, et al. An environmental bacterial taxon with a large and distinct metabolic repertoire. *Nature*. 2014;506:58-62.
[205] Gibbons SM, Gilbert JA. Microbial diversity-exploration of natural ecosystems and microbiomes. *Current Opinion in Genetics & Development*. 2015;35:66-72.
[206] Mullis MM, Rambo IM, Baker BJ, Reese BK. Diversity, ecology, and prevalence of antimicrobials in nature. *Frontiers in Microbiology*. 2019;10.
[207] Verma VC, Gond SK, Kumar A, Mishra A, Kharwar RN, Gange AC. Endophytic actinomycetes from *Azadirachta indica* A. Juss.: isolation, diversity, and anti-microbial activity. *Microbial Ecology*. 2009;57:749-56.
[208] Gebhardt K, Schimana J, Krastel P, Dettner K, Rheinheimer J, Zeeck A, et al. Endophenazines A-D, new phenazine antibiotics from the arthropod associated endosymbiont *Streptomyces anulatus*. *The Journal of Antibiotics*. 2002;55:794-800.
[209] Zipperer A, Konnerth MC, Laux C, Berscheid A, Janek D, Weidenmaier C, et al. Human commensals producing a novel antibiotic impair pathogen colonization. *Nature*. 2016;535:511.
[210] Challinor VL, Bode HB. Bioactive natural products from novel microbial sources. *Annals of the New York Academy of Sciences*. 2015;1354:82-97.
[211] Wolfe RS. Anaerobic life-a centennial view. *Journal of Bacteriology*. 1999;181:3317-20.
[212] Morris JG. Obligately anaerobic bacteria in biotechnology. *Applied Biochemistry and Biotechnology*. 1994;48:75-106.
[213] Stephen TC. Time to consider *Clostridium* probiotics? *Future Microbiology*. 2011;6:969-71.
[214] Nakanishi S, Tanaka M. Sequence analysis of a bacteriocinogenic plasmid of *Clostridium butyricum* and expression of the bacteriocin gene in *Escherichia coli*. *Anaerobe*. 2010;16:253-7.
[215] Takahashi M, Taguchi H, Yamaguchi H, Osaki T, Komatsu A, Kamiya S. The effect of probiotic treatment with *Clostridium butyricum* on enterohemorrhagic *Escherichia coli* O157:H7 infection in mice. *FEMS Immunology & Medical Microbiology*. 2004;41:219-26.
[216] Woo TDH, Oka K, Takahashi M, Hojo F, Osaki T, Hanawa T, et al. Inhibition of the cytotoxic effect of *Clostridium difficile* in vitro by *Clostridium butyricum* MIYAIRI 588 strain. *Journal of Medical Microbiology*. 2011;60:1617-25.
[217] Parte AC. LPSN—list of prokaryotic names with standing in nomenclature. *Nucleic Acids Research*. 2013;42:D613-D6.
[218] Madigan MT, Bender KS, Buckley DH, Sattley WM, Stahl DA. Brock biology of microorganisms. 15 ed: *NY, NY : Pearson,*; 2019.

[219] Wells CL, Wilkins TD. Clostridia: sporeforming anaerobic bacilli. In: Baron S, editor. Medical Microbiology. 4 ed. Galveston (TX): *University of Texas Medical Branch at Galveston*; 1996.
[220] Andre S, Vallaeys T, Planchon S. Spore-forming bacteria responsible for food spoilage. *Research in Microbiology*. 2017;168:379-87.
[221] Downes FP, Ito K. Compendium of methods for the microbiological examination of foods. 4 ed. Washington, D.C.: *American Public Health Association*; 2001.
[222] Sumi C, Yang B, Yeo I, Hahm Y. Antimicrobial peptides of the genus *Bacillus*: a new era for antibiotics. *Canadian Journal of Microbiology*. 2014;61:93-103.
[223] Storm DR, Rosenthal KS, Swanson PE. Polymyxin and related peptide antibiotics. *Annual Review of Biochemistry*. 1977;46:723-63.
[224] Craig LC, Weisiger JR, Hausmann W, Harfenist EJ. The seperation and characterization of bacitracin polypeptides. *Journal of Biological Chemistry*. 1952;199:259-66.
[225] Kawulka KE, Sprules T, Diaper CM, Whittal RM, McKay RT, Mercier P, et al. Structure of subtilosin A, a cyclic antimicrobial peptide from *Bacillus subtilis* with unusual sulfur to α-carbon cross-links: formation and reduction of α-thio-α-amino acid derivatives. *Biochemistry*. 2004;43:3385-95.
[226] Hongo M, Murata A, Ogata S, Kono K, Kato F. Characterization of a temperate phage and four bacteriocins produced by nonpathogenic *Clostridium* species. *Agricultural and Biological Chemistry*. 1968;32:773-80.
[227] Mahony DE, Butler ME. Bacteriocins of *Clostridium perfringens*. 1. Isolation and preliminary studies. *Canadian Journal of Microbiology*. 1971;17:1-6.
[228] Mahony DE. Bacteriocin susceptibility of *Clostridium perfringens*: a provisional typing schema. *Applied Microbiology*. 1974;28:172-6.
[229] Clarke DJ, Robson RM, Morris JG. Purification of two *Clostridium* bacteriocins by procedures appropriate to hydrophobic proteins. *Antimicrobial Agents and Chemotherapy*. 1974;7:256-64.
[230] Wolff A, Ionesco H. Purification and characterization of "*Clostridium perfringens*" BP6K-N5 strain bacteriocin N5. *Annales de Microbiologie (Paris)*. 1975;126:343-56.
[231] Li AW, Verpoorte JA, Lewis RG, Mahony DE. Characterization of bacteriocin 28 produced by *Clostridium perfringens*. *Canadian Journal of Microbiology*. 1982;28:860-73.
[232] Lau AHS, Hawirko RZ, Chow CT. Purification and properties of biotin-P produced by *Clostridium botulinum*. *Canadian Journal of Microbiology*. 1974;20:385.
[233] Ionesco H, Wolff A. The mode of action of bacteriocin N5 purified from *Clostridium perfringens*. *Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences*. 1975;281:2033-6.
[234] Garnier T, Cole ST. Characterization of a bacteriocinogenic plasmid from *Clostridium perfringens* and molecular genetic analysis of the bacteriocin-encoding gene. *Journal of Bacteriology*. 1986;168:1189-96.
[235] Dineen SS, Bradshaw M, Johnson EA. Cloning, nucleotide sequence, and expression of the gene encoding the bacteriocin boticin B from *Clostridium botulinum* Strain 213B. *Applied and Environmental Microbiology*. 2000;66:5480-3.
[236] Kemperman R, Kuipers A, Karsens H, Nauta A, Kuipers O, Kok J. Identification and characterization of two novel clostridial bacteriocins, circularin A and closticin 574. *Applied and Environmental Microbiology*. 2003;69:1589-97.

[237] Timbermont L, De Smet L, Van Nieuwerburgh F, Parreira VR, Van Driessche G, Haesebrouck F, et al. Perfrin, a novel bacteriocin associated with netB positive *Clostridium perfringens* strains from broilers with necrotic enteritis. *Veterinary Research*. 2014;45:40.

[238] Chiriac AI, Kloss F, Krämer J, Vuong C, Hertweck C, Sahl HG. Mode of action of closthioamide: the first member of the polythioamide class of bacterial DNA gyrase inhibitors. *Journal of Antimicrobial Chemotherapy*. 2015;70:2576-88.

[239] Royal Society of Chemistry. clostrubin. *ChemSpider*.

[240] Tracanna V, Medema MH, de Jong A, Kuipers OP. Mining prokaryotes for antimicrobial compounds: from diversity to function. *FEMS Microbiology Reviews*. 2017;41:417-29.

[241] Ortega MA, van der Donk WA. New insights into the biosynthetic logic of ribosomally synthesized and post-translationally modified peptide natural products. *Cell Chemical Biology*. 2016;23:31-44.

[242] Skinnider MA, Johnston CW, Edgar RE, Dejong CA, Merwin NJ, Rees PN, et al. Genomic charting of ribosomally synthesized natural product chemical space facilitates targeted mining. *Proceedings of the National Academy of Sciences*. 2016;113:E6343-E51.

[243] Donia MS, Cimermancic P, Schulze CJ, Wieland Brown LC, Martin J, Mitreva M, et al. A systematic analysis of biosynthetic gene clusters in the human microbiome reveals a common family of antibiotics. *Cell*. 2014;158:1402-14.

[244] Hetrick KJ, van der Donk WA. Ribosomally synthesized and post-translationally modified peptide natural product discovery in the genomic era. *Current Opinion in Chemical Biology*. 2017;38:36-44.

[245] Letzel A, Pidot SJ, Hertweck C. Genome mining for ribosomally synthesized and post-translationally modified peptides (RiPPs) in anaerobic bacteria. *BMC Genomics*. 2014;15:983-.

[246] Tushar L, Sasi Jyothsna TS, Sasikala C, Ramana CV. Draft genome sequence of antimicrobial-producing *Clostridium* sp. JC272, isolated from marine sediment. *Genome Announcements*. 2015;3:e00650-15.

[247] Seedorf H, Fricke WF, Veith B, Brüggemann H, Liesegang H, Strittmatter A, et al. The genome of *Clostridium kluyveri*, a strict anaerobe with unique metabolic features. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105:2128-33.

[248] Behnken S, Hertweck C. Cryptic polyketide synthase genes in non-pathogenic *Clostridium* spp. *PLOS ONE*. 2012;7:e29609.

[249] Donadio S, Monciardini P, Sosio M. Polyketide synthases and nonribosomal peptide synthetases: the emerging view from bacterial genomics. *Natural Product Reports*. 2007;24:1073-109.

[250] National Center for Biotechnology Information. 2-Hydroxy-4-methylvaleric acid, CID=92779. *PubChem Database*.

[251] Smit BA, Engels WJM, Wouters JTM, Smit G. Diversity of l-leucine catabolism in various microorganisms involved in dairy fermentations, and identification of the rate-controlling step in the formation of the potent flavour component 3-methylbutanal. *Applied Microbiology and Biotechnology*. 2004;64:396-402.

[252] Sakko M, Tjäderhane L, Sorsa T, Hietala P, Järvinen A, Bowyer P, et al. 2-hydroxyisocaproic acid (HICA): a new potential topical antibacterial agent. *International Journal of Antimicrobial Agents*. 2012;39:539-40.

[253] Mero AA, Ojala T, Hulmi JJ, Puurtinen R, Karila TA, Seppälä T. Effects of alfa-hydroxy-isocaproic acid on body composition, DOMS and performance in athletes. *Journal of the International Society of Sports Nutrition*. 2010;7:1-.

[254] Hoffer LJ, Taveroff A, Robitaille L, Mamer OA, Reimer MLJ. α-keto and α-hydroxy branched-chain acid interrelationships in normal humans. *The Journal of Nutrition*. 1993;123:1513-21.

[255] Hietala PK, Westermarck HW, Jaarma M. Identification of antimicrobial alpha-hydroxyacids in *Lactobacillus plantarum* fermented animal protein. *Annals of Nutrition and Metabolism*. 1979;23:227-34.

[256] Brooks JB, Nunez-Montiel OL, Wycoff BJ, Moss CW. Frequency-pulsed electron capture gas-liquid chromatographic analysis of metabolites produced by *Clostridium difficile* in broth enriched with amino acids. *Journal of Clinical Microbiology*. 1984;20:539-48.

[257] Hummel W, Schütte H, Kula M-R. D-2-hydroxyisocaproate dehydrogenase from *Lactobacillus casei*. *Applied Microbiology and Biotechnology*. 1985;21:7-15.

[258] Bernard N, Johnsen K, Ferain T, Garmyn D, Hols P, Holbrook JJ, et al. NAD+-dependent d-2-hydroxyisocaproate dehydrogenase of *Lactobacillus Delbrueckii* subsp. *Bulgaricus*. *European Journal of Biochemistry*. 1994;224:439-46.

[259] Yvon M, Rijnen L. Cheese flavour formation by amino acid catabolism. *International Dairy Journal*. 2001;11:185-201.

[260] Hamid A, Uematsu H, Sato N, Kota K, Iwaku M, Hoshino E. Inhibitory effects of metronidazole on anaerobic metabolism of phenylalanine and leucine by *Peptostreptococcus anaerobius*. *Journal of Antimicrobial Chemotherapy*. 1997;39:129-34.

[261] Park B, Hwang H, Chang JY, Hong SW, Lee SH, Jung MY, et al. Identification of 2-hydroxyisocaproic acid production in lactic acid bacteria and evaluation of microbial dynamics during kimchi ripening. *Scientific Reports*. 2017;7:10904.

[262] Wyk CJV, Kepner RE, Webb AD. Some volatile components of *Vitis vinifera* variety white riesling. 2. organic acids extracted from wine. *Journal of Food Science*. 1967;32:664-8.

[263] Selis D, Pande Y, Smoczer C, Wheater M, Alhabeil J, Paurazas S, et al. Cytotoxicity and genotoxicity of a new intracanal medicament, 2-hydroxyisocaproic acid–an *in vitro* study. *Journal of Endodontics*. 2019;45:578-83.

[264] Sakko M, Moore C, Novak-Frazer L, Rautemaa V, Sorsa T, Hietala P, et al. 2-hydroxyisocaproic acid is fungicidal for *Candida* and *Aspergillus* species. *Mycoses*. 2014;57:214-21.

[265] Nieminen MT, Novak-Frazer L, Rautemaa V, Rajendran R, Sorsa T, Ramage G, et al. A novel antifungal is active against *Candida albicans* biofilms and inhibits mutagenic acetaldehyde production in vitro. *PLOS ONE*. 2014;9:e97864.

[266] Sakko M, Tjäderhane L, Sorsa T, Hietala P, Rautemaa R. Antimicrobial 2-hydroxyisocaproic acid and chlorhexidine resist inactivation by dentine. *International Endodontic Journal*. 2016;49:352-60.

[267] Nieminen MT, Hernandez M, Novak-Frazer L, Kuula H, Ramage G, Bowyer P, et al. DL-2-hydroxyisocaproic acid attenuates inflammatory responses in a murine *Candida albicans* biofilm model. *Clinical and Vaccine Immunology* 2014;21:1240-5.

[268] Lang CH, Pruznak A, Navaratnarajah M, Rankine KA, Deiter G, Magne H, et al. Chronic α-hydroxyisocaproic acid treatment improves muscle recovery after immobilization-induced atrophy. *American Journal of Physiology-Endocrinology and Metabolism*. 2013;305:E416-E28.

[269] Gupta TB, Brightwell G. Farm level survey of spore-forming bacteria on four dairy farms in the Waikato region of New Zealand. *MicrobiologyOpen*. 2017;6:e00457.
[270] U.S. Food and Drug Administration. BAM R11: Butterfield's phosphate-buffered dilution water. In: Merker RI, editor. Bacteriological Analytical Manual. 8 ed: *Center for Food Safety and Applied Nutrition, FDA* 1995.
[271] Vijayakumar PP, Muriana PM. A microplate growth inhibition assay for screening bacteriocins against *Listeria monocytogenes* to differentiate their mode-of-action. *Biomolecules*. 2015;5:1178-94.
[272] Böddinghaus B, Wolters J, Heikens W, Böttger EC. Phylogenetic analysis and identification of different serovars of *Mycobacterium intracellulare* at the molecular level. *FEMS Microbiology Letters*. 1990;70:197-203.
[273] Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*. 2012;28:1647-9.
[274] Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*. 2007;73:5261-7.
[275] Lasik-Kurdyś M, Sip A. Evaluation of the antimicrobial activity of bacteriocin-like inhibitory substances of enological importance produced by *Oenococcus oeni* isolated from wine. *European Food Research and Technology*. 2019;245:375-82.
[276] Sprouffske K, Wagner A. Growthcurver: an R package for obtaining interpretable metrics from microbial growth curves. *BMC Bioinformatics*. 2016;17:172.
[277] Subbaraj AK, Kim YHB, Fraser K, Farouk MM. A hydrophilic interaction liquid chromatography–mass spectrometry (HILIC–MS) based metabolomics study on colour stability of ovine meat. *Meat Science*. 2016;117:163-72.
[278] Holman JD, Tabb DL, Mallick P. Employing ProteoWizard to convert raw mass spectrometry data. *Current Protocols in Bioinformatics*. 2014;46.
[279] Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G. XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Analytical Chemistry*. 2006;78:779-87.
[280] Ihaka R, Gentleman R. R: A language for data analysis and graphics. *Journal of Computational and Graphical Statistics*. 1996;5:299-314.
[281] Dunn WB, Wilson ID, Nicholls AW, Broadhurst D. The importance of experimental design and QC samples in large-scale and MS-driven untargeted metabolomic studies of humans. *Bioanalysis*. 2012;4:2249-64.
[282] Parsons HM, Ekman DR, Collette TW, Viant MR. Spectral relative standard deviation: a practical benchmark in metabolomics. *Analyst*. 2009;134:478-85.
[283] Xia J, Psychogios N, Young N, Wishart DS. MetaboAnalyst: a web server for metabolomic data analysis and interpretation. *Nucleic Acids Research*. 2009;37:W652-W60.
[284] Soni A, Oey I, Silcock P, Bremer PJ. Impact of temperature, nutrients, pH and cold storage on the germination, growth and resistance of *Bacillus cereus* spores in egg white. *Food Research International*. 2018;106:394-403.
[285] Artíguez ML, Martínez de Marañón I. Effect of pulsed light treatment on the germination of *Bacillus subtilis* spores. *Food and Bioprocess Technology*. 2015;8:478-85.
[286] Yu Z, Gunn L, Brennan E, Reid R, Wall PG, Gaora PÓ, et al. Complete genome sequence of *Clostridium estertheticum* DSM 8809, a microbe identified in spoiled vacuum packed beef. *Frontiers in Microbiology*. 2016;7:1764-.

[287] Coil D, Jospin G, Darling AE. A5-miseq: an updated pipeline to assemble microbial genomes from Illumina MiSeq data. *Bioinformatics*. 2014;31:587-9.
[288] Simão FA, Waterhouse RM, Ioannidis P, Kriventseva EV, Zdobnov EM. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics*. 2015;31:3210-2.
[289] Meier-Kolthoff JP, Göker M. TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. *Nature Communications*. 2019;10:2182.
[290] Lagesen K, Hallin P, Rødland EA, Stærfeldt H-H, Rognes T, Ussery DW. RNAmmer: consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Research*. 2007;35:3100-8.
[291] Meier-Kolthoff JP, Auch AF, Klenk H-P, Göker M. Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics*. 2013;14:60.
[292] Lefort V, Desper R, Gascuel O. FastME 2.0: A comprehensive, accurate, and fast distance-based phylogeny inference program. *Molecular Biology and Evolution*. 2015;32:2798-800.
[293] Desper R, Gascuel O. Theoretical foundation of the balanced minimum evolution method of phylogenetic inference and its relationship to weighted least-squares tree fitting. *Molecular Biology and Evolution*. 2004;21:587-98.
[294] Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*. 2018;35:1547-9.
[295] Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, Tiedje JM. DNA–DNA hybridization values and their relationship to whole-genome sequence similarities. *International Journal of Systematic and Evolutionary Microbiology*. 2007;57:81-91.
[296] Altermann E. Tracing lifestyle adaptation in prokaryotic genomes. *Frontiers in Microbiology*. 2012;3.
[297] Altermann E, Lu J, McCulloch A. GAMOLA2, a comprehensive software package for the annotation and curation of draft and complete microbial genomes. *Frontiers in Microbiology*. 2017;8:346-.
[298] Liu B, Zheng D, Jin Q, Chen L, Yang J. VFDB 2019: a comparative pathogenomic platform with an interactive web interface. *Nucleic Acids Research*. 2018;47:D687-D92.
[299] Bateman A, Martin M-J, Orchard S, Magrane M, Agivetova R, Ahmad S, et al. UniProt: the universal protein knowledgebase in 2021. *Nucleic Acids Research*. 2021;49:D480-D9.
[300] Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*. 1997;25:3389-402.
[301] Alqadeeri F, Rukayadi Y, Abbas F, Shaari K. Antibacterial and antispore activities of isolated compounds from *Piper cubeba* L. *Molecules (Basel, Switzerland)*. 2019;24:3095.
[302] Muheim C, Götzke H, Eriksson AU, Lindberg S, Lauritsen I, Nørholm MHH, et al. Increasing the permeability of *Escherichia coli* using MAC13243. *Scientific Reports*. 2017;7:17629-.
[303] Te Winkel JD, Gray DA, Seistrup KH, Hamoen LW, Strahl H. Analysis of antimicrobial-triggered membrane depolarization using voltage sensitive dyes. *Frontiers in Cell and Developmental Biology*. 2016;4:29-.

[304] Zhou R, Tseng C-L, Huan T, Li L. IsoMS: automated processing of LC-MS data generated by a chemical isotope labeling metabolomics platform. *Analytical Chemistry*. 2014;86:4675-9.
[305] Huan T, Li L. Counting missing values in a metabolite-intensity data set for measuring the analytical performance of a metabolomics platform. *Analytical Chemistry*. 2015;87:1306-13.
[306] Dahabiyeh LA, Malkawi AK, Wang X, Colak D, Mujamammi AH, Sabi EM, et al. Dexamethasone-induced perturbations in tissue metabolomics revealed by chemical isotope labeling LC-MS analysis. *Metabolites*. 2020;10:42.
[307] Kurokawa M, Ying B-W. Precise, high-throughput analysis of bacterial growth. *Journal of Visualized Experiments : JoVE*. 2017:56197.
[308] Zhou H, Fang J, Tian Y, Lu XY. Mechanisms of nisin resistance in Gram-positive bacteria. *Annals of Microbiology*. 2014;64:413-20.
[309] Prüß BM, Dietrich R, Nibler B, Märtlbauer E, Scherer S. The hemolytic enterotoxin HBL is broadly distributed among species of the *Bacillus cereus* group. *Applied and Environmental Microbiology*. 1999;65:5436-42.
[310] Ribeiro Júnior JC, de Oliveira AM, Silva FdG, Tamanini R, de Oliveira ALM, Beloti V. The main spoilage-related psychrotrophic bacteria in refrigerated raw milk. *Journal of Dairy Science*. 2018;101:75-83.
[311] Lim JY, Yoon J, Hovde CJ. A brief overview of *Escherichia coli* O157:H7 and its plasmid O157. *Journal of Microbiology and Biotechnology*. 2010;20:5-14.
[312] Arslan S, Eyi A, Özdemir F. Spoilage potentials and antimicrobial resistance of *Pseudomonas* spp. isolated from cheeses. *Journal of Dairy Science*. 2011;94:5851-6.
[313] Dhanani AS, Block G, Dewar K, Forgetta V, Topp E, Beiko RG, et al. Genomic comparison of non-typhoidal *Salmonella enterica* serovars Typhimurium, Enteritidis, Heidelberg, Hadar and Kentucky isolates from broiler chickens. *PLOS ONE*. 2015;10:e0128773.
[314] Paškevičius Š, Starkevič U, Misiūnas A, Vitkauskienė A, Gleba Y, Ražanskienė A. Plant-expressed pyocins for control of *Pseudomonas aeruginosa*. *PLOS ONE*. 2017;12:e0185782.
[315] Li Q, Montalban-Lopez M, Kuipers OP. Increasing the antimicrobial activity of nisin-based lantibiotics against Gram-negative pathogens. *Applied and Environmental Microbiology*. 2018;84:e00052-18.
[316] Goers L, Freemont P, Polizzi KM. Co-culture systems and technologies: taking synthetic biology to the next level. *Journal of the Royal Society, Interface*. 2014;11:20140065.
[317] Geesink P, Tyc O, Küsel K, Taubert M, van de Velde C, Kumar S, et al. Growth promotion and inhibition induced by interactions of groundwater bacteria. *FEMS Microbiology Ecology*. 2018;94.
[318] Kinkel LL, Schlatter DC, Xiao K, Baines AD. Sympatric inhibition and niche differentiation suggest alternative coevolutionary trajectories among Streptomycetes. *The ISME Journal*. 2014;8:249-56.
[319] Romero D, Traxler MF, LóPez D, Kolter R. Antibiotics as signal molecules. *Chemical Reviews*. 2011;111:5492-505.
[320] Liang L, Xu J, Zhou W-W, Brand E, Chen H-B, Zhao Z-Z. Integrating targeted and untargeted metabolomics to investigate the processing chemistry of polygoni multiflori radix. *Frontiers in Pharmacology*. 2018;9.
[321] Boccard J, Rudaz S. Harnessing the complexity of metabolomic data with chemometrics. *Journal of Chemometrics*. 2014;28:1-9.

[322] Sarker SD, Nahar L. Chapter 19 - Applications of high performance liquid chromatography in the analysis of herbal products. In: Mukherjee PK, editor. Evidence-based Validation of Herbal Medicine. Boston: *Elsevier*; 2015. p. 405-25.
[323] Aksenov AA, Schivo M, Bardaweel H, Zrodnikov Y, Kwan AM, Zamuruyev K, et al. Chapter 8 - Volatile organic compounds in human breath: biogenic origin and point-of-care analysis approaches. In: Amann A, Smith D, editors. Volatile Biomarkers. Boston: *Elsevier*; 2013. p. 129-54.
[324] Ebrahimpoor M, Goeman JJ. Inflated false discovery rate due to volcano plots: problem and solutions. *Briefings in Bioinformatics*. 2021.
[325] Mercolini L. New psychoactive substances: an overview. In: Dasgupta A, editor. Critical Issues in Alcohol and Drugs of Abuse Testing Second ed: *Academic Press*; 2019. p. 247-58.
[326] Scott-Ham M, Stark MM. Substance misuse: legal highs. In: Payne-James J, Byard RW, editors. Encyclopedia of Forensic and Legal Medicine. Second ed. Oxford: *Elsevier*; 2016. p. 394-9.
[327] Williams Brianna B, Van Benschoten Andrew H, Cimermancic P, Donia Mohamed S, Zimmermann M, Taketani M, et al. Discovery and characterization of gut microbiota decarboxylases that can produce the neurotransmitter tryptamine. *Cell Host & Microbe*. 2014;16:495-503.
[328] Campos P, Pichon E, Moriou C, Clerc P, Trépos R, Frederich M, et al. New antimalarial and antimicrobial tryptamine derivatives from the marine sponge *Fascaplysinopsis reticulata*. *Marine Drugs*. 2019;17:167.
[329] Chandrika K, Ian M. Inhibition of yeast growth by tryptamine and recovery with tryptophan. *Current Bioactive Compounds*. 2020;16:48-52.
[330] Dhakal R, Bajpai VK, Baek K-H. Production of gaba (γ - aminobutyric acid) by microorganisms: a review. *Brazilian Journal of Microbiology* 2012;43:1230-41.
[331] Lu X, Chen Z, Gu Z, Han Y. Isolation of γ-aminobutyric acid-producing bacteria and optimization of fermentative medium. *Biochemical Engineering Journal*. 2008;41:48-52.
[332] Cho YR, Chang JY, Chang HC. Production of gamma-aminobutyric acid (GABA) by *Lactobacillus buchneri* isolated from kimchi and its neuroprotective effect on neuronal cells. *Journal of Microbiology and Biotechnology*. 2007;17:104-9.
[333] Adeghate E, Ponery AS. GABA in the endocrine pancreas: cellular localization and function in normal and diabetic rats. *Tissue and Cell*. 2002;34:1-6.
[334] Alizadeh Behbahani B, Jooyandeh H, Falah F, Vasiee A. Gamma-aminobutyric acid production by *Lactobacillus brevis* A3: optimization of production, antioxidant potential, cell toxicity, and antimicrobial activity. *Food Science & Nutrition*. 2020;8:5330-9.
[335] National Center for Biotechnology Information. PubChem compound summary for CID 12122, 3-hydroxyphenylacetic acid. 2021.
[336] Zafra A, Juárez MJB, Blanc R, Navalón A, González J, Vílchez JL. Determination of polyphenolic compounds in wastewater olive oil by gas chromatography–mass spectrometry. *Talanta*. 2006;70:213-8.
[337] Xiong X, Liu D, Wang Y, Zeng T, Peng Y. Urinary 3-(3-hydroxyphenyl)-3-hydroxypropionic acid, 3-hydroxyphenylacetic acid, and 3-hydroxyhippuric acid are elevated in children with autism spectrum disorders. *BioMed Research International*. 2016;2016:9485412.

[338] Ozdemir OO, Soyer F. *Pseudomonas aeruginosa* presents multiple vital changes in its proteome in the presence of 3-hydroxyphenylacetic acid, a promising antimicrobial agent. *ACS Omega*. 2020;5:19938-51.
[339] National Center for Biotechnology Information. PubChem compound summary for CID 586, Creatine. 2021.
[340] Wyss M, Kaddurah-Daouk R. Creatine and creatinine metabolism. *Physiological Reviews*. 2000;80:1107-213.
[341] Hermann M, Knerr H-J, Mai N, Groß A, Kaltwasser H. Creatinine and N-methylhydantoin degradation in two newly isolated *Clostridium* species. *Archives of Microbiology*. 1992;157:395-401.
[342] Matthews RT, Ferrante RJ, Klivenyi P, Yang L, Klein AM, Mueller G, et al. Creatine and cyclocreatine attenuate MPTP neurotoxicity. *Experimental Neurology*. 1999;157:142-9.
[343] Sullivan PG, Geiger JD, Mattson MP, Scheff SW. Dietary supplement creatine protects against traumatic brain injury. *Annals of Neurology*. 2000;48:723-9.
[344] Ferrante RJ, Andreassen OA, Jenkins BG, Dedeoglu A, Kuemmerle S, Kubilus JK, et al. Neuroprotective effects of creatine in a transgenic mouse model of Huntington's disease. *The Journal of Neuroscience* 2000;20:4389-97.
[345] Guo P, Zhang K, Ma X, He P. *Clostridium* species as probiotics: potentials and challenges. *Journal of Animal Science and Biotechnology*. 2020;11:24-.
[346] Chai KF, Voo AYH, Chen WN. Bioactive peptides from food fermentation: a comprehensive review of their sources, bioactivities, applications, and future development. *Comprehensive Reviews in Food Science and Food Safety*. 2020;19:3825-85.
[347] Dunn WB, Erban A, Weber RJM, Creek DJ, Brown M, Breitling R, et al. Mass appeal: metabolite identification in mass spectrometry-focused untargeted metabolomics. *Metabolomics*. 2013;9:44-66.
[348] Lee N-K, Kim HW, Lee JY, Ahn DU, Kim C-J, Paik H-D. Antimicrobial effect of nisin against *Bacillus cereus* in beef jerky during storage. *Korean Journal for Food Science of Animal Resources*. 2015;35:272-6.
[349] Brasca M, Morandi S, Silvetti T. *Clostridium* spp. Reference Module in Food Science: *Elsevier*; 2020.
[350] Li C, Yan X, Lillehoj HS. Complete genome sequences of *Clostridium perfringens* Del1 strain isolated from chickens affected by necrotic enteritis. *Gut Pathogens*. 2017;9.
[351] Chen R, Feng Y, Wang X, Yang J, Zhang X, Lü X, et al. Whole genome sequences of three Clade 3 *Clostridium difficile* strains carrying binary toxin genes in China. *Scientific Reports*. 2017;7:43555.
[352] Varghese NJ, Mukherjee S, Ivanova N, Konstantinidis KT, Mavrommatis K, Kyrpides NC, et al. Microbial species delineation using whole genome sequences. *Nucleic Acids Research*. 2015;43:6761-71.
[353] Chun J, Rainey FA. Integrating genomics into the taxonomy and systematics of the bacteria and archaea. *International Journal of Systematic and Evolutionary Microbiology*. 2014;64:316-24.
[354] Chun J, Oren A, Ventosa A, Christensen H, Arahal DR, da Costa MS, et al. Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. *International Journal of Systematic and Evolutionary Microbiology*. 2018;68:461-6.

[355] Wassenaar TM, Zschüttig A, Beimfohr C, Geske T, Auerbach C, Cook H, et al. Virulence genes in a probiotic *E. coli* product with a recorded long history of safe use. *European Journal of Microbiology & Immunology*. 2015;5:81-93.

[356] Shokryazdan P, Faseleh Jahromi M, Liang JB, Kalavathy R, Sieo CC, Ho YW. Safety assessment of two new *Lactobacillus* strains as probiotic for human using a rat model. *PLOS ONE*. 2016;11:e0159851.

[357] *Clostridium botulinum* and *Clostridium perfringens*. Foodborne Microbial Pathogens: Mechanisms and Pathogenesis. New York, NY: *Springer New York*; 2008. p. 149-64.

[358] Ba-Thein W, Lyristis M, Ohtani K, Nisbet IT, Hayashi H, Rood JI, et al. The virR/virS locus regulates the transcription of genes encoding extracellular toxin production in *Clostridium perfringens*. *Journal of Bacteriology*. 1996;178:2514-20.

[359] Flühe L, Marahiel MA. Radical S-adenosylmethionine enzyme catalyzed thioether bond formation in sactipeptide biosynthesis. *Current Opinion in Chemical Biology*. 2013;17:605-12.

[360] Himes PM, Allen SE, Hwang S, Bowers AA. Production of sactipeptides in *Escherichia coli*: probing the substrate promiscuity of subtilosin A biosynthesis. *ACS Chemical Biology*. 2016;11:1737-44.

[361] Kawulka K, Sprules T, McKay RT, Mercier P, Diaper CM, Zuber P, et al. Structure of subtilosin A, an antimicrobial peptide from *Bacillus subtilis* with unusual posttranslational modifications linking cysteine sulfurs to α-carbons of phenylalanine and threonine. *Journal of the American Chemical Society*. 2003;125:4726-7.

[362] Flühe L, Knappe TA, Gattner MJ, Schäfer A, Burghaus O, Linne U, et al. The radical SAM enzyme AlbA catalyzes thioether bond formation in subtilosin A. *Nature Chemical Biology*. 2012;8:350-7.

[363] Haft DH, Basu MK. Biological systems discovery *In Silico*: radical s-adenosylmethionine protein families and their target peptides for posttranslational modification. *Journal of Bacteriology*. 2011;193:2745-55.

[364] Walsh CT. The chemical versatility of natural-product assembly lines. *Accounts of Chemical Research*. 2008;41:4-10.

[365] Kopp F, Marahiel MA. Macrocyclization strategies in polyketide and nonribosomal peptide biosynthesis. *Natural Product Reports*. 2007;24:735-49.

[366] Gontang EA, Gaudêncio SP, Fenical W, Jensen PR. Sequence-based analysis of secondary-metabolite biosynthesis in marine actinobacteria. *Applied and Environmental Microbiology*. 2010;76:2487-99.

[367] Huang W-C, Tang IC. Chapter 8 - Bacterial and yeast cultures – process characteristics, products, and applications. In: Yang S-T, editor. Bioprocessing for Value-added Products from Renewable Resources. Amsterdam: *Elsevier*; 2007. p. 185-223.

[368] Dengler U, Niefind K, Kiess M, Schomburg D. Crystal structure of a ternary complex of D-2-hydroxyisocaproate dehydrogenase from *Lactobacillus casei*, NAD+ and 2-oxoisocaproate at 1.9 A resolution. *Journal of Molecular Biology*. 1997;267:640-60.

[369] Bachmann H, Starrenburg MJC, Dijkstra A, Molenaar D, Kleerebezem M, Rademaker JLW, et al. Regulatory phenotyping reveals important diversity within the species *Lactococcus lactis*. *Applied and Environmental Microbiology*. 2009;75:5687-94.

[370] CLSI. Methods for determining bactericidal activity of antimicrobial agents. Approved guideline. CLSI document M26-A. 950 West Valley Roadn Suite 2500,Wayne, Pennsylvania 19087, USA: *Clinical and Laboratory Standards Institute*; 1998.

[371] Kim SA, Kim NH, Lee SH, Hwang IG, Rhee MS. Survival of foodborne pathogenic bacteria (*Bacillus cereus*, *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus*, and *Listeria monocytogenes*) and *Bacillus cereus* spores in fermented alcoholic beverages (beer and refined rice wine). *Journal of Food Protection*. 2014;77:419-26.

[372] Bagge D, Hjelm M, Johansen C, Huber I, Gram L. *Shewanella putrefaciens* adhesion and biofilm formation on food processing surfaces. *Applied and Environmental Microbiology*. 2001;67:2319-25.

[373] Ercolini D, Russo F, Nasi A, Ferranti P, Villani F. Mesophilic and psychrotrophic bacteria from meat and their spoilage potential in vitro and in beef. *Applied and Environmental Microbiology*. 2009;75:1990-2001.

[374] Liu Y-J, Xie J, Zhao L-J, Qian Y-F, Zhao Y, Liu X. Biofilm formation characteristics of *Pseudomonas lundensis* isolated from meat. *Journal of Food Science*. 2015;80:M2904-M10.

[375] Hamasaki Y, Kotoura S, Nakane M, Sugiyama M. Spoilage ability of psychrotrophic *Paenibacillus* spp. isolated from cooked food products. *Biocontrol Science*. 2006;11:43-7.

[376] Gopal N, Hill C, Ross PR, Beresford TP, Fenelon MA, Cotter PD. The prevalence and control of *Bacillus* and related spore-forming bacteria in the dairy industry. *Frontiers in Microbiology*. 2015;6.

[377] Papadopoulou OS, Iliopoulos V, Mallouchos A, Panagou EZ, Chorianopoulos N, Tassou CC, et al. Spoilage potential of *Pseudomonas* (*P. fragi*, *P. putida*) and LAB (*Leuconostoc mesenteroides*, *Lactobacillus sakei*) strains and their volatilome profile during storage of sterile pork meat using GC/MS and data analytics. *Foods (Basel, Switzerland)*. 2020;9:633.

[378] de Bentzmann S, Plésiat P. The *Pseudomonas aeruginosa* opportunistic pathogen and human infections. *Environmental Microbiology*. 2011;13:1655-65.

[379] Soni A, Oey I, Silcock P, Bremer P. *Bacillus* spores in the food industry: a review on resistance and response to novel inactivation technologies. *Comprehensive Reviews in Food Science and Food Safety*. 2016;15:1139-48.

[380] Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, et al. Cell viability assays. In: Markossian S, Grossman A, Brimacombe K, Arkin M, Auld D, Austin CP, et al., editors. Assay Guidance Manual. Bethesda (MD): *Eli Lilly & Company and the National Center for Advancing Translational Sciences*; 2004.

[381] Johnson KM, Busta FF. Heat-induced temperature sensitivity of outgrowing *Bacillus cereus* spores. *Applied and Environmental Microbiology*. 1984;47:768-74.

[382] Hartmann M, Berditsch M, Hawecker J, Ardakani MF, Gerthsen D, Ulrich AS. Damage of the bacterial cell envelope by antimicrobial peptides gramicidin S and PGLa as revealed by transmission and scanning electron microscopy. *Antimicrobial Agents and Chemotherapy*. 2010;54:3132-42.

[383] Delcour AH. Outer membrane permeability and antibiotic resistance. *Biochimica et Biophysica Acta*. 2009;1794:808-16.

[384] Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. *Microbiology and Molecular Biology Reviews*. 2003;67:593-656.

[385] Silver LL. Challenges of antibacterial discovery. *Clinical Microbiology Reviews*. 2011;24:71-109.
[386] Helander IM, Mattila-Sandholm T. Fluorometric assessment of Gram-negative bacterial permeabilization. *Journal of Applied Microbiology*. 2000;88:213-9.
[387] Maloney PC, Kashket ER, Wilson TH. A protonmotive force drives ATP synthesis in bacteria. *Proceedings of the National Academy of Sciences*. 1974;71:3896-900.
[388] Strahl H, Hamoen LW. Membrane potential is important for bacterial cell division. *Proceedings of the National Academy of Sciences*. 2010;107:12281-6.
[389] Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. *Pharmacological Reviews*. 2003;55:27-55.
[390] Wimley WC, Hristova K. Antimicrobial peptides: successes, challenges and unanswered questions. *The Journal of Membrane Biology*. 2011;239:27-34.
[391] Cushnie TPT, O'Driscoll NH, Lamb AJ. Morphological and ultrastructural changes in bacterial cells as an indicator of antibacterial mechanism of action. *Cellular and Molecular Life Sciences*. 2016;73:4471-92.
[392] Kovach KN, Fleming D, Rumbaugh KP, Gordon VD. Specific disruption of established *P. aeruginosa* biofilms using polymer-attacking enzymes. *bioRxiv*. 2019:598979.
[393] Ghai I, Ghai S. Understanding antibiotic resistance via outer membrane permeability. *Infection and Drug Resistance*. 2018;11:523-30.
[394] Gyawali R, Hayek SA, Ibrahim SA. 3 - Plant extracts as antimicrobials in food products: mechanisms of action, extraction methods, and applications. In: Taylor TM, editor. Handbook of Natural Antimicrobials for Food Safety and Quality. Oxford: *Woodhead Publishing*; 2015. p. 49-68.
[395] Stanojević-Nikolić S, Dimić G, Mojović L, Pejin J, Djukić-Vuković A, Kocić-Tanackov S. Antimicrobial activity of lactic acid against pathogen and spoilage microorganisms. *Journal of Food Processing and Preservation*. 2016;40:990-8.
[396] Alakomi HL, Skyttä E, Saarela M, Mattila-Sandholm T, Latva-Kala K, Helander IM. Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. *Applied and Environmental Microbiology*. 2000;66:2001-5.
[397] Smit G, Smit BA, Engels WJM. Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. *FEMS Microbiology Reviews*. 2005;29:591-610.
[398] Zhao S, Li H, Han W, Chan W, Li L. Metabolomic coverage of chemical-group-submetabolome analysis: group classification and four-channel chemical isotope labeling LC-MS. *Analytical Chemistry*. 2019;91:12108-15.
[399] Chen D, Su X, Wang N, Li Y, Yin H, Li L, et al. Chemical isotope labeling LC-MS for monitoring disease progression and treatment in animal models: plasma metabolomics study of osteoarthritis rat model. *Scientific Reports*. 2017;7:40543.
[400] Spaapen LJM, Ketting D, Wadman SK, Bruinvis L, Duran M. Urinary D-4-hydroxyphenyllactate, D-phenyllactate and D-2-hydroxyisocaproate, abnormalities of bacterial origin. *Journal of Inherited Metabolic Disease*. 1987;10:383-90.
[401] Beloborodov NV, Khodakova AS, Bairamov IT, Olenin AY. Microbial origin of phenylcarboxylic acids in the human body. *Biochemistry (Moscow)*. 2009;74:1350-5.
[402] Guimarães A, Santiago A, Teixeira JA, Venâncio A, Abrunhosa L. Anti-aflatoxigenic effect of organic acids produced by *Lactobacillus plantarum*. *International Journal of Food Microbiology*. 2018;264:31-8.

[403] Guimarães A, Venancio A, Abrunhosa L. Antifungal effect of organic acids from lactic acid bacteria on *Penicillium nordicum*. *Food Additives & Contaminants: Part A*. 2018;35:1803-18.
[404] Rauschenbach MO, Zharova EI, Sergeeva TI, Ivanova VD, Probatova NA. Blastomogenic activity of hydroxyphenyllactic acid in mice. *Cancer Research*. 1975;35:577-85.
[405] Zharova EI, Sergeeva TI, Makhalova NV, Romanenko VI, Chitiridi NG, Raushenbakh MO. Transplacental carcinogenic action of p-hydroxyphenyllactic acid. *Bulletin of Experimental Biology and Medicine*. 1979;87:46-8.
[406] Tammali R, Seenayya G, Reddy G. Fermentation of cellulose to acetic acid by *Clostridium lentocellum* SG6: induction of sporulation and effect of buffering agent on acetic acid production. *Letters in Applied Microbiology*. 2003;37:304-8.
[407] Turton LJ, Drucker DB, Ganguli LA. Effect of glucose concentration in the growth medium upon neutral and acidic fermentation end-products of *Clostridium bifermentans*, *Clostridium sporogenes* and *Peptostreptococcus anaerobius*. *Journal of Medical Microbiology*. 1983;16:61-7.
[408] Phillips I, Lobo AZ, Fernandes R, Gundara NS. Acetic acid in the treatment of superficial wounds infected by *Pseudomonas aeruginosa*. *The Lancet*. 1968;291:11-3.
[409] Halstead FD, Rauf M, Moiemen NS, Bamford A, Wearn CM, Fraise AP, et al. The antibacterial activity of acetic acid against biofilm-producing pathogens of relevance to burns patients. *PLOS ONE*. 2015;10:e0136190.
[410] U.S. Food and Drug Administration. Food additive status list. 2019.
[411] Delaquis PJ, Sholberg PL, Stanich K. Disinfection of mung bean seed with gaseous acetic acid. *Journal of Food Protection*. 1999;62:953-7.
[412] Entani E, Asai M, Tsujihata S, Tsukamoto Y, Ohta M. Antibacterial action of vinegar against food-borne pathogenic bacteria including *Escherichia coli* O157:H7. *Journal of Food Protection*. 1998;61:953-9.
[413] Heimann E, Nyman M, Pålbrink A-K, Lindkvist-Petersson K, Degerman E. Branched short-chain fatty acids modulate glucose and lipid metabolism in primary adipocytes. *Adipocyte*. 2016;5:359-68.
[414] Petrognani C, Boon N, Ganigué R. Production of isobutyric acid from methanol by *Clostridium luticellarii*. *Green Chemistry*. 2020;22:8389-402.
[415] U.S. Food and Drug Administration. Isobutyric acid. Substances added to food: *U.S. Food and Drug Administration*; 2021.
[416] Huang CB, Alimova Y, Myers TM, Ebersole JL. Short- and medium-chain fatty acids exhibit antimicrobial activity for oral microorganisms. *Archives of Oral Biology*. 2011;56:650-4.
[417] Nakamura K, O'Neill AM, Williams MR, Cau L, Nakatsuji T, Horswill AR, et al. Short chain fatty acids produced by *Cutibacterium acnes* inhibit biofilm formation by *Staphylococcus epidermidis*. *Scientific Reports*. 2020;10.
[418] Goldberg I, Rokem JS. Organic and fatty acid production, microbial. Encyclopedia of Microbiology. Third ed: *Elsevier*; 2009. p. 421-42.
[419] Stadtman ER, Stadtman TC, Barker HA. Tracer experiments on the mechanism of synthesis of valeric and caproic acids by *Clostridium kluyveri*. *Journal of Biological Chemistry*. 1949;178:677-82.
[420] Kovanda L, Zhang W, Wei X, Luo J, Wu X, Atwill ER, et al. *In vitro* antimicrobial activities of organic acids and their derivatives on several species of Gram-negative and Gram-positive bacteria. *Molecules*. 2019;24:3770.

[421] Gio-Batta M, Sjöberg F, Jonsson K, Barman M, Lundell A-C, Adlerberth I, et al. Fecal short chain fatty acids in children living on farms and a link between valeric acid and protection from eczema. *Scientific Reports*. 2020;10.
[422] Liu P, Wang Y, Yang G, Zhang Q, Meng L, Xin Y, et al. The role of short-chain fatty acids in intestinal barrier function, inflammation, oxidative stress, and colonic carcinogenesis. *Pharmacological Research*. 2021;165:105420.
[423] Usta-Gorgun B, Yilmaz-Ersan L. Short-chain fatty acids production by *Bifidobacterium* species in the presence of salep. *Electronic Journal of Biotechnology*. 2020;47:29-35.
[424] Markowiak-Kopeć P, Śliżewska K. The effect of probiotics on the production of short-chain fatty acids by human intestinal microbiome. *Nutrients*. 2020;12:1107.
[425] Gillor O, Etzion A, Riley MA. The dual role of bacteriocins as anti- and probiotics. *Applied Microbiology and Biotechnology*. 2008;81:591-606.
[426] Lopetuso LR, Scaldaferri F, Petito V, Gasbarrini A. Commensal Clostridia: leading players in the maintenance of gut homeostasis. *Gut Pathogens*. 2013;5:23.
[427] Lo Grasso L, Chillura-Martino D, Alduina R. Production of antibacterial compounds from actinomycetes. *IntechOpen*; 2016.
[428] Kuete V, Kamga J, Sandjo LP, Ngameni B, Poumale HMP, Ambassa P, et al. Antimicrobial activities of the methanol extract, fractions and compounds from *Ficus polita* Vahl. (Moraceae). *BMC Complementary and Alternative Medicine*. 2011;11:6.
[429] Bartmańska A, Wałecka-Zacharska E, Tronina T, Popłoński J, Sordon S, Brzezowska E, et al. Antimicrobial properties of spent hops extracts, flavonoids isolated therefrom, and their derivatives. *Molecules (Basel, Switzerland)*. 2018;23:2059.
[430] Panter F, Bader CD, Müller R. Synergizing the potential of bacterial genomics and metabolomics to find novel antibiotics. *Chemical Science*. 2021;12:5994-6010.
[431] Bader CD, Haack PA, Panter F, Krug D, Müller R. Expanding the scope of detectable microbial natural products by complementary analytical methods and cultivation systems. *Journal of Natural Products*. 2021;84:268-77.
[432] Kim Y-m, Farrah S, Baney RH. Membrane damage of bacteria by silanols treatment. *Electronic Journal of Biotechnology*. 2007;10:252-9.
[433] Hurdle JG, O'Neill AJ, Chopra I, Lee RE. Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. *Nature Reviews Microbiology*. 2011;9:62-75.
[434] Hao T, Xie Z, Wang M, Liu L, Zhang Y, Wang W, et al. An anaerobic bacterium host system for heterologous expression of natural product biosynthetic gene clusters. *Nature Communications*. 2019;10.
[435] Guo L, Wang C, Zhu W-C, Xu F-Q. Bioassay-guided fractionation and identification of active substances from the fungus *Aspergillus tubingensis* against *Vibrio anguillarum*. *Biotechnology & Biotechnological Equipment*. 2016;30:602-6.

Appendices

Appendix A1

A1.1 R script used to calculate growth parameters including the area under the experimental curve

```
library(growthcurver)
d <- read.csv(choose.files(), header = T)

num_analyses <- length(names(d)) - 1
d_gc <- data.frame(sample = character(num_analyses),
                   k = numeric(num_analyses),
                   n0  = numeric(num_analyses),
                   r = numeric(num_analyses),
                   t_mid = numeric(num_analyses),
                   t_gen = numeric(num_analyses),
                   auc_l = numeric(num_analyses),
                   auc_e = numeric(num_analyses),
                   sigma = numeric(num_analyses),
                   stringsAsFactors = FALSE)

trim_at_time <- 24

#par(mfcol = c(8,6))
#par(mar = c(0.25,0.25,0.25,0.25))
#par(mfrow = c(4,5))
y_lim_max <- max(d[,setdiff(names(d), "time")]) - min(d[,setdiff(names(d), "time")])

n <- 1
for (col_name in names(d)) {
  if (col_name != "time") {

    d_loop <- d[, c("time", col_name)]
```

```
    gc_fit <- SummarizeGrowth(data_t = d_loop[, "time"],
                              data_n = d_loop[, col_name],
                              t_trim = trim_at_time,
                              bg_correct = "none")

    d_gc$sample[n] <- col_name
    d_gc[n, 2:9] <- c(gc_fit$vals$k,
                      gc_fit$vals$n0,
                      gc_fit$vals$r,
                      gc_fit$vals$t_mid,
                      gc_fit$vals$t_gen,
                      gc_fit$vals$auc_l,
                      gc_fit$vals$auc_e,
                      gc_fit$vals$sigma)
    n <- n + 1

    n_obs <- length(gc_fit$data$t)
    idx_to_plot <- 1:24 / 24 * n_obs
    plot(gc_fit$data$t[idx_to_plot], gc_fit$data$N[idx_to_plot],
         pch = 20,
         xlim = c(0, trim_at_time),
         ylim = c(0, y_lim_max),
         cex = 0.6,
         xlab = "Time (hr)",
         ylab = "Absorbance (595 nm)",
         main = col_name) #, xaxt = "n", yaxt = "n"
    #text(x = trim_at_time / 4, y = y_lim_max, labels = col_name, pos = 1)
    lines(gc_fit$data$t, predict(gc_fit$model), col = "red")
  }
}
```

Appendix A2

A2.1 16S rRNA gene sequences of representative isolates

PT08

AGTCGAGCGATCTCTTCGGAGAGAGCGGCGGACGGGTGAGTAACGCGTGGGTAACCTGCCC
TGTACACACGGATAACATACCGAAAGGTATACTAATACGGGATAACATATGAAAGTCGCAT
GGCTTTTGTATCAAAGCTCCGGCGGTACAGGATGGACCCGCGTCTGATTAGCTAGTTGGTAA
GGTAATGGCTTACCAAGGCAACGATCAGTAGCCGACCTGAGAGGGTGATCGGCCACACTGG
AACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCG
AAAGCCTGATGCAGCAACGCCGCGTGAGCGATGAAGGCCTTCGGGTCGTAAAGCTCTGTCCT
CAAGGAAGATAATGACGGTACTTGAGGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCCGC
GGTAATACGTAGGGGGCTAGCGTTATCCGGAATTACTGGGCGTAAAGGGTGCGTAGGTGGT
TTTTTAAGTCAGAAGTGAAAGGCTACGGCTCAACCGTAGTAAGCTTTTGAAACTAGAGAACT
TGAGTGCAGGAGAGGAGAGTAGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAG
GAATACCAGTAGCGAAGGCGGCTCTCTGGACTGTAACTGACACTGAGGCACGAAAGCGTGG
GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAGGTGTCG
GGGGTTACCCCCCTCGGTGCCGCAGCTAACGCATTAAGTACTCCGCCTGGGAAGTACGCTCG
CAAGAGTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGTAGCGGAGCATGTGGTTTAA
TTCGAAGCAACGCGAAGAACCTTACCTAAGCTTGACATCCCACTGACCTCTCCCTAATCGGA
GATTTCCCTTCGGGGACAGTGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAG
ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGCCTTTAGTTGCCAGCATTAAGTTGGG
CACTCTAGAGGGACTGCCGAGGATAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCAT
GCCCCTTATGCTTAGGGCTACACACGTGCTACAATGGGTGGTACAGAGGGTTGCCAAGCCGC
GAGGTGGAGCTAATCCCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGAAACTCGCCTACA
TGAAGCTGGAGTTACTAGTAATCGCAGATCAGAATGCTGCGGTGAATGCGTTCCCGGGTCTT
GTACACACCGCCCGTCACACCATGGAAGTTGGGGGCGCCCGAAGCCGGTTAGCTAACCTTTT
AGGAAGCGGCCGTCGAAGGTGAAACCA

PT13

TCGAGCGATTTACTTCCGGTAAAGAGCGGCGGACGGGTGAGTAACGCGTGGGTAACCTGCC
TCATACACATGGATAACATACCGAAAGGTATGCTAATACAGGATAATATAAGAGATTCACA
TGGATTTCTTATCAAAGCTCCGGCGGTATGAGATGGACCCGCGTCTGATTAGCTAGTTGGTA
AGGTAATGGCTTACCAAGGCGACGATCAGTAGCCGACCTGAGAGGGTGATCGGCCACATTG
GAACTGAGACACGGTCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGC
GAAAGCCTGATGCAGCAACGCCGCGTGAGTGATGAAGGCCTTCGGGTCGTAAAACTCTGTC
CTCAAGGAAGATAATGACGGTACTTGAGGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCC
GCGGTAATACGTAGGGGGCTAGCGTTATCCGGATTTACTGGGCGTAAAGGGTGCGTAGGTG
GTTTTTTAAGTCAGGAGTGAAAGGCTACGGCTCAACCGTAGTAAGCTCTTGAAACTGGAAAA
CTTGAGTGCAGGAGAGGAAAGTGGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGG
AGGAACACCAGTAGCGAAGGCGGCTTTCTGGACTGTAACTGACACTGAGGCACGAAAGCGT
GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAGGTGT-
-
CGGGGGTTACCCCCCCTCGGTGCCGCAGCTAACGCATTAAGTACTCCGCCNTGGGGAGTACG
CTCGCAAGAGTGAAACTCAAAGGAATTGACGGGGGACCCGCACAAGTAGCGGAGCATGTNG
GTTTAATTCG-AAGCAACGCGAAGAACCTTACCTAAGCTTGACATCC-
TTTTGACCGATGCCTAATCGCANTTTTTCCCTTCGGGGACAGAAGTGACAGGTGGTGCATGG
TTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGCCT
TTAGTTGCCAGCATTAAGTTGGGCACTCTAGAGGGACTGCCAGGGATAACCTGGAGGAAGG
TGGGGATGACGTCAAATCATCATGCCCCTTATGCTTAGGGCTACACACGTGCTACAATGGGT
GGTACAGAGGGCAGCCAAGTCGTGAGGCCGAGCTAATCCCTTAAAGCCATTCTCAGTTCGG
ATTGTAGGCTGAAACTCGCCTACATGAAGCTGGAGTTACTAGTAATCGCAGATCAGAATGCT
GCGGTGAATGCGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGGAAGTTGGGGGCGC
CCGAAGCCACTTAGCTAACCCTTTTGGGAAGCGAGTGTCGAAGGTGAAATCAATAACTGGG
GTGAAG

PT15

GTCGAGCGAGGGAGCACCTTCGGGTGTGAACTAGCGGCGGACGGGTGAGTAACACGTGGGC
AACCTGCCTTACAGAGGGGGATAGCCTTCCGAAAGGAAGATTAATACCGCATATTATGATTT
TTCTGCATGGGAAAGTCATGAAAGGAGCAATCCGCTGTAAGATGGGCCCGCGGCGCATTAG
CTAGTTGGTGAGGTAAGGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATC
GGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG
CACAATGGGGGAAACCCTGATGCAGCAACGCCGCGTGAGTGATGACGGCCTTCGGGTTGTA
AAGCTCTGTCTTCAGGGACGATAATGACGGTACCTGAGGAGGAAGCCACGGCTAACTACGT
GCCAGCAGCCGCGGTAATACGTAGGTGGCGAGCGTTATCCGGATTTACTGGGCGTAAAGGA
TGCGTAGGTGGAATTTTAAGTGGGATGTGAAATACCCGGGCTCAACCTGGGAACTGCATTCC
AAACTGGAATTCTAGAGTGCAGGAGAGGAAAGCGGAATTCCTAGTGTAGCGGTGAAATGCG
TAGAGATTAGGAAGAACACCAGTGGCGAAGGCGGCTTTCTGGACTGTAACTGACACTGAGG
CATGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGG
GTACTAGGTGTAGGGGTTTCGATACCTCTGTGCCGCCGTAAACACAATAAGTACCCCGCCTG
GGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGTAGCGGA
GCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAGACTTGACATGTCCTGAATTA
CCTGTAATAAGGGAAGCTCTTTCGGGAGCAGGAACACAGGTGGTGCATGGTTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATAGTTAGTTGCTAAC
AGTAAGATGAGCACTCTAGCTAGACTGCCGTGGTTAACGCGGAGGAAGGTGGGGATGACGT
CAAATCATCATGCCCCTTATGTCTAGGGCTACACACGTGCTACAATGGCGAGAACAAAGAG
AAGCAAGACCGCGAGGTGGAGCAAAACTCATAAAACTCGTCCCAGTTCGGATTGCAGGCTG
CAACTCGCCTGCATGAAGCCGGAGTTACTAGTAATCGCGAATCAGAATGTCGCGGTGAATAC
GTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTGGCAATACCCAAAGTCCGT
GAGGTAACCGAAAGGAGCCAGCGGCCTAAGGTAGGGTCAGCGATTGG

LB02

GCGATGAAGCTTCTTCGGAAGTGGATTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCT
GCCTCATAGAGAGGGATAGCCTTTCGAAAGGAAGATTAATACCTCATAAGATTGTAGTTTCG
CATGAAACGGCAATAAAAGGAGCAATCCGCTATGAGATGGGCCCGCGTCGCATTAGCTAGT
TGGTAAGGTAATGGCTTACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCA
CATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAA
TGGGGGAAACCCTGATGCAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATTGTAAAACT
CTGTCTTTGGGGACGATAATGACGGTACCCAAGGAGGAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTACTGGGCGTAAAGGGAGCGT
AGGTGGATATTTAAGTGGGATGTGAAATACTCGGGCTTAACCTGAGTGCTGCATTCCAAACT
GGATATCTAGAGTGCAGGAGAGGAAAGTGGAATTCCTAGTGTAGCGGTGAAATGCGTAGAT
ATTAGGAAGAACACCAGTGGCGAAGGCGACTTTCTGGACTGTAACTGACACTGAGGCTCGA
AAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATACT
AGGTGTAGGAGCTGTCATGGCTTCTGTGCCGCCGCTAACGCATTAAGTATTCCGCCTGGGGA
GTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCAT
GTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAGACTTGACATCTCCTGAATTACTCT
TAATCGAGGAAGTCACTTCGGTGACAGGAAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCTACCATTTA
GTTGAGCACTCTAGCGAGACTGCCGTGGTTAACGCGGAGGAAGGTGGGGATGACGTCAAAT
CATCATGCCCCTTATGTCTAGGGCTACACACGTGCTACAATGGCTGGTACAAAGAGATGCAA
TACCGCGAGGTGGAGCTAAACTATAAAACCAGTCTCAGTTCGGATTGTAGGCTGAAACTCGC
CTACATGAAGCTGGAGTTGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGG
GCCTTGTACACACCGCCCGTCACACCATGAGAGTTGGCAATACCCAAAGTTCGTGAGCTAAC
CCGTAAGGGAGGCAGCGACCTAAGGTAGGGTCAG

LB03

GAGCGATTCACTTCGGTGAAGAGCGGCGGACGGGTGAGTAACGCGTGGGTAACCTGCCTCA
TACACATGGATAACATACCGAAAGGTATGCTAATACAGGATAACATAAGAGATTCACATGT
ATCTCTTATCAAAGCTCCGGCGGTATGAGATGGACCCGCGTCTGATTAGCTAGTTGGTAAGG
TAACGGCTTACCAAGGCGACGATCAGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGAA
CTGAGACACGGTCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAA
AGCCTGATGCAGCAACGCCGCGTGAGTGATGAAGGCCTTCGGGTCGTAAAACTCTGTCCTCA
AGGAAGATAATGACGGTACTTGAGGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGG
TAATACGTAGGGGGCTAGCGTTATCCGGATTTACTGGGCGTAAAGGGTGCGTAGGTGGTTTC
TTAAGTCAGGAGTGAAAGGCTACGGCTCAACCGTAGTAAGCTCTTGAAACTGGGAGACTTG
AGTGCAGGAGAGGAAAGTGGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGA
ACACCAGTAGCGAAGGCGGCTTTCTGGACTGTAACTGACACTGAGGCACGAAAGCGTGGGG
AGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAGGTGTCGGG
GGTTACCCCCCTCGGTGCCGCAGCTAACGCATTAAGTACTCCGCCTGGGGAGTACGCTCGCA
AGAGTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGTAGCGGAGCATGTGGTTTAATT
CGAAGCAACGCGAAGAACCTTACCTAAGCTTGACATCCTTTTGACCGATGCCTAATCGCATT
TTTCCCTTCGGGGACAGAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGAT
GTTNGGGTTAAGTCCCGCAACGAGCGCAACCCTTGCCTTTAGTTGCCAGCATTAAGTTGGGC
ACTCTAGAGGGACTGCCAGGGATAACCTGGAGGAAGGTGGGGATGACGTCAAATCATCATG
CCCCTTATGCTTAGGGCTACACACGTGCTACAATGGGTGGTACAGAGGGCAGCCAAGTCGTG
AGGCCGAGCTAATCCCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGAAACTCGCCTACAT
GAAGCTGGAGTTACTAGTAATCGCAGATCAGAATGCTGCGGTGAATGCGTTCCCGGGTCTTG
TACACACCGCCCGTCACACCACGGAAGTTGGGGGCGCCCGAAGCCACTTAGCTAACCCTTTT
GGGAAGCGAGTGTCGAAGG

LB12

GTCGAGCGAGGGAGCACCTTCGGGTGTGAACTAGCGGCGGACGGGTGAGTAACACGTGGGC
AACCTGCCTTACAGAGGGGGATAGCCTTCCGAAAGGAAGATTAATACCGCATATTATGATTT
TTCTGCATGGGAAAGTCATGAAAGGAGCAATCCGCTGTAAGATGGGCCCGCGGCGCATTAG
CTAGTTGGTGAGGTAAGGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATC
GGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG
CACAATGGGGGAAACCCTGATGCAGCAACGCCGCGTGAGTGATGACGGCCTTCGGGTTGTA
AAGCTCTGTCTTCAGGGACGATAATGACGGTACCTGAGGAGGAAGCCACGGCTAACTACGT
GCCAGCAGCCGCGGTAATACGTAGGTGGCGAGCGTTATCCGGATTTACTGGGCGTAAAGGA
TGCGTAGGTGGAATTTTAAGTGGGATGTGAAATACCCGGGCTCAACCTGGGAACTGCATTCC
AAACTGGAATTCTAGAGTGCAGGAGAGGAAAGCGGAATTCCTAGTGTAGCGGTGAAATGCG
TAGAGATTAGGAAGAACACCAGTGGCGAAGGCGGCTTTCTGGACTGTAACTGACACTGAGG
CATGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGG
GTACTAGGTGTAGGGGTTTCGATACCTCTGTGCCGCCGTAAACACAATAAGTACCCCGCCTG
GGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGTAGCGGA
GCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAGACTTGACATGTCCTGAATTA
CCTGTAATAAGGGAAGCTCTTTCGGGAGCAGGAACACAGGTGGTGCATGGTTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATAGTTAGTTGCTAAC
AGTAAGATGAGCACTCTAGCTAGACTGCCGTGGTTAACGCGGAGGAAGGTGGGGATGACGT
CAAATCATCATGCCCCTTATGTCTAGGGCTACACACGTGCTACAATGGCGAGAACAAAGAG
AAGCAAGACCGCGAGGTGGAGCAAAACTCATAAAACTCGTCCCAGTTCGGATTGCAGGCTG
CAACTCGCCTGCATGAAGCCGGAGTTACTAGTAATCGCGAATCAGAATGTCGCGGTGAATAC
GTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTGGCAATACCCAAAGTCCGT
GAGGTAACCGAAAGGAGCCAGCGGCCTAAGGTAGGGTCAGCGA

LB10

TCGAGCGATGAAGTTTCCTTCGGGAAACGGATTAGCGGCGGACGGGTGAGTAACACGTGGG
TAACCTGCCTCATAGAGTGGAATAGCCTTCCGAAAGGAAGATTAATACCGCATAACGTTGAA
AGATGGCATCATCATTCAACCAAAGGAGCAATCCGCTATGAGATGGACCCGCGGCGCATTA
GCTAGTTGGTGGGGTAACGGCCTACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGAT
CGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT
GCACAATGGGGGAAACCCTGATGCAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGT
AAAGCTCTGTCTTTGGGGAAGATAATGACGGTACCCAAGGAGGAAGCCACGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGTGGCGAGCGTTATCCGGATTTACTGGGCGTAAAGG
GAGCGTAGGCGGATGATTAAGTGGGATGTGAAATACCCGGGCTCAACTTGGGTGCTGCATTC
CAAACTGGTTATCTAGAGTGCAGGAGAGGAGAGTGGAATTCCTAGTGTAGCGGTGAAATGC
GTAGAGATTAGGAAGAACACCAGTGGCGAAGGCGACTCTCTGGACTGTAACTGACGCTGAG
GCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG
AATACTAGGTGTGGGGGTTTCAACACCTCCGTGCCGCCGCTAACGCATTAAGTATTCCGCCT
GGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGTAGCGG
AGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTACACTTGACATCCCTTGCATT
ACTCTTAATCGAGGAAATCCCTTCGGGGACAAGGTGACAGGTGGTGCATGGTTGTCGTCAGC
TCGTGTCGTGAGATG-
TTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCGTTAGTTACTACCATTAAGTTGAGGAC
TCTAGCGAGACTGCCTGGGTTAMCCAGGAGGAAGGTGGGGATGACGTCAAATCATCATGCC
CCTTATGTGTAGGGCTACACACGTGCTACAATGGCTGGTACAGAGAGATGCAATACCGCGA
GGTGGAGCCAAACTTAAAAACCAGTCTCAGTTCGGATTGTAGGCTGAAACTCGCCTACATGA
AGCTGGAGTTACTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGTCTTGTA
CACACCGCCCGTCACACCATGAGAGTTGGCAATACCCGAAGTCCGTGAGCTAACCGCAAGG
AGGCAGCGGCCGAAGGTAGGGTCAGCGA

LB17

CGAGCGATGAAGCTCCTTCGGGAGTGGATTAGCGGCGGACGGGTGAGTAACACGTGGGTAA
CCTGCCTCATAGAGGGGAATAGCCTTTCGAAAGGAAGATTAATACCGCATAAGATTGTAGTA
CCGCATGGTACAGCAATTAAAGGAGTAATCCGCTATGAGATGGACCCGCGTCGCATTAGCTA
GTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGC
CACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCAC
AATGGGGGAAACCCTGATGCAGCAACGCCGCGTGAGTGATGACGGTCTTCGGATTGTAAAG
CTCTGTCTTTAGGGACGATAATGACGGTACCTAAGGAGGAAGCCACGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTACTGGGCGTAAAGGGAGCG
TAGGTGGATATTTAAGTGGGATGTGAAATACCCGGGCTTAACCTGGGTGCTGCATTCCAAAC
TGGATATCTAGAGTGCAGGAGAGGAAAGGAGAATTCCTAGTGTAGCGGTGAAATGCGTAGA
GATTAGGAAGAATACCAGTGGCGAAGGCGCCTTTCTGGACTGTAACTGACACTGAGGCTCG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATAC
TAGGTGTAGGGGTTGTCATGACCTCTGTGCCGCCGCTAACGCATTAAGTATTCCGCCTGGGG
AGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCA
TGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAGACTTGACATCTCCTGAATTACTC
TGTAATGGAGGAAGCCACTTCGGTGGCAGGAAGACAGGTGGTGCATGGTTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCTACCA
TTTAGTTGAGCACTCTAGCGAGACTGCCCGGGTTAACCGGGAGGAAGGTGGGGATGACGTC
AAATCATCATGCCCCTTATGTCTAGGGCTACACACGTGCTACAATGGTCGGTACAATGAGAT
GCAACCTCGCGAGAGTGAGCAAAACTATAAAACCGATCTCAGTTCGGATTGTAGGCTGAAA
CTCGCCTACATGAAGCTGGAGTTGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTT
CCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTGGCAATACCCAAAGTTCGTGAG
CTAACCGCAAGGAGGCAGCGACCTAAGGTAGGGTCAGCGA

RT01

AGAGCGGCGGACGGGTGAGTAACGCGTGGGTAACCTGCCTCATACACATGGATAACATACC
GAAAGGTATGCTAATACAGGATAATATAAGAGATTCACATGGATTTCTTATCAAAGCTCCGG
CGGTATGAGATGGACCCGCGTCTGATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCGAC
GATCAGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGAACTGAGACACGGTCCAAACTC
CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCAACGCCG
CGTGAGTGATGAAGGCCTTCGGGTCGTAAAACTCTGTCCTCAAGGAAGATAATGACGGTACT
TGAGGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCTAGCG
TTATCCGGATTTACTGGGCGTAAAGGGTGCGTAGGTGGTTTTTTAAGTCAGGAGTGAAAGGC
TACGGCTCAACCGTAGTAAGCTCTTGAAACTGGAAAACTTGAGTGCAGGAGAGGAAAGTGG
AATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAACACCAGTAGCGAAGGCGGC
TTTCTGGACTGTAACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGAGTACTAGGTGTCGGGGGTTACCCCCCTCGGTGCCGC
AGCTAACGCATTAAGTACTCCGCCTGGGGAGTACGCTCGCAAGAGTGAAACTCAAAGGAAT
TGACGGGGACCCGCACAAGTAGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCT
TACCTAAGCTTGACATCCTTTTGACCGATGCCTAATCGCATTTTTCCCTTCGGGGACAGAAGT
GACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACG
AGCGCAACCCTTGCCTTTAGTTGCCAGCATTAAGTTGGGCACTCTAGAGGGACTGCCAGGGA
TAACCTGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGCTTAGGGCTACACA
CGTGCTACAATGGGTGGTACAGAGGGCAGCCAAGTCGTGAGGCCGAGCTAATCCCTTAAAG
CCATTCTCAGTTCGGATTGTAGGCTGAAACTCGCCTACATGAAGCTGGAGTTACTAGTAATC
GCAGATCAGAATGCTGCGGTGAATGCGTTCCCGGGTCTTGTACACACCGCCCGTCACACCAC
GGAAGTTGGGGGCGCCCGAAGCCACTTAGCTAACCCTTTTGGGAAGCGAGTGTCGAAGGTG
AAATCAATAACTGGG

RT09

GTCGAGCGAGGGAGCACCTTCGGGTGTGAACTAGCGGCGGACGGGTGAGTAACACGTGGGC
AACCTGCCTTACAGAGGGGGATAGCCTTCCGAAAGGAAGATTAATACCGCATATTATGATTT
TTCTGCATGGGAAAGTCATGAAAGGAGCAATCCGCTGTAAGATGGGCCCGCGGCGCATTAG
CTAGTTGGTGAGGTAAGGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATC
GGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG
CACAATGGGGGAAACCCTGATGCAGCAACGCCGCGTGAGTGATGACGGCCTTCGGGTTGTA
AAGCTCTGTCTTCAGGGACGATAATGACGGTACCTGAGGAGGAAGCCACGGCTAACTACGT
GCCAGCAGCCGCGGTAATACGTAGGTGGCGAGCGTTATCCGGATTTACTGGGCGTAAAGGA
TGCGTAGGTGGAATTTTAAGTGGGATGTGAAATACCCGGGCTCAACCTGGGAACTGCATTCC
AAACTGGAATTCTAGAGTGCAGGAGAGGAAAGCGGAATTCCTAGTGTAGCGGTGAAATGCG
TAGAGATTAGGAAGAACACCAGTGGCGAAGGCGGCTTTCTGGACTGTAACTGACACTGAGG
CATGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGG
GTACTAGGTGTAGGGGTTTCGATACCTCTGTGCCGCCGTAAACACAATAAGTACCCCGCCTG
GGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGTAGCGGA
GCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAGACTTGACATGTCCTGAATTA
CCTGTAATAAGGGAAGCTCTTTCGGGAGCAGGAACACAGGTGGTGCATGGTTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATAGTTAGTTGCTAAC
AGTAAGATGAGCACTCTAGCTAGACTGCCGTGGTTAACGCGGANGGAAGGTGGGGATGACG
TCAAATCATCATGCCCCTTATGTCTAGGGCTACACACGTGCTACAATGGCGAGAACAAAGAG
AAGCAAGACCGCGAGGTGGAGCAAAACTCATAAAACTCGTCCCAGTTCGGATTGCAGGCTG
CAACTCGCCTGCATGAAGCCGGAGTTACTAGTAATCGCGAATCAGAATGTCGCGGTGAATAC
GTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTGGCAATACCCAAAGTCCGT
GAGGTAACCGAAAGGAGCCAGCGGCCTAAGGTAGGGTCAGCGATTGGG

RT10

CTCTTCGGAGAGAGCGGCGGACGGGTGAGTAACGCGTGGGTAACCTGCCCTGTACACACGG
ATAACATACCGAAAGGTATACTAATACGGGATAACATATGAAAGTCGCATGGCTTTTGTATC
AAAGCTCCGGCGGTACAGGATGGACCCGCGTCTGATTAGCTAGTTGGTAAGGTAATGGCTTA
CCAAGGCAACGATCAGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACG
GTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGC
AGCAACGCCGCGTGAGCGATGAAGGCCTTCGGGTCGTAAAGCTCTGTCCTCAAGGAAGATA
ATGACGGTACTTGAGGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA
GGGGGCTAGCGTTATCCGGAATTACTGGGCGTAAAGGGTGCGTAGGTGGTTTTTTAAGTCAG
AAGTGAAAGGCTACGGCTCAACCGTAGTAAGCTTTTGAAACTAGAGAACTTGAGTGCAGGA
GAGGAGAGTAGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAATACCAGTA
GCGAAGGCGGCTCTCTGGACTGTAACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAG
GATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAGGTGTCGGGGGTTACCCCC
CTCGGTGCCGCAGCTAACGCATTAAGTACTCCGCCTGGGAAGTACGCTCGCAAGAGTGAAA
CTCAAAGGAATTGACGGGGACCCGCACAAGTAGCGGAGCATGTGGTTTAATTCGAAGCAAC
GCGAAGAACCTTACCTAAGCTTGACATCCCACTGACCTCTCCCTAATCGGAGATTTCCCTTCG
GGGACAGTGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTGCCTTTAGTTGCCAGCATTAAGTTGGGCACTCTAGAGGG
ACTGCCGAGGATAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGCTT
AGGGCTACACACGTGCTACAATGGGTGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTA
ATCCCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGAAACTCGCCTACATGAAGCTGGAGT
TACTAGTAATCGCAGATCAGAATGCTGCGGTGAATGCGTTCCCGGGTCTTGTACACACCGCC
CGTCACACCATGGAAGTTGGGGGCGCCCGAAGCCGGTTAGCTAACCTTTTAGGAAGCGGCC
GTCGAAGGTGAAACCAATGAC

RT24

TAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTCATAGAGGGGAATAGCCTTTCG
AAAGGAAGATTAATACCGCATAAGATTGTAGTACCGCATGGTACAGCAATTAAAGGAGTAA
TCCGCTATGAGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCG
ACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCAACGC
CGCGTGAGTGATGACGGCCTTCGGGTTGTAAAACTCTGTCTTTGGGGACGATAATGACGGTA
CCCAAGGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAG
CGTTGTCCGGATTTACTGGGCGTAAAGGGAGCGTAGGTGGATATTTAAGTGGGATGTGAAAT
ACTCGGGCTTAACCTGGGTGCTGCATTCCAAACTGGATATCTAGAGTGCAGGAGAGGAAAG
GAGAATTCCTAGTGTAGCGGTGAAATGCGTAGAGATTAGGAAGAATACCAGTGGCGAAGGC
GCCTTTCTGGACTGTAACTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTAGGGGTTGTCATGACCTCTGTG
CCGCCGCTAACGCATTAAGTATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAG
GAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGA
ACCTTACCTAGACTTGACATCTCCTGAATTACTCTGTAATGGAGGAAGCCACTTCGGTGGCA
GGAAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTTGGGTTAAGTCCCG
CAACGAGCGCAACCCTTATTGTTAGTTGCTACCATTTAGTTGAGCACTCTAGCGAGACTGC

RT26

CTCCTTCGGGAGTGGAATAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTCATAG
AGGGGAATAGCCTTCCGAAAGGGAGATTAATACCGCATAACATAACTTTATCGCATGGTAG
AGTTATCAAAGGAGTAATCCGCTATGAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGT
AACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGAAC
TGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGGGAAA
CCCTGACGCAGCAACGCCGCGTGAGTGAAGAAGGCCTTAGGGTTGTAAAGCTCTGTCATATG
GGAAGATAATGACGGTACCATAAGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGTGGCGAGCGTTGTCCGGATTTACTGGGCGTAAAGGATGCGTAGGCGGATATT
TAAGTGGGATGTGAAATCCCCGAGCTTAACTCGGGGGCTGCATTCCAAACTGGATATCTAGA
GTGTCGGAGGGGAAAGTGGAATTCCTAGTGTAGCGGTGAAATGCGTAGAGATTAGGAAGAA
CACCAGTGGCGAAGGCGACTTTCTGGACGATAACTGACGCTGAGGCATGAAAGCGTGGGGA
GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTAGGAG
GTATCGACTCCTTCTGTGCCGCAGTTAACACATTAAGTATTCCGCCTGGGGAGTACGATCGC
AAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGTTTAAT
TCGAAGCAACGCGAAGAACCTTACCTAGACTTGACATCTCCTGCATTACTCTTAACAGAGGA
AGTCCTTCGGGACAGGATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTT-
GGGTTAAGTCCCGCAACGAGCGCAACCCTTATCATTAGTTGCTACCATTAAGTTGAGCACTC
TAGTGAGACTGCCCGGGTTAACCGGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC
TTATGTCTAGGGCTACACACGTGCTACAATGGTAGGTACAACAAGATGCAATACCGTGAGGT
GGAGCTAAACTCAAAAACCTATCCCAGTTCGGATTGTAGGCTGAAACTCGCCTACATGAAGC
CGGAGTTGCTAGTAATCGCGAATCAGCATGTCGCGGTGAATACGTTCCCGGGCCTTGTACAC
ACCGCCCGTCACACCATGAGAGCCGATAACACCCGAAGTCCGTGAGGTAACCGTAAGGAGC
CAGCGGCC

FS01

TCGAGCGATCTCTTCGGAGAGAGCGGCGGACGGGTGAGTAACGCGTGGGTAACCTGCCCTG
TACACACGGATAACATACCGAAAGGTATACTAATACGGGATAAYATAYGAAAGTCGCATGG
CTTTTGTATCAAAGCTCCGGCGGTACAGGATGGACCCGCGTCTGATTAGCTAGTTGGTAAGG
TAATGGCTTACCAAGGCAACGATCAGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAA
CTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAA
AGCCTGATGCAGCAACGCCGCGTGAGCGATGAAGGCCTTCGGGTCGTAAAGCTCTGTCCTCA
AGGAAGATAATGACGGTACTTGAGGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGG
TAATACGTAGGGGGCTAGCGTTATCCGGAATTACTGGGCGTAAAGGGTGCGTAGGTGGTTTT
TTAAGTCAGAAGTGAAAGGCTACGGCTCAACCGTAGTAAGCTTTTGAAACTAGAGAACTTG
AGTGCAGGAGAGGAGAGTAGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGA
ATACCAGTAGCGAAGGCGGCTCTCTGGACTGTAACTGACACTGAGGCACGAAAGCGTGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAGGTGTCGGG
GGTTACCCCCCTCGGTGCCGCAGCTAACGCATTAAGTACTCCGCCTGGGAAGTACGCTCGCA
AGAGTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGTAGCGGAGCATGTGGTTTAATT
CGAAGCAACGCGAAGAACCTTACCTAAGCTTGACATCCCACTGACCTCTCCCTAATCGGAGA
TTTCCCTTCGGGGACAGTGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGAT
GTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGCCTTTAGTTGCCAGCATTAAGTTGGGCA
CTCTAGAGGGACTGCCGAGGATAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGC
CCCTTATGCTTAGGGCTACACACGTGCTACAATGGGTGGTACAGAGGGTTGCCAAGCCGCGA
GGTGGAGCTAATCCCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGAAACTCGCCTACATG
AAGCTGGAGTTACTAGTAATCGCAGATCAGAATGCTGCGGTGAATGCGTTCCCGGGTCTTGT
ACACACCGCCCGTCACACCATGGAAGTTGGGGGCGCCCGAARCCGGTTAGCTAACCTTTTAG
GAAGCGGCCGTCGAAGGTGAAACCA

FS2.2

GTCGAGCGAGGGAGCACCTTCGGGTGTGAACTAGCGGCGGACGGGTGAGTAACACGTGGGC
AACCTGCCTTACAGAGGGGGATAGCCTTCCGAAAGGAAGATTAATACCGCATATTATGATTT
TTCTGCATGGGAAAGTCATGAAAGGAGCAATCCGCTGTAAGATGGGCCCGCGGCGCATTAG
CTAGTTGGTGAGGTAAGGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATC
GGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG
CACAATGGGGGAAACCCTGATGCAGCAACGCCGCGTGAGTGATGACGGCCTTCGGGTTGTA
AAGCTCTGTCTTCAGGGACGATAATGACGGTACCTGAGGAGGAAGCCACGGCTAACTACGT
GCCAGCAGCCGCGGTAATACGTAGGTGGCGAGCGTTATCCGGATTTACTGGGCGTAAAGGA
TGCGTAGGTGGAATTTTAAGTGGGATGTGAAATACCCGGGCTCAACCTGGGAACTGCATTCC
AAACTGGAATTCTAGAGTGCAGGAGAGGAAAGCGGAATTCCTAGTGTAGCGGTGAAATGCG
TAGAGATTAGGAAGAACACCAGTGGCGAAGGCGGCTTTCTGGACTGTAACTGACACTGAGG
CATGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGG
GTACTAGGTGTAGGGGTTTCGATACCTCTGTGCCGCCGTAAACACAATAAGTACCCCGCCTG
GGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGTAGCGGA
GCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAGACTTGACATGTCCTGAATTA
CCTRTAATAAGGGAAGCTCTTTCGGGAGCAGGAACACAGGTGGTGCATGGTTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATAGTTAGTTGCTAAC
AGTAAGATGAGCACTCTAGCTAGACTGCCGTGGTTAACGCGGAGGAAGGTGGGGATGACGT
CAAATCATCATGCCCCTTATGTCTAGGGCTACACACGTGCTACAATGGCGAGAACAAAGAG
AAGCAAGACCGCGAGGTGGAGCAAAACTCATAAAACTCGTCCCAGTTCGGATTGCAGGCTG
CAACTCGCCTGCATGAAGCCGGAGTTACTAGTAATCGCGAATCAGAATGTCGCGGTGAATAC
GTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTGGCAATACCCAAAGTCCGT
GAGGTAACCGAAAGGAGCCAGCGGCCTAAGGTAGG

FS03

TTACTTCGGTAAAGAGCGGCGGACGGGTGAGTAACGCGTGGGTAACCTGCCTCATACACAT
GGATAACATACCGAAAGGTATGCTAATACAGGATAATATAAGAGATTCACATGGATTTTTTA
TCAAAGCTCCGGCGGTATGAGATGGACCCGCGTCTGATTAGCTAGTTGGTAAGGTAATGGCT
TACCAAGGCGACGATCAGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGAACTGAGACA
CGGTCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGAT
GCAGCAACGCCGCGTGAGTGATGAAGGCCTTCGGGTCGTAAAACTCTGTCCTCAAGGAAGA
TAATGACGGTACTTGAGGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACG
TAGGGGGCTAGCGTTATCCGGATTTACTGGGCGTAAAGGGTGCGTAGGTGGTTTTTTAAGTC
AGGAGTGAAAGGCTACGGCTCAACCGTAGTAAGCTCTTGAAACTGGAAAACTTGAGTGCAG
GAGAGGAAAGTGGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAACACCAG
TAGCGAAGGCGGCTTTCTGGACTGTAACTGACACTGAGGCACGAAAGCGTGGGGAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAGGTGTCGGGGGTTACCC
CCCTCGGTGCCGCAGCTAACGCATTAAGTACTCCGCCTGGGGAGTACGCTCGCAAGAGTGAA
ACTCAAAGGAATTGACGGGGACCCGCACAAGTAGCGGAGCATGTGGTTTAATTCGAAGCAA
CGCGAAGAACCTTACCTAAGCTTGACATCCTTTTGACCGATGCCTAATCGCATTTTTCCCTTC
GGGGACAGAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTGCCTTTAGTTGCCAGCATTAAGTTGGGCACTCTAGAGG
GACTGCCAGGGATAACCTGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGC
TTAGGGCTACACACGTGCTACAATGGGTGGTACAGAGGGCAGCCAAGTCGTGAGGCCGAGC
TAATCCCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGAAACTCGCCTACATGAAGCTGGA
GTTACTAGTAATCGCAGATCAGAATGCTGCGGTGAATGCGTTCCCGGGTCTTGTACACACCG
CCCGTCACACCACGGAAGTTGGGGGCGCCCGAAGCCACTTAGCTAACCCTTTTGGGAAGCG
AGTGTCGAAGGTGAAATCAATAAC

FS04

TCCTTCGGGAGTGGAATAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTCATAGA
GGGGAATAGCCTTCCGAAAGGGAGATTAATACCGCATAACATAACTTTATCGCATGGTAGA
GTTATCAAAGGAGTAATCCGCTATGAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTA
ACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGAACT
GAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGGGAAAC
CCTGACGCAGCAACGCCGCGTGAGTGAAGAAGGCCTTAGGGTTGTAAAGCTCTGTCATATG
GGAAGATAATGACGGTACCATAAGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGTGGCGAGCGTTGTCCGGATTTACTGGGCGTAAAGGATGCGTAGGCGGATATT
TAAGTGGGATGTGAAATCCCCGAGCTTAACTCGGGGGCTGCATTCCAAACTGGATATCTAGA
GTGTCGGAGGGGAAAGTGGAATTCCTAGTGTAGCGGTGAAATGCGTAGAGATTAGGAAGAA
CACCAGTGGCGAAGGCGACTTTCTGGACGATAACTGACGCTGAGGCATGAAAGCGTGGGGA
GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTAGGAG
GTATCGACTCCTTCTGTGCCGCAGTTAACACATTAAGTATTCCGCCTGGGGAGTACGATCGC
AAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGTTTAAT
TCGAAGCAACGCGAAGAACCTTACCTAGACTTGACATCTCCTGCATTACTCTTAACAGAGGA
AGTCCTTCGGGACAGGATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCTTATCATTAGTTGCTACCATTAAGTTGAGCACTC
TAGTGAGACTGCCCGGGTTAACCGGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC
TTATGTCTAGGGCTACACACGTGCTACAATGGTAGGTACAACAAGATGCAATACCGTGAGGT
GGAGCTAAACTCAAAAACCTATCCCAGTTCGGATTGTAGGCTGAAACTCGCCTACATGAAGC
CGGAGTTGCTAGTAATCGCGAATCAGCATGTCGCGGTGAATACGTTCCCGGGCCTTGTACAC
ACCGCCCGTCACACCATGAGAGCCGATAACACCCGAAGTCCGTGAGGTAACCGTAAGGAGC
CAGCGGCCGAAGGTGGGAT

PH1

GAGTAACACGTGGGTAACCTGCCTCATAGAGGGGAATAGCCTTCCGAAAGGAAGATTAATA
CCGCATAATGTAGTACCTTCGCATGAAGGAACTATCAAAAGAGCAATCTGCTATGAGATGG
ACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCG
ACCTGAGAGGGTGATCGGCCACATTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA
GCAGTGGGGAATATTGCGCAATGGGGGAAACCCTGACGCAGCAACGCCGCGTGAATGAAGA
AGGCCTTCGGGTTGTAAAGTTCTGTCTTATGGGAAGATAATGACGGTACCATAGGAGGAAGC
CACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTA
CTGGGCGTAAAGGATGCGTAGGCGGATATTTAAGTGGGATGTGAAATACCTGAGCTCAACT
CGGGTGCTGCATTCCAAACTGGATATCTAGAGTGTCGGAGGGGAAAGCGGAATTCCTAGTGT
AGCGGTGAAATGCGTAGAGATTAGGAAGAACACCAGTGGCGAAGGCGGCTTTCTGGACGAT
AACTGACGCTGAGGCATGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
GCCGTAAACGATGAATACTAGGTGTAGGAGGTATCGACTCCTTCTGTGCCGCAGTTAACACA
TTAAGTATTCCGCCTGGGGAGTACGATCGCAAGATTAAAACTCAAAGGAATTGACGGGGGC
CCGCACAAGCAGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAGACT
TGACATCCCCTGAATCACTCTTAATCGAGGAAGTCCTTCGGGACAGGGAGACAGGTGGTGCA
TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTA
TCATTAGTTGCTACCATTAAGTTGAGCACTCTAGTGAGACTGCCCGGGTTAACCGGGAGGAA
GGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTAGGGCTACACACGTGCTACAATGG
TAGGTACAAATAGACGCAATACCGCGAGGTGGAGCAAAACTCTAAAACCTATCCCAGTTCG
GATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAGTTGCTAGTAATCGCGAATCAGCATGT
CGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGCCGATAACA
CCCGAAGTCCGTGAGGTAACCGTAAGGAGCCAGCGGCCGAAGG

PH05

GGGTGAGTAACGCGTGGGTAACCTGCCTCATACACATGGATAACATACCGAAAGGTATGCT
AATACAGGATAATATAAGAGATTCACATGGATTTCTTATCAAAGCTCCGGCGGTATGAGATG
GACCCGCGTCTGATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCGACGATCAGTAGCCG
ACCTGAGAGGGTGATCGGCCACATTGGAACTGAGACACGGTCCAAACTCCTACGGGAGGCA
GCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCAACGCCGCGTGAGTGATGA
AGGCCTTCGGGTCGTAAAACTCTGTCCTCAAGGAAGATAATGACGGTACTTGAGGAGGAAG
CCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCTAGCGTTATCCGGATTT
ACTGGGCGTAAAGGGTGCGTAGGTGGTTTTTTAAGTCAGGAGTGAAAGGCTACGGCTCAAC
CGTAGTAAGCTCTTGAAACTGGGAAACTTGAGTGCAGGAGAGGAAAGTGGAATTCCTAGTG
TAGCGGTGAAATGCGTAGATATTAGGAGGAACACCAGTAGCGAAGGCGGCTTTCTGGACTG
TAACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGAGTACTAGGTGTCGGGGGTTACCCCCCTCGGTGCCGCAGCTAACGCAT
TAAGTACTCCGCCTGGGGAGTACGCTCGCAAGAGTGAAACTCAAAGGAATTGACGGGGACC
CGCACAAGTAGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAAGCTT
GACATCCTTTTGACCGATGCCTAATCGCATTTTTCCCTTCGGGGACAGAAGTGACAGGTGGT
GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTGCCTTTAGTTGCCAGCATTAAGTTGGGCACTCTAGAGGGACTGCCAGGGATAACCTGGAG
GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGCTTAGGGCTACACACGTGCTACAA
TGGGTGGTACAGAGGGCAGCCAAGTCGTGAGGCCGAGCTAATCCCTTAAAGCCATTCTCAGT
TCGGATTGTAGGCTGAAACTCGCCTACATGAAGCTGGAGTTACTAGTAATCGCAGATCAGAA
TGCTGCGGTGAATGCGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGGAAGTTGGGG
GCGCCCGAAGCCGACTTAGCTAACCCTTT

PH08

GTCGAGCGATGAAGTTTCCTTCGGGAAACGGATTAGCGGCGGACGGGTGAGTAACACGTGG
GTAACCTGCCTCATAGAGTGGAATAGCCTTCCGAAAGGAAGATTAATACCGCATAATGTTGA
AAGATGGCATCATCATTCAACCAAAGGAGCAATCCGCTATGAGATGGACCCGCGGCGCATT
AGCTAGTTGGTGGGGTAACGGCCTACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGA
TCGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT
TGCACAATGGGGGAAACCCTGATGCAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCG
TAAAGCTCTGTCTTTGGGGAAGATAATGACGGTACCCAAGGAGGAAGCCACGGCTAACTAC
GTGCCAGCAGCCGCGGTAATACGTAGGTGGCGAGCGTTATCCGGATTTACTGGGCGTAAAG
GGAGCGTAGGCGGATGATTAAGTGGGATGTGAAATACCCGGGCTCAACTTGGGTGCTGCAT
TCCAAACTGGTTATCTAGAGTGCAGGAGAGGAGAGTGGAATTCCTAGTGTAGCGGTGAAAT
GCGTAGAGATTAGGAAGAACACCAGTGGCGAAGGCGACTCTCTGGACTGTAACTGACGCTG
AGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGA
TGAATACTAGGTGTGGGGGTTTCAACACCTCCGTGCCGCCGCTAACGCATTAAGTATTCCGC
CTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGTAGC
GGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTACACTTGACATCCCTTGCA
TTACTCTTAATCGAGGAAATCCCTTCGGGGACAAGGTGACAGGTGGTGCATGGTTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCGTTAGTTACT
ACCATTAAGTTGAGGACTCTAGCGAGACTGCCTGGGTTAACCAGGAGGAAGGTGGGGATGA
CGTCAAATCATCATGCCCCTTATGTGTAGGGCTACACACGTGCTACAATGGCTGGTACAGAG
AGATGCAATACCGCGAGGTGGAGCCAAACTTAAAAACCAGTCTCAGTTCGGATTGTAGGCT
GAAACTCGCCTACATGAAGCTGGAGTTACTAGTAATCGCGAATCAGAATGTCGCGGTGAAT
ACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGAGAGTTGGCAATACCCGAAGTCC
GTGAGCTAACCGCAAGGAGGCAGCGGCCGAAGGTAGGGTCAGCGA

PH10

TCTTCGGAGAGAGCGGCGGACGGGTGAGTAACGCGTGGGTAACCTGCCCTGTACACACGGA
TAACATACCGAAAGGTATACTAATACGGGATAACATACGAAAGTCGCATGGCTTTTGTATCA
AAGCTCCGGCGGTACAGGATGGACCCGCGTCTGATTAGCTAGTTGGTAAGGTAATGGCTTAC
CAAGGCAACGATCAGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGG
TCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCA
GCAACGCCGCGTGAGCGATGAAGGCCTTCGGGTCGTAAAGCTCTGTCCTCAAGGAAGATAA
TGACGGTACTTGAGGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
GGGGCTAGCGTTATCCGGAATTACTGGGCGTAAAGGGTGCGTAGGTGGTTTTTTAAGTCAGA
AGTGAAAGGCTACGGCTCAACCGTAGTAAGCTTTTGAAACTAGAGAACTTGAGTGCAGGAG
AGGAGAGTAGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAATACCAGTAG
CGAAGGCGGCTCTCTGGACTGTAACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAGGTGTC--
GGGGGTTACCCCCCCTCGGTGCCGCAGCTAACGCATTAAGTACTCCGCCNTGGGAAGTACGC
TCGCAAGAGTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGTAGCGGAGCATGTGGTT
TAATTCGAAGCAACGCGAAGAACCTTACCTAAGCTTGACATCCCACTGACCTCTCCCTNAAT
CGGAGATTTCCCTTCGGGGGACAGTGGTGACAGGTGGTGNCATGNGTTGTCGTCAGCTCGTG
TCGTGAG-
ATGTTTGGGGTTAAGTCCNCGCAACGAGCGCAACCCTTNGCCTTTAGTTGCCAGCATTAAGT
TGGGCACTCTAGAGGGACTGCCGAGGATAACTCGGAGGAAGGTGGGGATGACGTCAAATCA
TCATGCCCCTTATGCTTAGGGCTACACACGTGCTACAATGGGTGGTACAGAGGGTTGCCAAG
CCGCGAGGTGGAGCTAATCCCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGAAACTCGCC
TACATGAAGCTGGAGTTACTAGTAATCGCAGATCAGAATGCTGCGGTGAATGCGTTCCCGGG
TCTTGTACACACCGCCCGTCACACCATGGAAGTTGGGGGCGCCCGAAGCCGGTTAGCTAACC
TTTTAGGAAGCGGCCGTCGAAGGTGAAACC

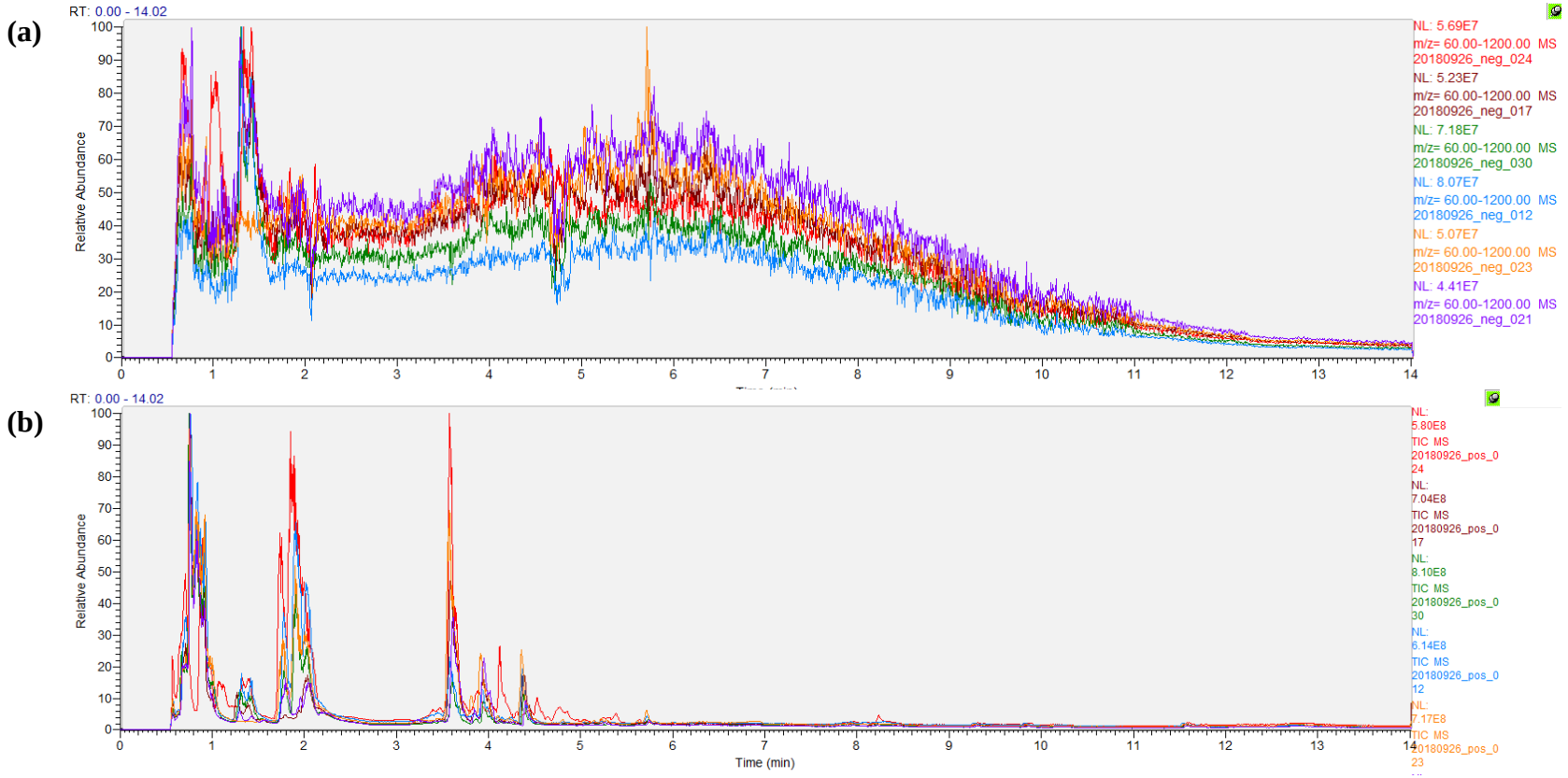
PH21

CGAGCGAGGGAGCACCTTCGGGTGTGAACTAGCGGCGGACGGGTGAGTAACACGTGGGCAA
CCTGCCTTACAGAGGGGGATAGCCTTCCGAAAGGAAGATTAATACCGCATATTATGATTTTT
CTGCATGGGAAAGTCATGAAAGGAGCAATCCGCTGTAAGATGGGCCCGCGGCGCATTAGCT
AGTTGGTGAGGTAAGGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGG
CCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA
CAATGGGGGAAACCCTGATGCAGCAACGCCGCGTGAGTGATGACGGCCTTCGGGTTGTAAA
GCTCTGTCTTCAGGGACGATAATGACGGTACCTGAGGAGGAAGCCACGGCTAACTACGTGC
CAGCAGCCGCGGTAATACGTAGGTGGCGAGCGTTATCCGGATTTACTGGGCGTAAAGGATG
CGTAGGTGGAATTTTAAGTGGGATGTGAAATACCCGGGCTCAACCTGGGAACTGCATTCCAA
ACTGGAATTCTAGAGTGCAGGAGAGGAAAGCGGAATTCCTAGTGTAGCGGTGAAATGCGTA
GAGATTAGGAAGAACACCAGTGGCGAAGGCGGCTTTCTGGACTGTAACTGACACTGAGGCA
TGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGGT
ACTAGGTGTAGGGGTTTCGATACCTCTGTGCCGCCGTAAACACAATAAGTACCCCGCCTGGG
GAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGTAGCGGAGC
ATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAGACTTGACATGTCCTGAATTACC
TGTAATAAGGGAAGCTCTTTCGGGAGCAGGAACACAGGTGGTGCATGGTTGTCGTCAGCTCG
TGTCGTGAGATG-
TTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATAGTTAGTTGCTAACAGTAAGATGAGCAC
TCTAGCTAGACTGCCGTGGTTAACGCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCC
CCTTATGTCTAGGGCTACACACGTGCTACAATGGCGAGAACAAAGAGAAGCAAGACCGCGA
GGTGGAGCAAAACTCATAAAACTCGTCCCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATG
AAGCCGGAGTTACTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGT
ACACACCGCCCGTCACACCATGAGAGTTGGCAATACCCAAAGTCCGTGAGGTAACCGAAAG
GAGCCATGCGGCCTAAGGTAGGGTCAGCGATTGG

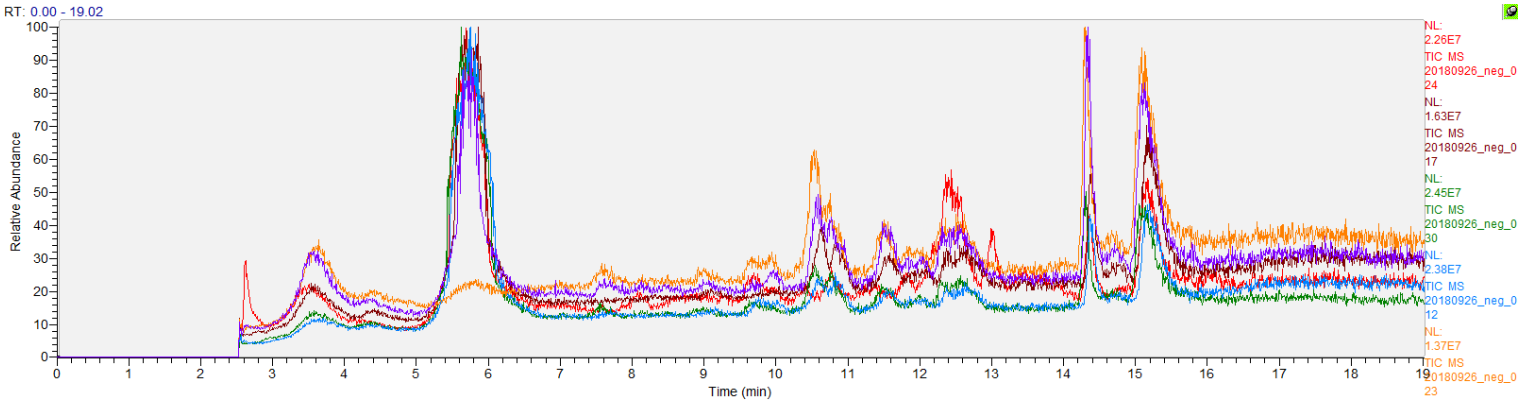
Appendix A3

A3.1 Total ion chromatograms (TICs) of CMGS and five soil CMs

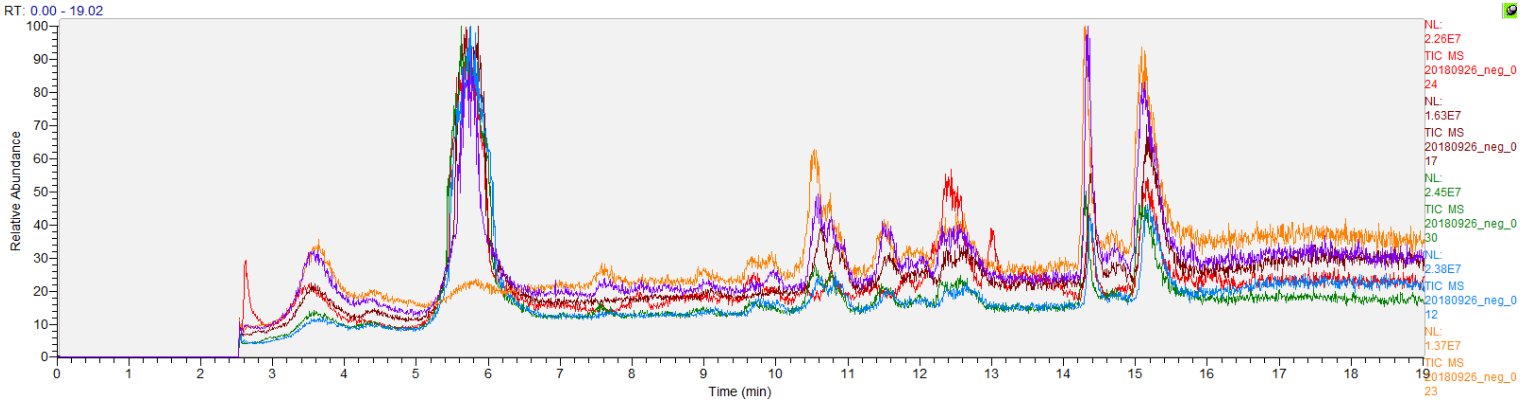
Representative LC-MS chromatograms of CMGS (red), F1SCM (brown), F2SCM (green), F3SCM (blue), F4SCM (orange) and F5SCM (purple) in C18 negative **(a)**, C18 positive **(b)**, HILIC negative **(c)**, and HILIC positive **(d)** ionization modes.



(c)

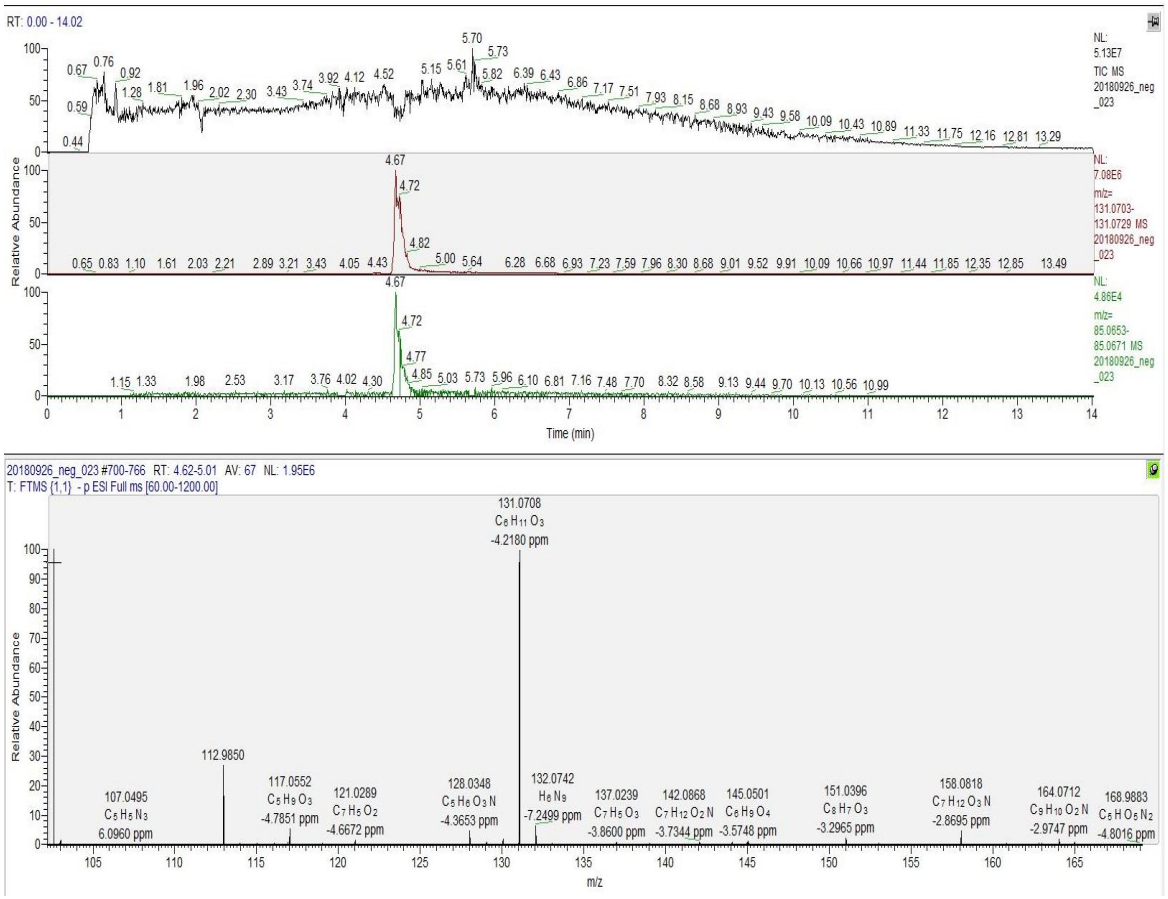

RT: 0.00 - 19.02
Relative Abundance
Time (min)
NL:
2.26E7
TIC MS
20180926_neg_024
NL:
1.63E7
TIC MS
20180926_neg_017
NL:
2.45E7
TIC MS
20180926_neg_030
NL:
2.38E7
TIC MS
20180926_neg_012
NL:
1.37E7
TIC MS
20180926_neg_023

(d)

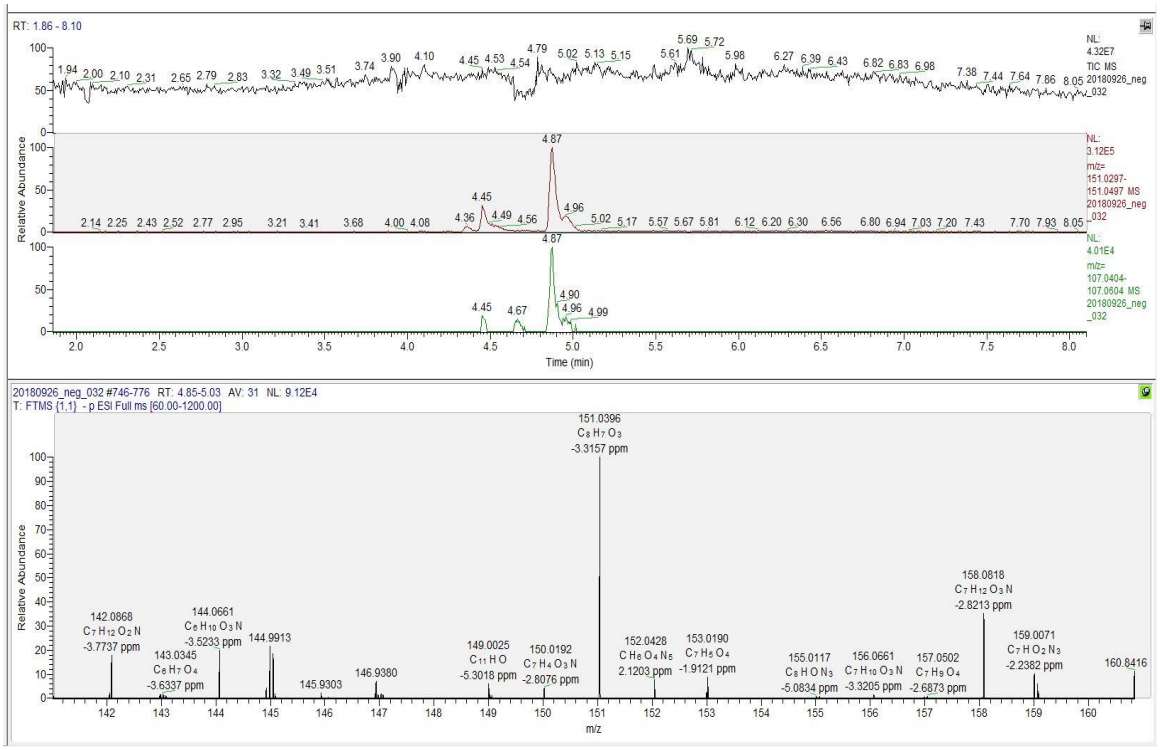

RT: 0.00 - 19.02
Relative Abundance
Time (min)
NL:
2.26E7
TIC MS
20180926_neg_024
NL:
1.63E7
TIC MS
20180926_neg_017
NL:
2.45E7
TIC MS
20180926_neg_030
NL:
2.38E7
TIC MS
20180926_neg_012
NL:
1.37E7
TIC MS
20180926_neg_023

A3.2 Extracted ion chromatograms for parent masses and co-eluting diagnostic fragments of putatively identified compounds

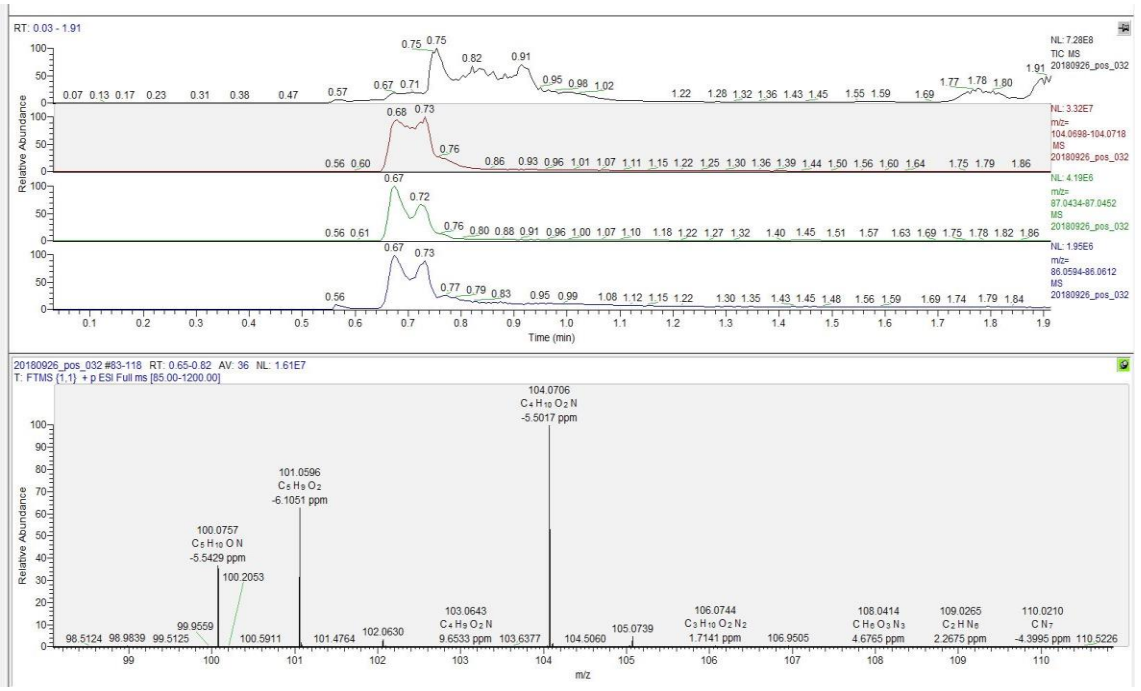
Compound name	*m/z*	RT (s)	Molecular formula	Adduct	Fragments (*m/z*)	Level of identification
2-hydroxyisocaproic acid	131.0708	284.2	C6H12O3	[M-H]-	85.0662	2



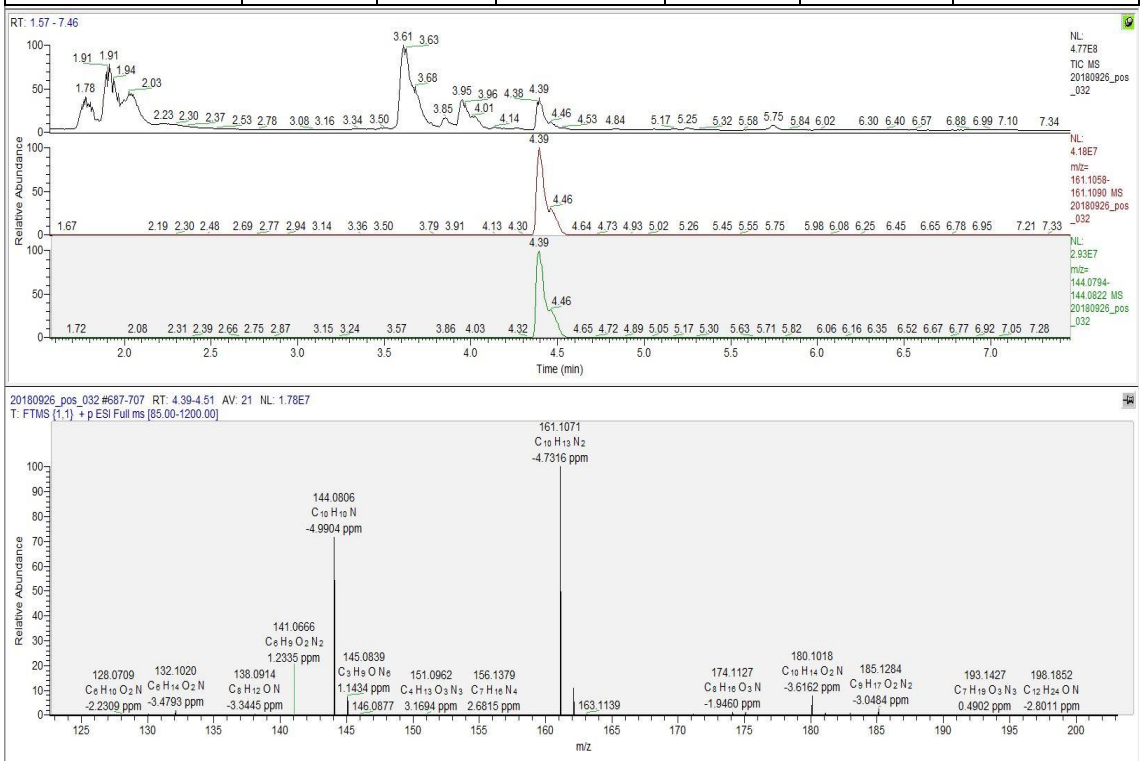
Compound name	*m/z*	RT (s)	Molecular formula	Adduct	Fragments (*m/z*)	Level of identification
3-Hydroxyphenylacetic acid	151.0395	295.3	C8H8O3	[M-H]-	107.0504	2



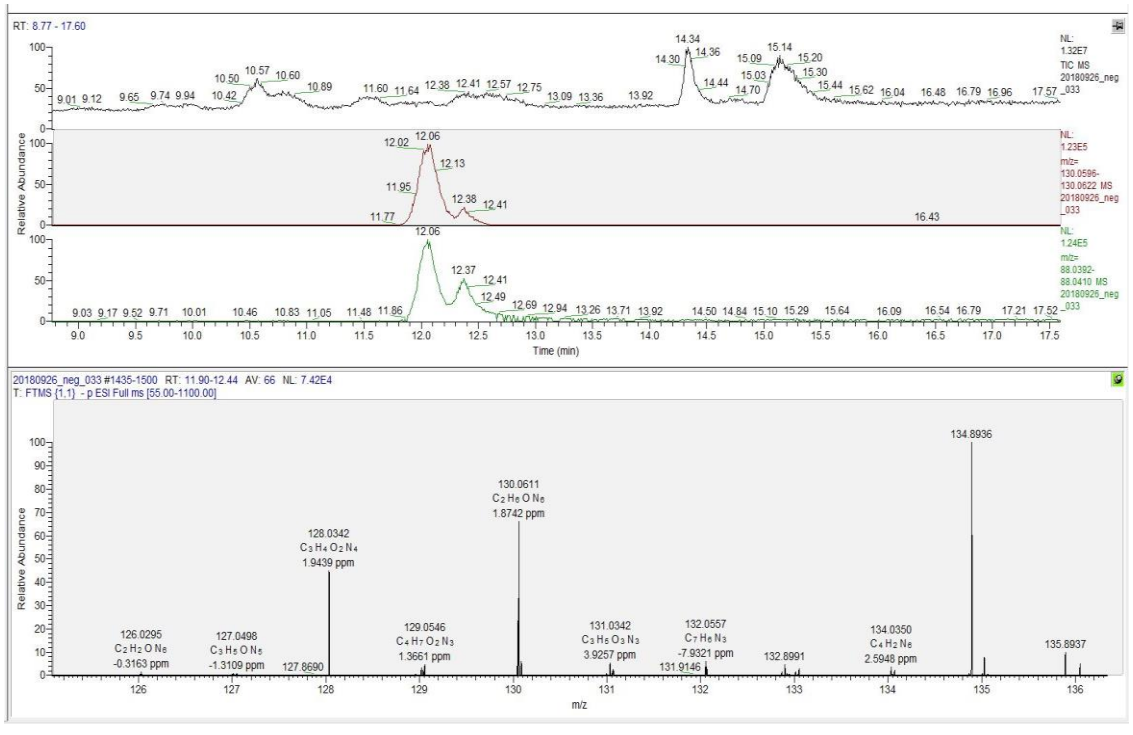
Compound name	*m/z*	RT (s)	Molecular formula	Adduct	Fragments (*m/z*)	Level of identification
γ-Aminobutyric acid	104.0706	42.41	C4H9NO2	[M+H]+	87.0443, 86.0603	2



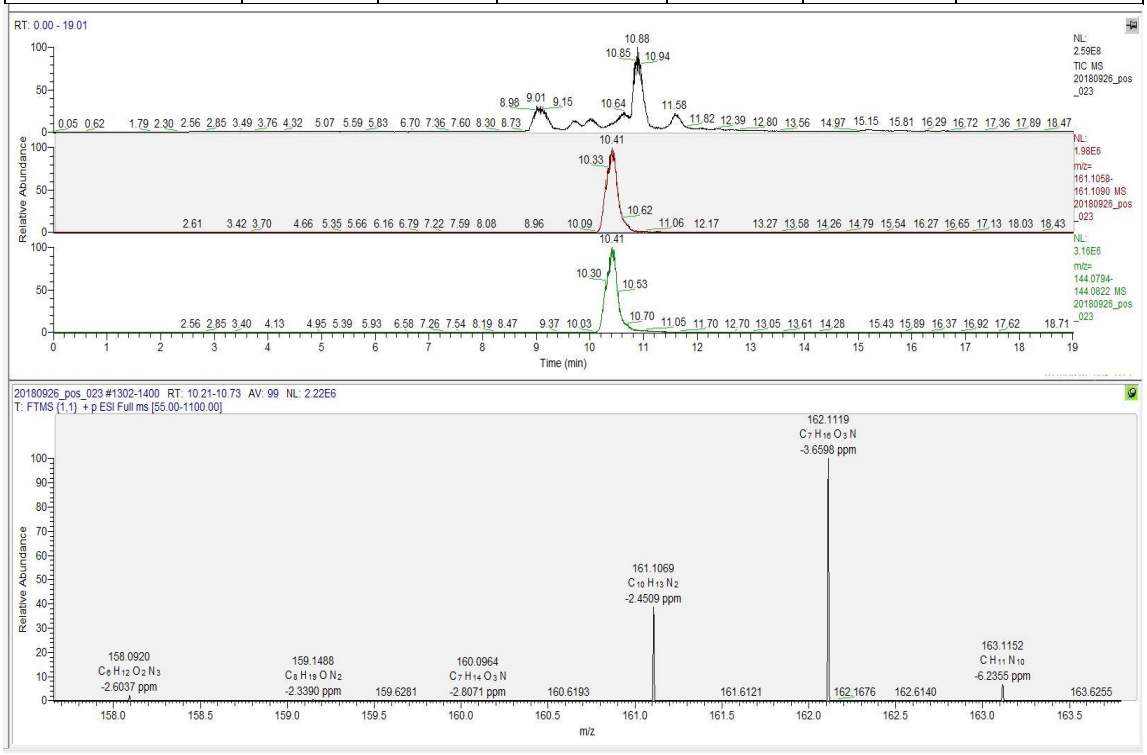
Compound name	*m/z*	RT (s)	Molecular formula	Adduct	Fragments (*m/z*)	Level of identification
Tryptamine	161.1071	262.45	C10H12N2	[M+H]+	144.0808	2



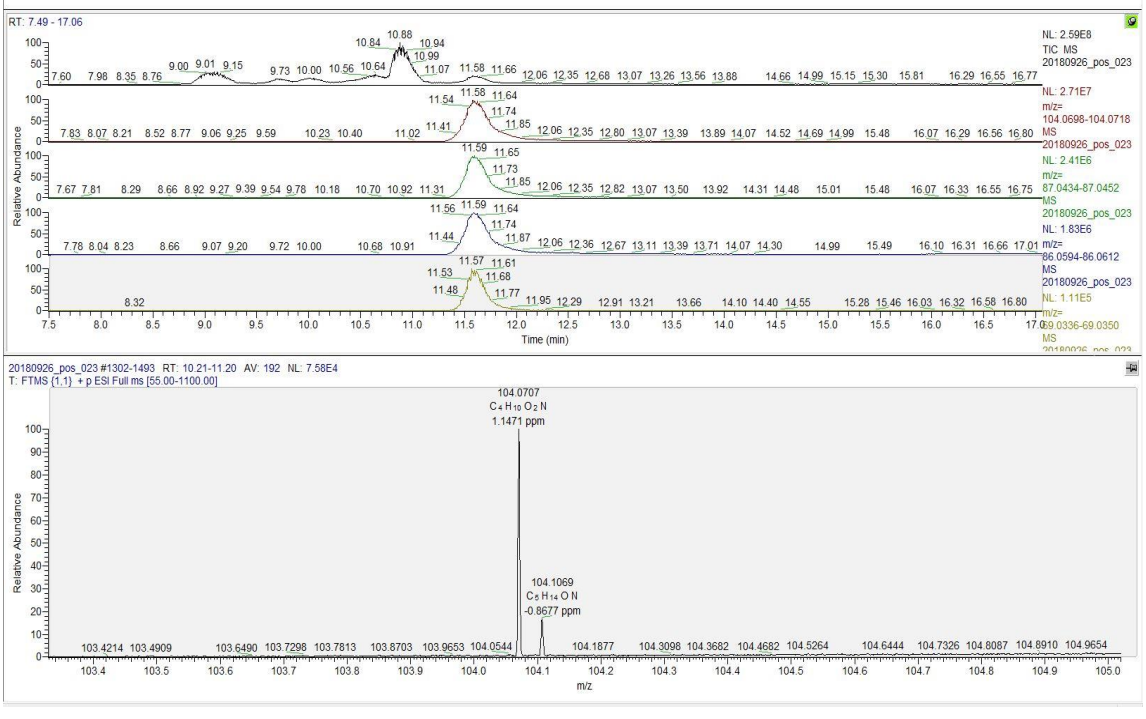
Compound name	*m/z*	RT (s)	Molecular formula	Adduct	Fragments (*m/z*)	Level of identification
Creatine	161.1071	723.24	C4H9N3O2	[M-H]-	88.0401	2



Compound name	*m/z*	RT (s)	Molecular formula	Adduct	Fragments (*m/z*)	Level of identification
Tryptamine	161.107	622.03	C10H12N2	[M+H]+	427.0	2



Compound name	*m/z*	RT (s)	Molecular formula	Adduct	Fragments (*m/z*)	Level of identification
γ-Aminobutyric acid	104.0706	692.68	C4H9NO2	[M+H]+	87.0443, 86.0603, 69.0343	2



A3.3 List of putatively characterized compounds with level 3 identification confidence from F4SCM

Compound name	***m/z***	**Mass difference**	**Molecular formula**	**Stream**	**Adduct**	***p*-value**	**FC**
Acetolactic acid	131.0344	6.23×10^{-4}	$C_5H_8O_4$	C18_Neg	M-H[-]	2.72×10^{-7}	14.9
p-cresol	107.0496	6.23×10^{-4}	C_7H_8O	C18_Neg	M-H[-]	1.36×10^{-7}	4.9
Valeric acid	101.0601	2.93×10^{-4}	$C_5H_{10}O_2$	C18_Neg	M-H[-]	2.93×10^{-7}	427.0
3-Methyl-2-buten-1-ol	85.0655	4.32×10^{-4}	$C_5H_{10}O$	C18_Neg	M-H[-]	4.14×10^{-7}	9.3
Ascorbic acid	175.0243	5.23×10^{-4}	$C_6H_8O_6$	C18_Neg	M-H[-]	6.38×10^{-7}	37.8
Leucine/Isoleucine	130.0867	6.23×10^{-4}	$C_6H_{13}NO_2$	C18_Neg	M-H[-]	8.97×10^{-7}	2.1
3-(2-Hydroxyphenyl)propionic acid	165.0551	6.23×10^{-4}	$C_9H_{10}O_3$	C18_Neg	M-H[-]	1.25×10^{-6}	44.7
Kynurenic acid	188.0347	6.23×10^{-4}	$C_{10}H_7NO_3$	C18_Neg	M-H[-]	2.19×10^{-6}	7.3
D-Arabinono-1,4-lactone	147.0294	5.23×10^{-4}	$C_5H_8O_5$	C18_Neg	M-H[-]	2.14×10^{-5}	2.3
Niacin/Nicotinic acid	122.0242	5.23×10^{-4}	$C_6H_5NO_2$	C18_Neg	M-H[-]	2.19×10^{-4}	2.6
Putrescine	89.1075	2.24×10^{-4}	$C_4H_{12}N_2$	C18_Pos	M+H[1+]	1.63×10^{-7}	77.5
Indoleacetic acid	176.0707	1.24×10^{-4}	$C_{10}H_9NO$	C18_Pos	M+H[1+]	4.13×10^{-7}	431.8
Dihydrouracil	115.0504	2.24×10^{-4}	$C_4H_6N_2O_2$	C18_Pos	M+H[1+]	4.62×10^{-6}	21.3
Serotonin	177.1027	4.24×10^{-4}	$C_{10}H_{12}N_2O$	C18_Pos	M+H[1+]	5.81×10^{-6}	59.2
Methylhistidine	170.0926	2.24×10^{-4}	$C_7H_{11}N_3O_2$	C18_Pos	M+H[1+]	1.63×10^{-5}	8.5
Tyramine	138.0913	7.65×10^{-5}	$C_8H_{11}NO$	C18_Pos	M+H[1+]	1.66×10^{-5}	180.6
Leucine/Isoleucine	132.102	1.24×10^{-4}	$C_6H_{13}NO_2$	C18_Pos	M+H[1+]	2.80×10^{-5}	2.4
Aspartic acid	132.0293	9.24×10^{-4}	$C_4H_7NO_4$	HILIC_Neg	M-H[-]	1.50×10^{-5}	2.8
Ascorbic acid	175.024	8.24×10^{-4}	$C_6H_8O_6$	HILIC_Neg	M-H[-]	9.42×10^{-6}	4.2
Itaconic/Mesaconic acid	175.024	9.24×10^{-4}	$C_5H_6O_4$	HILIC_Neg	M-H[-]	2.39×10^{-5}	2.9
Glutamate	146.0449	1.02×10^{-3}	$C_5H_9NO_4$	HILIC_Neg	M-H[-]	2.68×10^{-5}	3.0
D-Arabinono-1,4-lactone	147.0288	1.12×10^{-3}	$C_5H_8O_5$	HILIC_Neg	M-H[-]	3.69×10^{-5}	3.0
O-Succinyl-L-homoserine	218.0663	7.24×10^{-4}	$C_8H_{13}NO_6$	HILIC_Neg	M-H[-]	6.52×10^{-5}	2.1
Citrulline	174.0875	9.24×10^{-4}	$C_6H_{13}N_3O_6$	HILIC_Neg	M-H[-]	2.62×10^{-3}	2.4
Niacinamide	123.0553	2.35×10^{-5}	$C_6H_6N_2O$	HILIC_Pos	M+H[1+]	7.29×10^{-9}	5452.1
3-Methyl-L-histidine	170.0921	2.76×10^{-4}	$C_7H_{11}N_3O_2$	HILIC_Pos	M+H[1+]	6.02×10^{-8}	38.8
Aminopentanoic acid	118.086	2.76×10^{-4}	$C_5H_{11}NO_2$	HILIC_Pos	M+H[1+]	1.72×10^{-7}	4.4
Phenylacetaldehyde	121.0648	2.35×10^{-5}	$C_8H_8O_5$	HILIC_Pos	M+H[1+]	4.94×10^{-7}	188.54
Tyramine	138.0909	4.76×10^{-5}	$C_8H_{11}NO$	HILIC_Pos	M+H[1+]	7.04×10^{-7}	181.6

L-Ornithine	133.0971	7.65×10^{-5}	$C_5H_{12}N_2O_2$	HILIC_Pos	M+H[1+]	2.66×10^{-5}	3.9
Cyclohexylammonium	100.112	7.65×10^{-5}	$C_6H_{13}N$	HILIC_Pos	M+H[1+]	2.91×10^{-5}	4.0
Proline	116.0707	1.24×10^{-4}	$C_5H_9NO_2$	HILIC_Pos	M+H[1+]	2.43×10^{-4}	5.1

m/z – mass to charge ratio, *p*-value- t-test between F4SCM and CMGS (Cooked meat glucose starch broth) groups, FC-fold change value (F4SCM/CMGS)

Appendix A4

A4.1 The best sequence alignments between FS01 isolate and hydroxyisocaproate acid dehydrogenases (HicDs).

UniProt ID of query HicD enzyme: A0A125V2B4

```
> scaffold_1
Length=293798

 Score = 425 bits (1092),  Expect = 2e-136, Method: Compositional matrix adjust.
 Identities = 204/332 (61%), Positives = 261/332 (79%), Gaps = 1/332 (0%)
 Frame = -1

Query  1      MKILVFGARDYEEPVIKKWSEEH-KDVQVDIYPENMTEENVVKAKGYDGISIQQTNYIDN  59
              MKIL+F  R++E P I KW EE+  +VQVD   E ++   V KAKGYDGISIQQTN ID+
Sbjct  40220  MKILMFSTREHELPAIAKWIEENGNNVQVDTIEEGLSSVTVDKAKGYDGISIQQTNPIDD  40041

Query  60     PYIYETLKDAGVKVIASRTAGVDMIHFDLVNENGLIVTNVPSYSPNAIAELAVTQAMNLL  119
                IYE LK+ G+K IASR AG DMI  DL  +N L++TNVP+YSPN++AELAVTQ MNLL
Sbjct  40040  AIIYEKLKEFGIKQIASRAAGFDMIDLDLATKNDLVITNVPAYSPNSVAELAVTQTMNLL  39861

Query  120    RKTPLVKKKVCEGDYRWIAELLGTEVRSITVGVIGTGKIGATSAKLFKGLGANVIAFDQY  179
              R   L+ + V  GD+RW A L+  E+RS TVG++GTGKIG+T+A+LFKGLGANVIA+D Y
Sbjct  39860  RNMHLINRNVNTGDFRWSANLIAREIRSTTVGIVGTGKIGSTAAQLFKGLGANVIAYDAY  39681

Query  180    PNSDLNDILTYKDSLEDLLKEADLITLHTPLLEGTKHMINKDTLAIMKDGAYIVNTGRGG  239
              PN DL +ILTYKDSL+DL+KEAD+++LHTPL E TKHMINKD L +MK  A++VNTGRGG
Sbjct  39680  PNEDLKEILTYKDSLDDLMKEADVVSLHTPLNENTKHMINKDNLKLMKKDAFLVNTGRGG  39501

Query  240    LINTGDLIEALESGKIRAAALDTFETEGLFLNKKMNPGELTDPEINKLLSMEQVIFTHHL  299
              ++ T DLIEALE+  + AAALDTFETEG FLNK +   +LTDP++ KLL+ME V+FTHH+
Sbjct  39500  VVCTDDLIEALENKDLCAAALDTFETEGTFLNKVIPHHDLTDPQVKKLLNMENVLFTHHI  39321

Query  300    GFFTSTAIENIVYSSLSSAVEVIKTGTATNRV  331
              G+FT+TA++N+V ++L+   EV+ TG + N V
Sbjct  39320  GYFTTTAVDNLVSTALNCVKEVLATGDSVNNV  39225
```

UniProt ID of query HicD enzyme: A0A0H3MZ08

```
> scaffold_1
Length=293798

 Score = 425 bits (1092),  Expect = 2e-136, Method: Compositional matrix adjust.
 Identities = 204/332 (61%), Positives = 261/332 (79%), Gaps = 1/332 (0%)
 Frame = -1

Query  1      MKILVFGARDYEEPVIKKWSEEH-KDVQVDIYPENMTEENVVKAKGYDGISIQQTNYIDN  59
              MKIL+F  R++E P I KW EE+  +VQVD   E ++   V KAKGYDGISIQQTN ID+
Sbjct  40220  MKILMFSTREHELPAIAKWIEENGNNVQVDTIEEGLSSVTVDKAKGYDGISIQQTNPIDD  40041

Query  60     PYIYETLKDAGVKVIASRTAGVDMIHFDLVNENGLIVTNVPSYSPNAIAELAVTQAMNLL  119
                IYE LK+ G+K IASR AG DMI  DL  +N L++TNVP+YSPN++AELAVTQ MNLL
Sbjct  40040  AIIYEKLKEFGIKQIASRAAGFDMIDLDLATKNDLVITNVPAYSPNSVAELAVTQTMNLL  39861

Query  120    RKTPLVKKKVCEGDYRWIAELLGTEVRSITVGVIGTGKIGATSAKLFKGLGANVIAFDQY  179
              R   L+ + V  GD+RW A L+  E+RS TVG++GTGKIG+T+A+LFKGLGANVIA+D Y
Sbjct  39860  RNMHLINRNVNTGDFRWSANLIAREIRSTTVGIVGTGKIGSTAAQLFKGLGANVIAYDAY  39681

Query  180    PNSDLNDILTYKDSLEDLLKEADLITLHTPLLEGTKHMINKDTLAIMKDGAYIVNTGRGG  239
              PN DL +ILTYKDSL+DL+KEAD+++LHTPL E TKHMINKD L +MK  A++VNTGRGG
Sbjct  39680  PNEDLKEILTYKDSLDDLMKEADVVSLHTPLNENTKHMINKDNLKLMKKDAFLVNTGRGG  39501

Query  240    LINTGDLIEALESGKIRAAALDTFETEGLFLNKKMNPGELTDPEINKLLSMEQVIFTHHL  299
              ++ T DLIEALE+  + AAALDTFETEG FLNK +   +LTDP++ KLL+ME V+FTHH+
Sbjct  39500  VVCTDDLIEALENKDLCAAALDTFETEGTFLNKVIPHHDLTDPQVKKLLNMENVLFTHHI  39321

Query  300    GFFTSTAIENIVYSSLSSAVEVIKTGTATNRV  331
              G+FT+TA++N+V ++L+   EV+ TG + N V
Sbjct  39320  GYFTTTAVDNLVSTALNCVKEVLATGDSVNNV  39225
```

UniProt ID of query HicD enzyme: C9YIH1

```
> scaffold_1
Length=293798

 Score = 425 bits (1092),  Expect = 2e-136, Method: Compositional matrix adjust.
 Identities = 204/332 (61%), Positives = 261/332 (79%), Gaps = 1/332 (0%)
 Frame = -1

Query  1      MKILVFGARDYEEPVIKKWSEEH-KDVQVDIYPENMTEENVVKAKGYDGISIQQTNYIDN  59
              MKIL+F  R++E P I KW EE+  +VQVD   E ++   V KAKGYDGISIQQTN ID+
Sbjct  40220  MKILMFSTREHELPAIAKWIEENGNNVQVDTIEEGLSSVTVDKAKGYDGISIQQTNPIDD  40041

Query  60     PYIYETLKDAGVKVIASRTAGVDMIHFDLVNENGLIVTNVPSYSPNAIAELAVTQAMNLL  119
                IYE LK+ G+K IASR AG DMI  DL  +N L++TNVP+YSPN++AELAVTQ MNLL
Sbjct  40040  AIIYEKLKEFGIKQIASRAAGFDMIDLDLATKNDLVITNVPAYSPNSVAELAVTQTMNLL  39861

Query  120    RKTPLVKKKVCEGDYRWIAELLGTEVRSITVGVIGTGKIGATSAKLFKGLGANVIAFDQY  179
              R   L+ + V  GD+RW A L+  E+RS TVG++GTGKIG+T+A+LFKGLGANVIA+D Y
Sbjct  39860  RNMHLINRNVNTGDFRWSANLIAREIRSTTVGIVGTGKIGSTAAQLFKGLGANVIAYDAY  39681

Query  180    PNSDLNDILTYKDSLEDLLKEADLITLHTPLLEGTKHMINKDTLAIMKDGAYIVNTGRGG  239
              PN DL +ILTYKDSL+DL+KEAD+++LHTPL E TKHMINKD L +MK  A++VNTGRGG
Sbjct  39680  PNEDLKEILTYKDSLDDLMKEADVVSLHTPLNENTKHMINKDNLKLMKKDAFLVNTGRGG  39501

Query  240    LINTGDLIEALESGKIRAAALDTFETEGLFLNKKMNPGELTDPEINKLLSMEQVIFTHHL  299
              ++ T DLIEALE+  + AAALDTFETEG FLNK +   +LTDP++ KLL+ME V+FTHH+
Sbjct  39500  VVCTDDLIEALENKDLCAAALDTFETEGTFLNKVIPHHDLTDPQVKKLLNMENVLFTHHI  39321

Query  300    GFFTSTAIENIVYSSLSSAVEVIKTGTATNRV  331
              G+FT+TA++N+V ++L+   EV+ TG + N V
Sbjct  39320  GYFTTTAVDNLVSTALNCVKEVLATGDSVNNV  39225
```

UniProt ID of query HicD enzyme: J7TF96

```
> scaffold_1
Length=293798

 Score = 459 bits (1181),  Expect = 2e-148, Method: Compositional matrix adjust.
 Identities = 222/334 (66%), Positives = 270/334 (81%), Gaps = 3/334 (1%)
 Frame = -1

Query  1      MKILMYSVREHEKPAIKKWLEANPE-VQIDLSNEALSEDTVCKVKGYDGIAIQQTNSIGG  59
              MKILM+S REHE PAI KW+E N   VQ+D   E LS  TV K KGYDGI+IQQTN I
Sbjct  40220  MKILMFSTREHELPAIAKWIEENGNNVQVDTIEEGLSSVTVDKAKGYDGISIQQTNPIDD  40041

Query  60     ETVYSTLKEYGIKQIASRTAGVDMIDLKMASENNILVTNVPAYSPNAIAELAVTHTMNLL  119
                +Y  LKE+GIKQIASR AG DMIDL +A++N++++TNVPAYSPN++AELAVT TMNLL
Sbjct  40040  AIIYEKLKEFGIKQIASRAAGFDMIDLDLATKNDLVITNVPAYSPNSVAELAVTQTMNLL  39861

Query  120    RNIKTVNKRIAFGDYRWSADLIAREVRSITVGVVGTGKIGRTSAKLFKGLGANVIGYDAY  179
              RN+  +N+ +  GD+RWSA+LIARE+RS TVG+VGTGKIG T+A+LFKGLGANVI YDAY
Sbjct  39860  RNMHLINRNVNTGDFRWSANLIAREIRSTTVGIVGTGKIGSTAAQLFKGLGANVIAYDAY  39681

Query  180    PDKKLEENNLLTYKESLEDLLKEADVVTLHTPLTENTKHMINKNNLKYMKPDAFIVNTGR  239
              P++ L+E  +LTYK+SL+DL+KEADVV+LHTPL ENTKHMINK+NLK MK DAF+VNTGR
Sbjct  39680  PNEDLKE--ILTYKDSLDDLMKEADVVSLHTPLNENTKHMINKDNLKLMKKDAFLVNTGR  39507

Query  240    GGIINTEDLIEALEENKIAGAALDTFENEGLFLNKVVDPTKIPDPQLDKLLKMDQVLITH  299
              GG++ T+DLIEALE   +  AALDTFE EG FLNKV+    + DPQ+ KLL M+ VL TH
Sbjct  39506  GGVVCTDDLIEALENKDLCAAALDTFETEGTFLNKVIPHHDLTDPQVKKLLNMENVLFTH  39327

Query  300    HVGFFTTTAVQNMVDTSLDSVVEVLKTSDSVNKV  333
              H+G+FTTTAV N+V T+L+ V EVL T DSVN V
Sbjct  39326  HIGYFTTTAVDNLVSTALNCVKEVLATGDSVNNV  39225
```

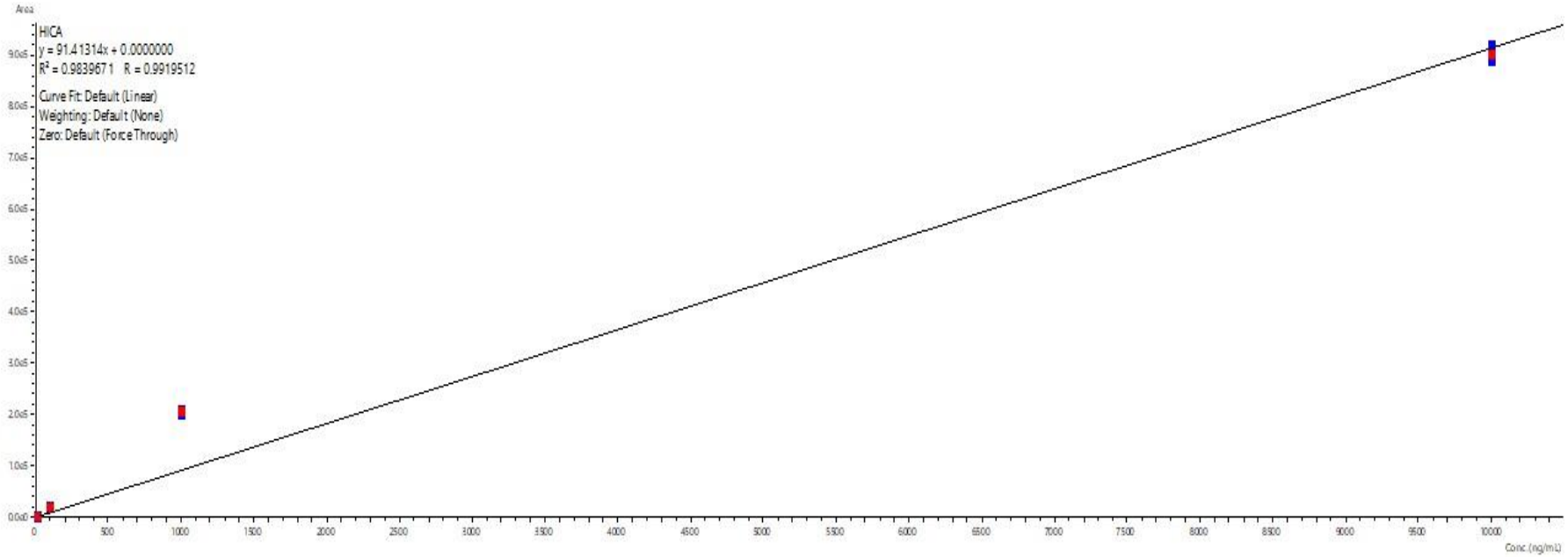
UniProt ID of query HicD enzyme: D5PZU0

```
> scaffold_1
Length=293798

 Score = 425 bits (1093),  Expect = 1e-136, Method: Compositional matrix adjust.
 Identities = 203/332 (61%), Positives = 262/332 (79%), Gaps = 1/332 (0%)
 Frame = -1

Query  1      MKILVFGARDYEEPVIKKWSEEH-KDVQVDIYPENMTEENIVKAKGYDGISIQQTNYIDN  59
              MKIL+F  R++E P I KW EE+  +VQVD   E ++   + KAKGYDGISIQQTN ID+
Sbjct  40220  MKILMFSTREHELPAIAKWIEENGNNVQVDTIEEGLSSVTVDKAKGYDGISIQQTNPIDD  40041

Query  60     PYIYETLKDAGVKVIASRTAGVDMIHFDLVNENGLIVTNVPSYSPNAIAELAVTQAMNLL  119
                IYE LK+ G+K IASR AG DMI  DL  +N L++TNVP+YSPN++AELAVTQ MNLL
Sbjct  40040  AIIYEKLKEFGIKQIASRAAGFDMIDLDLATKNDLVITNVPAYSPNSVAELAVTQTMNLL  39861

Query  120    RKTPLVKKKVCEGDYRWIAELLGTEVRSITVGVIGTGKIGATSAKLFKGLGANVIAFDQY  179
              R   L+ + V  GD+RW A L+  E+RS TVG++GTGKIG+T+A+LFKGLGANVIA+D Y
Sbjct  39860  RNMHLINRNVNTGDFRWSANLIAREIRSTTVGIVGTGKIGSTAAQLFKGLGANVIAYDAY  39681

Query  180    PNSDLNDILTYKDSLEDLLKEADLITLHTPLLEGTKHMINKDTLAIMKDGAYIVNTGRGG  239
              PN DL +ILTYKDSL+DL+KEAD+++LHTPL E TKHMINKD L +MK  A++VNTGRGG
Sbjct  39680  PNEDLKEILTYKDSLDDLMKEADVVSLHTPLNENTKHMINKDNLKLMKKDAFLVNTGRGG  39501

Query  240    LIKTEDLIEALESGKIRAAALDTFETEGLFLNKKMNPGELTDPEINKLLSMEQVIFTHHL  299
              ++ T+DLIEALE+  + AAALDTFETEG FLNK +   +LTDP++ KLL+ME V+FTHH+
Sbjct  39500  VVCTDDLIEALENKDLCAAALDTFETEGTFLNKVIPHHDLTDPQVKKLLNMENVLFTHHI  39321

Query  300    GFFTSTAIENIVYSSLSSAVEVIKTGTATNRV  331
              G+FT+TA++N+V ++L+   EV+ TG + N V
Sbjct  39320  GYFTTTAVDNLVSTALNCVKEVLATGDSVNNV  39225
```

UniProt ID of query HicD enzyme: Q5U922

```
> scaffold_1
Length=293798

 Score = 422 bits (1085),  Expect = 1e-135, Method: Compositional matrix adjust.
 Identities = 203/331 (61%), Positives = 260/331 (79%), Gaps = 1/331 (0%)
 Frame = -1

Query  1      KILVFGARDYEEPVIKKWSEEH-KDVQVDIYPENMTEENVVKAKGYDGISIQQTNYIDNP  59
              KIL+F  R++E P I KW EE+  +VQVD   E ++   V KAKGYDGISIQQTN ID+
Sbjct  40217  KILMFSTREHELPAIAKWIEENGNNVQVDTIEEGLSSVTVDKAKGYDGISIQQTNPIDDA  40038

Query  60     YIYETLKDAGVKVIASRTAGVDMIHFDLVNENGLIVTNVPSYSPNAIAELAVTQAMNLLR  119
               IYE LK+ G+K IASR AG DMI  DL  +N L++TNVP+YSPN++AELAVTQ MNLLR
Sbjct  40037  IIYEKLKEFGIKQIASRAAGFDMIDLDLATKNDLVITNVPAYSPNSVAELAVTQTMNLLR  39858

Query  120    KTPLVKKKVCEGDYRWIAELLGTEVRSITVGVIGTGKIGATSAKLFKGLGANVIAFDQYP  179
                 L+ + V  GD+RW A L+  E+RS TVG++GTGKIG+T+A+LFKGLGANVIA+D YP
Sbjct  39857  NMHLINRNVNTGDFRWSANLIAREIRSTTVGIVGTGKIGSTAAQLFKGLGANVIAYDAYP  39678

Query  180    NSDLNDILTYKDSLEDLLKEADLITLHTPLLEGTKHMINKDTLAIMKDGAYIVNTGRGGL  239
              N DL +ILTYKDSL+DL+KEAD+++LHTPL E TKHMINKD L +MK  A++VNTGRGG+
Sbjct  39677  NEDLKEILTYKDSLDDLMKEADVVSLHTPLNENTKHMINKDNLKLMKKDAFLVNTGRGGV  39498

Query  240    INTGDLIEALESGKIRAAALDTFETEGLFLNKKMNPGELTDPEINKLLSMEQVIFTHHLG  299
              + T DLIEALE+  + AAALDTFETEG FLNK +   +LTDP++ KLL+ME V+FTHH+G
Sbjct  39497  VCTDDLIEALENKDLCAAALDTFETEGTFLNKVIPHHDLTDPQVKKLLNMENVLFTHHIG  39318

Query  300    FFTSTAIENIVYSSLSSAVEVIKTGTATNRV  330
              +FT+TA++N+V ++L+   EV+ TG + N V
Sbjct  39317  YFTTTAVDNLVSTALNCVKEVLATGDSVNNV  39225
```

A4.2 Standard calibration curve of HICA determined using UHPLC system coupled to a Q-TOF mass spectrometer



Appendix A5

A5.1 Putatively identified significantly abundant metabolites from FS03CM (Tier 2 identification level)

External identifier	Compound name	Neutral mass (Da)	Normalized RT (s)	Fold change	*p*-value	Level of identification
C00612	N1-Acetylspermidine	187.1668	1084.9	12.18	4.17×10^{-7}	2
C07086	Toluic acid	136.0519	408.4	99.27	4.03×10^{-6}	2
C00077	L-Ornithine /D-Ornithine	132.0874	1033.1	3.09	1.61×10^{-5}	2
C00242	Guanine	151.0488	432.3	3.37	2.25×10^{-5}	2
C00437	N-Acetylornithine	174.1007	320.6	3.08	2.82×10^{-5}	2
C20827	3-Hydroxyisovaleric acid	118.0632	294.5	5.66	4.30×10^{-5}	2
C00077	(2R,4S)-2,4-Diaminopentanoic acid	132.0933	1026.7	2.75	4.52 × 10-5	2
C00246	Butanoic acid	88.0517	384.7	5.55	1.23×10^{-4}	2
C11004	2,6-Dimethylaniline	121.0897	1217.3	19.14	1.26×10^{-4}	2
C00979	O-Acetyl-L-serine	147.0534	262	3.46	1.94×10^{-4}	2
C00439	N-Formimino-L-glutamic acid	174.0636	237.6	26.57	2.80×10^{-4}	2
C00803	Ethylmethylacetic acid	102.0676	442.7	7.66	2.80×10^{-4}	2
C07086	Phenylacetic acid	136.0529	395.2	534.73	3.45×10^{-4}	2
C05715	gamma-Amino-gamma-cyanobutanoic acid	128.0587	273.6	2.60	5.88×10^{-4}	2
C00490	Itaconic acid	130.0258	340.3	5.30	6.56×10^{-4}	2
C03351	4-Hydroxybenzyl alcohol	124.0524	991	2.34	8.64×10^{-4}	2
C14602	3-Hydroxyaminophenol	125.0453	1613.3	3.18	9.04×10^{-4}	2
C01799	Norvaline	117.0798	570.9	16.68	9.57×10^{-4}	2
C06044	4-Hydroxyphenylethanol	138.0666	1034.5	3.95	1.40×10^{-3}	2
C00555	4-Aminobutyraldehyde	87.0685	578	3.01	1.40×10^{-3}	2
C00232	2-Methyl-3-oxopropanoic acid	102.0319	237.9	2.30	1.70×10^{-3}	2
C08276	3-(Methylthio)propanoic acid	120.0244	339.7	384.10	2.35×10^{-3}	2
C21762	4-Hydroxytryptamine	176.095	869.6	3.035	2.45×10^{-3}	2

C02501	2-Hydroxymuconic acid	158.0201	549.6	2.78	3.18×10^{-3}	2
C15987	4-Methylaminobutyric acid	117.0794	65.2	3.71	3.20×10^{-3}	2
C03722	Quinolinic acid	167.0214	394.1	4.45	3.71×10^{-3}	2
C00990	5-Aminopentanamide	116.0949	372.8	2.19	1.30×10^{-2}	2
C14452	Morpholine	87.0677	700.3	2.16	1.49×10^{-2}	2
C06052	5-Pyridoxolactone	165.0421	553.6	2.44	1.83×10^{-2}	2
C00632	3-Amino-4-hydroxybenzoic acid	153.0443	1440.6	2.70	1.91×10^{-2}	2
C04043	3,4-Dihydroxyphenylacetaldehyde	152.0453	1478.7	3.75	2.38×10^{-2}	2
C09131	Chanoclavine	256.1576	1224.3	2.54	2.72×10^{-2}	2

External identifier - KEGG/HMDB entry of the identified compound; Neutral mass (Da) - The neutral monoisotope mass of the metabolite (i.e., labeled mass - the mass of the labeling group); Normalized RT (s) - The corrected retention time of the peak pair with universal RT Calibrant data; Fold change - The ratio of the average peak ratio values (FS03CM/CMGS); *p*-value - From student's t-test (FS03CM Vs CMGS)

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Amila S N W Pahalagedara
Name/title of Primary Supervisor:	Prof. Steve Flint
Name of Research Output and full reference:	
Pahalagedara ASNW, Flint S, Palmer J, Brightwell G, Gupta TB: Antimicrobial production by strictly anaerobic Clostridium spp. International Journal of Antimicrobial Agents 2020:105910.	
In which Chapter is the Manuscript /Published work:	Chapter 2
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	80
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
The candidate performed the lab work and drafted the manuscript	
For manuscripts intended for publication please indicate target journal:	
Candidate's Signature:	Amila Srilal Nawarathna Weligala Pahalagedara Digitally signed by Amila Srilal Nawarathna Weligala Pahalagedara Date: 2021.08.19 14:07:37 +12'00'
Date:	19/08/2021
Primary Supervisor's Signature:	Steve Flint Digitally signed by Steve Flint DN: cn=Steve Flint, c=NZ, email=s.h.flint@massey.ac.nz Date: 2021.08.19 14:20:12 +12'00'
Date:	19th August 2021

(This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis)

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Amila S N W Pahalagedara
Name/title of Primary Supervisor:	Prof. Steve Flint
Name of Research Output and full reference:	
Pahalagedara ASNW, Flint S, Palmer J, Subbaraj A, Brightwell G, Gupta TB: Antimicrobial activity of soil Clostridium enriched conditioned media against Bacillus mycoides, Bacillus cereus, and Pseudomonas aeruginosa. Frontiers in Microbiology 2020,	
In which Chapter is the Manuscript /Published work:	Chapter 4 and 5
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	80
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
The candidate performed the lab work and drafted the manuscript.	
For manuscripts intended for publication please indicate target journal:	
Candidate's Signature:	Amila Srilal Nawarathna Weligala Pahalagedara Digitally signed by Amila Srilal Nawarathna Weligala Pahalagedara Date: 2021.08.19 14:12:27 +12'00'
Date:	19/08/2021
Primary Supervisor's Signature:	Steve Flint Digitally signed by Steve Flint DN: cn=Steve Flint, c=NZ, email=s.h.flint@massey.ac.nz Date: 2021.08.19 14:22:17 +12'00'
Date:	19th August 2021

(This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis)

GRS Version 4– January 2019

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Amila S N W Pahalagedara
Name/title of Primary Supervisor:	Prof. Steve Flint
Name of Research Output and full reference:	
Pahalagedara ASNW, Ruy J, Maclean P, Altermann E, Flint S, Palmer J, Bilgiwell G, Gupta TB: Culture and genome-based analysis of four soil Clostridium isolates reveal their potential for antimicrobial production. BMC Genomics 2021, 22, 686.	
In which Chapter is the Manuscript /Published work:	Chapter 6
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	80
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
The candidate performed the lab work, analysis and drafted the manuscript.	
For manuscripts intended for publication please indicate target journal:	
Candidate's Signature:	Amila Srilal Nawarathna Weligala Pahalagedara — Digitally signed by Amila Srilal Nawarathna Weligala Pahalagedara Date: 2021.12.07 20:25:49 +01'00'
Date:	07/12/2021
Primary Supervisor's Signature:	Steve Flint — Digitally signed by Steve Flint DN: cn=Steve Flint, c=NZ, email=s.h.flint@massey.ac.nz Date: 2021.12.08 08:51:02 +13'00'
Date:	

(This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis)

GRS Version 4– January 2019