EVALUATION OF THE MOLLUSCICIDAL ACTIVITY OF *BACILLUS* SPP. ISOLATES TO CONTROL AQUATIC INTERMEDIATE HOST SNAILS OF LIVER FLUKE (*FASCIOLA* SPP.)

by

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Doctor of Philosophy

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PREFACE

The research contained in this thesis was completed by the candidate while based in the Discipline of Plant Pathology, School of Agriculture, Earth and Environmental Science, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg Campus, South Africa. The research was financially supported by the National Research Foundation (Grant UID: 118198) and Milk SA.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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Signed: Prof. M. D. Laing (Supervisor)

Date: February 2024

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Signed: Dr. C. H. Hunter (Co-supervisor)

Date: February 2024

DECLARATION 1: PLAGIARISM

I, Matthew George Dennis van Wyngaard, declare that:

(i) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;

(ii) this dissertation has not been submitted in full or in part for any degree or examination to any other university;

(iii) this dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;

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a) their words have been re-written but the general information attributed to them has been referenced;

b) where their exact words have been used, their writing has been placed inside quotation marks, and referenced;

(v) where I have used material for which publications followed, I have indicated in detail my role in the work;

(vi) this dissertation is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

(vii) this dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.

Signed: M. G. D. van Wyngaard (Candidate)

Date: February 2024

DECLARATION 2: PUBLICATIONS

Details of contribution to publications that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication).

van Wyngaard, M. G. D., Hunter, C. H., Laing, M. D. (2024) Molluscicidal activity of surfactin against aquatic snail species *Physella acuta* and *Pseudosuccinea columella*. Pest Management Science. Submitted.

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Date: February 2024

THESIS SUMMARY

Aquatic snails are involved in harmful disease cycles of *Fasciola* (liver fluke) that affect both humans and livestock in agriculture. The focus of this study was to isolate and identify candidate bacterial isolates antagonistic to aquatic snails, with the ultimate goal of controlling the host snails responsible for the transmission of liver flukes in South Africa. A bacterial antagonist would offer a novel means of snail population control and reduce the dependence on chemicals, and the growing risk of resistance. Due to their molluscicidal capabilities reported in the literature, and the benefits of being endospore-formers, strains within the family *Bacillaceae* were targeted as candidate biocontrol agents. A population of the freshwater snail *Physella acuta* (Draparnaud, 1805) was established and used for screening bacterial candidates as an easily-reared, proxy species for the *Fasciola* spp. intermediate host snails. Aerobic endospore-forming bacteria were isolated from aquatic soil collected primarily in the KwaZulu-Natal province, South Africa, utilising several approaches, including a general endospore heat-shock isolation method and two *Bacillus thuringiensis* (Bt) (Berliner, 1915) specific isolation methods. Initial screening for molluscicidal activity of 1180 isolates did not yield any strong performers; however, a subset of 124 isolates demonstrated some evidence of potential activity in preliminary screening, that in the absence of any strong molluscicidal isolates, warranted a more in-depth investigation. Subsequently, when the bioassays were repeated none of the 124 isolates showed strong molluscicidal activity; however, the eight isolates causing the highest mortality rates (16.7 – 50%) were selected for further analysis to determine whether these isolates exhibited weak molluscicidal activity. When spent culture supernatant was evaluated in molluscicidal bioassays 12 of the 124 isolates demonstrated strong molluscicidal activity.

Selected isolates underwent identification and characterisation using DNA-based methods for the purposes of species identification and de-duplication of isolates on the basis of sequencing data and end point PCR. Isolates were identified using the benchmark technique of 16S rDNA gene fragment sequencing. For discrimination of closely related isolates, sequence analysis of two additional housekeeping genes was performed using *rpoB* and *dnaJ*. In addition, a number of end point PCRs were used to support sequence data by the presence or absence of PCR products for the lipopeptide marker genes for surfactin, fengycin, bacillomycin and iturin, as well as a *Bacillus velezensis* (Ruiz-García *et al.*, 2005) specific primer. All the isolates were

confirmed to be members of the *Bacillaceae*. Isolates selected for their production of molluscicidal supernatants were found to be closely related and identified as strains of *B. velezensis*, *Bacillus amyloliquefaciens* (Priest *et al.*, 1987) and *Bacillus subtilis* (Ehrenberg, 1835). Isolates with potential molluscicidal activity were found to be more diverse, with representatives of *B. velezensis*, *Bacillus cereus* (Frankland and Frankland, 1887), *Bacillus mycoides* (Flügge, 1886), *Paenibacillus* sp., *Priestia* sp., and *Gottfriedia* sp. being identified. Isolates were deduplicated by removal of replicates with identical results for the various gene sequence data and end point PCRs. From the original 12 isolates producing molluscicidal supernatant, 6 were retained and all 8 isolates with potential molluscicidal activity were carried forward for further investigation.

Molluscicidal supernatant producers underwent lipopeptide extraction procedures and bioassays to determine whether this class of compounds may be responsible for their observed molluscicidal activity. The 8 potential molluscicidal isolates, along with the 6 molluscicidal supernatant producers, were assayed for molluscicidal activity against *P. acuta* snails in a long-term (i.e. 2 weeks feeding endospore-impregnated food pellets followed by 4 weeks observation) endospore exposure assay. Molluscicidal active fractions were successfully extracted and concentrated from broth culture supernatants via a lipopeptide acid precipitation extraction protocol. This suggested that lipopeptides, or compounds extractable by acid precipitation, were responsible for the observed molluscicidal activity. Lipopeptide extracts showed molluscicidal activity at between 25 and 200 $\mu g.mL^{-1}$ concentrations. In contrast, extended endospore exposure assays yielded no significant molluscicidal results. For the 8 isolates brought forward based on their potential molluscicidal activity, this result confirmed the lack of direct molluscicidal activity identified in the initial screening. Additionally, for isolates that produced molluscicidal supernatant, exposure of snails to these isolates' endospores did not result in any observable molluscicidal activity, suggesting that only the excreted metabolites were responsible for their molluscicidal activity and that this did not occur at sufficient concentrations in the snail-tank system to demonstrate an effect.

Subsequent investigations focused on the extracted molluscicidal compounds, their characterisation and identification, as well as the measurement of their lethal concentrations. Crude lipopeptide extracts from Landy and TSB growth media with molluscicidal activity against *P. acuta* underwent UPLC-ESI-MS to identify the lipopeptide components. In addition,

the dose response curves were measured for 24 h and 72 h contact times. The LC_{50} for crude lipopeptide extracts with a 24 h exposure ranged between 16.37 µg.mL^{-1} and 36.16 µg.mL^{-1}, and the LC_{95} was between 20.05 and 48.68 µg.mL^{-1}. The LC_{50} for the crude extracts with a 72 h exposure was between 11.89 µg.mL^{-1} and 27.21 µg.mL^{-1}, and the LC_{95} was between 15.09 and 30.93 µg.mL^{-1}. Analysis of the UPLC-ESI-MS spectra for each molluscicidal crude extract indicated the presence of various lipopeptide isoforms, including surfactin, iturin, fengycin and bacillomycin-D and -L. Surfactin was common to all molluscicidal crude lipopeptide extracts examined, which suggests that surfactin was a major contributor to the observed molluscicidal activity.

The molluscicidal properties of pure surfactin was evaluated against two aquatic snail species, *P. acuta,* which was used in initial screening, and secondly against *Pseudosuccinea columella* (Say, 1817), a host snail of *Fasciola* liver flukes. The molluscicidal efficacy of the crude lipopeptide extracts and pure surfactin were assessed against a test fish species, *Danio rerio* (F. Hamilton, 1822), as a first step in evaluating ecotoxicity. Commercially-available pure surfactin was found to be molluscicidal against *P. acuta* and *P. columella*, with LC_{50} values of 10.04 µg.mL^{-1} and 16.58 µg.mL^{-1}, respectively, and LC_{90} of 12.29 µg.mL^{-1} and 19.15 µg.mL^{-1}, respectively, with a 24 h contact time. Crude lipopeptide extracts demonstrated lethal effects on 24 hpf *D. rerio* embryos with an LC_{50} of between 10.19 and 44.93 µg.mL^{-1} with a 24 h contact time. *Danio rerio* exposure to pure surfactin was found to be lethal, with LC_{50} and LC_{90} values of 7.96 and 11.45 µg.mL^{-1}, respectively, with a 24 h exposure period. In comparison to 24 hpf embryos, 24 hpf eggs are still encased in the chorion. The 96 hpf embryos showed lower sensitivity to surfactin with LC_{50} values of 13.96 and 12.26 µg.mL^{-1}, and LC_{90} values of 17.61 and 16.87 µg.mL^{-1}, respectively, with 24 h contact times. This is the first report of surfactin having molluscicidal characteristics but it does raise some ecotoxicological concerns for its potential use as a molluscicide.

This research is the first broad-scale screening of aerobic endospore-forming bacteria for use as molluscicidal biocontrol agents. An initial screening of 1180 isolates did not reveal any strong molluscicidal isolates with direct activity on aquatic snails. However, an investigation into secreted metabolites determined that lipopeptide extracts exhibited molluscicidal effects, of which surfactin was common to all active fractions. Pure surfactin assays proved that this lipopeptide has molluscicidal activity at low concentrations, with LC_{50} values of

10.04 µg.mL^{-1} and 16.58 µg.mL^{-1} for *P. acuta* and *P. columella*, respectively, with a 24 h exposure time. This is a novel finding, increasing the range of activity of this biotechnologically useful compound. While no isolates from this research have demonstrated utility as biocontrol agents, the finding of molluscicidal lipopeptides opens a new avenue for the biocontrol of aquatic snail pest species, which may impact the neglected tropical diseases fascioliasis and schistosomiasis, with further potential for the control of other aquatic snail pest species.

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TABLE OF CONTENTS

THESIS INTRODUCTION

Liver flukes are the causal agent of fascioliasis, an economically damaging disease of livestock and humans caused by two trematode species, *Fasciola hepatica* (Linnaeus, 1758) and *Fasciola gigantica* (Cobbold, 1855). Flukes have a complicated life cycle that involves an intermediate host snail species and a definitive mammalian host species (e.g. cattle, sheep, goats, horses, and humans). Fascioliasis is classified as a neglected tropical disease by the World Health Organization (WHO), and currently there is only a limited pool of overutilized chemical-based control methods and treatments (Skuce and Zadoks, 2013; Fairweather *et al.*, 2020). Current *Fasciola* mitigation strategies involve the application of molluscicidal and anthelmintic products, in combination with the management of high-risk pastures (e.g., pasture draining and vegetation clearing), and restriction of animal host access to high-risk areas (Marif et al., 2016; Skuce and Zadoks, 2013). Chemical control of infected animals is both expensive and toxic to the treated animals, and the chemicals persist as residues in meat and milk (Skuce and Zadoks, 2013). Furthermore, the necessary widespread use of flukicidal and molluscicidal compounds has resulted in the development of resistance in flukes (Marif et al., 2016).

Snail control represents an avenue for mitigation of trematode transmission (King and Bertsch, 2015). The amphibious intermediate snail hosts of *Fasciola* liver fluke are found worldwide. In South Africa *Galba truncatula* (Müller, 1774) are hosts for *F. hepatica*, while *Radix natalensis* (Krauss, 1848) is host to *F. gigantica* (Marif et al., 2016; Appleton, 2002). The invasive species *Pseudosuccinea columella* (Say, 1817) may be host to both *Fasciola* species, and further invasive species, *Radix rubiginosa* (Michelin, 1831) and *Radix auricularia* (Linnaeus, 1758) have the potential to host *F. gigantica* (Appleton, 2002; Appleton and Miranda, 2015; Malatji *et al.*, 2019; Nyagura *et al.*, 2022). Incidences of fascioliasis in both humans and livestock in South Africa are underreported, and accurate statistics are hard to come by (De Kock and Wolmarans, 2008). Nevertheless, the widespread nature of the intermediate snail host species, and the fact that many rural populations are forced to share their water sources with their livestock, suggests that fascioliasis is of economic concern, and poses a threat to human and livestock health in South Africa.

Although chemical control of snails remains an effective option, it is also difficult, expensive, carries a range of non-target effects and poses risks to environmental health (Abd El-Ghany

and Abd El-Ghany, 2017). Biocontrol approaches represent environmentally friendly and sustainable means of pest snail control, whether used alone or as part of integrated pest management strategies. Certain species of the aerobic endospore-forming bacteria, in particular *Bacillus thuringiensis* (Berliner, 1915) and *Paenibacillus* sp., have shown antagonism towards the intermediate snail hosts of trematodes (Halima *et al.*, 2006; Ali *et al.*, 2010; Duval *et al.*, 2015; Abd El-Ghany and Abd El-Ghany, 2017). Members of the *Bacillaceae* have shown utility in a range of biocontrol applications against microbial and insect pests (Osman *et al.*, 2015; Tran *et al*., 2022). This group of bacteria then presents an opportunity to source molluscicidal species that can be used against the *Fasciola*-transmitting snail species. Furthermore, endospores present an easy-to-formulate option for commercial products, which are easy to handle and have a long shelf life.

This research project was undertaken with the aims of isolating and screening aerobic endospore-forming bacteria species as antagonists of the snails that act as intermediate hosts of *Fasciola* species. The objectives for this study can be summarised as follows: -

1) Isolate aerobic endospore-forming bacteria from aquatic environments;
2) Screen these bacterial isolates for molluscicidal potential against liver fluke host snails;
3) Characterise antagonistic isolates and measure their biocontrol potential;
4) Determine the mode of action of the molluscicidal isolates.

This thesis has been subdivided into 7 chapters. Chapter 1 is a literature review of the impacts of *Fasciola* sp. and the current state of mitigation measures. Chapter 2 details the isolation and initial screening of aerobic endospore-forming bacterial isolates. Chapter 3 presents the identification by DNA-based methods and de-duplication of molluscicidal isolates. Chapter 4 investigates the role of lipopeptide compounds in molluscicidal potential. Chapter 5 characterises and identifies the lipopeptide fractions in the molluscicidal extracts and measures activity against aquatic snails. Chapter 6 investigates the molluscicidal activity of pure surfactin against two snail species and its impact on zebrafish embryos. Final conclusions and a general overview of the research undertaken are presented in Chapter 7.

The referencing system used in the chapters of this thesis is based on the Harvard system of referencing, and follows the specific style used in the journal *Biological Control.* The thesis is in the form of discrete research chapters, each following the format of a stand-alone research paper. This is the dominant thesis format adopted by the University of KwaZulu-Natal because

it facilitates the publishing of research from a thesis far more easily than the older monograph form of a thesis. As such, there is some unavoidable repetition of references and some introductory information between chapters.

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CHAPTER ONE: LITERATURE REVIEW: *FASCIOLA* LIVER FLUKE FROM THE SOUTH AFRICAN PERSPECTIVE AND THE POTENTIAL APPLICATION OF THE MEMBERS OF THE GENUS *BACILLUS* AS BIOCONTROL AGENTS

1.1. Introduction

Fascioliasis is an economically damaging foodborne zoonotic trematodiases caused by two liver fluke species: *Fasciola hepatica* (Linnaeus, 1758) and *Fasciola gigantica* (Cobbold, 1855). These parasitic flukes have a life cycle that involves an intermediate snail host and a definitive mammalian host (e.g. cattle, sheep, goats, horses and humans) (Mas-Coma *et al*., 2019). In the mammalian host, flukes cause damage to the liver parenchyma and bile ducts, which causes a range of symptoms and a decline in animal health and productivity, and in some cases, may result in death (Marif *et al*., 2016). While economic harm can be partially restricted to livestock, fascioliasis also represents a human health risk in certain areas. Control efforts currently revolve around a limited number of chemical treatments, interventions to control intermediate hosts, and land management practices (Skuce and Zadoks, 2013).

Fascioliasis has been declared, a "Neglected Tropical Disease", by the World Health Organization. Neglected tropical disease are a diverse group of diseases prevalent in tropical and subtropical regions, commonly associated with poverty. This group of diseases impacts over 1.5 billion people but has received disproportionately less focus from global health entities (WHO, 2024). Adema *et al.* (2012) discuss the many shortfalls surrounding snail-borne disease research and highlight that, in spite of our lack of knowledge, research on trematodiases remains underfunded and understudied.

The question of the extent of *Fasciola* liver flukes and their economic and human health costs is only a partially answered one, both globally and in South Africa. Fascioliasis has often been relegated to a disease of concern only for livestock production. Research on this disease is primarily focused on the livestock aspects, with the human impacts is only recently being explored in depth (Mas-Coma *et al.*, 2009). While statistics on *Fasciola* incidence in both

humans and animals in South Africa are scarce, figures reported for African and other endemic countries help to provide a glimpse of the possible threat South Africa faces from this parasite. This review intends to summarise the disease, the risks *Fasciola* poses to human and livestock health, current treatments and preventative measures, and their shortfalls. Looking to the future, avenues of research that may offer some solutions to the liver fluke problem are addressed.

1.2 Fascioliasis causal agents

Fascioliasis is a foodborne zoonotic disease caused by infection by a trematode parasite of which two species, namely *Fasciola hepatica* and *F. gigantica* are described (Figure 1.1). Both species are able to cause disease in many mammal species and are of economic concern primarily for their effects on livestock and human health. Of the two *Fasciola* species, *F. hepatica* has historically prevailed in areas where their amphibious lymnaeid hosts access small bodies of water which are frequently dependant on rainfall, while *F. gigantica* is more common in areas where their preferred lymnaeid hosts utilise larger water bodies richer in aquatic vegetation (Mas-Coma *et al*., 2005). In South Africa, both fluke species are present, though *F. hepatica* is more prevalent (64%), while in neighbouring Zimbabwe *F. gigantica* comprises 99% of livestock and wild animal infections (Mucheka *et al*., 2015).

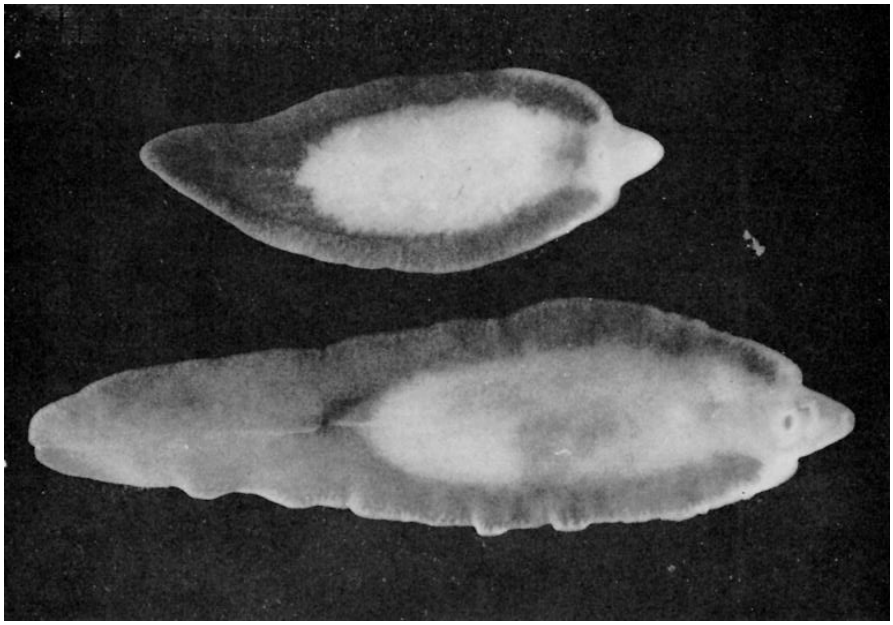

Figure 1.1. Liver flukes *F. hepatica* (top) and *F. gigantica* (bottom). (Source: Palmer and Reeder, URL: https://info.isradiology.org/tropical_deseases/tmcr/chapter21/otherfas.htm, accessed 14 January 2024).

1.3 Host snails

Liver flukes, along with similar trematode parasites, require a snail host as a part of their life cycle of which there are several snail species able to host *Fasciola* flukes. A consequence of the extended host range is that *Fasciola* flukes have spread to many countries worldwide owing to their adaptation to autochthonous lymnaeid species (Mas-Coma *et al*., 2005). There is also a correlation of fascioliasis incidence, and the climate preferences and ecological range of these various host snail species (Mas-Coma *et al*., 2005). Climate change will likely further impact the distribution and presence of these snail hosts, and consequently of fascioliasis incidence (Fox *et al.,* 2011).

In South Africa the indigenous snail species *Galba truncatula* (Müller, 1774) are intermediate hosts for *F. hepatica*, while *Radix natalensis* (Krauss, 1848) is host to *F. gigantica* (Appleton, 2002; Marif *et al*., 2016). Invasive snail species in South Africa may also serve as hosts to liver fluke. *Radix rubiginosa* (Michelin, 1831) and *Radix auricularia* (Linnaeus, 1758) are invasive species that have been recently introduced to South Africa, though their current prevalence and role in the wild has not been defined (Appleton and Miranda, 2015; Malatji *et al.,* 2019; Nyagura *et al.,* 2022). A further invasive species, *Pseudosuccinea columella* (Say, 1817), was reported in South Africa early in the 1940s, and it has since spread throughout the country (De Kock and Wolmarans, 2008). *Pseudosuccinea columella* is a known intermediate host for liver fluke in several parts of the world, where it has been identified as a host to both *Fasciola* species, although, this has not yet been conclusively established in the South African context (Appleton, 2002; Mas-Coma *et al.,* 2005; De Kock and Wolmarans, 2008; Appleton and Miranda, 2015). However, a publication has reported *F. gigantica* in wild populations of *P. columella* using PCR from two provinces in South Africa, KwaZulu-Natal and the Eastern Cape (Malatji and Mukaratirwa, 2020). *Pseudosuccinea columella* is known to be rapidly-colonising and is highly tolerant of a range of environmental conditions (Mas-Coma *et al*., 2005). The spread of invasive species, such as *P. columella,* have been implicated in the spread of *Fasciola* outside the host ranges of indigenous snail species (Grabner *et al*., 2014). A large number of other snail species and cryptic species found worldwide are known to be carriers of fluke and may be important in their local contexts (Correa *et al.,* 2010; Mahulu *et al.,* 2019). Two intermediate snail hosts, *P. columella* and *R. natalensis*, that had been identified in the KwaZulu-Natal area are shown in Figure 1.2

Figure 1.2. Fasciola intermediate host snail species, *Pseudosuccinea columella* (A) and *Radix natalensis* (B) identified from water bodies in the KwaZulu-Natal area.

1.4 Life cycle

The life cycle of *F. hepatica* and *F. gigantica* are very similar, with both requiring aquatic snails as a vital stage in their life cycle, although the two liver fluke species typically exploit different intermediate snail hosts (Appleton, 2002) (Figure 1.3). Adult flukes parasitize the large biliary passages and gall bladder of the mammalian host, where they cause the symptoms of fascioliasis (Mas-Coma *et al*., 2019). The hermaphroditic adult flukes can produce on average 21 000 to 24 000 eggs per day per mature fluke (Boray, 1969; Skuce and Zadoks, 2013). The eggs move into the small intestine and are then excreted in the faeces (Marif *et al*., 2016). Eggs may remain viable but dormant for several months in undesirable conditions (Mas-Coma *et al*., 2019). Once in water, eggs become embryonated and hatch within 1-4 weeks, to release motile miracidia, which must infect a host snail within 3-12 hours of hatching (Andrews, 1999; Skuce and Zadoks, 2013; Mas-Coma *et al*., 2019).

In the snail host, miracidia develop over a few life stages (sporocyst, redial generations, production of cercariae), culminating in the production of motile cercariae that are released from the snail (Skuce and Zadoks 2013; Mas-Coma *et al*., 2019). The cercariae attach onto plant material and encyst to form infective metacercaria (Marif *et al*., 2016). This cyst stage is hardy and may persist for several months on the plant and may even survive on silage (Skuce and Zadoks, 2013). The mammalian host then ingests the metacercaria, which resist acid digestion in the stomach owing to their resistant outer covering (Marif *et al*., 2016). In the hours following ingestion, the juvenile flukes excyst in the duodenum and migrate through the gut wall and into the peritoneal cavity and into the liver (Andrews, 1999). Immature flukes travel through the liver towards the bile ducts over the course of weeks, feeding directly off the liver tissue and initiating symptoms in the host (Kaplan, 2001; Mas-Coma *et al*., 2019). Flukes mature over several months in the bile ducts as obligate blood feeders and then begin to produce eggs (Andrews, 1999; Kaplan, 2001; Skuce and Zadoks, 2013; Mas-Coma *et al*., 2019).

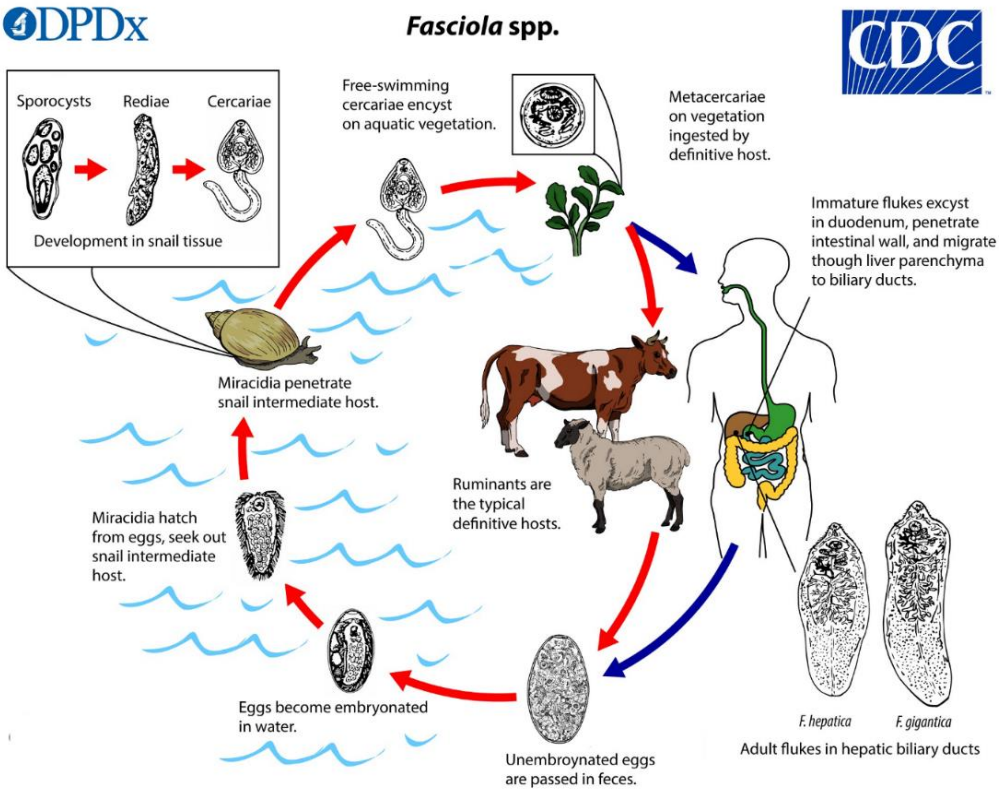

Figure 1.3. Life cycle of *Fasciola* spp. (Adapted from: Centres for Disease Control, DPDx - Laboratory Identification of Parasites of Public Health Concern, URL: https://www.cdc.gov/dpdx/fascioliasis/index.html, accessed 14 January 2024).

Adult liver flukes have leaf-shaped bodies varying in size between the species: *F. hepatica* measures up to 29 mm long and 14.1 mm wide; *F. gigantica* measures up to 52.3 mm long and 11.8 mm wide. Hybrid forms may exist in regions where these species overlap (Mas-Coma *et al*., 2019). The appearance of hybrid varieties has implications for resistance transfer between species and potentially more virulent varieties (Cwiklinski *et al*., 2016). The exact nature of the hybrids found, and the full potential for their impact, are currently unclear (Cwiklinski *et al*., 2016).

A full *Fasciola* life cycle is accomplished in approximately 14-23 weeks, with a substantial part of this cycle occurring external to the hosts, where its progression is dependent on the prevailing conditions and fluke species (Skuce and Zadoks, 2013; Mas-Coma *et al*., 2019). Seasonal variation in prevalence of the disease has also been noted, with a higher incidence during the summer months than in the winter months (Jaja *et al*., 2017a). There is concern that the incidence of infection may increase with global warming, which has been reported to increase the survival and breeding rate of the liver fluke snail hosts (Fox *et al.,* 2011; Skuce and Zadoks, 2013).

1.5 Animal impact of fascioliasis

Fasciola infection causes a range of symptoms in animals, resulting in a decline in animal health and productivity, and in severe cases may result in death (Marif *et al*., 2016). In livestock, the clinical signs of fascioliasis are categorised as acute, sub-acute, and chronic, based on fluke maturity (Marif *et al*., 2016). Disease symptoms are associated with immature flukes and manifest after extensive damage to hepatic parenchyma by the migration of juvenile flukes (Kaplan, 2001; Marif *et al*., 2016). Sub-acute and chronic disease in cattle and sheep are characterised by anaemia, hypoalbuminemia, emaciation, ascites and submandibular oedema (Marif *et al*., 2016). Fluke-infected cattle only rarely show signs of clinical disease, though sub-clinical infections impact on productivity in terms of feed efficiency, growth, and fertility (the latter by altering metabolism and/or sex hormones) (Kaplan, 2001). Acute clinical disease or sudden death is rare in cattle, owing to the toughness of their liver, although milk production suffers, as does fat content in milk, and calving intervals increase in dairy cattle (Saleha, 1991; Skuce and Zadoks, 2013). The risk of death is higher in animals with lower levels of natural

resistance such as sheep (Dalton *et al*., 2013). *Fasciola hepatica* may persist untreated in sheep for up to 20 years and in cattle for 1 to 2 years (Andrews, 1999).

In wild animals, both *Fasciola* species have been found to varying degrees, worldwide (Mas-Coma *et al*., 2019). The presence of wild reservoirs of parasites has implications for any control measures, particularly in areas where domesticated and wild animals are spatially associated (van Wyk and Boomker 2011; Skuce and Zadoks, 2013). In Southern Africa, wild species of antelope have also been reported as being susceptible to liver fluke infection (Mucheka *et al*., 2015).

1.6 Human impact of fascioliasis

Human populations are at risk from *Fasciola* species through the consumption of wild and domesticated plants exposed to contaminated water sources, as well as the consumption of raw liver and drinking and domestic use of contaminated water (Mas-Coma *et al.,* 2018). *Fasciola* liver fluke infection of humans has been reported on all continents (barring Antarctica), with some countries in Asia and Africa being considered as having endemic infections (Mas-Coma, 2005; Nyindo and Lukambagire, 2015). Accurate estimates for human infection numbers are hard to come by, but the WHO estimated in 1990 that at least 2.4-12 million people are infected worldwide (Hopkins, 1992; Mas-Coma *et al*., 2019). In Egypt, human fascioliasis incidence has been measured as high as 19% (Esteban *et al.*, 2003). Keiser and Utzinger (2005) estimated that 91.1 million people were at risk of infection globally. Global estimates from 2005 suggest that 2.6 million people are infected by fluke, resulting in 83 699 years of life lost or disability-adjusted life years (Fürst *et al*., 2012). Endemic areas are considered to include countries in the South Americas (Cuba, Peru, Bolivia, Chile, and Ecuador), western Europe (Portugal, France, and Spain), Iran, and Egypt (Mas-Coma *et al*., 2005).

In South Africa, there are very few cases of fascioliasis in humans have been recorded, although the incidence may well be underdiagnosed (Black *et al*., 2013; Frean and Mendelson, 2013). No broad-scale studies are available, except for an intestinal parasite study conducted amongst schoolchildren in KwaZulu-Natal in 1981, which found 22 out of 7 569 children to be infected with *F. hepatica* (Schutte *et al*., 1981). Nevertheless, the widespread nature of the intermediate snail host species, and the fact that many rural populations are forced to share their water

sources with their livestock, suggests that fascioliasis may pose a greater threat to human health in South Africa than is currently recognised (De Kock and Wolmarans, 2008). However, high incidence of fasciolosis in animals does not necessarily correlate to similar incidence in humans (Mas-Coma *et al*., 2005).

Complications resulting from long-term *Fasciola* infection are of particular concern due to parasite persistence in the human host, which has been reported at greater than 10 years (Marcos *et al*., 2008). Long-term infections have potential repercussions on liver health, with risks of fibrosis, cirrhosis, and cancer developing (Machicado *et al*., 2016). Confirmation of these effects is lacking, although there is some degree of meta-analysis evidence for association with liver fibrosis and cirrhosis (Machicado *et al*., 2016).

1.7 Economic impact of fascioliasis

Global economic losses to fascioliasis have been estimated at over US$3.2 billion annually, representing lost income and productivity, and expenditure on both curative and preventative measures (Spithill *et al*., 1999). Livestock infections compromise the health and feed efficiency of the animal, and negatively impact meat and milk production and quality (Saleha, 1991; Kaplan, 2001; Skuce and Zadoks, 2013). Quayle *et al*. (2010) estimated that anthelmintic treatment costs in South Africa reach ZAR15-30 per animal per annum, with milk losses amounting to ZAR500 per animal yearly and liver condemnation at harvest amounting up to ZAR300 per animal.

Many studies provide numbers focussed on small, specific localities, and there is a paucity of nationwide comprehensive studies. This makes comparing numbers between regions and countries more complicated, and are not necessarily fully representative or sufficient to accurately gauge the true economic impact of the disease. Statistics of fascioliasis in both livestock and humans in South Africa are limited (De Kock and Wolmarans, 2008). Some studies conducted in Southern Africa have shed some light on fascioliasis prevalence and its impact on livestock production: A study conducted in the Eastern Cape Province of South Africa examined abattoir records from three abattoirs between 2010-2012, and found that fascioliasis presence varied from 2.0% to 14.4% over the period (Jaja *et al*., 2017b). A Zimbabwean study of five abattoirs between 1990-1999 found *F. gigantica* incidence at 37%

in cattle inspected (Pfukenyi and Mukaratirwa, 2004). In contrast, a similar study in Botswana, a much drier country, from 2001-2010 examined meat inspection records and found that less than 0.1% of slaughtered cattle were infected with *F. gigantica* (Mochankana and Robertson, 2016). A further Botswanan study from 2011-2013 examined faecal samples and found an overall *F. gigantica* incidence of less than 1% overall, with the highest value recorded at 9% (Mochankana and Robertson, 2018). In Mongu, Zambia, a 2013-2017 study reported that 64.4% of cattle livers were found to be infected, this resulted in economic losses of 164 tons liver condemned, equivalent to US$592 560 in direct losses at the time (Nyirenda *et al*., 2019). In Ethiopia, a study found approximately 20% of cattle infected at a single abattoir, amounting to a direct loss of US$43 000 annually for condemned livers (Zewde *et al*., 2019). A study from Ghana, reported that in 2018, at a single abattoir, 10.27% of cattle were infected with *F. gigantica* (Addy *et al*., 2020).

In endemic regions such as Mexico, fascioliasis incidences can be as high as 99.3% in dairy cattle, with an estimated lost milk yield of 0.51–1.0 kg per cow per day. The annual cost of treating cows was estimated at between US$64-118 per cow, with estimated losses resulting from flukes of US$119 million in production (Villa-Mancera and Reynoso-Palomar, 2019). At a single abattoir in Peru, 55.72% of livers were condemned due to fascioliasis, which corresponded to a direct loss of 16.45 tons of liver worth US$35 000, and an indirect estimated lost body weight gain of US$35 570 (Arias-Pacheco *et al*., 2020).

1.8 Drug treatment and resistance

Conventional animal treatment depends on the livestock species, the fluke stage of life cycle, and regulatory issues surrounding the compounds and residues present in meat and milk products (Skuce and Zadoks, 2013). In milk, residues persist for weeks or months; and the concentrations of undesirable compound residues increases with processing for example: accumulation in cheese was concentrated at a rate of 13-fold (Skuce and Zadoks, 2013). Triclabendazole (TCBZ) is the only treatment option for the early stages of liver fluke infection in humans and animals. This is especially important as it is the only treatment able to target the tissue damaging stages of liver fluke infection (Skuce and Zadoks, 2013; Cwiklinski *et al*., 2016). Other drug treatments are available that are effective at the later stages of fluke development for cattle and sheep infections, and constitute a small group of chemical

compounds: albendazole (ABZ), ricobendazole, oxyclozanide (OXYCLO), nitroxynil, and closantel (CLOS). Clorsulon (CLORS) and rafoxanide (RAFOX) are also used in cattle (Skuce and Zadoks, 2013; Fairweather *et al*., 2020). Human treatment of *F. hepatica* and *F. gigantica* with TCBZ is effective and is currently the only treatment recommended by the WHO (Villegas *et al*., 2012; Gandhi *et al*., 2019). Nitazoxanide and ABZ have been applied as alternative treatments in cases of TCBZ resistance (Cwiklinski *et al*., 2016; Ramadan *et al*., 2019; Khan *et al*., 2020).

Triclabendazole remains the most commonly used chemical flukicide, despite the fact that incidence of fluke resistance has been on the increase since this was first reported in 1995 (Overend and Bowen, 1995). Resistance to TCBZ in human infections was first reported in 2012 in the Netherlands, and subsequently in other countries (Peru, Turkey, Chile) (Winkelhagen *et al*., 2012; Gil *et al*., 2014; Belgin *et al*., 2015; Cabada *et al*., 2016; Ramadan *et al*., 2019). Currently, resistance to TCBZ has only been detected in *F. hepatica*, while *F. gigantica* remains susceptible (Cwiklinski *et al*., 2016). However, if resistance does not emerge spontaneously for *F. gigantica*, the emergence of hybrid *Fasciola* spp. forms may facilitate the interspecies transfer of resistance capabilities (Cwiklinski *et al*., 2016). Resistance has also been reported for some of the alternative livestock treatments. A collection of resistance data from a variety of studies was outlined in Fairweather *et al*. (2020), indicating resistance to ABZ, CLORS in sheep, and to ABZ, RAFOX, OXYCLO and CLOS in cattle. As resistance becomes more common, the presence of resistant infections in humans can be expected to increase, representing a growing risk to human life.

1.9 Alternative control measures

With the emergence of resistance for the primary treatments of fascioliasis, alternative management strategies become more significant for managing all aspects of this parasite (Fairweather *et al*., 2020). Control measures have been recommended that are preventative rather than curative strategies, which aim to reduce fluke numbers and prevalence of eggs in pastures, and to reduce the numbers of intermediate hosts (Marif *et al*., 2016). Primary strategies currently include applying anthelminthic compounds to kill flukes, snail control via chemical compounds, and habitat management such as draining and fencing of intermediate host habitats to restrict livestock access to high-risk areas (Skuce and Zadoks, 2013; Marif *et*

al., 2016). Other avenues with potential application are the use of host snail populations that are naturally resistant to liver fluke. A *P. columella* population in Cuba has been found to naturally resist infection with *F. hepatica* miracidia (Gutierrez *et al*., 2003a; Gutierrez *et al*., 2003b; Alba *et al.*, 2018).

A promising theoretical control method is to vaccinate susceptible animal populations. However, as yet, there are no commercialized vaccines for *Fasciola* infection (Dalton *et al*., 2013). Helminths, like *Fasciola,* are known to release protein and carbohydrate mediator molecules that modulate the host immune response, thereby protecting the parasite (Dalton *et al*., 2013). Furthermore, the host immune response elicited by *F. hepatica* is different from the response to *F. gigantica,* hence, different pathways of development may be required to develop viable vaccines (Dalton *et al*., 2013; Toet *et al.*, 2014). The role of immunomodulatory molecules produced by *Fasciola,* as well as their potential as vaccine candidates, was reviewed by Dalton *et al*. (2013). Nevertheless, without a firmer understanding of the pathways that govern the livestock immune system mechanisms, the development of a livestock vaccine is difficult (Toet *et al.*, 2014). The current understanding of vaccine development targets and progress, as well as prospects on *Fasciola* spp. have been reviewed by several authours (Spithill *et al*., 2012; Toet *et al*., 2014; Molina-Hernandez *et al*., 2015).

Measures that have been applied to schistosomiasis control may be transferable to the host snails of *Fasciola* spp., in particular the control of snail populations. The first control measures for schistosomiasis revolved around snail population control before being replaced by chemical control in the form of anthelminthic compounds. However, snail control is now being revisited (King and Bertsch 2015; Sokolow *et al.*, 2018). The control of snail populations has been reported as an effective means of reducing schistosomiasis incidence, but to date, the most common strategy for snail control remains the use of expensive and toxic molluscicides (Sokolow *et al*., 2016). Many of the conventional effective molluscicides have an environmental impact. For example, the molluscicidal compound niclosamide is a known environmental pollutant (Ross *et al*., 2017). In light of the toxic nature of conventionally-applied molluscicidal compounds, alternative snail control methods are being explored. One avenue is the use of predation using natural snail predators, for example, the use of prawn species, *Macrobrachium vollenhovenii* (Herklots, 1857) and *M. rosenbergii* (De Man, 1879) and the crayfish species *Procambarus clarkii* (Girard, 1852) to control schistosomiasis

intermediate host snails (Khalil and Sleem, 2011; Hoover *et al.*, 2020). The utilisation of predatory snail species to control the fluke intermediate host snails has also been demonstrated using terrestrial snail species *Zonitoides nitidus* (Müller, 1774) against *G. truncatula* (Rondelaud *et al*., 2006). There have also been reports of molluscicidal compounds being produced in culture media from two *Aspergillus* species with activity against *Oncomelania hupensis* (Gredler, 1881), a host of *Schistosoma japonicum* (Chen *et al.*, 2009; Guo *et al.*, 2010).

As alternatives to synthetic compounds, many types of plant extracts have potential as molluscicides, as comprehensively reviewed by Marston and Hostettmann (1985). Mandefro *et al.* (2017) reported that an aqueous extract of *Achyranthes aspera* (Linnaeus, 1753) showing activity against *Schistosoma* intermediate host snail *Biomphalaria pfeifferi* (Krauss, 1848) and *Fasciola* intermediate host snail *R. natalensis*. Abdel-Haleem (2013) showed molluscicidal activity of *Euphorbia splendens* (Bojer ex Hook, 1829), *Ziziphus spinachristi* (Linnaeus, 1798) and *Ambrosia maritima* (Linnaeus, 1753) on two *Schistosoma* host snail species. Clark (1994) identified three South African plant species with acceptable molluscicidal characteristics namely: *Warburgia salutaris* (Bertol.f.) Chiov., *Gardenia thunbergia* (Thunberg, 1780) and *Apodytes dimidiate (*E.Mey. ex Arn.). Yadav and Singh (2011) evaluated *Euphorbia hirta* (Linnaeus, 1753) latex in conjunction with other purified plant-derived active compounds against *Radix acuminata* (Lamarck, 1822) and found acceptable control was possible. Okunji and Iwu (1988) reported 13 plant species with activity against *Bulinus globosus* (Morelet, 1866), *B. pfeifferi* and *R. natalensis*. Plant extracts have not yet been commercialised, but have potential because of their accessibility and lower costs, in the local context. A disadvantage of such extracts is the loss of activity over time, as reported by Clark (1994) and Yadav and Singh (2011). The risk of these compounds to other non-target aquatic species also remains to be investigated fully, and some compounds have already been reported to have such effects (Clark, 1994). Integrated strategies have also been suggested for the use of plant extracts. Despite the promise of alternative control methods against snail hosts of *Schistosoma* and *Fasciola*, the majority of these have only been explored in small-scale research studies.

1.10 Potential of isolates of the genus *Bacillus* as biocontrol agents of the host snails transmitting fascioliasis

Isolates of the bacterial genus *Bacillus* have a long history of utility as biocontrol agents against several pest species, including microorganisms and insects (Ongena and Jacques, 2008; Geetha *et al.,* 2012; Denoirjean *et al.,* 2021; Koim-Puchowska *et al.,* 2023). Additionally, the production of a resistant endospore as part of the life cycle offers ease of commercialisation, storage, and distribution of any formulated biocontrol product. If a molluscicidal *Bacillus* isolate could be found, this would greatly simplify practical application as compared to less hardy species. This is especially important when considering that fascioliasis is common to tropical and sub-tropical regions of the world where cold storage is absent or difficult to maintain. A number of *Bacillus* species are strong biocontrol candidates with a broad range of target pest groups and modes of action. While there has been limited exploration of the molluscicidal properties of *Bacillus* species, identification of a snail pathogen species is not outside of the realm of possibility. There may also be classes of compounds produced by *Bacillus* spp. which may have molluscicidal properties.

A number of studies have investigated the potential of *Bacillus thuringiensis* (Bt) (Berliner, 1915) with antagonism to a variety of terrestrial and aquatic snail species, including *Fasciola* and *Schistosoma* host snails. *Bacillus thuringiensis* purified endotoxin has shown activity against *Oncomelania sp.* snails, which are *Schistosoma* host species (Ali *et al*., 2010). Genena and Mostafa (2008) demonstrated activity in freshly prepared Bt cell suspensions when applied to soil and snail food in combination against land snail species. A number of authors have reported molluscicidal activity of Bt spore-crystal products against a number of snail targets including land snails, as well as schistosomiasis host snails *O. hupensis* and *Biomphalaria alexandrina* (Ehrenberg, 1831) (Halima *et al.,* 2006; Osman *et al.,* 2011; Abd El-Ghany and Abd El-Ghany 2017; Gaber *et al.,* 2022). Furthermore, activity of the Bt strain Dipel-2X has been reported to act against *R. natalensis* by Abdel-Rahman and Hassanian (1999). Outside of Bt, the characterisation and identification of a pathogenic strain of *Paenibacillus* was reported by Duval *et al*. (2015). Duval *et al.* (2015) described a new species of *Paenibacillus*, preliminarily named *P. glabratella*, which was found in nodules of the schistosomiasis intermediate host snail *Biomphalaria glabrata* (Say, 1818), and which caused high mortality rates in adults and eggs of these snails. However, this bacterial species was not amenable to general microbiological cultivation methods.

The genus *Bacillus* and associated genera in the *Bacillaceae* family are ubiquitous organisms within the environment and play roles in a wide range of ecological niches (Gupta *et al.*, 2020). Within the genus *Bacillus* itself there are several species with well-developed pathogenic and antagonistic behaviour to a wide range of species. The most famous example of *Bacillus* spp. with widespread biocontrol use is the insecticidal *Bacillus thuringiensis*. The Bt toxins were reviewed comprehensively by Palma *et al*. (2014) and Osman *et al*. (2015). Briefly, Bt produces insecticidal protein crystals during spore formation, of which there are two broad classes – Cry and Cyt. Examples of Cry proteins are toxic to a range of insect orders, including Lepidoptera, Coleoptera, Hymenoptera, Hemiptera and Diptera, as well as nematodes, while Cyt proteins are generally known for toxicity against Dipteran insects (Palma *et al.*, 2014; Osman *et al.*, 2015). *Bacillus thuringiensis* is also known for the production of VIPs (Vegetative Insecticidal Proteins) and SIPs (Secreted Insecticidal Proteins), which are distinct from the Cry and Cyt proteins, but with similar toxicity to insects, which are produced during vegetative growth instead of during sporulation (Palma *et al.*, 2014; Osman *et al.*, 2015).

Significant research into biocontrol has also focused on *Bacillus subtilis* (Ehrenberg 1835) Cohn 1872, and other closely related species, which have been developed as bio-fungicides, -bactericides, and -insecticides (Li *et al.*, 2020; Denoirjean *et al.*, 2021; Labiadh *et al.*, 2021; Bouchard-Rochette *et al.*, 2022; Ramesar and Hunter, 2023; Koim-Puchowska *et al.*, 2023). Common to these species, and implicated in their efficacy, are classes of compounds known as lipopeptides. Lipopeptides are widely synthesised among members of the *B. subtilis* group of related taxa (Raaijmakers *et al.*, 2010). These compounds comprise a lipid tail linked to a cyclic oligopeptide, are amphiphilic, function as biosurfactants capable of reducing surface tension, and reduce membrane stability through pore formation (Ongena and Jacques, 2008; Raaijmakers *et al.*, 2010; Jacques, 2011). *Bacillus*-produced lipopeptides are classified into three main variants: surfactin, iturin, and fengycin, which are differentiated by specific residues at positions in the peptide fraction, and different lengths and compositions of the fatty acid tail (Ongena and Jacques, 2008). These compounds assist the bacteria in spreading, colonising, and persisting within their environment, and act as signalling molecules in biofilm formation and development (Raaijmakers *et al.*, 2010). The pore-forming and surfactant properties of lipopeptides have further potential applications in industry and agriculture (Meena and Kanwar, 2015).

Lipopeptide-producing *Bacillus* spp. are valued in agriculture for their roles in pathogen antagonism, plant resistance stimulation, and other plant growth promotion activities (Ongena *et al.,* 2007; Fan *et al.,* 2018; Crouzet *et al.,* 2020; Malviya *et al.,* 2020). Fengycins and iturins are primarily associated with antifungal and antibacterial properties. In addition to these characteristics, surfactin is a potent biosurfactant and has shown activity against a number of insect species (Ongena and Jacques, 2008; Geetha *et al.,* 2012; Denoirjean *et al.,* 2021; Koim-Puchowska *et al.,* 2023). Given the wide range of applications for lipopeptides and their structural diversity, these compounds may have utility in mollusc biocontrol.

1.11 Conclusions

Liver flukes, and *Fasciola* in particular, remain a neglected tropical disease in many parts of the world. Without accurate and up-to-date statistics of fascioliasis incidence and production losses in South Africa, it is hard to gauge the size of the problem in the South African context. Nevertheless, studies of *Fasciola* in small areas throughout South Africa, and in neighbouring countries, suggest that this is not a problem that can be ignored. The broadening consequences of climate change and the already widespread distribution of the fluke host snails indicate that increasing fascioliasis incidences are very likely in the coming decades.

Current fluke management practices remain only partially effective. In the face of emerging fluke resistance to chemical treatments, and the lack of new anthelmintic compounds, the development of a vaccine would be the ideal development for future control of this disease. With a vaccine having an indeterminate development timeline, control of the host snails may be a more achievable option in the near term. Biocontrol strategies, often comprising integrated measures, are on the rise for the control of a variety of agricultural pests, given impetus by phenomena such as consumer demand for products without agrochemical residues, increasing concerns of environmental stewardship, and the threat of emerging parasite resistance. Microbial antagonists of the snail host species, therefore, present an appealing avenue for snail control. *Bacillus* species have widely shown utility in several agricultural contexts and are considered safe organisms that are easily commercialised and formulated. With the reports of *Bacillus* spp. with molluscicidal properties, there is a strong argument to be made to investigate these endospore-formers to control the snail species which host *Fasciola* liver fluke.

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CHAPTER TWO: *BACILLACEAE* ISOLATION AND HIGH-THROUGHPUT SCREENING FOR MOLLUSCICIDAL POTENTIAL

Abstract

Aquatic snails are involved in harmful disease cycles of *Fasciola* (liver fluke) both in humans and in livestock agriculture. The focus of this study was to isolate and identify candidate bacterial isolates antagonistic to aquatic snails, with the ultimate goal of controlling the host snails responsible for the transmission of liver fluke in South Africa. A bacterial antagonist would offer a novel means of snail population control and reduce society's dependence on chemicals. Due to their molluscicidal capabilities reported in the literature, and the benefits of being endospore-formers, strains within the family *Bacillaceae* were targeted as candidate biocontrol agents. A population of the freshwater snail *Physella acuta* (Draparnaud, 1805) was established and used for screening bacterial candidates as an easily-reared proxy species for the *Fasciola* spp. intermediate host snails. Aerobic endospore-forming bacteria were isolated from aquatic soil, collected primarily in the KwaZulu-Natal province, South Africa utilising several approaches including a general endospore heat-shock isolation method and two *Bacillus thuringiensis* (Bt) (Berliner, 1915) specific isolation methods. Initial screening for molluscicidal activity in the 1180 isolates did not yield any strong performers; however, a subset of 124 isolates demonstrated potential activity in preliminary screening, that in the absence of any strong molluscicidal isolates, warranted a more in-depth investigation. Subsequently, when the bioassays were repeated, none of the 124 isolates showed strong molluscicidal activity; however, eight isolates with the highest mortality rates (16.7 – 50%) were selected for further analysis in a later chapter to determine whether these isolates exhibit potential molluscicidal activity. When spent culture supernatant was evaluated in molluscicidal bioassays 12 of the 124 isolates demonstrated strong molluscicidal activity, these isolates were selected for further investigation and characterisation in a later chapter.

2.1 Introduction

Aquatic snails present a problem for agricultural and human health by virtue of their role in a number of disease life cycles (Lu *et al.*, 2018). In particular, fascioliasis is a neglected global tropical disease present worldwide, which is able to utilise a number of snail species as

intermediate hosts (Lu *et al.,* 2018). Current control measures revolve around chemical control of the snails and treatment of affected livestock. This has resulted in environmental concerns associated with the use of synthetic molluscicides, as well as resistance concerns regarding the chemical flukicides in common use (Fairweather *et al*., 2020). An avenue for the control of fascioliasis is the development of a biological control agent for the liver fluke transmitting snails, for example *Galba truncatula* (Müller, 1774), *Radix natalensis* (Krauss, 1848) and *Pseudosuccinea columella* (Say, 1817) - snail species implicated in *Fasciola* transmission in South Africa (Appleton, 2002; Marif *et al*., 2016; Malatji and Mukaratirwa, 2020). Development of a snail biocontrol agent could also have applications in the control of other snail species, such as those responsible for the transmission of schistosomiasis, another neglected tropical disease. Biocontrol has also been advocated for the control of invasive aquatic snails such as the Golden apple snail (*Pomacea canaliculata*, Lamarck, 1822), which have an impact on water-associated food crops such as *Colocasia esculenta* (Linnaeus, Schott) (taro) and *Oryza sativa* (Linnaeus) (rice) (Yang *et al.,* 2018). Isolation of a bacterial biological control agent antagonistic to aquatic snails may present a novel biocontrol solution for *Fasciola* and potentially other aquatic snail pest species.

A number of bacterial species have been identified with some degree of molluscicidal activity, including *B. thuringiensis* (Abdel-Rahman and Hassanian, 1999; Halima *et al.,* 2006; Osman *et al.,* 2011; Abd El-Ghany and Abd El-Ghany 2017; Gaber *et al.,* 2022) and a *Paenibacillus* sp. (Duval *et al.,* 2015). Members of the *Bacillaceae* have an in-built advantage relative to other microbial species in that they form a resistant endospore, which ensures a long shelf-life for a formulated biological control product. In addition, *Bacillus* spp. are generally easily cultivated and formulated, which should translate into a financially viable product if a molluscicidal strain can be identified. For the above reasons, we targeted endospore-formers for isolation from aquatic sources in KwaZulu-Natal, South Africa and surrounding provinces. The aim of this project was to develop and implement a high throughput screening methodology for the rapid isolation and screening of *Bacillaceae* that could parasitize and kill aquatic snails. A broad screening strategy was adopted, targeting Bt strains, in particular, with a parallel screening of other endospore-forming species also being undertaken.

2.2 Materials and Methods

2.2.1 Aquatic snail population maintenance and breeding

A breeding population of the *Fasciola* transmitting snail *P. columella* was gifted by Dr J.A. van Wyk, University of Pretoria, Gauteng, South Africa. Wild-collected populations of *P. columella* and *R. natalensis* were also collected (KwaZulu-Natal, Nottingham Road, Strathdean Farm dam, - 29.3579, 30.02334). A common invasive snail species, *P. acuta* (synonym, *Physa acuta*) was sourced from a local pet shop (Pets World & Aquatics, Pietermaritzburg, KwaZulu-Natal, South Africa, 3201).

Snails were housed in 60 and 100 L glass aquaria kept at approximately 25°C in the School of Life Sciences Small Animal House (UKZN, Pietermaritzburg, South Africa) for breeding and maintenance purposes. Snails were fed a diet of lettuce leaves, which were made available in the tank continuously, covering approximately 30% of the tank surface. Fish food (approximately 5 g per tank) (Regular Pond pellets, Qualipet, Phoenix, Durban, KwaZulu-Natal, South Africa, 4068) was fed weekly. Water changes were performed monthly, with approximately 80% of the water volume being replaced with fresh water. Filtration was provided to each tank using an air lift foam filter. A substrate of coral pieces was provided to buffer pH and to provide a source of calcium. In addition, calcium carbonate powder was added with each water change (5 g per tank).

2.2.2 Aquatic snail identification

Identification of snails used in this project was confirmed by sequencing a portion of the cytochrome oxidase 1 gene (COX-1) (Lawton *et al.*, 2015). DNA extraction was performed using NucleoSpin™ Tissue Mini kits (Macherey-Nagel™, Düren, Nordrhein-Westfalen, Germany). Snails were euthanised in 5% ethanol (v/v) for 30 minutes before immersion in 95% ethanol 5 minutes before dissection (Gilbertson and Wyatt, 2016). Approximately 25 mg of tissue from the snail foot was removed using a sterile scalpel for DNA extraction following the standard protocol for animal tissue recommended by the manufacturer. Samples were pre-lysed for until complete digestion for 4 h at 56°C in a dry bath, with hourly vortexing. The remainder of the extraction was performed as per the kit's instructions. Snails selected for DNA extraction

included two representatives from each *P. columella* colony, two *R. natalensis* snails and four individuals, putatively identified as *P. acuta.*

PCR reactions were performed using Dreamtaq Green PCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Reactions were performed in 30 µL reaction volumes with final primer concentration of 0.3 mM per primer, with 1 µL of template DNA. Primers were sourced from Inqaba Biotech (Muckleneuk, Pretoria, Gauteng, South Africa, 0002). Primers targeting a 710 bp fragment of the Cox1 gene were used:
LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3')
HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al.*, 1994).

The PCR reactions were run in a G-Storm GS1 Thermal Cycler (G-Storm, Somerset, U.K.) with the following cycle parameters: Initial DNA denaturation at 95°C for 3 minutes; 40 cycles of: denaturation at 95°Cfor 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 60 seconds; and, a final extension of 72°C for 5 minutes before cooling to 4°C for storage.

Amplified product (2-5 µL) was visualised on agarose gel (1% w/v) made up with sodium boric acid buffer (SB) (Brody and Kern, 2004), stained using SYBR Safe (Thermo Fisher Scientific, Waltham, Massachusetts, United States) at 0.5x concentration (0.5 µL per 10 mL gel volume). Product size was visually estimated using a GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Gels were run for 80 minutes at 80 V before visualising and image capture using a SynGene G:Box and Syngene GeneSnap software (version 7.09) (Syngene, Cambridge, England).

The PCR reactions were sent to Inqaba Biotech for sequencing in a single direction using the LCO1490 primer. Sequences were base called using Chromas Lite (Version 2.01). Sequences were then processed using NCBI blast for identification purposes (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Zhang *et al.*, 2000).

2.2.3 Aerobic endospore-former isolation

Soil samples were collected from February 2018 to December 2020 from several locations primarily around the KwaZulu-Natal Province, South Africa, with some samplings from the

Free State Province. Location details are provided in Appendix A. Soil samples were collected from sites associated with aquatic environments (i.e. river banks, dams' edges, wetlands and underwater substrates) and allowed to dry under ambient conditions in clean plastic containers and stored at room temperature before processing. Other sources included deceased *P. columella* snails sourced from a dam located at Nottingham Road, KwaZulu-Natal (KwaZulu-Natal, Nottingham Road, Strathdean Farm dam, - 29.3579, 30.02334), as well as unidentified, deceased land snail carcasses (probably in the *Achatinidae* family) (University of KwaZulu Natal, Pietermaritzburg, Agriculture campus, 3201).

A number of bacterial isolates were also obtained from University of KwaZulu-Natal culture collections from the Discipline of Microbiology (School of Life Sciences, College of Agriculture, Engineering & Science) as well as from the Discipline of Plant Pathology (School of Agricultural, Earth and Environmental Sciences, College of Agriculture, Engineering & Science) located at the UKZN Pietermaritzburg, KwaZulu-Natal campus. These bacterial isolates were included based on previous knowledge of their metabolites, which merited inclusion as general species representatives, and which have potential utility as biological control agents for a range of other target species (*viz*. bacterial, fungal, insect).

Several isolation methods were applied to select for a range of *Bacillaceae* species. Initial screening used a Bt isolation utilizing the characteristic of Bt spores to not germinate in the presence of sodium acetate. While selective media for Bt was used, selection was not exclusive to Bt colony morphology (i.e. white to cream coloured, usually granular in surface texture and circular to irregular in colony shape with variable margins: undulate, crenate or fimbriate) (Logan and De Vos, 2009). Isolate selection was based on selecting for the most representative colony morphologies from each sample. As the project progressed other isolation methods were applied. These include a more general isolation for endospores using a heat-shock method and an additional Bt enrichment method exploiting the fast growth rate of Bt strains. For a single sample of deceased land snails, a general isolation method was applied with no heat-shock endospore selection.

2.2.3.1 Sodium acetate isolation

A Bt isolation procedure utilising sodium acetate to inhibit Bt spore germination, as developed by Travers *et al.* (1987), was used for the initial isolation of Bt and other endospore-formers. A 1 g portion of dry soil was added aseptically to 10 mL of sterile sodium acetate Luria-Bertani medium in a 125 mL Erlenmeyer flask (10 $g.L^{-1}$ tryptone, 5 $g.L^{-1}$yeast extract, 5 $g.L^{-1}$ NaCl, 20.5 $g.L^{-1}$ anhydrous sodium acetate, pH adjusted to 7.0 before sterilisation at 121°C for 15 minutes). This was Incubated at 30°C for 4 h in a rotary incubator at 150 rpm. After incubation, a 1 mL liquid sample was removed from the flask to a sterile 1.5 mL microcentrifuge tube and heat-shocked at 80°C for 10 minutes in a dry bath. Heat-shocked media was subjected to a dilution series and 100 µL of various dilutions was aseptically spread with a glass hockey stick onto 90 mm Tryptic Soy Agar (TSA) (Neogen Corporation, Lansing, Michigan, United States) plates. Dilutions of 10^{-0} and 10^{-1} were plated out in quadruplicate. These plates were incubated inverted at 30°C for 24 h in the dark.

2.2.3.2 Endospore heat-shock isolation

A general endospore isolation, utilising heat-shock to select for endospores, was applied to isolate *Bacillaceae*. A 1 g dry portion of dry soil was added aseptically to 10 mL of sterile ¼-strength Ringer's solution (Sisco Research Laboratories Pvt Ltd, Mumbai, India) in a 125 mL Erlenmeyer flask. This was shaken in a rotary incubator at 150 rpm at 30°C for 15 minutes to homogenise. After homogenisation, a 1 mL liquid sample was removed from the flask to a 1.5 mL microcentrifuge tube and heat-shocked at 80°C for 10 minutes in a dry bath. Heat-shocked media was subjected to a dilution series and 100 µL of various dilutions was aseptically spread with a glass hockey stick onto 90 mm TSA plates. Dilutions plated out were at 10^{-1} and 10^{-2}, in quadruplicate. These plates were incubated inverted at 30°C for 24 h in the dark.

2.2.3.3 Bacillus thuringiensis enrichment isolation

A Bt enrichment technique was used that exploited the fast growth rate of Bt strains, as described by Patel *et al*. (2013). A 1 g portion of dry soil was added aseptically to 20 mL GYS media in a 125 mL Erlenmeyer flask (1 $g.L^{-1}$ glucose, 2 $g.L^{-1}$ yeast extract, 2 $g.L^{-1}$ $(NH_4)_2SO_4$, 0.2 $g.L^{-1}$ $MgSO_4$, 0.08 $g.L^{-1}$ $CaCl_2.2H_20$, 0.05 $g.L^{-1}$ $MnSO_4.H_20$, 0.5 $g.L^{-1}$ K_2HPO_4 adjusted to

pH 7.3 before sterilisation at 121°C for 15 minutes). This was incubated at 30°C in a rotary incubator at 150 rpm for 48 h. After incubation, a 1 mL liquid sample was removed from the flask to a 1.5 mL microcentrifuge tube and heat-shocked at 80°C for 10 minutes in a dry bath. Heat-shocked media was subjected to a dilution series and 100 µL of various dilutions was aseptically spread with a glass hockey stick onto 90 mm TSA plates. Dilutions at 10^{-4}, 10^{-5}, and 10^{-6} were plated out in quadruplicate. These plates were incubated inverted at 30°C for 24 h in the dark.

2.2.3.4 Alternative isolation procedure for deceased snails

When the opportunity presented itself, several snail corpses were processed as potential sources of molluscicidal bacterial isolates.

A dead, dried land snail corpse (UKZN, sample AI) was processed by blending the corpse along with associated soil in 50 mL sterile ¼-strength Ringer's using a stick blender sanitised with 70% ethanol for five minutes. This homogenate was processed using the methods for sodium acetate isolation (Section 2.2.3.1) and Endospore heat-shock isolation (Section 2.2.3.2) with 1 mL homogenate used in place of 1 g dry soil. Dilutions were performed as described in the respective sections and plated at the same dilutions as in the referenced sections. Plates were incubated inverted at 30°C for 24 h in the dark.

A freshly dead land snail (UKZN, sample AH) was processed by blending of the corpse along with 50 mL sterile ¼ Ringers with a sanitised stick blender. This homogenate was processed using the methods for sodium acetate isolation (Section 2.2.3.1) and Endospore heat-shock isolation (Section 2.2.3.2) with 1 mL homogenate used in place of 1 g dry soil. Dilutions were performed as described in the respective sections and plated at the same dilutions, before being incubated at 30°C for 24 h. In addition, isolation from the homogenate without a heat-shock selection step or enrichment was performed. A dilution series of untreated homogenate was performed, and 100 µL of various dilutions were plated aseptically by spreading with a glass hockey stick onto 90mm TSA plates. Dilutions of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} were plated out in quadruplicate. These plates were incubated inverted at 30°C for 24 h in the dark.

Eight freshly deceased, wild-caught *P. columella* (Andermatt PHP, Strathdean Farm dam, Gowrie Avenue, Nottingham Road, 3280) (Sample AR) were processed by crushing the whole snails along with 5 mL sterile ¼-strength Ringer's solution in a sterilised mortar and pestle. This homogenate was processed using the method for Endospore heat-shock isolation (Section 2.2.3.2) with 1 mL homogenate used in place of 1 g dry soil. Dilutions of 10^{-1}, 10^{-2}, and 10^{-3} were plated out in quintuplicate by plating of 100 µL of each dilution aseptically by spreading with a glass hockey stick onto 90mm TSA plates. These plates were incubated inverted at 30°C for 24 h in the dark.

2.2.3.5 Isolate selection and purification

Incubated plates (Sections 2.2.3.1 through 2.2.3.4) underwent selection and re-streaking of individual isolates to obtain pure cultures on TSA via three-way streaking. Selection of isolates was based on selecting a diverse range of the available colony morphologies in each sample and isolation procedure. In general, isolates with very similar colony appearance was restricted to 3 isolates per sample, per isolation procedure. Selection for Bt type colony morphology was prioritised. However, non-Bt morphologies were also selected in large numbers as these morphologies were more frequently found.

2.2.4 High throughput snail assay and initial screening

Isolates from the screening methods in Section 2.2.3 were grown on TSA for 5 days at 30°C, culture material was scraped from the plate using a flame-sterilised inoculating loop and aseptically placed into a microfuge tube containing 200 µL of sterile 0.35% saline (0.35% w/v NaCl). Approximately 50 µL of culture material per tube was targeted. Typically, three passes of the inoculating loop were sufficient to transfer this volume, depending on the texture and adherence of each respective isolate's colony material.

Resulting bacterial suspensions were homogenised by pipetting up-and-down immediately before use. Initial screening of isolates from soil samples A through AQ involved inoculating 100 µL of bacterial homogenate onto approximately 8 autoclaved food pellets (Regular Pond pellets, Qualipet, Phoenix, Durban, KwaZulu-Natal, South Africa, 4068) placed in a single well of sterilised 12-hole ceramic palette (Figure 2.1 A). This was sufficient to coat the food pellets,

which were then dried in a glass desiccator (Non-vacuum) containing freshly dried silica gel desiccant overnight before being fed to the assay snails. Subsequently, to simplify and streamline the assay preparation process (from sample AR until cessation of screening) the pellet preparation step was omitted and instead 100 µL of bacterial homogenate was added directly to the assay tank.

Snail assay tanks comprised 500 mL PET bottles with perforated screw caps for aeration (Figure 2.1 B). These were filled with approximately 400 mL of water sourced from the tanks from which harvested snails were reared. Each assay tank was populated with three *P. acuta* snails between 8 and 15 mm in length. Each isolate tested was tested in single replicates. Tanks were kept for observation for 24 h without food before bacteria were applied and the assay initiated, in order to mitigate any snail mortality from the assay tank preparation process.

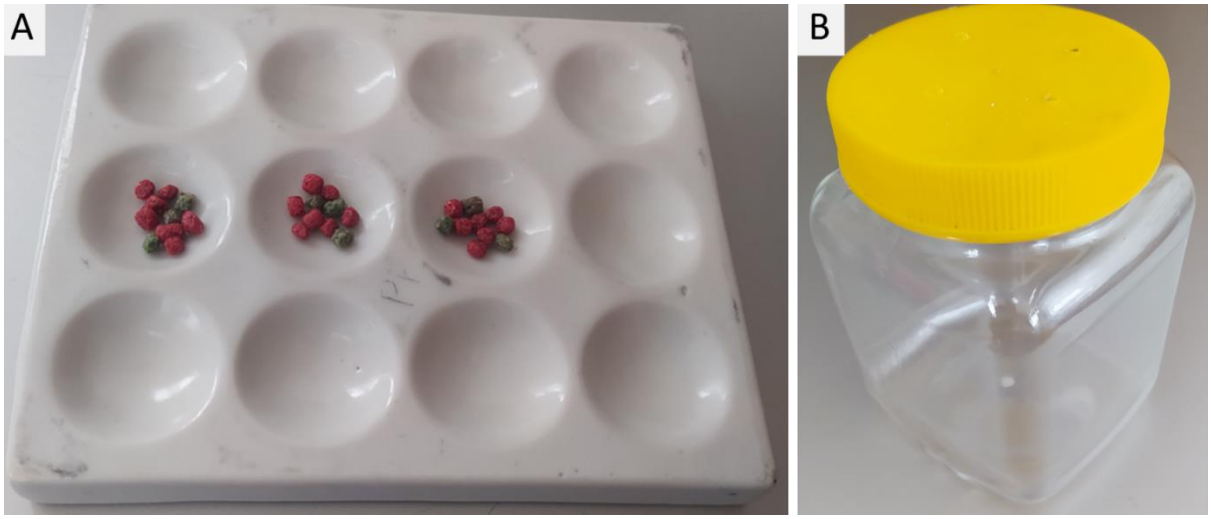

Figure 2.1. Ceramic palette (A) and 500 mL PET assay jar (B) used in bacterial pellet impregnation and snail assays respectively

Assays began with the feeding of approximately three culture-coated food pellets per tank for initial samples, while for later sample isolates, 100 µL of bacterial homogenate was added directly into the assay tank at assay commencement. Control tanks without exposure to bacterial isolates were included in each batch of assays. These were fed with unadulterated food pellets and then maintained in the same manner and location as the assay tanks. All assay tanks were stored together in 100 L clear plastic storage containers under ambient laboratory

conditions. Tanks were maintained with approximately 5 cm^2 of lettuce always available as a food source for the entirety of the observation period.

At the culmination of each assay, surviving snails were transferred into a 100 L quarantine tank maintained in a separate lab to the assay snail breeding population. This tank was maintained over the course of the project. To this tank was also added excess soil samples remaining after bacterial isolation. Snails in this tank were observed for any behavioural or physical changes that suggested the presence of a pathogen that was not detected in the initial assay or was not isolated from the original soil samples.

Assay tanks were observed for a total of 15 days after initial exposure to bacterial isolates. Tanks were observed at least 3 times a week for dead snails. Water was not changed, and dead snails were not removed during the course of the assay. In cases where an assay tank showed any snail mortality, that bacterial isolate was rescreened under identical conditions for a 21-day observation period. In addition, these respective bacterial isolates were stored in glycerol at -80°C. Long-term glycerol storage was performed by scraping approximately 50 µL of culture material from 24 h old TSA plates into 500 µL of sterile 20% glycerol in 0.35% saline in 1.5 mL microcentrifuge tubes. Tubes were homogenised by vortexing, before freezing at -80°C until needed.

An estimated quantification of bacterial cell additions was performed on a subset of 25 isolates. Bacterial homogenate (200 µL) was prepared in the same manner as used for the initial screening assay in Section 2.2.4. After appropriate dilution in 0.35% saline, bacterial cells were counted on a Petroff-Hausser counting chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) of 0.02 mm depth with a counted area of 0.04 mm^2 averaged over five replicates.

2.2.5 Further evaluation of selected isolates

Isolates associated with snail mortality in the screening assays (Section 2.2.4) were re-tested for pathogenicity, as well as for the presence of any molluscicidal metabolites excreted into spent broth culture. Bacteria from 24 h and 120 h cultures grown on TSA at 30^oC were used

and compared. The presence of active metabolites excreted into growth media was assessed from the 120 h culture using cell-free supernatant, produced via centrifugation.

Several isolates were included as reference strains in the screening process. Three *B. thuringiensis* strains, ATCC 10792 and ATCC 33679 and *B. thuringiensis* subsp. *kurstaki* isolated from a commercial formulation of Margaret Roberts Biological Caterpillar Insecticide (Dipel) (Kirchhoff's Margaret Roberts, Reg.no L7112 Act 36/47). Three *Bacillus velezensis* strains were also included namely, R16, FZB42 and PHP 1601, which are known to produce a range of bioactive lipopeptide compounds associated with biological control applications (Hunter, 2016; Fan *et al.,* 2018; Ramesar and Hunter, 2023)

2.2.5.1 Inoculum production

Isolates were revived from -80°C glycerol stocks (Section 2.2.4) onto fresh TSA and incubated at 30°C. After 24 h incubation a single colony was re-streaked aseptically onto fresh TSA and incubated at 30°C. In the case of mixed cultures, isolates were purified and designated as sub-sets of the initial culture designation. Glycerol stocks were made of the purified isolates following the method in Section 2.2.4.

In order to standardise bacterial dosage between isolates, bacterial inoculum was prepared at a set absorbance value using a spectrophotometer. Cell counts were conducted via Petroff-Hausser counting chamber to determine approximate dosage rates. Inoculum for assays was generated by inoculating pure cultures into 20 mL Tryptic soy broth (TSB) (Neogen Corporation, Lansing, Michigan, United States) in 125 mL Erlenmeyer and incubated at 30°C with agitation at 150 rpm for 24 or 120 h before processing. At the end of the incubation period, 2 mL of culture was centrifuged in a sterile microcentrifuge tube for 5 minutes at 10 000 *x g* in a benchtop centrifuge. After supernatant removal, the cell pellet was resuspended in 1 mL sterile 0.35% saline. After homogenising by vortexing, absorbance readings were measured at 600 nm using a Shimadzu UVmini-1240 Spectrophotometer (Shimadzu Corporation, Kyoto, Japan), diluting as needed with 0.35% saline to achieve a measurement of approximately OD_{600} 0.7. The spectrophotometer was blanked with sterile 0.35% saline.

Using the calculated absorbance value of the original broth, a volume of 1 mL standardised inoculum was prepared with an extrapolated absorbance of OD_{600} 5.0. This inoculum was prepared by either diluting starting broth appropriately with 0.35 % saline or by using centrifugation to concentrate the original broth (5 minutes at 10 000 *x g*). Inoculum in microcentrifuge tubes, were then centrifuged at 5 minutes at 10 000 *x g* and the supernatant discarded. The cell pellet generated was used immediately in the snail assays. Duplicate samples were prepared for each isolate at each time interval. Cell counts were performed as per Section 2.2.4 on the saline homogenate after appropriate dilution. Bacterial cell concentrations were recorded, and the average calculated.

2.2.5.2 Bacterial assay procedure

Assay tanks (500 mL) containing approximately 400 mL aquarium tank water containing 3 snails each were prepared 24 h in advance as per Section 2.2.4. Assays of 24 and 120 h were inoculated in duplicate assay tanks for a total of 6 individual snails per culture time interval per isolate. Uninoculated control tanks were run in parallel with each assay batch.

Prepared cells (Section 2.2.5.1, 1 mL with a calculated absorbance of OD_{600} 5.0) were resuspended directly into the assay tanks. Snails were exposed to the bacterial suspension for 48 h. Thereafter, the water was changed with fresh aquarium tank water. Assay tanks were observed over a period of 3 weeks for snail mortality. Dead snails were not removed from the assay tank. Water was changed on a weekly basis and fresh lettuce (approximately 5 cm^2) was provided as needed for the duration of the observation period (as per Section 2.2.4).

2.2.5.3 Supernatant processing and assay

For the supernatant assay, 3 mL of cell-free supernatant was produced by centrifugation of 120 h old TSB (Section 2.2.5.1) at 10 000 *x g* for 5 minutes. This supernatant was used to make a 10% (v/v) solution of 30 mL by dilution with snail aquaria water and then pH adjusted to pH 7.2 using 0.1M HCl. A volume of 10 mL of the 10% solution was removed and diluted 1:1 with aquaria tank water to create a 5% (v/v) solution. A TSB control was prepared to account for any potential molluscicidal media components by preparing sterile TSB at 20% and 10% concentrations in aquaria water, and pH adjusted as described above. In addition, baseline mortality controls using unamended aquaria tank water were also prepared.

Supernatant assays were performed in 50 mL plastic sample jars with perforated lids. Six *P. acuta* snails between 8–15 mm in size were placed in each jar. Jars were emptied of excess water and 20 mL of the appropriate concentration of pH-adjusted supernatant solution was added. Each isolate and each concentration were tested as a single replicate. These 50 mL assay tanks were stored under ambient laboratory conditions for 24 h before transfer of the snails into 500 mL PET assay jars containing 400 mL aquarium tank water. Snails were provided with a supply of lettuce (approximately 5 cm^2) and observed for one week, assessing recovery from exposure to the supernatant.

2.3 Results

Molluscicidal assays require large populations of aquatic snail species to accommodate high throughput screening of a large number of bacterial isolates. Initial attempts to establish a viable breeding population of South African *Fasciola* intermediate host snails, *R. natalensis* and *P. columella* were not successful. While a population of *P. columella* sourced from Dr J.A. van Wyk was maintained over several years, egg laying and successful maturation of juvenile snails to adulthood was a continuous bottleneck. Wild-caught populations of *R. natalensis* and *P. columella* failed to establish in captivity. The decision was then taken to make use of the rapidly breeding population of *Physella acuta* for this initial screening. This species proved to be easy to cultivate in freshwater aquaria, and readily provided the number of adult snails required for screening purposes. While screening on the specific target snails relevant to South African *Fasciola* transmission would have been preferred, this was not possible with the

resources at hand. Instead, *P. acuta* was chosen as a proxy species for the purpose of these screening assays.

2.3.1 Snail identification by COX-1 PCR partial gene sequencing

The products of COX-1 PCR were visualised using agarose gel electrophoresis, which yielded a single band of approximately 700 bp, gel image shown in Appendix B. After sequencing using the forward primer and base calling using Chromas Lite, sequence data (588 bp) was submitted to the NCBI BLAST database and the closest matched database entries were collated as shown in Table 2.1. Sequence data confirmed the identification of the wild-caught snails as *R. natalensis* and *P. columella*, as well as confirmed the identity of the *P. columella* colony sourced from Dr J.A. van Wyk. The identity of the proxy snail species used in the screening assays was confirmed as *P. acuta*. A comparative phylogenetic tree was generated (Figure 2.2.) that illustrates the similarities of the snails sequenced with known species representatives from the NCBI database.

Table 2.1. Identities of snail species as determined by COX-1 partial DNA sequences (588 bp) submitted to the NCBI BLAST database (December 2022) using the MegaBLAST algorithm

Snail Sample	Species Identity	Percentage Identity	Query Cover
Strathdean 1 Strathdean 2 Dr. van Wyk 1 Dr. van Wyk 2	*Pseudosuccinea columella*	100%	100%
Strathdean 3 Strathdean 4	*Radix natalensis*	98.55%	99%
Aquarium 1 Aquarium 2 Aquarium 3 Aquarium 4	*Physella acuta*	100%	99%

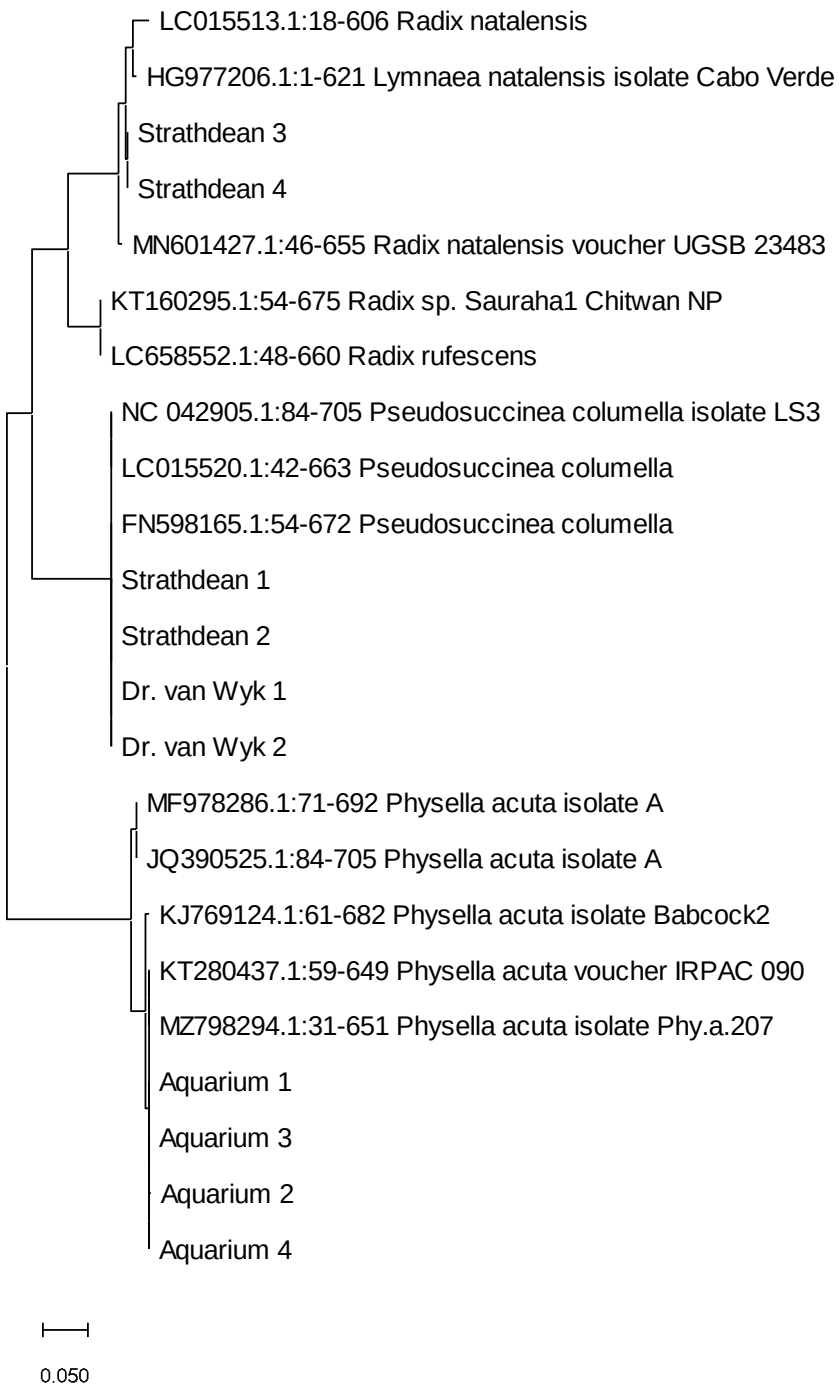

Figure 2.2. Phylogenetic tree of aquatic snails based on COX-1 partial DNA sequence data generated using MEGA11 software (Tamura *et al.*, 2021)

The tree was inferred using the Maximum Likelihood Method and the Tamura-Nei Model (Tamura and Nei, 1993). The tree shown represents the topology with the highest log likelihood value from initial trees obtained by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances, estimated using the Tamura-Nei Model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis applied 23 nucleotide sequences with total of 588 positions in the final dataset.

2.3.2 Bacterial isolates from environmental samples

A total of 63 samples were processed with 60 soil samples and 3 samples of dead snails. The number of isolates yielded from each isolation method are shown in Figure 2.3. Sodium acetate isolations yielded 529 isolates from 60 soil samples and 22 isolates from deceased land snail samples. Endospore heat-shock isolation yielded 347 isolates from 43 soil samples and a further 60 isolates from deceased snail samples. The Bt enrichment isolation yielded 189 isolates from 18 soil samples. Non-selective isolation from the freshly deceased land snail sample (UKZN, AI) resulted in a further 10 isolates. In addition to isolates from natural sources, 23 isolates from two culture collections from previous post-graduate studies were also assayed. A total of 1180 isolates underwent screening for molluscicidal activity during the course of screening as outlined in Section 2.2.4.

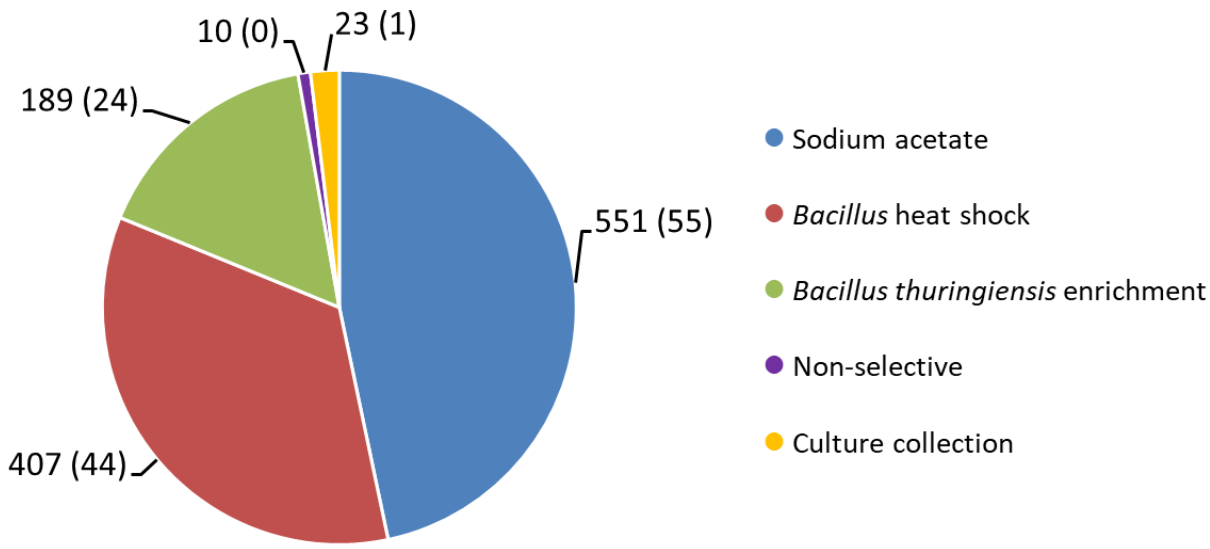

Figure 2.3. Graphical representation of the number of bacterial isolates obtained from each respective isolation procedure. Numbers in brackets represent the number of isolates associated with snail mortality in the initial screening assays.

2.3.3 Initial screening results

During the course of the initial screening assays, 124 control tanks, comprising 372 individual snails, were run in parallel. Only two deaths were noted in total, equating to a baseline mortality rate of 0.54% over the 15-day observation period. Of the 1180 isolates screened, 124 isolates

(10.5% of isolates) were associated with one or more deaths during the 15-day exposure period. When these 124 isolates were re-screened, only 16 isolates reproduced snail mortality over the follow up three-week observation period. The quarantine tank of surviving snails from the initial assays, along with excess soil sample, did not yield any observable impacts on the population that would suggest a pathogen was present.

Of the 124 isolates selected in the preliminary screen: 55 (9.98% of those initially isolated) were isolated using the sodium acetate isolation method; 44 (10.81 % of those initially isolated) were isolated using the endospore heat-shock isolation method; 24 (12.70% of those initially isolated) isolated using the Bt enrichment isolation method; and one isolate from a culture collection. The small sample of non-heat-shocked isolates (10) yielded no isolates with molluscicidal activity.

2.3.3.1 Quantification estimate of bacterial exposure

For the purposes of estimating bacterial dose rate (Section 2.2.4), a selection of isolates (n=25) were prepared to generate bacterial homogenate from 5-day old TSA plates. Counting of the homogenates with a Petroff-Hausser counting chamber gave an average of 2.72 $x10^{10}$ cells per mL ± 9.77 $x10^{9}$ (95% CI). Using the averaged count value, initial assay methodology (Samples A through AQ) using coated pellets, would have had on average a total of ~1.02 $x10^{9}$ cells applied per assay tank. Later assays (AR through BD2), using 100 µL of homogenate directly, would have had on average a total of ~2.72 $x10^{9}$ cells applied per assay tank. This corresponds to approximate total cell concentrations of between 2.55 $x10^{6}$ and 6.80 $x10^{6}$ cells per mL in each assay tank.

2.3.4 Further evaluation of selected isolates

Follow-up assays were performed on 124 isolates selected as potentially molluscicidal in initial screening (Section 2.2.4). A further 4 isolates were generated from a separation of isolates determined to be mixed cultures. Additionally, 3 Bt reference strains and 3 *B. velezensis* reference strains were tested (Section 2.2.5). Screening focused on the comparison of 24 h and 120 h old bacterial cells in a direct antagonistic challenge, as well molluscicidal activity of 120 h cell-free supernatant.

In order to determine if isolates identified were pathogenic to snails, snails were exposed to culture of either 24 h or 120 h old. In the control tanks, totalling 87 tanks with a population of 261 snails, only 4 deaths occurred, for a baseline mortality rate of 1.53% over the 3 weeks of observation.

2.3.4.1 Direct bacterial application of 24-hour bacterial culture

For the 24 h bacterial assay, deaths were noted for 15 isolates. One snail death occurred (16.7% mortality) for 13 tested isolates and for 2 isolates, 3 deaths (50% mortality) were recorded. For the reference strains tested, only 1 death was recorded associated with isolate R16. The remaining 119 isolates showed no evidence of snail mortality. Average cells applied (n=126), measured by Petroff-Hausser counting chamber was determined to be $1.37 \times 10^9 \pm 1.61 \times 10^8$ (95% CI) for a total concentration of 3.43×10^6 cells per mL per tank. Clumping of bacteria was an issue when conducting cell counts, hence the lower number of counted samples than samples tested.

2.3.4.2 Direct bacterial application of 120-hour bacterial culture

For the 120 h bacterial assay, deaths were noted for 19 isolates. One snail death occurred (16.7% mortality) for 17 isolates and for 2 isolates, 2 deaths were observed (33.3% mortality). No deaths were noted to result from the 6 reference strains. The remaining 115 isolates caused zero deaths. Average cells applied (n=130), measured by Petroff-Hausser counting chamber, was determined to be $1.50 \times 10^9 \pm 1.68 \times 10^8$ (95% CI) for a total concentration of 3.75×10^6 cells per mL per tank.

2.3.4.3 Cell free culture supernatant assay

A cell-free supernatant from a 120 h culture broth was assayed against snails at 5 and 10% concentrations. Of the supernatants tested, from 134 isolates, a total of 41 caused greater than 80% mortality (5 or greater deaths out of 6 snails) at the 10% concentration. Of these 41 isolates, 15 (including 3 *B. velezensis* reference strains R16, PHP 1601, FZB42) caused greater than 80% mortality rate at the 5% supernatant concentration. The 12 isolates with the greatest activity at 5%, are shown in Table 2.2. The 3 Bt reference strains caused greater than 80%

mortality at the 10% concentration, but no deaths were caused at the 5% concentration. The baseline mortality of sterile TSB at 20% was 1 dead snail out of 60 (1.66%), and at 10% TSB no deaths were recorded out of 60 snails. For snails in untreated tank water out of 132 snails, no deaths were observed.

2.3.4.4 Comparison of assay results

A comparison of the 24 h and 120 h bacterial assay results show an overlap of snail mortality in both time intervals for only 4 isolates (i.e. Isolates AX56, BB1-156a, BA1-53 and BC8-151) with a single death recorded each for the 24 h and 120 h cultures (Table 2.2). A total of 8 isolates caused more than 1 death in either culture time intervals (i.e. ≥2/12), namely Isolates H8, C1, AX56, BA1-10, BA1-53, BB1-1, BB1-156a and BC8-151. For these 8 isolates, only two overlap with the 15 active supernatant isolates that showed greater than 80% mortality rate at the 5% supernatant concentration (Isolates AX56 and BB1-156a). Of the remaining 6 isolates, 5 isolates showed low to no activity in the 10% cell-free supernatant, while a single isolate caused greater than 80% mortality at the 10% concentration, but none at the 5% supernatant concentration (Isolate BC8-151).

Table 2.2. Comparison of results from selected isolates assayed against *P. acuta* selected for further screening

Activity	Isolate	Bacterial Culture Age[€]		TSB Supernatant Concentration[#]		Location
		24 h	120 h	5%	10%	
Supernatant	O8b	0	0	100	100	KZN, Pietermaritzburg, Botanical Garden
	R9	0	0	83.3	100	KZN, Pietermaritzburg, Msunduzi River
	S3	0	0	100	100	KZN, Pietermaritzburg, Patricia Road
	U7	0	0	83.3	100	KZN, Pietermaritzburg, Hatcheries Park, Chase Valley
	AS2	0	0	100	100	KZN, Nottingham Road, Strathdean Farm dam
	AS54	0	0	83.3	100	KZN, Nottingham Road, Strathdean Farm dam
	AS61	0	0	100	100	KZN, Nottingham Road, Strathdean Farm dam
	AS62	0	0	100	100	KZN, Nottingham Road, Strathdean Farm dam
	AY11	0	0	83.3	100	KZN, Nottingham Road, Strathdean Farm dam
	BB1-156b	0	0	100	100	KZN, Zenzani, Hopewell Farm
Both	AX56	16.7	16.7	100	100	KZN, Nottingham Road, Strathdean Farm dam
	BB1-156a	16.7	16.7	100	83.3	KZN, Zenzani, Hopewell Farm
Potential direct molluscicidal	C1	50	0	0	0	KZN, Pietermaritzburg, UKZN Disease Garden
	H8	0	33.3	0	0	KZN, Pietermaritzburg, Botanical Garden
	BA1-10	0	33.3	0	50	KZN, Zenzani, Hopewell Farm
	BA1-53	16.7	16.7	16.7	0	KZN, Zenzani, Hopewell Farm
	BB1-1	50	0	0	33.3	KZN, Zenzani, Hopewell Farm
	BC8-151	16.7	16.7	16.7	83.3	KZN, Zenzani, Hopewell Farm

[€] Percentage mortality of snails exposed to bacterial culture of 24 and 120 hours respectively
[#]Percentage mortality of snails exposed to cell-free culture supernatant at 5% (v/v) or 10% (v/v)
KZN = KwaZulu-Natal Province of South Africa

2.4 Discussion

The initial aim of this study was to screen for *Bacillaceae* antagonistic towards the aquatic snail species responsible for the transmission of *Fasciola* in South Africa. Populations of *P. columella* and *R. natalensis* were sourced, and identified via COX-1 sequencing, but were not amenable to rearing on a scale suitable for screening purposes. Instead, a proxy aquatic snail species was selected for the initial screening. This proxy species was identified as *P. acuta* via COX-1 sequencing and is an invasive species that is now found world-wide, but originated from North America (Vinarski, 2017). Although this species is not responsible for *Fasciola* transmission, it provided an easy to rear proxy species, which made the initial screening possible. While screening directly on the target snail's species responsible for *Fasciola* spread

would have been the ideal, the inability to lab rear these populations and a lack of access to local wild caught populations of sufficient availability to allow screening made the use of a proxy species necessary to continue this study. *Physella acuta* with its rapid growth rates, fecundity and ease of rearing in large populations made it an ideal candidate for screening purposes. *Physella acuta* (Family *Physidae*) is taxonomically related to the *Fasciola* fluke snail host species within the family *Lymnaeidae*, both falling within the order *Lymnaeoidea* (Saadi *et al.*, 2020). In addition, these snails occupy similar ecological niches and exhibit similar breeding patterns and feeding behaviour.

The isolation procedures aimed to isolate endospore-forming aerobic bacteria from aquatic-associated soil sediments and to screen them for any molluscicidal activity. *Bacillus thuringiensis* was targeted via the screening methodology. However, non-Bt isolates were also selected. To this end, a total of 1180 isolates were screened. While 10.5% of isolates caused snail mortality in the single replicate high-throughput trials, the majority of these isolates showed no activity when re-tested under identical conditions. This suggests the initial positive candidates recorded were either false-positives or represent a weak level of molluscicidal activity. The absence of any mortality in the post assay quarantine tank would also suggest that no truly pathogenic isolate was present either in the isolates tested or in the soil samples.

The baseline mortality rate for control snails was found to be 0.54% during the course of the initial and follow-up screening. Lower baseline mortality allows for a higher confidence in any positive results and suggests that the overall structure of the screening process was not artificially inflated by baseline mortality. This initial screening was not focused on measuring high levels of molluscicidal activity but instead to cast a wide net to identify isolates meriting inclusion in follow-up screening. A comparison between the different endospore isolation methods used showed similar percentage positive hits of approximately 10% of the isolate pools tested.

Further evaluation of isolates identified during initial screening showed that for the majority of isolates, no snail deaths were recorded in the direct bacterial antagonism assays using either 24 h or 120 h bacterial cultures. Where some snail deaths were recorded, there was little overlap between exposures to either culture age, nor high percentages of mortality. This experiment did not identify any strongly molluscicidal isolates. However, some isolates showed levels of

potential molluscicidal activity. The highest recorded mortality rates were 50% for Isolates C-1 and BB1-1, both for their 24 h culture age. This assay was repeated with these isolates with the same methodology with 7 replicates each. However, Isolate C-1 caused no deaths, and Isolate BB1-1 only caused 1 death out of 21 snails (data not shown). This assay had a similar bacterial dosing rate (2.5-6.8 $x10^6$ cells per mL) in the assay tanks as that used for the initial bioassay, the only difference being that the culture introduced was washed of any excreted metabolites, and that the initial exposure time was decreased to allow for tank water changes.

The supernatant assay was the first method to give a strong, demonstrable molluscicidal effect, with 30.6 % of isolates tested causing a strong response with the 10% spent broth concentration. This was a stark difference to the control tanks, as well as the assays with other isolates, which did not show any molluscicidal activity, i.e., zero mortality. The sterile TSB medium controls suggest that there are no constitutive components in TSB with apparent molluscicidal activity at the concentrations tested or higher. While fresh TSB has a different medium composition to depleted TSB, the spent media of 93 isolates (equating to 69.4% of those tested) did not cause a strong response at a 10% concentration. This suggests that spent media itself is also not molluscicidal. Twelve isolates showed strong activity in their 5% spent broth concentration, along with three of the reference strains.

The observation of putative molluscicidal activity resulting from bacterial culture medium supernatants raises the question of whether the presence of metabolites could be the cause of deaths noted in the original screening assays. The active fraction concentrations present in the supernatant assays are probably higher than the small amount of carryover from culture homogenate in the initial screening assays, although this does not confirm metabolites as a cause of mortality. Activity by supernatants suggests a mode of action linked to excreted metabolites, rather than any direct bacterial pathogenicity against snails. This is perhaps unsurprising considering that the genus *Bacillus* is known to produce biologically active metabolites, which have been widely studied for biotechnological applications (Tran *et al.*, 2022).

The limited number of positive isolates detected in the initial screening highlights the innate challenges of testing with biological entities and the need for repeated assays to obtain reliable results. This process becomes complicated when high-throughput screening is embarked upon,

where resources and time are demanding aspects of these studies. This isolation and screening procedure deliberately targeted a broad range of endospore-forming species in the hopes of identifying either molluscicidal isolates or indication of which species may be useful for future screening assays. The approach taken for this project was one of a broad screening using low snail numbers per isolate based on the premise that this would increase the likelihood of identifying a molluscicidal isolate, as opposed to more in-depth testing with a necessarily decreased isolate pool. Ultimately the decision was made to focus on high throughput assays with the primary limitation being the number of snails available for screening, despite utilising a fast-reproducing proxy species. While the small scale of the assays would have reduced the chance of detecting weakly molluscicidal isolates, the premise was that by increasing the throughput, we would increase the chances of discovering a potent anti-molluscicidal bacterial isolate.

Given the utility of various *Bacillaceae* in a number of biocontrol strategies, this family of bacteria seems worth screening for molluscicidal activity. Without strong molluscicidal isolates being detected, and without similar broad screening investigations being reported in literature, it is difficult to determine how successful this approach was or whether a different screening methodology is required. A review of the literature does not reveal similar research using broad screening approaches against snails nor general targeting of *Bacillaceae*. Instead, previous work has focused on selected Bt isolates or has revolved around detection of diseased snails in lab snail populations (Halima *et al.,* 2006; Osman *et al.,* 2011; Duval, *et al.,* 2015; Abd El-Ghany and Abd El-Ghany, 2017: Gaber *et al.,* 2022). Whether this absence of strong findings is due to the rarity of molluscicidal isolates or whether the screening methodology required amendments is unclear. Nevertheless, this work has selected 8 isolates which may have weak molluscicidal activity and warrant further evaluation of any molluscicidal potential. In addition, the broad detection of molluscicidal activity in spent culture supernatant opens an avenue of study not originally envisaged, and has brought forward 12 isolates for characterisation of the metabolites responsible for observed molluscicidal activity. While the intention was to find an isolate which directly affects snail populations the detection of excreted molluscicidal compound/s offers an avenue worth further exploration.

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CHAPTER THREE: IDENTIFICATION AND DEDUPLICATION OF ENDOSPORE-FORMING BACTERIAL ISOLATES EXHIBITING MOLLUSCICIDAL ACTIVITY

Abstract

Initial screening for molluscicidal endospore-forming bacteria resulted in a subset of 18 isolates being selected that demonstrated either potential molluscicidal activity and/or were able to produce secondary metabolites with molluscicidal activity. Two isolates fitted into both categories. Isolates underwent identification and characterisation using DNA-based methods for the purposes of species identification and deduplication on the basis of sequencing data and end point PCRs. The first step of molecular identification was to use 16S rDNA gene fragment sequencing; subsequently, additional discrimination of selected isolates was performed using *rpoB* and *dnaJ* gene fragment sequencing, and selected end-point PCR approaches. Isolates were all confirmed to be members of the *Bacillaceae*. Isolates selected for their production of molluscicidal supernatants were found to be closely related and identified as strains of *Bacillus velezensis* (Ruiz-García *et al.*, 2005), *Bacillus amyloliquefaciens* (Priest *et al.*, 1987) and *Bacillus subtilis* (Ehrenberg, 1835). Isolates with potential molluscicidal activity were found to be more diverse, with representatives of *B. velezensis*, *Bacillus cereus* (Frankland and Frankland, 1887), *Bacillus mycoides* (Flügge, 1886), *Paenibacillus* sp., *Priestia* sp., and *Gottfriedia* sp. being identified. Isolates were deduplicated by removal of replicates with identical results for the various gene sequence data and end point PCRs. From the original 12 isolates producing molluscicidal supernatant, 6 were retained and all 8 isolates with potential molluscicidal activity were carried forward for further investigation.

3.1 Introduction

Members of the *Bacillaceae* are potent biological control agents, with the advantage that endospore formation results in a robust propagule, which is ideal for the storage of a commercial biocontrol product. The present study targeted and screened endospore-forming bacteria for molluscicidal properties (Chapter 2). The *Bacillaceae* were targeted, not only for the benefits an endospore-former has for a biopesticide formulation, but also due to the literature describing molluscicidal activity of a number of aerobic endospore-forming bacteria,

including *Paenibacillus sp*. (Duval *et al*., 2015) and *Bacillus thuringiensis* (Bt) (Berliner, 1915) (Abd El-Ghany and Abd El-Ghany, 2017, Osman *et al*., 2011, Ali *et al*., 2010, and Salem *et al*., 2006).

In the present study, a number of isolates were selected on the basis of either molluscicidal spent-culture supernatant (12 isolates) or potential molluscicidal activity (8 isolates) (Chapter 2). Due to the high-throughput nature of the initial screening, no tests were performed initially to confirm whether these isolates were from the *Bacillaceae*. As such, confirmation of isolate identity was necessary. In addition, species-level identification also affords the opportunity to infer possible modes of action based on capabilities of the respective species. *Bacillus* species have a number of groups with well-defined capabilities, such as *B. thuringiensis,* which produce insecticidal CRY endotoxins and VIP vegetative insecticidal proteins (Melo *et al.,* 2016). A *Paenibacillus* species has also been described as being pathogenic to the aquatic snail intermediate hosts of schistosomiasis, *Biomphalaria glabrata* (Say, 1818) (Duval *et al.*, 2015). Additionally, there are a number of species, for example, *B subtilis* and *B. velezensis,* that are producers of potent lipopeptides with antifungal and antimicrobial activity (Dame *et al.*, 2021).

The research presented in this chapter aimed to identify the bacteria of interest, selected for their molluscicidal potential. To this end, gene fragment sequencing (16S rDNA, *rpoB* and *dnaJ*) was used for isolate identification, and relatedness between isolates was determined using a number of end-point PCRs. 16S rDNA sequencing is still considered the benchmark housekeeping gene sequence for bacterial identification, however in the case of closely related *Bacillus* spp. this approach does not offer sufficient discrimination (Fritze, 2004). As such, the sequences of the housekeeping genes *rpoB* and *dnaJ* have been used to better discriminate amongst these more closely related species (Rooney *et al.*, 2009; Liu *et al.*, 2022)

Another approach utilised for discrimination of isolates is end point PCR where the presence of a PCR fragment or its absence can be diagnostic of similarity between isolates without the expense of sequencing. As *B. velezensis* reference strains (R16, FZB42 and 1601) had produced similarly molluscicidal supernatants as the best performing isolates in Chapter 2, an end point PCR specific to *B. velezensis* was chosen in an attempt to establish whether some of these isolates could be identified as such (Dunlap, 2019). In addition, lipopeptides have been identified as playing a number of roles in *Bacillus* spp. biocontrol efficacy; end point PCR was

performed targeting genes responsible for the synthesis of a number of lipopeptide compounds common to *B. subtilis* and other closely related species. A representative of each of four lipopeptide variants were selected representing surfactin, fengycin, iturin and bacillomycin classes (Jacques, 2011). The comparison between isolates also offered the opportunity for deduplication of any closely related isolates amongst those initially selected, and then to focus further research on representative candidates.

3.2 Material and Methods

3.2.1 Glycerol stocks

Fresh glycerol stocks were made for long term storage of isolates selected in Chapter 2. Isolates were revived from glycerol stocks (Section 2.2.4) by three-way-streaking onto tryptic soy agar (TSA) (Neogen Corporation, Lansing, Michigan, United States) and incubated inverted for 24 h at 30 °C. A single colony was picked off, inoculated into 20 mL tryptic soy broth (TSB) (Neogen Corporation, Lansing, Michigan, United States) in 125 mL Erlenmeyer flasks and incubated overnight at 30 °C at 120 rpm in a rotary shaking incubator (MRC, Holon, Israel). After incubation, 500 µL of sterile 40% (v/v) glycerol in distilled water was decanted into sterile 2.0 mL cryovials and 500 µL of broth culture was added, for a final glycerol concentration of 20% (v/v). The cryovials were shaken for 30 minutes before freezing at -80 °C for long term storage.

3.2.2 DNA extraction

Bacterial DNA was extracted from pure cultures that were inoculated into 10 mL Luria-Bertani (LB) broth in 125 mL Erlenmeyer flasks and incubated at 30 °C for 16 h at 120 rpm in a rotary incubator. Aliquots of 500 µL of broth culture was removed aseptically to DNAse-free 1.5 mL microcentrifuge tubes and centrifuged at 15,000 *x g* for 5 minutes in a benchtop centrifuge (Labnet Spectrafuge 16M, USA) and the supernatant discarded. The cell pellet was washed by resuspending in 200 µL nuclease-free water, centrifuged at 15,000 *x g* for 5 minutes and the supernatant discarded. Pelleted cells were suspended in 200 µL TE buffer (Macherey-Nagel™) and frozen at -80 °C for 30 minutes, followed by 10 minutes at 95 °C in a dry block (Operon OPR-HB-100, Korea). This heating/cooling cycle was repeated three times in total. After the

final heating step, samples were allowed to return to room temperature before centrifugation at 15,000 *x g* for 5 minutes, after which 100 µL of supernatant was transferred into a sterile DNAse-free 1.5 mL microcentrifuge tube. Absorbance, at 260 nm and 280 nm, of these samples were measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, Massachusetts, U. S. A.). Dilutions were calculated to give a final absorbance of 1.0 at 260 nm and diluted accordingly. Both diluted and undiluted samples were stored at -20 °C. All PCR reactions were performed using diluted template DNA.

3.2.3 PCR and gel visualisation

PCR reactions were performed using Dreamtaq Green PCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Reactions were performed in 30 µL reaction volumes with a final primer concentration of 0.3 µM per primer, with 1 µL of standardised template DNA. Primers were sourced from Inqaba Biotech (Muckleneuk, Pretoria, Gauteng, South Africa, 0002). The PCR reactions were run in a G-Storm GS1 Thermal Cycler (G-Storm, Somerset, U.K.) with the following cycle parameters: Initial DNA denaturation at 95 °C for 3 minutes; variable number of cycles of denaturation at 95 °C for 30 seconds, annealing at primer determined temperature for 30 seconds, extension at 72 °C for 60 seconds; and a final extension of 72 °C for 5 minutes before cooling to 4 °C for storage. Details of the primers used, the optimised number of cycles and the annealing temperatures are found in Table 3.1.

PCR products were visualised on agarose gel (1% w/v) made up with sodium boric acid (SB) buffer (Brody and Kern, 2004), stained using SYBR Safe (Thermo Fisher Scientific, Waltham, Massachusetts, United States) at 0.5x concentration (0.5 µL per 10 mL gel volume). Amplicon size was determined using a GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Gels were run for 80 minutes at 80 V before visualizing and image capture using a SynGene G:Box and Syngene GeneSnap software (version 7.09) (Syngene, Cambridge, England).

Table 3.1. Primers and respective PCR reaction conditions

Gene Target	Primer	Sequence 5'-3'	Annealing Temperature	No. Cycles
16S rDNA	BacF (Heuer *et al*., 1997) R1378 (Garbeva *et al*., 2003)	GGG AAA CCG GGG CTA ATA CCG GAT CGG TGT GTA CAA GGC CCG GGA ACG	68 °C	30
16S rDNA (Alternative)	fD1 (Weisburg *et al.,* 1997) rP2 (Weisburg *et al.,* 1997)	AGA GTT TGA TCC TGG CTC AG ACG GCT ACC TTG TTA CGA CTT	55 °C	35
Bacillus velezensis specific primer	Bvel-F (Dunlap, 2019) Bvel-F (Dunlap, 2019)	CCT TTG CGT TTT GTT ACC C CAC ATC AAT TCC TTC TCC	60 °C	30
DNA-J	DNAJ-F (Connor *et al*., 2010) DNAJ-R (Connor *et al*., 2010)	GGG GTA GGT AAG AGC GCT TC CGG CAT TTC GCA GTA AAT ATC	58 °C	35
RPO-B	RPOB-F (Roberts *et al*., 1994) RPOB-R (Roberts *et al*., 1994)	AGG TCA ACT AGT TCA GTA TGG ACG GTC CTA CAT TGG CAA GAT CGT ATC	48 °C	35
SUR3	SUR3F (Ramarathnam, 2007) SUR3R (Ramarathnam, 2007)	ACA GTA TGG AGG CAT GGT C TTC CGC CAC TTT TTC AGT TT	63 °C	35
ITUD1	ITUD1F (Ramarathnam, 2007) ITUD1R (Ramarathnam, 2007)	GAT GCG ATC TCC TTG GAT GT ATC GTC ATG TGC TGC TTG AG	63 °C	35
BACC1	BACC1F (Ramarathnam, 2007) BACC1R (Ramarathnam, 2007)	GAA GGA CAC GGC AGA GAG TC CGC TGA TGA CTG TTC ATG CT	59 °C	35
FENG	FENG-F (Hunter, 2016) FENG-R (Hunter, 2016)	GGA ACG TCA CGG AAA TGA AG CAG CTG AAA CGG TAC GCT GT	67 °C	35

3.2.3.1 16S rDNA PCR and partial gene sequencing

All isolates underwent 16S rDNA gene amplification and gene fragment sequencing because this gene region is an important benchmark for prokaryote genetic identification. All but one isolate was successfully amplified using the BacF Heuer *et al.* (1997) and R1378 Garbeva *et al.* (2003) primer sets. The remaining isolate was amplified with other 16S primers, fD1 and rP2 (Weisberg et al 1997). The 16S PCR amplicons were sent to Inqaba Biotech for sequencing with both forward and reverse primers. Sequences were base-called using Chromas Lite (Version 2.01). Forward and reverse sequences were aligned in Bioedit version 7.2.5 (Hall,

1999) and a consensus sequence created for each isolate. Sequences were then processed using NCBI Blast for identification purposes (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Zhang *et al.*, 2000). Phylogenetic trees were generated using Mega 11.0.13 using the Maximum Likelihood Method and the Tamura-Nei Model (Tamura and Nei, 1993, Tamura et al., 2021).

3.2.3.2 Bacillus velezensis end point PCR

A number of isolates showed sequence similarity to *B. velezensis* after 16S rDNA partial gene sequencing (Section 3.2.3.1). A set of end-point primers specific to *B. velezensis* (Dunlap, 2019) were used to confirm this identification and this was applied to all isolates.

3.2.3.3 dnaJ and rpoB PCR and partial gene sequencing

Due to the high level of similarity of isolates responsible for molluscicidal supernatants from 16S rDNA partial gene sequencing (Section 3.2.3.1), and the results of the *Bacillus velezensis* specific primers, further gene fragments were generated and sequenced to aid identification and discrimination between isolates. Two gene targets were selected for amplification and sequencing because these have been applied to distinguish between closely related *Bacillus* spp.: *dnaJ* (Connor *et al.,* 2010) and *rpoB* (Roberts *et al.,* 1994). The PCR reactions were sent to Inqaba Biotech for sequencing with the forward primer set. Sequences were base-called using Chromas Lite (Version 2.01) and then processed using NCBI BLAST for identification purposes (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Zhang *et al.*, 2000).

3.2.3.4 Lipopeptide end point PCR

The high degree of sequence similarity (16S, *dnaJ*, *rpoB*) between isolates responsible for molluscicidal supernatant, along with the literature pointing to the *B. subtilis* group of species being potent producers of lipopeptides, indicated that lipopeptides may have been a candidate for the molluscicidal activity observed. In light of this, several lipopeptide markers were selected to give an indication of the presence of common lipopeptide gene markers. While not an exhaustive analysis of lipopeptide gene presence, this served as a rapid, economical method of comparing between isolates on the basis of qualitative differences in a simple end-point PCR.

Twelve molluscicidal supernatant candidate isolates, along with the three reference strains (*viz*. R16, PHP1601, FZB42) underwent end-point PCR with the SUR3, FENG, ITUD1, and BACC1 primer sets (Ramarathnam, 2007; Hunter, 2016). These primers selected for a single example of the lipopeptide classes surfactin, fengycin, iturin and bacillomycin respectively. As an additional step, representatives underwent sequencing for positive lipopeptide genes to determine whether amplification was attributable to the gene of interest.

3.3 Results

3.3.1 16S rDNA partial gene sequencing

The products of 16S rDNA PCR were visualised using agarose gel electrophoresis, which yielded a single band of approximately 1400 bp. One isolate (BA1-53) did not amplify with the primary set of primers (Bacf, R1378). However, it did amplify with the alternate set (fD1, rP3) and produced a single band of approximately 1400 bp. PCR product was sequenced using the forward and reverse primers, base-calling used Chromas Lite, followed by sequence alignment and consensus sequence generation using Bioedit 7.2.5. A partial 16S rDNA gene sequence of 1150 bp was generated for each isolate (Gel image in Appendix C). Sequence data was submitted to the NCBI BLAST database and the closest matched database entries were collated, as shown in Table 3.2.

The 16S rDNA partial gene sequence data shows a high degree of similarity between the molluscicidal supernatant isolates. For 9 of the 12 isolates (S3, AS2, AS54, AS61, AS62, AX56, AY11, BB1-156a, BB1-156b) 100% sequence homology was observed. A further two isolates (R9, U7) differ from this first group by only 1 bp. The final molluscicidal supernatant isolate, O8b, differed from the first group by only 4 bp. This high degree of similarity is reflected in the phylogenetic tree in Figure 3.1.

For the isolates selected for potential molluscicidal activity, the identifications fall in a more diverse range of *Bacillus* species than those of the molluscicidal supernatant isolates. The 8 isolates in this group can be divided into 5 distinct groups: 3 *B. cereus* group isolates, 2 *B. velezensis* isolates and one representative each of *Paenibacillus*, *Priestia*, and *Gottfriedia*. This diversity is evident in the distinct groupings amongst these isolates observed in the

phylogenetic tree in Figure 3.1. Within this group, two isolates (BA1-10, BB1-1) show identical sequences, along with the two overlapping isolates with the 12 (AX56, BB1-156a) already mentioned. Isolate H8 is similar to isolates BA1-1 and BB1-1 with a 5 bp difference. However, Isolate H8 has a distinct colony morphology that is consistent with a *Bacillus mycoides* (Schleifer, 2009), which is lacking from the BA1-1 and BB1-1.

Table 3.2. Identities of bacterial isolates as determined by 16S rDNA partial gene sequences (1156 bp) submitted to the NCBI BLAST database (December 2022) using the MegaBLAST algorithm

Isolate	**Species Identity**	**Query Cover**	**Identity**
R9	*Bacillus velezensis*	100%	100%
S3 AS2 AS54 AS61 AS62 AX56 AY11 BB1-156a BB1-156b	*Bacillus velezensis*	100%	100%
O8b	*Bacillus subtilis*	100%	100%
U7	*Bacillus amyloliquefaciens*	100%	100%
H8	*Bacillus mycoides*	100%	99.48%
BA1-10 BB1-1	*Bacillus cereus*	100%	100%
C1	*Bacillus acidiceler* (Syn. *Gottfriedia acidiceleris*)	100%	99.74%
BA1-53	*Paenibacillus polysaccharolyticus*	100%	99.84%
BC8-151	*Bacillus megaterium* (Syn. *Priestia megaterium*)	100%	100%

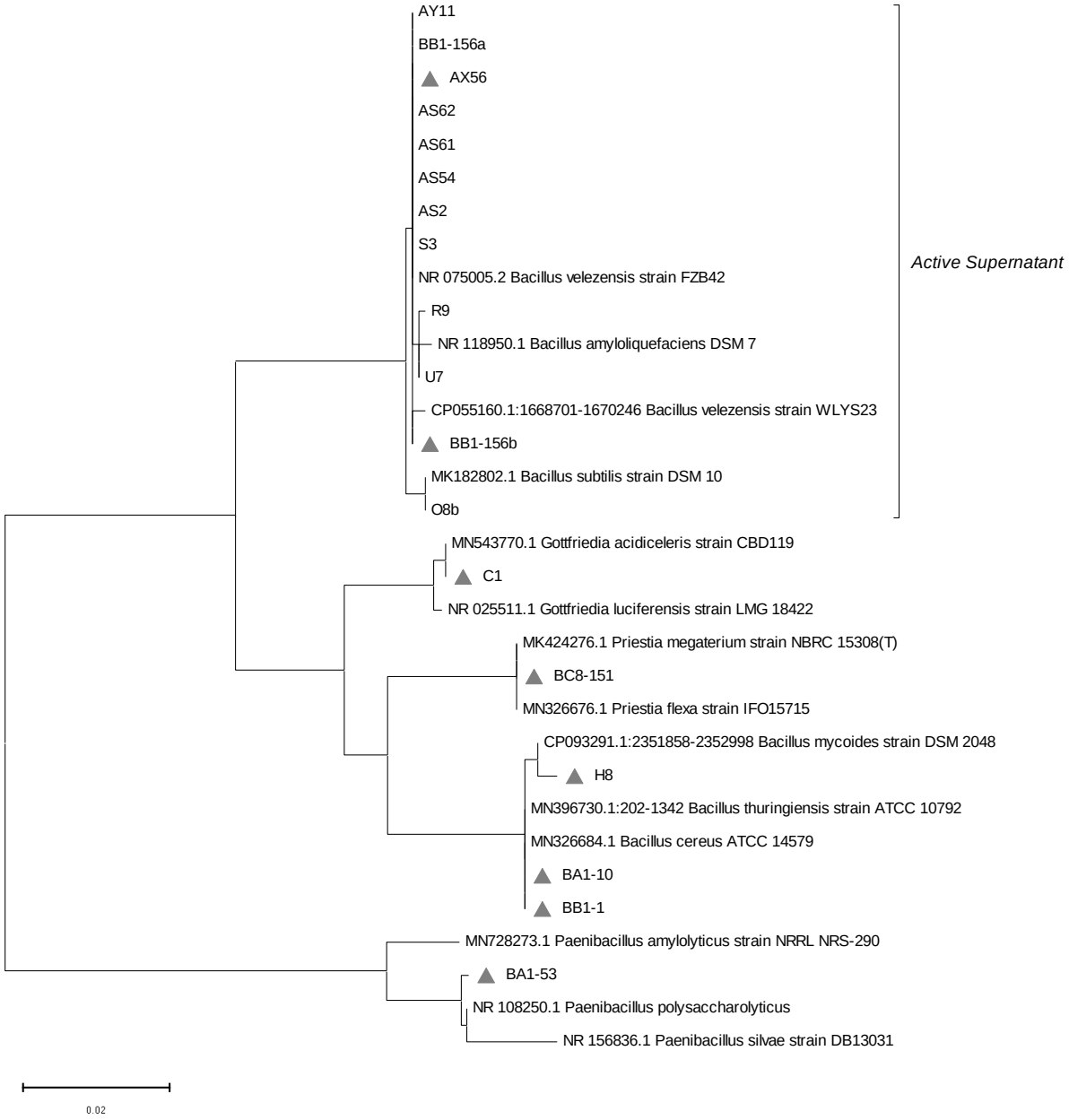

Figure 3.1. Phylogenetic tree of bacterial isolates based on 16S rDNA partial gene sequence data generated using MEGA11 software (Tamura *et al*., 2021)

The tree was inferred using the Maximum Likelihood Method and the Tamura-Nei Model (Tamura and Nei, 1993). The tree shown represents the topology with the highest log likelihood value from initial trees obtained by applying Neighbour-Joining and BioNJ algorithms to a matrix of pairwise distances, estimated using the Tamura-Nei Model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 32 nucleotide sequences. There were a total of 1156 positions in the final dataset. Isolates marked with a triangle (▲) indicate those showing potential molluscicidal activity.

3.3.2 *Bacillus velezensis* end point PCR

All isolates producing molluscicidal supernatant, along with 3 *B. velezensis* reference strains (R16, FZB42 and PHP1601) underwent a *B. velezensis* specific end-point PCR (Dunlap, 2019). The PCR products were visualised using agarose gel electrophoresis, which yielded a single band of approximately 180 bp (Gel image in Appendix D). As expected, all 3 *B. velezensis* reference strains produced a band of the expected size. For the 12 isolates with molluscicidal supernatant, 10 produced a band of the correct size (R9, S3, AS2, AS54, AS61, AS62, AX56, AY11, BB1-156a, BB1-156b), while Isolates U7 and O8b produced no amplicon. Of the 8 isolates selected on the basis of potential molluscicidal activity (H8, C1, BA1-10, BA1-53, BB1-1, BC8-151) 6 did not produce the *B. velezensis* specific amplicon. However, the two overlapping isolates, AX56 and BB1-156a, did so. Amongst the potentially molluscicidal isolates, this primer set did not show any amplification, which was an expected result on the basis of the 16S identities.

3.3.3 *dnaJ* and *rpoB* partial gene sequencing

Further discrimination between molluscicidal supernatant isolates was attempted by partial gene sequencing of the *rpoB* and *dnaJ* genes. The products of both *rpoB* and *dnaJ* PCR were visualised using agarose gel electrophoresis, which yielded a single band of approximately 900 bp for both products (Gel images in Appendix E and F respectively). NCBI BLAST hits for both the *rpoB* and *dnaJ* were consistent for all isolates, with the combined BLAST data shown in Table 3.3. Percentage identities and query coverage was 99% or greater for all sequences analysed. For both *rpoB* and *dnaJ*, significant sequence similarity between isolates was observed. For Isolates AS2, AS54, AS61, AS62, AX56, AY11 sequences were identical to each other for both *rpoB* and *dnaJ* and also identical to the reference strain PHP 1601. Isolates BB1-156a and BB1-156b also showed identical sequences for both of the *rpoB* and *dnaJ* partial gene sequences. A Maximum Likelihood phylogenetic tree utilizing concatenated sequences of *rpoB* and *dnaJ* is shown in Figure 3.2. BLAST matches for both partial gene sequences support the identification based on data generated for the 16S rDNA partial gene sequences (Section 3.3.1), as well as for the *B. velezensis* specific primer results (Section 3.3.2).

Table 3.3. Identities of bacterial isolates as determined by *rpoB* (720bp) and *dnaJ* (621 bp) partial gene sequences submitted to the NCBI BLAST database (December 2022) using the MegaBLAST algorithm

Isolate	Species Similarity
R9	*Bacillus velezensis*
S3	*Bacillus velezensis*
O8b	*Bacillus subtilis*
U7	*Bacillus amyloliquefaciens*
AS2 AS54 AS61 AS62 AX56 AY11	*Bacillus velezensis*
BB1-156a BB1-156b	*Bacillus velezensis*

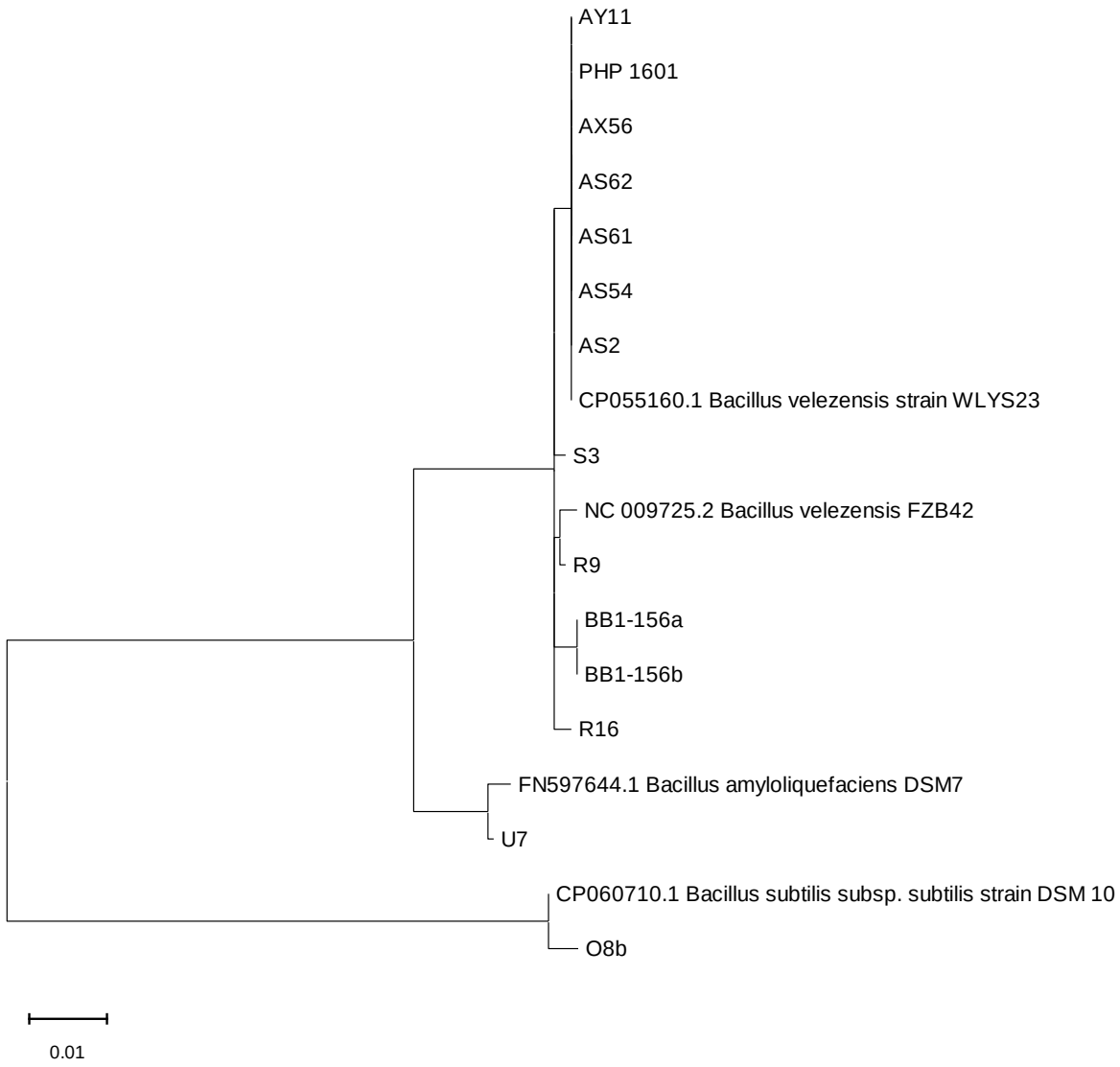

Figure 3.2. Phylogenetic tree of bacterial isolates based on concatenated *rpoB* and *dnaJ* partial gene sequence data generated using MEGA11 software (Tamura *et al.*, 2021)

The tree was inferred using the Maximum Likelihood Method and the Tamura-Nei Model (Tamura and Nei, 1993). The tree shown represents the topology with the highest log likelihood value from initial trees obtained by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances, estimated using the Tamura-Nei Model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 18 nucleotide sequences. There were a total of 1341 positions in the final dataset.

3.3.4 Lipopeptide gene endpoint PCR

Several lipopeptide markers were selected to give an indication of the presence of common lipopeptide genes amongst molluscicidal supernatant-producing isolates. While not an exhaustive analysis of lipopeptide gene presence, this serves as a rapid and cost-effective method of comparing between isolates on the basis of qualitative differences in a simple end-point PCR. The PCR products were visualised using agarose gel electrophoresis, which yielded single bands of the following sizes: FENG approximately 550 bp; ITUD1 approximately 650 bp; SUR3 approximately 450 bp; and BACC1 approximately 900 bp. The comparison of lipopeptide end-point PCRs is shown in Table 3.4 and a comparative gel image in Appendix G. Only two isolates stand out as unique from this data set: Isolate O8b with no positive reactions; and Isolate U7 with only positive reactions for SUR3 and ITUD1. The remaining isolates tested fall into only two groups, those positive for all four gene markers (Isolates R9, S3, and refence strains R16 and FZB42) and a second group positive for SUR3, ITUD1 and FENG but negative for BACC1 (AS2, AS54, AS61, AS62, AX56, AY11, BB1-156a, BB1-156b and the reference strain PHP1601). Partial gene fragments submitted for sequencing confirmed the PCR products to reflect the respective lipopeptide marker gene regions (Data not shown).

Table 3.4. End-point PCR results for lipopeptide marker genes (SUR3, FENG, ITUD1, and BACC1) for isolates and reference strains with molluscicidal broth supernatant

Isolate	PCR Primer			
	SUR3	**ITUD1**	**BACC1**	**FENG**
R9	+	+	+	+
S3	+	+	+	+
O8b	-	-	-	-
U7	+ weak	+	-	-
AS2	+	+	-	+
AS54	+	+	-	+
AS61	+	+	-	+
AS62	+	+	-	+
AX56	+	+	-	+
AY11	+	+	-	+
BB1-156a	+	+	-	+
BB1-156b	+	+	-	+
Reference Strains				
R16	+	+	+	+
FZB42	+	+	+	+
PHP 1601	+	+	-	+

(+) indicates a positive PCR (Gene presence); (-) indicates no PCR (Gene absence)

3.4 Discussion

This chapter sought to identify isolates of interest using DNA sequencing and PCR approaches. Identification of the 12 isolates with molluscicidal supernatants showed that these are closely related isolates. These isolates belong primarily to the species *B. velezensis*, with a representative each of *B. amyloliquefaciens* (U7) and *B. subtilis* (O8b). The results for the *B. velezensis* end point primer was in agreement with the 16S rDNA partial gene sequence data for a number of closely related isolates identified via 16S sequencing. Amongst the molluscicidal supernatant isolates, some representatives were indistinguishable from each

other over three gene fragment sequences (viz. 16S, *rpoB*, *dnaJ*) and the four lipopeptide marker end-point PCRs.

The largest group of isolates (AS2, AS54, AS61, AS62, AX56, AY11) were all isolated from the same location (Strathdean Farm, PHP). In addition, these isolates are indistinguishable from the PHP1601 reference strain also originating from this location. As such, it appears that these isolates may be environmental re-isolations of the original PHP1601 strain. The other group of indistinguishable isolates (BB1-156a, BB1-156b) shows the same pattern of identical gene fragment sequences and the lipopeptide marker end-point PCRs. This pair of isolates originate from a single glycerol stock culture which was split into two isolates due to apparent differences in colony morphology. On re-examination, there are no obvious differences in colony morphology between these two isolates; the original decision to separate them may have been due to apparent differences in morphology that *Bacillus* sp. can present when cultured on solid media (Fujikawa, 1994).

The 8 isolates with potential molluscicidal activity are far more diverse with a wide range of endospore-forming species represented, namely *B. cereus*, *B. velezensis*, *Gottfriedia sp.*, *Prestia sp.* and *Paenibacillus sp.* Out of these species only the genus *Paenibacillus* has had any reports of molluscicidal activity (Duval, 2019). Similarities between the 8 potentially molluscicidal isolates was limited with only isolates BB1-1 and BA1-10 having identical sequences. However, only 16S partial gene sequencing was performed for these isolates and 16S gene homology is particularly highly conserved in *Bacillus* spp. Even though both of these isolates were isolated from the same location (Hopewell), this is inadequate to determine if these are separate isolations of the same strain. *Bacillus* is known for high degrees of 16S sequence homology, so species level identification is difficult using this technique (Mandić-Mulec *et al.*, 2015).

In order to enable a more detailed examination of isolates in the chapters to come, the decision was made to deduplicate these isolates and only bring forward unique isolates for further testing. Out of the AS2, AS54, AS61, AS62, AX56, AY11 group, isolate AX56 was chosen to represent these apparently very closely related isolates. Selection was determined due to its overlap with the potentially molluscicidal isolates also characterised. For the same reason isolate BB1-156a was also selected for further characterisation. This brings the totals of isolates

for metabolite screening to six: R9, S3, O8b, U7, AX56, and BB1-156a. These isolates offer the greatest diversity within the original set of 12 and should maximise a diversity of any compounds when examined in detail.

For the 8 isolates with potential molluscicidal activity, no deduplication was possible. While isolates BA1-10 and BB1-1 had identical 16S rDNA partial gene sequences, this was not sufficient grounds to remove an isolate from future screening, nor would such a change significantly increase the ability to analysis these isolates in terms of resources and time spent per isolate.

It is interesting to note that the isolates that produced molluscicidal metabolites were all very closely related to one another. *Bacillus velezensis* is particularly well known for production of lipopeptides, and this trait has also been reported in the other two species identified here (*B. amyloliquefaciens* and *B. subtilis*) (Jacques, 2011). This suggests that lipopeptides may have been responsible for the observed molluscicidal activity in spent culture supernatant. However, no lipopeptides have been identified as molluscicidal in the literature, with the described range of activity encompassing antibacterial, antifungal and insecticidal properties (Ongena and Jacques, 2008; Geetha *et al.,* 2012; Denoirjean *et al.,* 2021; Koim-Puchowska *et al.,* 2023). Lipopeptides are amphiphilic compounds which reduce surface tension and disrupt biological membranes (Jacques, 2011). It is possible that the pore formation ability of lipopeptides could cause a lethal effect for aquatic molluscs (Meena and Kanwar, 2015).

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CHAPTER FOUR: LIPOPEPTIDE EXTRACTION AND EVALUATION OF SPENT CULTURE SUPERNATANT ACTIVITY, AND EVALUATION OF EXTENDED EXPOSURE OF SNAILS TO MOLLUSCICIDAL CANDIDATE ENDOSPORES

Abstract

Initial screening of bacterial samples yielded two subsets of isolates: Six isolates selected for production of molluscicidal spent culture supernatant, and 8 isolates with potential pathogenic molluscicidal activity. Molluscicidal supernatant producers underwent lipopeptide extraction procedures and bioassays to determine whether this class of compounds may be responsible for observed molluscicidal activity. The 8 potential molluscicidal isolates, along with the 6 molluscicidal supernatant producers, were assayed for molluscicidal activity against *Physella acuta* (Draparnaud, 1805*)* snails in a long-term (i.e. 2 weeks feeding endospore impregnated food pellets followed by 4 weeks observation) endospore exposure assay. Molluscicidal active fractions were successfully extracted and concentrated from broth culture supernatants via a lipopeptide acid precipitation extraction protocol. This suggests that lipopeptides, or compounds extractable by acid precipitation, are responsible for the observed molluscicidal activity. Lipopeptide extracts showed molluscicidal activity at between 25 and 200 $\mu g.mL^{-1}$ concentrations. Extended endospore exposure assays yielded no significant molluscicidal results. For the 8 isolates brought forward based on potential molluscicidal activity, this result confirms the lack of direct molluscicidal activity identified in initial screening. Additionally, for isolates that produced molluscicidal supernatant, exposure of snails to these isolates' endospores did not result in observable molluscicidal activity, suggesting that only the excreted metabolites are responsible for molluscicidal activity and that this did not occur at sufficient concentrations in the snail-tank system to demonstrate an effect.

4.1 Introduction

In Chapter 2, 12 isolates produced strong molluscicidal activity when snails were exposed to cell-free spent culture supernatant. After identification and deduplication (Chapter 3), six isolates were selected for further investigation (O8b, R9, S3, U7, AX56, BB1-156a). Due to the identification of these isolates as members of *Bacillus velezensis* (Ruiz-García *et al.*, 2005),

or the closely related species, *Bacillus subtilis* (Ehrenberg, 1835) and *Bacillus amyloliquefaciens* (Priest *et al.,* 1987), a lipopeptide extraction of spent culture supernatant was performed. Production of lipopeptides is a hallmark of these species and it is reasonable to suspect that these compounds may be the active compounds responsible for observed molluscicidal activity (Fira *et al.,* 2018, Maksimov *et al.,* 2020). This research aimed to screen lipopeptide extracts against snails to determine whether these compounds could be associated with observed molluscicidal activity. Lipopeptide extractions are generally accomplished by several methods such as acid precipitation, liquid-liquid extraction and column chromatography (Maksimov *et al.,* 2020). For this work, an acid precipitation protocol was employed.

Initial screening of *Bacillaceae* isolates intended to select for antagonistic species, with the intention of identifying a pathogenic strain such as the unculturable *Paenibacillus* sp. isolated by Duval *et al.,* (2015) but with more amenable culture handling characteristics. Use of bacterial strains pathogenic to pest species is an established strategy of biocontrol, most notably with *Bacillus thuringiensis* (Bt) (Berliner, 1915) antagonistic to several insect species (Kumar *et al.,* 2021). Initial screening for this research project did not yield any strongly performing isolates, however, 8 isolates that approached a statistically significant effect were selected for more in-depth screening in order to confirm the absence of detected molluscicidal strains. These 8 isolates (C1, H8, AX56, BA1-10, BA1-53, BB1-156a, BB1-1, BC8-151) underwent extended exposure assays in order to determine whether these isolates have any potential pathogenic activity. These assays involved an extended exposure period to bacterial endospores applied to food pellets, as well as an extended observation period post-exposure. In this manner, a combination of prolonged incubation of the snails with these bacterial species in the environment, as well as likely ingestion of spores and vegetative cells, would be achieved in parallel. This would serve to determine whether the exposure to the bacteria or their spores would cause snail death by a direct mechanism. Quantification of direct molluscicidal activity would allow for determination of whether this research avenue within this project has any utility in pursuing it further, or whether further research should continue to focus on molluscicidal spent culture supernatant. In addition, identification of even a weakly pathogenic strain would enable future work to focus on a smaller pool of candidate strains to find a viable species for snail biocontrol. In parallel to the extended endospore exposure assays, six isolates (O8b, R9, S3, U7, AX56, BB1-156a), along with three *B. velezensis* reference strains (R16,

FZB42, PHP1601) that generated molluscicidal supernatants were included in the endospore exposure assays. While molluscicidal supernatant producing isolates had not shown strong activity in this study when snails were exposed to the bacteria themselves, longer-term exposure has the potential to allow the production of molluscicidal compounds that may either build up in concentration or have an effect over time. Determination of whether efficacious concentrations of molluscicidal metabolites can be achieved naturally, or whether applications of extracted, concentrated metabolites are necessary to achieve desired pest control levels, would be a significant milestone in this study.

The research in this chapter sought to determine if lipopeptides are candidate compounds for observed molluscicidal supernatants. Furthermore, to determine if any potential pathogenic molluscicidal activity had been identified in initial screening. A secondary aim was to determine whether isolates that produce molluscicidal metabolites could yield an observable effect in the absence of exposure to spent culture metabolites and instead with extended exposure to these isolates under assay tank conditions.

4.2 Material and methods

4.2.1 Lipopeptide extraction and assay

Isolates selected for their ability to produce molluscicidal cell free culture supernatant underwent extraction of active metabolites from cell-free broth supernatant using lipopeptide extraction techniques.

4.2.1.1 Crude lipopeptide extraction from TSB medium

Isolates selected for lipopeptide extraction were 4 isolates identified as *B. velezensis* (R9, S3, AX56, and BB1-156a) as well as a *B. subtilis* (O8b) and a *B. amyloliquefaciens* (U7). Inoculum of 20 mL TSB was prepared in 125 mL Erlenmeyer flasks and inoculated with a fresh, single colony (24-48 h TSA, 30°C). This inoculum was grown overnight at 30°C, in an orbital shaker at 150 rpm. The entire volume was then aseptically transferred to fresh sterile 200 mL TSB in a 1 L Erlenmeyer and incubated for 5 days at 30°C at 150 rpm in a rotary shaker. Typically, lipopeptides use a culture media such as Landy's, however the initial molluscicidal activity

was from TSB culture and so this media was retained for initial lipopeptide extractions (Landy *et al.*, 1948). TSB broth growth conditions were identical to that which demonstrated molluscicidal activity in the initial screening in Chapter 2.

An acid precipitation procedure for lipopeptide extraction was modified from a number of sources (Hunter, 2016, Xu *et al.*, 2018). Broth culture was centrifuged at 8 000 *x g* for 15 minutes. The supernatant was transferred into a sterile 250 mL Schott bottle for acidification and a 10 mL sample was removed and stored at -20°C for later assay. Acidification of the broth supernatant involved adjusting it to pH 2.0 with 32 wt. % HCl and then storing it for 18 h at 4°C. This acidified supernatant was then centrifuged at 12 000 *x g* for 30 minutes and the supernatant was removed. A 10 mL sample of acidified supernatant was stored at -20°C for later assays. The crude lipopeptide pellet was washed twice with 40 mL of cold (4°C) 0.01M HCl (pH 2.1) in distilled water, centrifuged at 12 000 *x g* for 30 minutes, with the supernatant discarded each time. The pellet was then washed twice with 5 mL aliquots of HPLC grade methanol. This methanol solution was decanted into 2 mL microcentrifuge tubes and centrifuged at 15 000 *x g* for 5 minutes to remove any solid material. The supernatant was carefully decanted into clean glass polytop vials that had been pre-weighed on a fine balance (OHAUS Adventurer, Parsippany, NJ 07054 USA). Samples were allowed to dry (lid off) in a fume hood at ambient temperature for 48 h, followed by 24 h in a glass desiccator (non-vacuum) containing a silica gel desiccant, after which the vials were weighed again to determine the total mass of recovered crude lipopeptides.

Crude lipopeptide extract was dissolved in phosphate buffer (10 mM phosphate buffer, pH 7.0) and made up to the final volume in a volumetric flask, targeting between 1 $mg.mL^{-1}$ and 2.5 $mg.mL^{-1}$ final concentration. This crude lipopeptide extract was frozen at -20°C until needed.

4.2.1.2 Bioassay of crude lipopeptides extracts from TSB

Bioassays on initial broth, extracted broth, and crude lipopeptide extract were performed to determine whether the active fraction was successfully extracted using acid precipitation. For initial broth supernatant and extracted broth supernatant, 8 mL of supernatant was used to make a 10% (v/v) solution of 80 mL total volume by dilution with snail aquaria water (typical pH 7.2) and then pH adjusted to pH 7.2 using 0.1 M HCl for unextracted broth supernatant, and

with 1M NaOH for acidified extracted broth. A volume of 40 mL of the 10% solution was removed and diluted 1:1 with aquaria tank water to create a 5% (v/v) solution. A volume of 40 mL of the 5% solution was removed and diluted 1:1 with aquaria tank water to create a 2.5% (v/v) solution. All 3 concentrations were tested (10%, 5% and 2.5% (v/v) unextracted broth in tank water). Lipopeptide extract in 10 mM phosphate buffer was used to make 40 mL aliquots of aquaria tank water at 25, 50, 100 and 200 $\mu g.mL^{-1}$ concentrations.

Supernatant assays were performed in 50 mL plastic sample jars with perforated lids. Six *P. acuta* snails between 8–15 mm in size were placed in each jar. Jars were emptied of excess water and 20 mL of the appropriate solution was added. Each isolate and each concentration were tested in duplicate. Assay tanks (50 mL) were stored under ambient laboratory conditions for 24 h of exposure before transfer of the snails into 500 mL PET assay jars containing 400 mL aquarium tank water. Snails were provided with a supply of lettuce (approximately 5 cm^2) and observed for 3 days, assessing for recovery.

To determine the effects of phosphate buffer on snails, 12 tanks were prepared using 10 mM phosphate buffer (pH 7.0) diluted to final concentrations of 0.25, 0.5, 1.0, and 3 mM phosphate buffer in aquarium tank water. Phosphate control tanks were prepared in triplicate at each tested concentration, each tank contained 6 snails each.

4.2.2.1 Crude lipopeptide extraction from Landy medium

The six isolates used in the TSB lipopeptide extraction assay (Section 4.2.1.1) were also evaluated on Landy medium (20 $g.L^{-1}$ Glucose, 5 $g.L^{-1}$ L-Glutamic acid, 1.02 $g.L^{-1}$ $MgSO_4.7H_2O$, 0.5 $g.L^{-1}$ KCl, 1 $g.L^{-1}$ K_2HPO_4 and 1 $mL.L^{-1}$ solution containing: 5 $g.L^{-1}$ $MnSO_4.H_2O$, 1.6 $g.L^{-1}$ $CuSO_4.5H_2O$ and 0.15 $g.L^{-1}$ $FeSO_4.7H_2O$), which was prepared according to Landy *et al*. (1948). The base medium was acidified to pH 6.1 with 1M HCl. Inoculum of 20 mL Landy medium was prepared in a 125 mL Erlenmeyer flask and inoculated with a fresh, single colony (24-48 h TSA, 30°C). This inoculum was grown overnight at 30°C at 150 rpm in a rotary shaker. The entire volume was then aseptically transferred to fresh sterile 200 mL Landy medium in 1 L Erlenmeyer flasks and incubated for 3 days at 30°C 150 rpm in a rotary shaker. Lipopeptide acid precipitation was performed as per the procedure followed in Section 4.2.1.1.

4.2.2.2 Bioassay of crude lipopeptide extracts from Landy medium

Bioassays of Landy lipopeptide extracts as well as extracted and unextracted broth were performed in an identical manner to those in Section 4.2.1.2.

4.2.3 Endospore production

Isolate selected for potential molluscicidal activity underwent further assays using purified endospores to establish whether these isolates do in fact have molluscicidal abilities. Initial culture growth and endospore harvesting utilised the methods described by Nicholson and Setlow (1990), with a modified version of the 2X SG broth media being used. Modified 2X SG broth was made up in 100 mL aliquots in 500 mL Erlenmeyer flasks and comprised the following: 2 $g.L^{-1}$ Yeast extract, 7 $g.L^{-1}$ Bactopeptone, 7 $g.L^{-1}$ tryptone, 2 $g.L^{-1}$ KCl, and 0.5 $g.L^{-1}$ $MgSO_4.7H_2O$, adjusted to pH 7.0 before autoclaving. After autoclaving, 1 $mL.L^{-1}$ each of the following filter-sterilized solutions were added: 1M $Ca(NO_3)_2$, 0.1M $MnCl_2.4H_2O$, 1 mM FeSO4 and 2 $mL.L^{-1}$ 50% (w/v) glucose. Broth media was inoculated with freshly grown culture from 24-48 h old TSA plates (Neogen Corporation, Lansing, Michigan, United States, Tryptic Soy Agar, 37 $g.L^{-1}$) and incubated at 37°C for 5 days at 150 rpm in a rotary shaking incubator. Culture media was checked for sporulation before harvesting under phase contrast microscopy at 1000x.

4.2.3.1 Modified 2X SG endospore harvesting

Endospore purification from cells and cell debris was performed as described by Nicholson and Setlow (1990). After incubation, 2X SG broth was harvested aseptically and centrifuged at 10 000 *x g* for 10 minutes and the supernatant was discarded. The cell pellet was washed by resuspending in 25 mL 4°C sterile distilled water and centrifuging at 10 000 *x g* for 10 minutes, discarding the supernatant. This washing step was repeated 3 times. After the final wash, the cell pellet was resuspended in 25 mL 4°C sterile distilled water and stored at 4°C overnight.
Over the next 5 days, the endospore suspension underwent daily centrifugation and washing by centrifugation at 20 000 *x g* for 20 minutes with the supernatant discarded. The resultant pellet was then resuspended in 25 mL 4°C sterile distilled water and stored overnight at 4°C. After this period of daily washing, the upper layer of cell debris was gently washed off with

4°C sterile distilled water, preserving the layer of endospores underneath. Confirmation of endospore purity was conducted using phase contrast microscopy at 1000x. Endospores were then stored suspended in sterile distilled water at 4°C, until use within one week of production. During the storage period, the endospore suspension was washed twice weekly by 10 000 *x g* centrifugation for 10 minutes, discarding the supernatant and resuspending the endospore pellet in fresh 4°C sterile distilled water.

For isolates that did not produce clean endospores with this approach, an extended incubation of 13 days at 37°C, as well as extended daily washing steps over 13 days, was performed.

4.2.3.2 Alternative endospore production and harvesting

For isolates that did not produce endospores using the method described in Section 4.2.3, a modified nutrient agar technique was used (Wang, 2009). Briefly, Nutrient Agar (Neogen Corporation, Lansing, Michigan, United States) (NA, 28 $g.L^{-1}$) was amended with 0.05 $g.L^{-1}$ $MnSO_4.H_20$. Plates were inoculated via streaking from fresh culture from TSA plates (24-48 h at 30°C) and incubated at 30°C for 19 days. After incubation, 4 mL sterile distilled water was added to the Petri dish, and colony matter was then allowed to hydrate for 2 minutes. The bacterial cell material was then mixed into a slurry using a sterile glass hockey stick and removed from the agar aseptically by pipetting it into sterile 2 mL microcentrifuge tubes for processing.

Culture material suspensions were centrifuged at 10 000 *x g* for 10 minutes and the supernatant was discarded. The cell pellet was then washed by resuspending in 1 mL sterile distilled water (4°C) and centrifuged at 10 000 *x g* for 10 minutes, discarding the supernatant. This washing step was repeated 3 times after which the cell pellet was resuspended in 1 mL sterile distilled water (4°C) and stored at 4°C overnight. The endospore suspension was washed daily over the next 8 days by centrifugation at 15 000 *x g* for 10 minutes, discarding of supernatant and resuspending in 1 mL sterile 4°C distilled water each time before storing at 4°C overnight. After 8 days, the upper layer of cell debris was removed by gentle pipetting, and the lower layer of endospores retained. Storage and maintenance procedures were then followed as described previously in Section 4.2.3.1.

4.2.4 Non-sporulating isolate BA1-53

One isolate was identified as a *Paenibacillus sp.* (Chapter 3), however this isolate did not produce visible spores under either of the methods used in Section 4.2.3. Instead, this isolate was formulated using Tryptic Soy Broth vegetative culture (Neogen Corporation, Lansing, Michigan, United States) (TSB, 30 $g.L^{-1}$). The TSB broth (20 mL) was inoculated using fresh TSA culture (24-48 h at 30°C) and incubated at 30°C for 24 h at 150rpm in an orbital shaking incubator. After incubation, 2 mL samples were transferred aseptically into 2 mL microcentrifuge tubes and centrifuged at 5000 *x g* for 10 minutes. The cell pellets were washed once with sterile 0.35% (w/v) saline and re-centrifuged at 5000 *x g* for 10 minutes. The washed cell pellets were resuspended in 1 mL sterile 0.35% (w/v) saline and used immediately after preparation.

4.2.5 Endospore pellet assay

4.2.5.1 Endospore pellet preparations

To evaluate potential molluscicidal isolates, snail food pellets were prepared with a defined number of endospores. Stored endospore suspensions prepared as above (Section 4.2.3), as well as fresh vegetative culture (Section 4.2.4), were enumerated using a Petroff-Hausser counting chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) of 0.02 mm depth with a counted area of 0.04 mm^2 with each count averaged over five replicates. After appropriate dilutions in sterile distilled water, a 2 mL final volume was prepared with a final concentration of ~$5x10^8$ endospores per mL. This suspension was applied to 2 g of autoclaved snail food pellets (Regular Pond pellets, Qualipet, Phoenix, Durban, KwaZulu-Natal, South Africa, 4068) in a sterile Petri dish. After approximately 2 minutes, the food pellets had completely absorbed the endospore suspension and could be shaken to distribute the individual pellets across the Petri dish for drying. Petri dishes and contents were dried, lid removed, in a glass desiccator (non-vacuum) containing a silica gel desiccant overnight in ambient laboratory conditions. The dried pellets were stored in the dark in a fresh glass desiccator. Dry pellets were then used for the snail antagonism assays and endospore and vegetative cell enumeration.

4.2.5.2 Pellet endospore and vegetative cell enumeration

As a measure of viability, prepared food pellets underwent an enumeration procedure using Colony Forming Units (CFU). Pellet endospore content enumeration was performed after 2-3 days of desiccation. Five randomly selected food pellets were aseptically removed and placed into a weighed microcentrifuge tube in order to determine the total pellet weight of the sample. The pellets were suspended in 1 mL of 0.35% (w/v) sterile saline and vortexed vigorously for 30 minutes to homogenise. A dilution series was then prepared using sterile 0.35% (w/v) saline and 100 µL aliquots spread plated using a sterile glass hockey stick in triplicate onto NA in order to determine total CFU. The prepared dilutions were then heat-treated using a dry block (Operon OPR-HB-100, Korea) for 10 minutes at 80°C and spread-plated on NA using a sterile glass hockey stick, in triplicate, to determine total endospore CFU numbers.

4.2.5.3 Endospore pellet snail assay

Snail tanks were prepared the day before each assay commenced. Tanks were prepared, as described previously in Chapter 2.2.3.4. Briefly, snail assay tanks comprised 500 mL PET bottles with perforated screw caps for aeration that were filled with approximately 400 mL of water sourced from the tanks from which harvested snails were reared. Each assay tank was populated with three *P. acuta* snails 8-15 mm in length. Tanks were kept for observation for 24 h without food before endospore-impregnated food pellets were provided, and the assay was initiated. Seven tanks were prepared for each isolate's endospore preparation, along with 20 control tanks fed with sterile, unamended food pellets. At assay commencement, tanks were fed 2-3 food pellets each (approximately 0.03 g). For 2 weeks tanks were fed as many amended food pellets as the snails would eat over 2 to 3 days (approximately 2-3 pellets per feed). Water was changed once weekly with fresh aquarium tank water. After 2 weeks of exposure to endospore-impregnated food pellets, 4 weeks of observation commenced, with weekly water changes and a constant supply of lettuce (approximately 5 cm^2) being provided. Observations of snail mortality were taken at least twice weekly. Dead snails were not removed during the course of the assay.

Statistical analysis was performed using MedCalc Software Ltd. Version 22.005 using the Fisher exact probability calculator. Tanks were pooled for this analysis for a total of 21 snails per isolate compared against the control tanks with 90 snails.

4.3 Results

4.3.1 TSB crude lipopeptide bioassay

Results for the assays of TSB broth supernatants and crude lipopeptide extracts are summarised in Table 4.1. In all cases, when snail mortality was observed at >80% (10 out of 12 snails dead) for a sample, if a higher concentration was tested mortality would be at that level or higher. Mortality percentages for each concentration tested are shown in Appendix H and I. For this reason, the lowest concentration with recorded mortality above >80% is reported for Table 4.1. The phosphate buffer control assays with concentrations between 0.25 and 3 mM tested in triplicate, caused no mortality or behavioural changes over the assay period. The extracted broth supernatant at 10% (v/v) had similar mortality to controls with the highest mortality recorded of 16.7% for isolate BB1-156a. The remaining isolate had either 0% mortality or 8.3%. For extracted broth supernatant this was not considered substantially different from the control.

Table 4.1. Results of *P. acuta* snail bioassays of TSB broth supernatants and crude lipopeptide extract

Isolate	TSB supernatant (%)[1]	Crude extract (µg/mL)[2]
S3	2.5	50
R9	10	50
O8b	10	200
U7	2.5	50
AX56	2.5	25
BB1-156a	10	100

[1] Percentage broth supernatant at which >80% snails die after 24-hours exposure.
[2]Concentration of crude extract at which >80% of snails died after 24-hours exposure.

Three isolates (R9, O8b, BB1-156a) caused a >80% mortality at 10% (v/v) of their spent culture supernatant, which is lower than the cut-off of 5% selected for initial isolate selection. Three isolates (S3, U7, AX56) showed >80% mortality at 2.5% (v/v) concentration. While there was some variability in the lowest concentration in which >80% mortality was recorded from cell free culture supernatant was observed between isolates, molluscicidal activity present in broth supernatant was universally absent from spent broth that has been extracted via acid precipitation. The crude lipopeptide extracts showed molluscicidal activity with >80% mortality at between 25 and 200 $\mu g.L^{-1}$ concentrations. Isolate R9 demonstrated relatively poor activity in the whole broth supernatant but had a relatively high activity in the crude lipopeptide extract.

4.3.2 Landy medium crude lipopeptide bioassay

Results for the assays of Landy broth supernatant and crude lipopeptide extracts are summarised in Table 4.2. In all cases, when snail mortality was observed at >80% (10 out of 12 snails dead) for a sample, if a higher concentration was tested mortality would be at that level or higher. Mortality percentages for each concentration tested are shown in Appendix H and I. For this reason, the lowest concentration with recorded mortality above >80% is reported for Table 4.2. The measured activity in the Landy broth supernatant was in general weaker than that for the TSB broth lipopeptide extraction shown in Table 4.1. The crude lipopeptide extracts from Landy media showed a similar range of activity to that of TSB crude lipopeptide extracts of 25 and 200 $\mu g.L^{-1}$. Isolates U7 and AX56 demonstrated the lowest activity recorded, showing no molluscicidal activity at 10% (v/v) broth supernatant, although the crude lipopeptide extract for these isolates were still active at between 100 and 200 $\mu g.L^{-1}$. A similar pattern was observed for the TSB lipopeptide extraction assay where the activity observed in the broth supernatant was absent from the extracted broth supernatant but appeared in the crude lipopeptide extract. The extracted broth supernatant at 10% (v/v) produced only a single snail death at 10% for isolate U7, the remaining isolates had no observed snail deaths.

Table 4.2. Results of *P. acuta* snail bioassays of Landy broth supernatant and crude lipopeptide extracts

Isolate	Landy supernatant (%)[1]	Crude extract (µg/mL)[2]
S3	5	100
R9	10	25
O8b	5	25
U7	-	100
AX56	-	200
BB1-156a	10	50

[1] Percentage broth supernatant at which >80% snails died after 24 hours exposure.
[2]Concentration of crude extract at which >80% of snails died after 24 hours exposure.
(-) less than 17% mortality at the highest concentration tested (10% v/v).

4.3.3 Endospore production

The majority of isolates (AX56, BB1-156a, BC8-151, O8b, R9, S3, U7), as well as the reference strains (R16, FZB42, and PHP 1601), produced clean endospore concentrates using the method outlined in Section 4.2.3. However, isolates C1, BA1-10, BB1-1 and BA1-53 did not produce visible endospores under these conditions, and, for isolate H8, the spores remained internal to chains of vegetative cells. For these five isolates, an extended incubation period (13 days) was conducted in modified 2x SG media, as well as an extended daily washing phase (13 days) with similar results. A second endospore production medium was attempted using amended NA (Wang, 2009). Isolates C1, H8, BA1-10 and BB1-1 produced clean endospore suspensions using this method. However, isolate BA1-53 did not produce any endospores visible under phase contrast microscopy and was instead assayed using a fresh vegetative cell suspension.

4.3.3.1 Endospore enumeration

Despite attempts to standardise endospore dosage with Petroff-Hausser counting chamber endospore counts, the actual recovered CFU's showed variability between isolates (Table 4.3). Differing endospore responses to hydration and drying in a high nutrient environment was also apparent, with the varying ratios of spores to vegetative cells. This may be exacerbated by variable bacterial recovery between isolates from the impregnated pellets. Nevertheless,

recovered cells were within an order of magnitude of their initial dosage and should be sufficient for a moderately effective molluscicidal isolate to be identified. The exception to this was isolate C1, with significantly lower numbers of recovered cells. During isolate culture work this isolate appeared to be more sensitive, readily becoming non-viable on solid media (data not shown).

Table 4.3. Enumeration of recovered spores and vegetative cells from impregnated snail food pellets

Isolate	CFU per gram food pellet	Endospores (%)	Vegetative cells (%)
C1	6.53E+05	98	2
H8	2.94E+07	31	69
Ax56	3.11E+07	41	59
BA1-10	8.21E+07	12	88
BA1-53	1.54E+07	0	100
BB1-156a	5.81E+07	55	45
BB1-1	7.94E+07	9	91
BC8-151	8.77E+07	3	97
O8b	4.43E+07	18	82
R9	4.10E+07	53	47
S3	4.92E+07	46	54
U7	7.05E+07	40	60
R16	3.15E+08	34	66
FZB42	2.08E+08	77	23
PHP 1601	3.12E+08	57	43
Average	9.49E+07	38	62

4.3.3.2 Endospore bioassay

Results for the endospore exposure assay in Table 4.4 show no statistically significant mortality when any isolate is compared to the control snail tanks. The highest mortality rate was for

isolate BC8-151, which caused a 14.3% mortality over the 6-week observation period. However, this result was not statistically significant, with a P-value of 0.123.

Table 4.4. Mortality rates of *P. acuta* in the prolonged endospore exposure bioassay

Isolate	Deaths	Total population	Mortality (%)	P-value*
C1	1	21	4.8	1
H8	0	21	0.0	1
Ax56	0	21	0.0	1
BA1-10	0	21	0.0	1
BA1-53	0	21	0.0	1
BB1-156a	2	21	9.5	0.317
BB1-1	0	21	0.0	1
BC8-151	3	21	14.3	0.123
O8b	1	21	4.8	1
R9	1	21	4.8	1
S3	2	21	9.5	0.317
U7	1	21	4.8	1
R16	0	21	0.0	1
FZB42	0	21	0.0	1
PHP1601	2	21	9.5	0.317
Control	4	90	4.4	

* (Fischer exact probability, MedCalc Version 22.005).

4.4 Discussion

With the identification of isolates that produced spent culture supernatant with molluscicidal properties, determining the characteristics of the responsible compounds became an important research question. Identification of the isolates exhibiting this property yielded *Bacillus* species known for the production of lipopeptides, the production of which is responsible for a number of beneficial characteristics (Zhao *et al.*, 2017, Penha *et al.*, 2020). While lipopeptides have not previously been identified as molluscicidal compounds, similarities in surfactant-like characteristics to saponins, plant extracts with a history of molluscicidal activity study, made these compounds a viable target to test for molluscicidal activity (Marston and Hostettmann,

1985, Singh, *et al.*, 1996). Snail bioassay results of the lipopeptide extracts from both TSB and Landy medium suggest that the compounds responsible for the observed molluscicidal activity were successfully extracted and concentrated using a lipopeptide extraction technique. While this does not confirm that lipopeptides are the compounds responsible for the observed activity, this method of production, extraction, and concentration appears effective for future research to identify the active fractions. Extraction and concentration of an active fraction is an important first step in determining what class of compounds is responsible for observed molluscicidal activity.

Molluscicidal, cell-free supernatant was initially produced from cultures grown in TSB broth, however not all isolates produced levels of activity consistent with the initial screening on this media when repeated in this chapter (Chapter 2, Section 2.3.4.3). This suggests some variability in the production of molluscicidal compounds and may indicate that some isolates may have been disadvantaged by relying on the results of a single screening. It is difficult to ascertain why there is this discrepancy, however standardisation of inoculum age and quantity may help remove this variable. Three isolates R9, O8b and BB1-156a, produced a level of activity (>80% lethality at 10% v/v cell free supernatant in tank water) that was lower than that used as the cut-off for selection in the initial assay. This exposes the shortfall of single replicate measurement of activity in a general-purpose medium. Stricter inoculation parameters may result in a higher degree of reproducibility in initial screening; however, this was not conducted in order to facilitate a higher throughput of isolates. Of the 128 isolates evaluated in Chapter 2, more than 40 isolates were identified with strong molluscicidal activity at this 10% (v/v) concentration. An additional data point of interest is the demonstration of activity at spent culture concentrations of 2.5% (v/v) for isolates S3, U7 AX56. Future screening of similar isolates may use a lower effective concentration to better screen for the strongest performers.

A comparison between the TSB broth lipopeptide extractions and those from Landy medium shows some differences in the observed molluscicidal activity levels of these spent broth supernatants (Table 4.1 and 4.2). While Landy medium is a common medium for lipopeptide production, it is not ideal for all lipopeptide producers, as these results suggest. Most strikingly, the isolates U7 and AX56 showed very poor activity when grown in Landy medium, with no mortality at 10% (v/v) broth supernatant and below average crude lipopeptide extract activity levels of 100 and 200 $\mu g.L^{-1}$. However, these isolates were amongst the most potent performers

in the TSB assay, with activity of broth supernatant at 2.5% (v/v) and crude lipopeptide activity of 25 and 50 $\mu g.L^{-1}$. In contrast, isolates O8b and BB1-156a show a reversed pattern, with better broth supernatant activity and crude lipopeptide activity when grown in Landy medium than when grown in TSB. Differences in the responses of various isolates to various growth media and incubation conditions are not unexpected. There is a large body of literature devoted to improving growth conditions for the production of various lipopeptides (Willenbacher *et al.*, 2015, Sun *et al.*, 2019, Maksimov *et al.*, 2020). Within the selected six isolates, there had been no attempt to optimise growth conditions to improve molluscicidal activity, and instead, a reasonable baseline for lipopeptide production was selected. Before any optimisation for growth conditions and media can be undertaken, identification of active fractions will be performed in Chapter 5.

From Chapter 3, eight isolates were selected for further screening for potential molluscicidal activity. Screening to date had depended on short periods of exposure of snails to bacterial culture material, followed by an observation period of 2-3 weeks, which had either yielded weak, or inconsistently positive results. The initial screening protocols did not provide for the measurement of weak levels of molluscicidal activity. For this chapter, it was decided to increase the exposure time frame, as well as that of the observation period. In addition, the decision was made to expose the snails to the potential antagonists in the form of food pellets coated with bacterial endospores in order to better match any potential commercial application of molluscicidal *Bacillaceae* isolates. While identification of a weak molluscicidal strain may not prove useful in snail biocontrol, the information could be useful in designing future screening procedures targeting a narrower range of *Bacillus* isolates. Alongside this extended screening, the representative isolates producing molluscicidal supernatant were run in parallel. While these isolates had not demonstrated any direct molluscicidal activity, these were included to determine whether a longer exposure and observation period would allow for a molluscicidal effect to be observed; either by long term exposure to a produced molluscicidal compound or by production of molluscicidal secondary metabolites reaching a concentration sufficient to cause mortality with prolonged incubation.

Results from this direct exposure bioassay using endospore exposure in food pellets showed no statistically significant differences between any isolate and the control (Table 4.4), with the highest recorded value for isolate BC8-151 of 14.3% mortality (P=0.123) compared to the 4.4%

mortality of the control. This is approaching significance, which would have been reached with 19% mortality rate (P=0.041) in the population size screened. Yet, even if significance had been reached at this low (19%) level of mortality, it is doubtful this would result in an acceptable level of snail biocontrol. It is apparent that extended exposure to endospore-impregnated food pellets did not increase molluscicidal activity, nor did the extended observation period post-exposure. From these bioassay results, research into the eight potential molluscicidal isolates can be concluded. Additionally, it appears that under these screening conditions, isolates that produced molluscicidal compounds in spent growth media are either not producing these compounds under these growth conditions, or are producing them in concentrations that do not demonstrate an effect.

Any future iterations of this work require improvements to be made to the formulation of dosed snail food pellets. The variabilities in responses between the different isolates' endospores to hydration and drying on a nutrient-rich media may pose further problems. Ideally, an entirely dry formulation, or one with less hydration or a shorter drying time, could serve to avoid this issue. Nevertheless, endospore dose rates were relatively consistent and ultimately endospores would likely revive rapidly after hydration in assay tank water.

The ideal for a biological control agent is for sufficient activity to be achieved with a minimum of investment in dosing or application, with the best-case scenario of biocontrol being the establishment of a stable population able to effect control from a single application of the appropriate species. The mode of action of these isolates with confirmed molluscicidal activity seems to reflect the production of a molluscicidal metabolite, i.e., antibiosis (Köhl *et al.*, 2019). Unfortunately, from the endospore exposure assays, it appears that production of these compounds at sufficient concentrations to demonstrate an effect on snails did not occur under tank conditions. The ideal would be to achieve a biological control effect by application of live bacteria producing molluscicidal compounds *in situ*, however, these results suggest that this is unlikely to be achieved. Greater potential may lie in the production and application of extracted metabolites. The next phase of this research will therefore focus on the characterisation of the compounds present, as well as determining the concentrations of extracts required for effective snail control.

4.5. References

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CHAPTER FIVE: CHARACTERISATION OF CRUDE LIPOPEPTIDE EXTRACTS FROM SELECTED *BACILLUS* STRAINS AND QUANTIFICATION OF THEIR MOLLUSCICIDAL ACTIVITY

Abstract

Bacillus spp. are known to produce lipopeptide compounds which are amphiphilic and have surfactant properties. Lipopeptides play a role in the biocontrol capabilities of these species and act against a range of pest species such as insects, fungi and bacteria. Crude lipopeptide extracts from Landy and TSB growth media with molluscicidal activity against *Physella acuta* (Draparnaud, 1805) underwent UPLC-ESI-MS to identify lipopeptide components. In addition, the dose response curves were measured for 24 h and 72 h contact times. The LC_{50} for crude lipopeptide extracts at 24 h exposure ranged between 16.37 $\mu g.mL^{-1}$ and 36.16 $\mu g.mL^{-1}$ while the LC_{95} was between 20.05 and 48.68 $\mu g.mL^{-1}$. The LC_{50} for 72 h exposure was calculated between 11.89 $\mu g.mL^{-1}$ and 27.21 $\mu g.mL^{-1}$ while the LC_{95} was between 15.09 and 30.93 $\mu g.mL^{-1}$. Analysis of the UPLC-ESI-MS spectra for each molluscicidal crude extract indicated the presence of various lipopeptide isoforms, including surfactin, iturin, fengycin and bacillomycin-D and -L. Surfactin was common to all molluscicidal crude lipopeptide extracts examined, which suggests that surfactin was a major contributor towards observed molluscicidal activity.

5.1 Introduction

Bacillus spp. produce a range of enzymes and secondary metabolites, most notably a group of compounds known as lipopeptides (Jacques, 2011; Ongena and Jacques, 2008). Lipopeptides are widely synthesised among members of the Bacillaceae, as well as other fungal and bacterial genera (Raaijmakers *et al.,* 2010). These compounds comprised of a lipid tail linked to a cyclic oligopeptide, are amphiphilic, function as biosurfactants capable of reducing surface tension, and reduce membrane stability through pore formation (Raaijmakers *et al.,* 2010). *Bacillus*-produced lipopeptides are classified into three main families: surfactin, iturin, and fengycin. These families are defined by specific amino acids at positions in the peptide fraction, and different lengths and compositions of the fatty acid tail (Ongena and Jacques, 2008). In natural ecosystems, these compounds assist the bacteria in spreading, colonising, and persisting within

their habitat, and also function as signalling molecules in biofilm formation and development (Raaijmakers *et al.,* 2010). These pore-forming and surfactant properties have seen lipopeptides investigated for potential applications in industry and agriculture (Meena and Kanwar, 2015; Zhu, *et al.,* 2021; Ledger, *et al.,* 2022; Sani *et al.,* 2024; Xia *et al.,* 2024)

In agricultural applications, lipopeptide-producing *Bacillus* are frequently credited for pathogen antagonism, plant resistance stimulation, and other plant growth promotion activities (Ongena *et al.,* 2007; Fan *et al.,* 2018; Crouzet *et al.,* 2020; Malviya *et al.,* 2020). Fengycins and iturins are primarily associated with antifungal and antibacterial properties; in addition to these characteristics, surfactin is a potent biosurfactant and has shown activity against a number of insect species (Ongena and Jacques, 2008; Geetha *et al.,* 2012; Denoirjean *et al.,* 2021; Koim-Puchowska *et al.,* 2023). Identification of lipopeptide compounds is commonly accomplished by a range of techniques, including chromatography and mass spectroscopy-based methods (Wang *et al.,* 2004; Bie *et al.,* 2009; Gong *et al.,* 2014; Pathak and Keharia, 2014; Luo *et al.,* 2015).

Six isolates identified for their molluscicidal potential (Chapter 4) were cultured to produce molluscicidal crude lipopeptide extracts in Landy and TSB growth media. Activity of these crude extracts was estimated using *P. acuta* snail bioassays at fixed exposure concentrations of 25, 50, 100, and 200 $\mu g.mL^{-1}$ with a 24 h exposure. This chapter discusses the dose response curves of these active extracts, and the identification of lipopeptides in the extracts. The crude extracts were produced using a lipopeptide extraction technique and a UPLC-ESI-MS procedure was used to identify lipopeptide compounds present in these molluscicidal extracts.

5.2 Materials and Methods

5.2.1 Crude lipopeptide extracts from TSB and Landy medium

Lipopeptide crude extracts dissolved in 10 mM phosphate buffer (pH 7.0) were produced from 5-day 30°C cultures of TSB and 3-day 30°C culture grown on Landy medium, as detailed in Chapter 4 (Sections 4.2.1.1 and 4.2.2.1).

5.2.2 UPLC-ESI-MS of crude extracts and lipopeptide standards

Crude lipopeptide extracts exhibiting molluscicidal activity underwent UPLC-ESI-MS analysis to identify lipopeptides present. Crude extracts were dissolved in methanol at a final concentration of 125 µg.mL^{-1}. Two lipopeptide standards, Surfactin (≥98.0%, purity, Sigma, USA) and Iturin A (≥95%, purity, Sigma, USA) were included to aid in lipopeptide identification. Reference standards were suspended in HPLC-grade methanol to a concentration of 100 µg.mL^{-1}. The UPLC-ESI-MS procedure was performed using previously-developed protocols (Hunter, 2016).

A Waters Acquity UPLC system was used, fitted with a Waters Acquity BEH C18 column (2.1 x 100 mm, particle size of 1.7 µm, 45°C). Two solvents were used during the course of each run: 0.2% (v/v) acetic acid and 100% methanol. Solvent flow rate was maintained at 300 µL.min^{-1} for the entirety of the run. The solvent ratio for the first 30 minutes was 9:1 acetic acid to methanol; after 30 and 38 minutes, the percentage of methanol was increased to a final concentration of 100%, after which the ratio was again changed to the original 9:1 for the remainder of the 40-minute run. Detection and measurement of eluted compounds was performed using a Waters Micromass LCT Premier TOF-MS, ionised using electrospray-ionisation with a capillary voltage of 5000 V and a cone voltage of 35 V. Positive mode ESI was used for detection over a *m/z* range of between 900 and 2000. Desolvation at 350°C was used with a gas flow rate of 400 L.h^{-1}. Sample aliquots of 2 µL were loaded for each run.

Identification of lipopeptides was based on the surfactin and Iturin standards used as well as previously described elution times with the above protocol and UPLC-ESI-MS system (Hunter, 2016), as well as the characterised mass spectra described in the literature (Wang *et al.,* 2004; Bie *et al.,* 2009; Gong et al 2014; Pathak and Keharia, 2014; Barale *et al.,* 2022).

5.2.3 Dose response of *P. acuta* to crude lipopeptide extracts

5.2.3.1 Dose response assays with 24-hour exposure

Crude isolates that demonstrated greater than 80% mortality when snails were exposed to a 50 µg.mL^{-1} extract concentration for 24 h underwent a series of bioassays to accurately measure the dose response curve. This included the crude lipopeptide extracts of isolates R9, O8b and

BB1-156a grown on Landy broth, and isolates S3, R9, U7 and AX56 grown in TSB. Crude extracts with activity at concentrations higher than 50 µg.mL^{-1} (Chapter 4) were not included in these assays due to the greatly increased mass of extract required to perform these tests at these concentrations, which would render these products unusable in practice.

An iterative approach was adopted, whereby each assay was performed for a concentration interval, the results of which informed the concentration intervals for the next round of bioassays. Concentration intervals targeted were 5 µg.mL^{-1} incremental steps between 10 and 55 µg.mL^{-1}, depending on the activity of the individual crude extract. Assays targeted concentration ranges giving mortality rates of between 0 and 100%. Crude extract test concentrations were prepared in sterile 250 mL glass beakers; 35 mL of aquarium tank water was transferred into the glass beaker and then a volume of crude lipopeptide concentrate in 10 mM phosphate buffer (pH 7.0) was added, to bring the new total volume to the target concentration.

Assays were performed in 50 mL plastic sample jars with perforated lids. Six *P. acuta* snails between 8-15 mm in size were placed in each jar. Jars were emptied of excess water and 10 mL of the appropriate crude lipopeptide preparation was added. Each concentration for each isolate was tested in triplicate. Assay tanks were stored under ambient laboratory conditions for 24 h of exposure before transfer of the snails into 500 mL PET assay jars containing 400 mL aquarium tank water. The snails were observed for 72 h, assessing their health status. Control tanks were prepared in an identical fashion but without the addition of any lipopeptide solution and were run in parallel.

5.2.3.2 Dose response assays with 72-hour exposure

In order to determine the effects of longer-term exposure of snails to crude lipopeptide extracts, assays were performed with an exposure time for 72 h. These assays were performed in a similar fashion to those of the 24 h assays (Section 5.2.3.1). To support 6 snails for 3 days, a larger volume water was provided (100 mL), as well as lettuce as a food source during the exposure period. Target concentrations ranged from between 5 and 35 µg.mL^{-1} and a similar iterative approach was used to avoid wastage of crude lipopeptide extracts. Results of the 24 h exposure assays were used to infer appropriate starting concentrations for these assays. Crude

extract test concentrations were prepared in sterile 500 mL glass beakers, 325 mL of aquarium tank water was transferred to the glass beaker and then a calculated volume of crude lipopeptide concentrate in 10 mM phosphate buffer (pH 7.0) was added, to bring the new total volume to the target concentration.

Assays were performed in 500 mL PET plastic jars with perforated lids. Six *P. acuta* snails between 8-15 mm in size were placed in each jar. Jars were emptied of excess water and 100 mL of the appropriate crude lipopeptide preparation was added. Each concentration for each isolate was tested in triplicate. Assay tanks (500 mL) were stored under ambient laboratory conditions for 72 h of exposure with a small supply of lettuce (approximately 5 cm^2) provided constantly. Once every 24 h during the exposure period, snails that had climbed above the water level were returned into the water either by gentle swirling of the assay tank, or by hand. After 72 h of exposure the amended tank water was removed, retaining all snails, but discarding any remaining lettuce. Assay tanks were then topped up with approximately 400 mL aquarium tank water. Snails were provided with a supply of lettuce and observed for 72 h, assessing their health status. Control tanks were prepared in an identical fashion but without any addition of a lipopeptide solution.

5.2.3.3 Statistical analysis

Dose response curve calculations were performed using MedCalc® Statistical Software version 22.006 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2023). A probit regression analysis was performed using grouped data format. Replicates were pooled, and due to low mortality rates in control tanks, no baseline mortality adjustment was performed.

5.3 Results

5.3.1 UPLC-ESI-MS

5.3.1.1 UPLC-ESI-MS of surfactin and iturin standards

UPLC-ESI-S analysis of the lipopeptide standards were performed first to establish a baseline for identification of lipopeptides present in crude extract. As shown in Figure 5.1, surfactin

peaks began eluting between 27 minutes run time until 29 minutes, with multiple peaks visible. These were identified as a number of surfactin isoforms, with representatives of C12 through to C16 surfactin. Mass spectra for these peaks are shown in Figure 5.2, with two peaks corresponding to protonated $[M+H]^+$ and sodium adducts $[M+Na]^+$ with the expected $[M+H]^+$ values of 994.7, 1008.7, 1022.7, 1036.7 and 1050.8 *m/z* representing C12 through to C16 isoforms of surfactin (Pathak and Keharia, 2014; Barale *et al.*, 2022). The protonated $[M+H]^+$ and sodium adducts $[M+Na]^+$ were shown as two distinct peaks with the expected 22 *m/z* mass increase for the sodium adduct.

For the iturin standard, also shown in Figure 5.1, iturin peaks begin eluting between 19 minutes and 23 minutes, with isoforms of C14 through to C17 iturin being detected. Mass spectra for these peaks are shown in Figure 5.2, with protonated $[M+H]^+$ and sodium adducts $[M+Na]^+$ with the expected $[M+H]^+$ values of 1043.6, 1057.6, 1071,6 and 1085.6 *m/z* representing C14 through to C17 isoforms of iturin (Pathak and Keharia, 2014). Several peaks identifiable as surfactin isoforms were also visible in Figure 5.1, alongside a number of unidentified peaks, that did not have mass spectra associated with described lipopeptides and are thus likely contaminants.

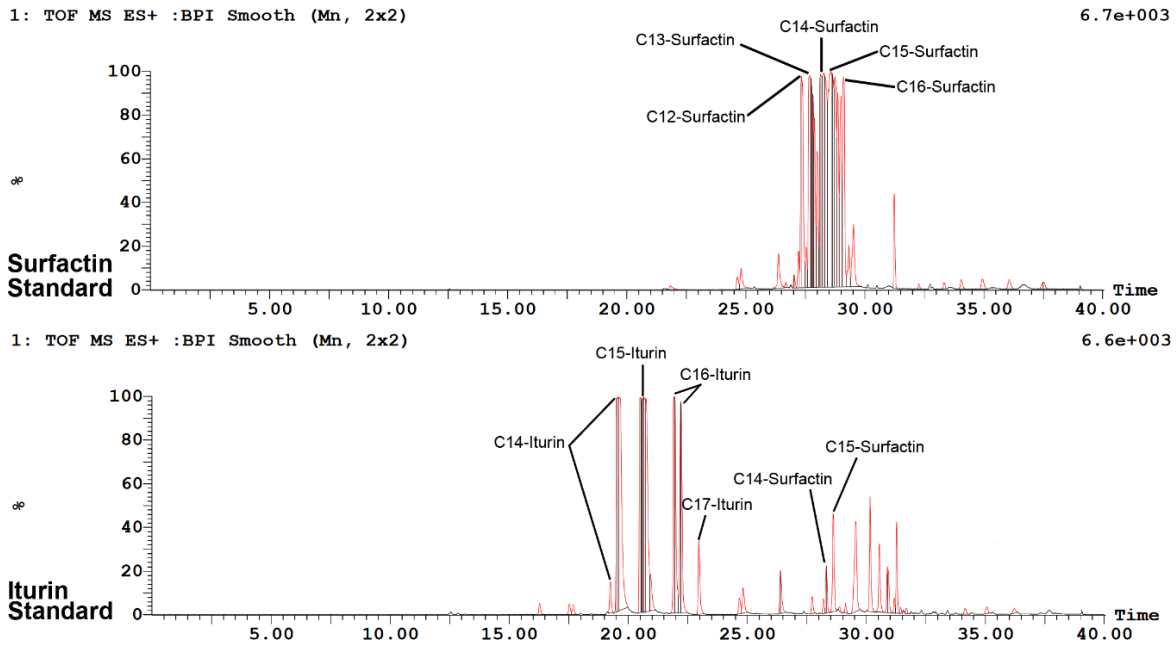

Figure 5.1. UPLC-ESI-MS chromatogram of the lipopeptide standards surfactin and iturin (Sigma Aldrich)

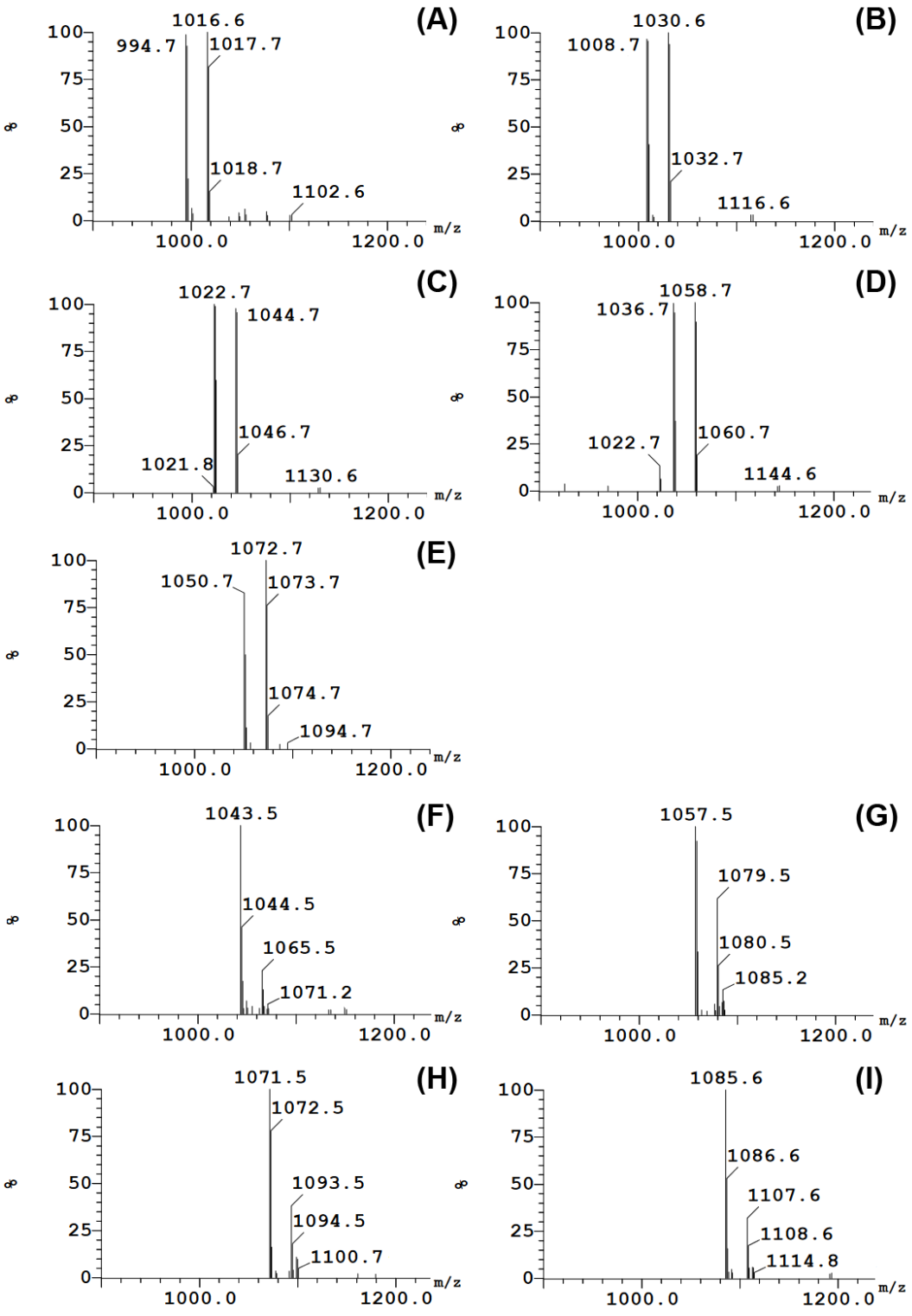

Figure 5.2. Mass spectra of surfactin and iturin isoforms identified from lipopeptide reference standards: (A) C12-Surfactin, (B) C13-Surfactin, (C) C14-Surfactin, (D) C15-Surfactin, (E) C16-Surfactin, (F) C14-Iturin, (G) C15-Iturin, (H) C16-Iturin, (I) C17-Iturin

5.3.1.2 Crude lipopeptide extract characterisation and identification

Analysis of the UPLC-ESI-MS data enabled a number of lipopeptides to be identified in the crude lipopeptide extracts. Comparison of elution times and mass spectra for surfactin and iturin isoforms from the standards allowed these compounds to be identified within this work. For lipopeptides for which standards were not sourced or unavailable, a comparison to the mass spectra values reported in the literature was used (Wang *et al.*, 2004; Bie *et al.*, 2009; Gong et al 2014; Pathak and Keharia, 2014; Barale *et al.*, 2022).

In general, protonated peaks $[M+H]^+$ were observed for all isoforms detected, with sodium adducts $[M+Na]^+$ being detected for surfactin, iturin, bacillomycin-D and -L but not for fengycin. For surfactin, iturin, bacillomycin-D and -L, a common pattern was observed with a 14 *m/z* unit difference between lipopeptide peaks which correlates with addition or removal of CH2 groups common with these compounds. For the purposes of this study, only the primary protonated peaks $[M+H]^+$ were reported here.

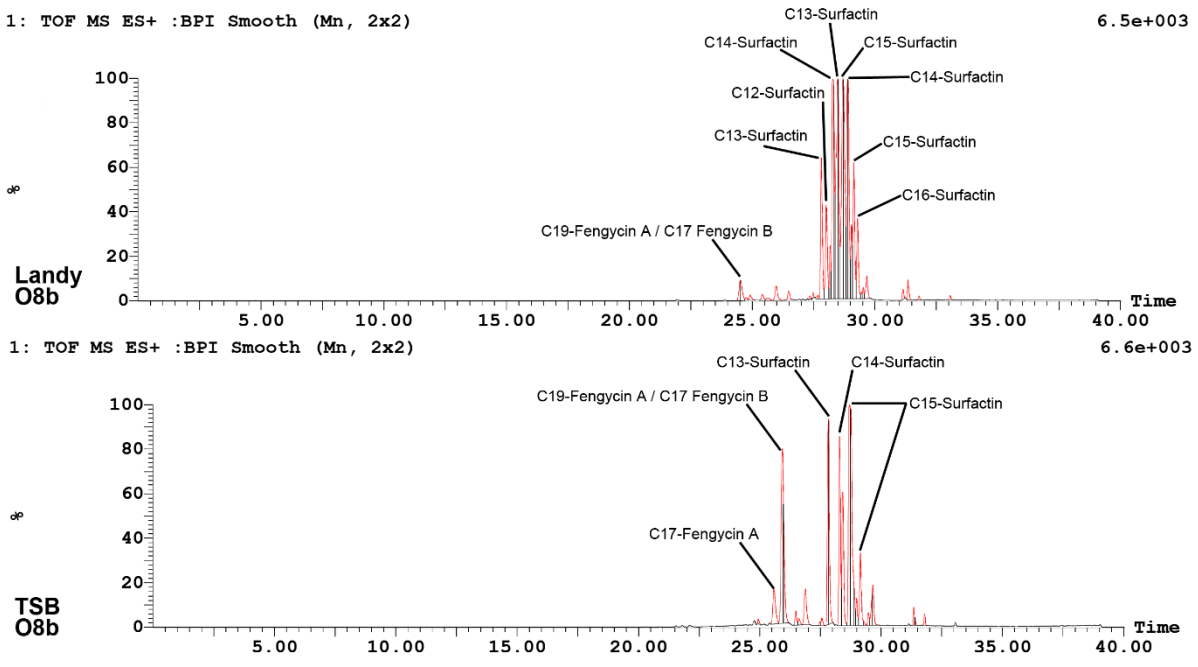

Figure 5.3. UPLC-ESI-MS chromatogram of lipopeptide extracts of isolate O8b grown in TSB and Landy medium

In Figure 5.3, the Landy medium lipopeptide extract for isolate O8b appears to primarily produce peaks correlating with surfactin isoforms of C12 through C16 surfactin was detected, although a small peak identified as either fengycin A C19 or fengycin B C17 was visible. In contrast, in the TSB lipopeptide extract, peaks of fengycin A C17 and either fengycin A C19 or fengycin B C17 were more prevalent while surfactin isoforms were less diverse and apparently at a lower peak intensity.

Figure 5.4 shows the mass spectra for individual isoform peaks for fengycin isoforms detected from isolates O8b and BB1-156a. Only protonated $[M+H]^+$ peaks appear, with no sodium adducts $[M+Na]^+$, although these have been detected by others (Luo *et al.*, 2015). Fengycin A C16 and C17 isoforms produce a unique *m/z* value of 1463.8 and 14.77.9, respectively. However, the mass spectra for fengycin A C19 or fengycin B C17 both have a *m/z* value of 1505.9, requiring further analysis to differentiate them (Wang *et al.*, 2004; Bie *et al.*, 2009).

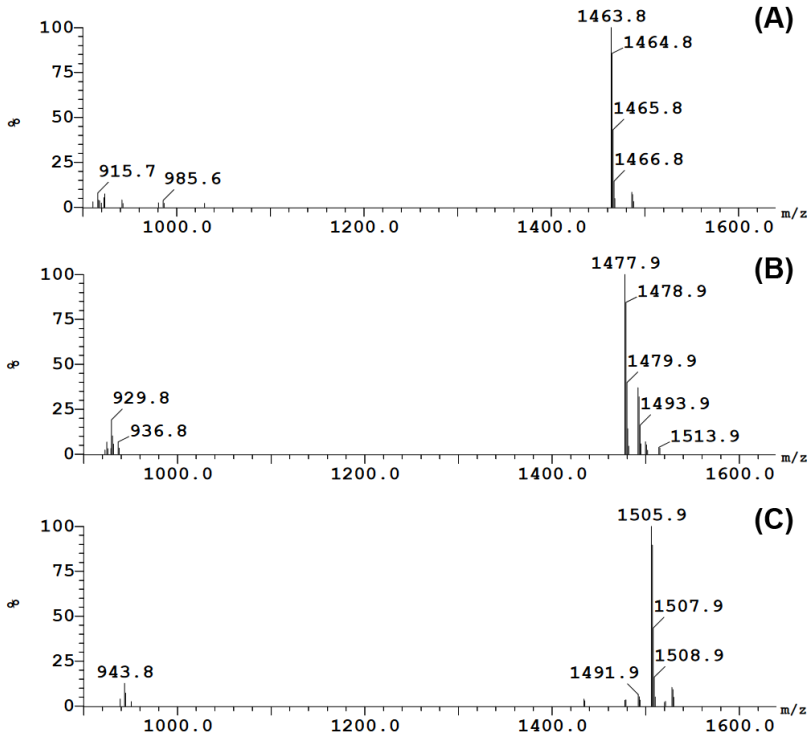

Figure 5.4. Mass spectra of putative fengycin isoforms identified in isolate O8b, TSB extract and isolate BB1-156a Landy medium extract: (A) C16-Fengycin A, (B) C17-Fengycin A, (C) C19-Fengycin A or C17-Fengycin B

In Figure 5.5, isolate R9 shows the majority of peaks identified as C13 through to C16 surfactin for both TSB and Landy medium extracts. However, there were a number of peaks appearing between 20- and 24-minutes elution, which were putatively identified as bacillomycin L, based on mass spectra results in the literature (Gu *et al.*, 2014; Luo *et al.*, 2015). Bacillomycin L was mostly noted in the Landy medium extract, with fewer lower intensity peaks recorded in the TSB extract.

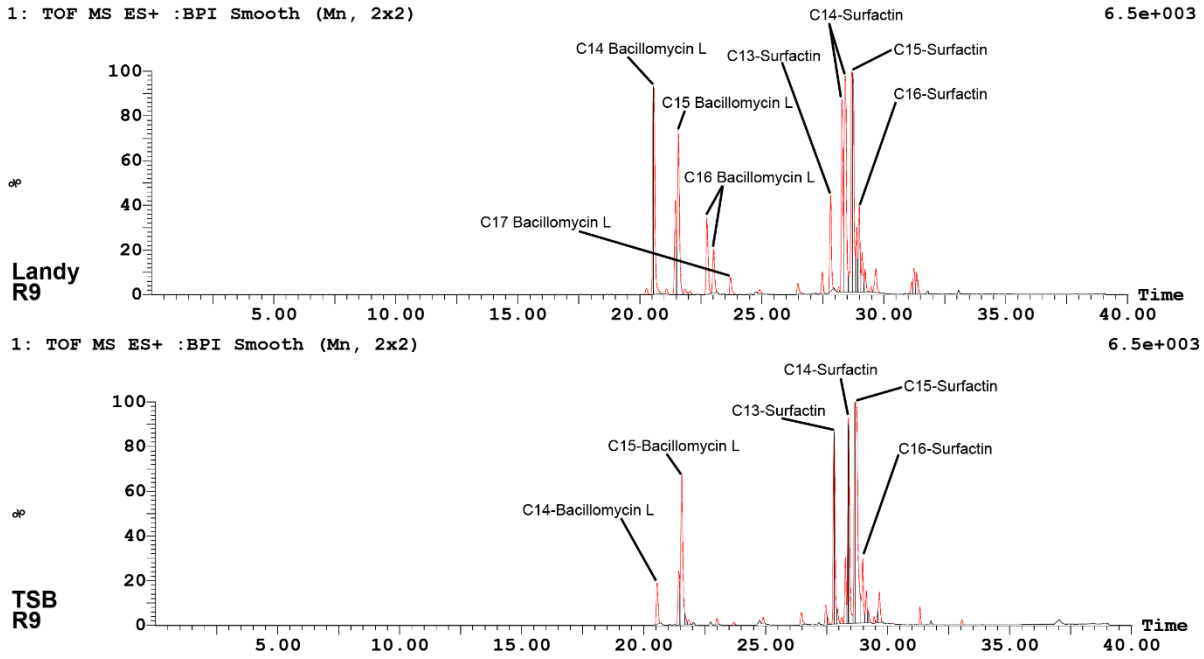

Figure 5.5. UPLC-ESI-MS chromatogram of lipopeptide extracts of isolate R9 grown in TSB and Landy medium

Figure 5.6 shows presumptive bacillomycin L mass spectra corresponding to C14 through C17 isoforms. Mass spectra for these peaks correspond to protonated $[M+H]^+$ and sodium adducts $[M+Na]^+$ (+22 *m/z*) with the expected $[M+H]^+$ values of 1021.5, 1035.6, 1049.6 and 1063.6 *m/z* corresponding to C14 through C17 bacillomycin L respectively (Luo *et al.*, 2015).

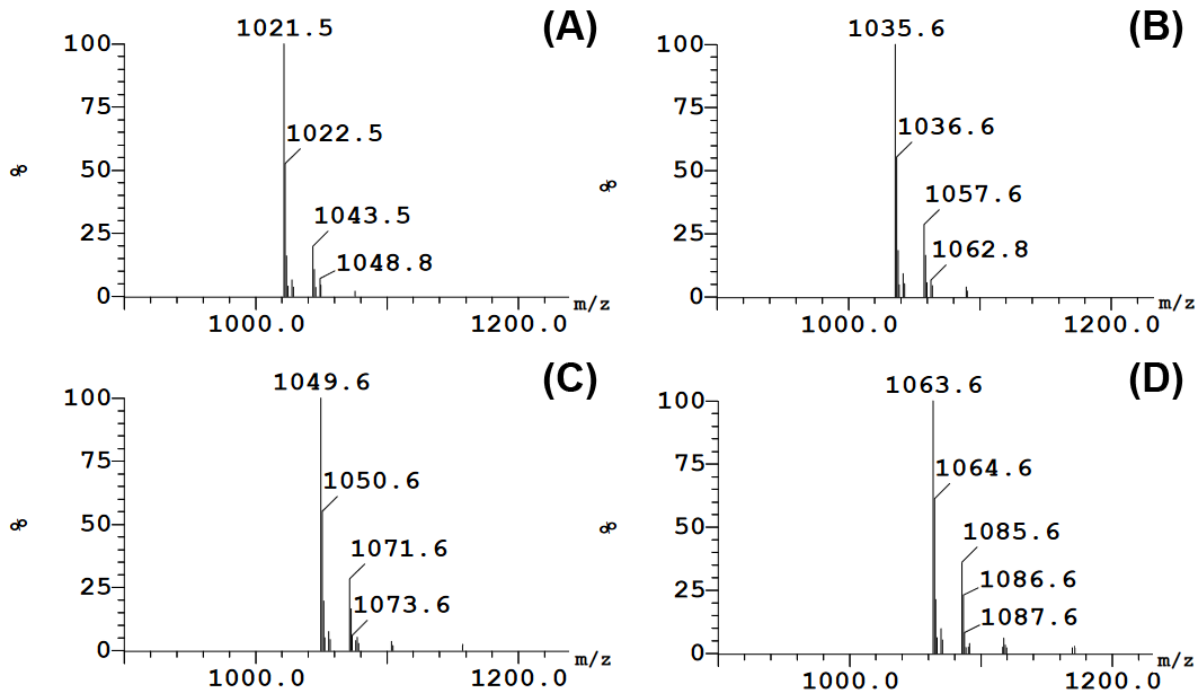

Figure 5.6. Mass spectra of putative bacillomycin L isoforms identified from isolate R9 TSB and Landy lipopeptide extract: (A) C14-Bacillomycin L, (B) C15-Bacillomycin L, (C) C16-Bacillomycin L, (D) C17-Bacillomycin L

For isolate S3 (Figure 5.7), both Landy medium and TSB extracts produce peaks putatively identified as bacillomycin D, eluting between 21 and 24 minutes, although the total peak area for these peaks in TSB extracts were larger than those of the Landy medium extracts. In addition, a number of peaks of fengycin were found in Landy medium extract but not in the TSB extracts. Presence of surfactin was noted for both extracts.

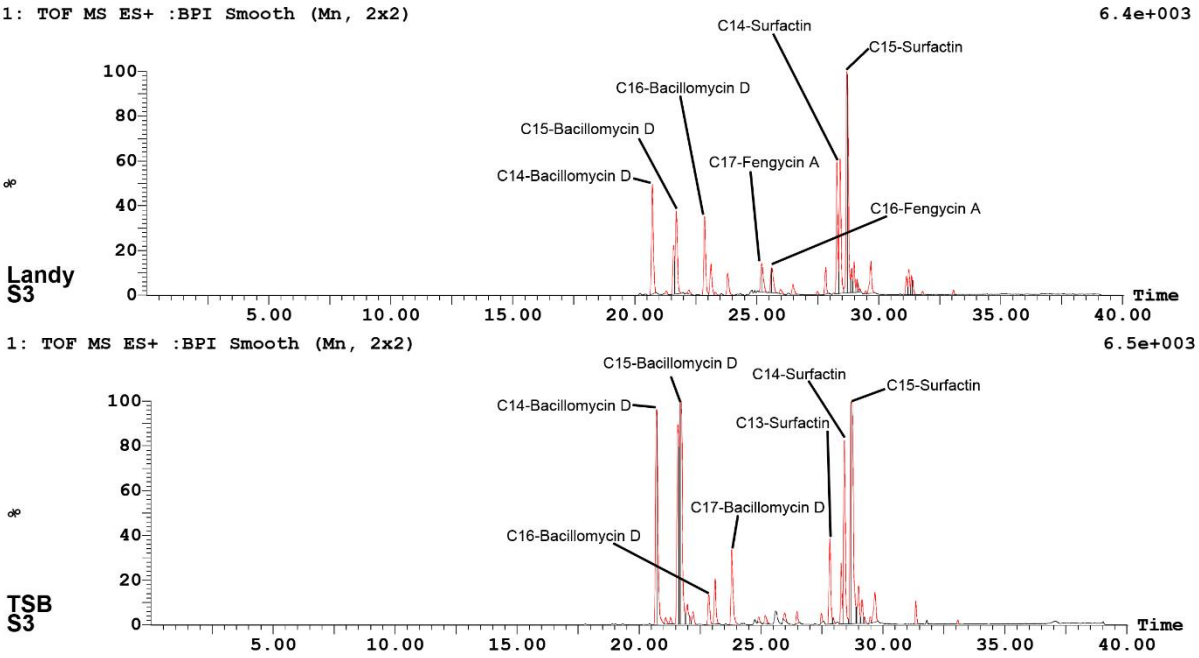

Figure 5.7. UPLC-ESI-MS chromatogram of lipopeptide extracts of isolate S3 grown in TSB and Landy medium

Figure 5.8 shows presumptive bacillomycin D mass spectra corresponding to C14 through to C17 isoforms. Mass spectra for these peaks correspond to protonated $[M+H]^+$ and sodium adducts $[M+Na]^+$ (+22 *m/z*) with the expected $[M+H]^+$ values of 1031.6, 1045.6, 1059.6 and 1073.6 *m/z* corresponding to C14 through C17 bacillomycin D, respectively (Gong *et al.*, 2014).

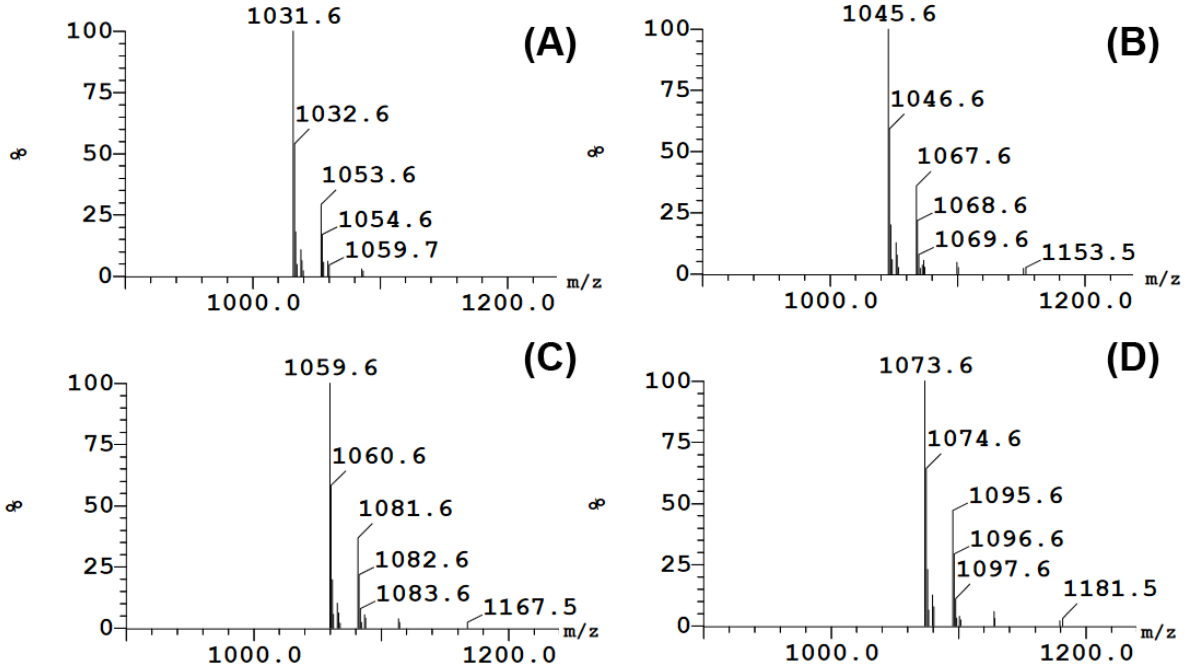

Figure 5.8. Mass spectra of bacillomycin D isoforms identified from isolate S3 TSB extract and Landy medium lipopeptide extract: (A) C14-Bacillomycin D, (B) C15-Bacillomycin D, (C) C16-Bacillomycin D, (D) C17-Bacillomycin D

For isolate U7 (Figure 5.9) both TSB and Landy medium extracts show primarily surfactin mass spectra peaks. A number of small iturin peaks were noted in the TSB extract.

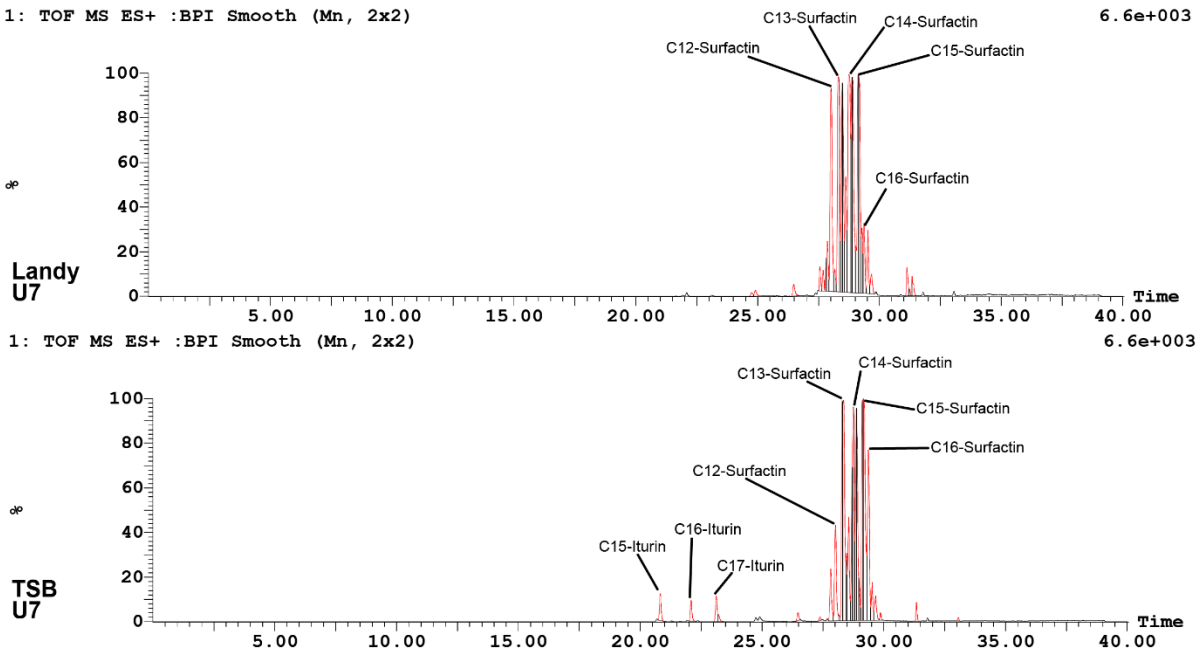

Figure 5.9. UPLC-ESI-MS chromatogram of lipopeptide extracts of isolate U7 grown in TSB and Landy medium

For isolate AX56 (Figure 5.10), both the TSB and Landy medium extracts show primarily peaks, which can be identified as surfactin. While a single peak of iturin (C14) was noted in the Landy medium extract, two (C14 and C15) were found in the TSB extract. Fengycin peaks were found in the Landy medium extract only.

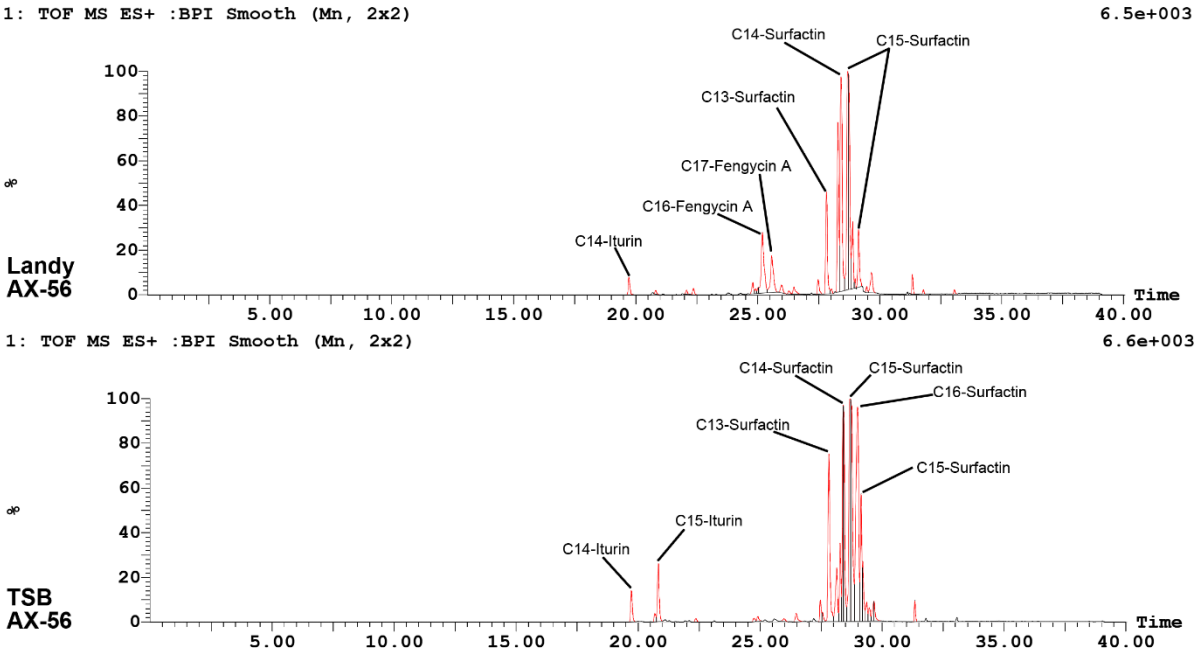

Figure 5.10. UPLC-ESI-MS chromatogram of lipopeptide extracts of isolate AX56 grown in TSB and Landy medium

For isolate BB1-156a (Figure 5.11), surfactin was present at apparently lower peak area than other lipopeptide components. For Landy medium, the largest peak was that of iturin. This was in contrast to any previous chromatograms where surfactin has been the dominant peak. Peaks of iturin were present for both extracts, although primarily for the Landy medium extract. The Landy medium extract also contained fengycin isoforms.

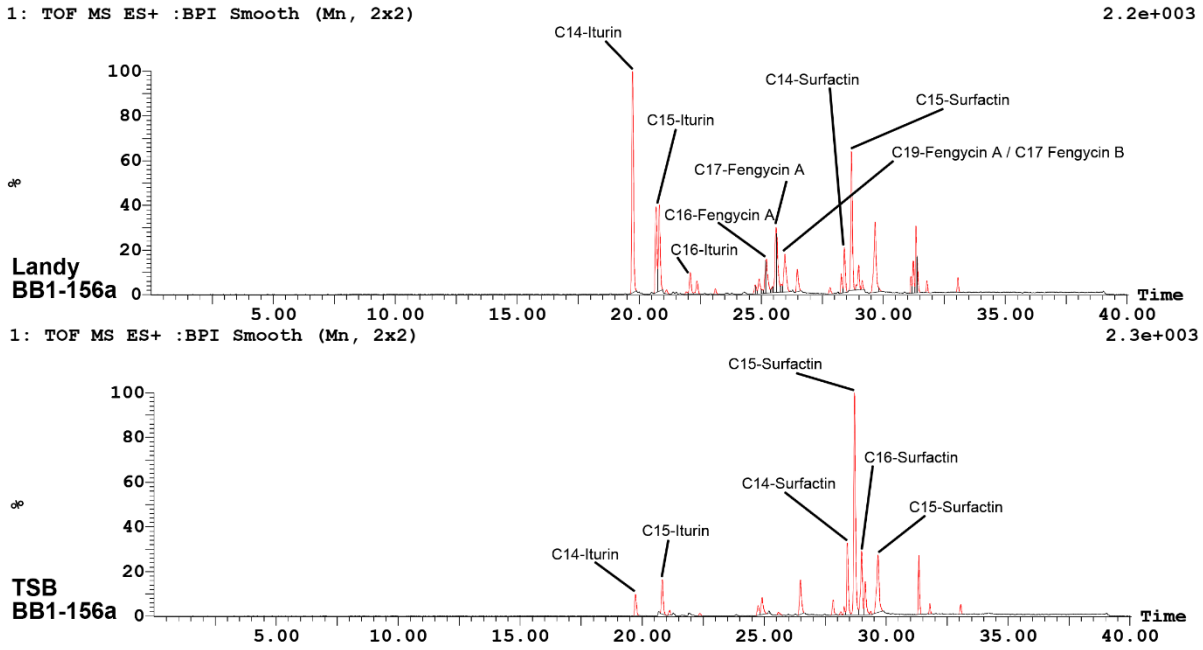

Figure 5.11. UPLC-ESI-MS chromatogram of lipopeptide extracts of isolate BB1-156a grown in TSB and Landy medium

Table 5.1 shows a comparison of the identified lipopeptides from all six isolates across both growth media. Although relative peak area is only semi-quantitative, this does provide some useful information as to the prevalence of the compounds detected. This shows the ubiquity of surfactin in the extracts tested.

Table 5.1. Putative identification of lipopeptides in crude lipopeptide extracts of TSB and Landy broth media

		LANDY						TSB					
m/z	**Isoform**	**O8b**	**R9**	**S3**	**U7**	**AX56**	**BB1-156a**	**O8b**	**R9**	**S3**	**U7**	**AX56**	**BB1-156a**
994.7	C12-surfactin	+ (4.9)	-	-	+ (12.8)	+ (1.1)	-	-	+ (1.2)	-	+ (7.4)	-	-
1008.7	C13-surfactin	+ 24.3)	+ (4.5)	+ (1.9)	+ (29.1)	+ (7.8)	-	+ (14.6)	+ (15.2)	+ (4.3)	+ (18.8)	+ (10.2)	+ (2.0)
1022.7	C14-surfactin	+ (32.1)	+ (23.0)	+ (19.4)	+ (28.3)	+ (31.2)	+ (4.8)	+ (18.8)	+ (19.1)	+ (11.5)	+ (31.1)	+ (22.7)	+ (9.1)
1036.7	C15-surfactin	+ (25.7)	+ (20.0)	+ (12.2)	+ (16.6)	+ (37.5)	+ (11.3)	+ (34.0)	+ (31.6)	+ (20.7)	+ (22.1)	+ (26.1)	+ (38.5)
1050.8	C16-surfactin	+ (3.4)	+ (5.9)	-	+ (4.8)	-	+ (2.0)	-	+ (4.4)	+ (2.1)	+ (10.6)	+ (19.27)	+ (9.9)
1031.6	C14-bacillomycin D	-	-	+ (9.4)	-	-	-	-	-	+ (14.6)	-	-	-
1045.6	C15-bacillomycin D	-	-	+ (11.7)	-	-	-	-	-	+ (31.3)	-	-	-
1059.6	C16-bacillomycin D	-	-	+ (9.7)	-	-	-	-	-	+ (4.6)	-	-	-
1073.6	C17-bacillomycin D	-	-	+ (1.9)	-	-	-	-	-	+ (4.2)	-	-	-
1463.8	C16-fengycin A	-	-	+ (3.5)	-	+ (7.6)	+ (4.6)	-	-	-	-	-	-
1477.9	C17-fengycin A	-	-	+ (3.3)	-	+ (4.7)	+ (9.1)	+ (3.3)	-	-	-	-	-
1505.9	C19 fengycin A/C17 fengycin B						+ (4.6)	+ (12.9)					
1043.6	C14-Iturin	-	-	-	-	+ (1.2)	+ (18.9)	-	-	-	-	+ (1.7)	+ (3.1)
1057.6	C15-Iturin	-	-	-	-	-	+ (14.5)	-	-	-	+ (1.2)	+ (3.8)	+ (5.5)
1071.6	C16-Iturin	-	-	-	-	-	+ (3.3)	-	-	-	-	-	-
1085.6	C17-Iturin	-	-	-	-	-	-	-	-	-	+ (1.4)	-	-
1021.5	C14-Bacillomycin L	-	+ (14.2)	-	-	-	-	-	+ (2.9)	-	-	-	-
1035.6	C15-Bacillomycin L	-	+ (14.2)	-	-	-	-	-	+ (15.6)	-	-	-	-
1049.6	C16-Bacillomycin L	-	+ (6.5)	-	-	-	-	-	-	-	-	-	-
1063.6	C17-Bacillomycin L	-	+ (1.0)	-	-	-	-	-	-	-	-	-	-

Numbers in brackets were the peak area percentage for that isoform. Only identified peaks with an area of ≥ 1% included.

5.3.2 Dose response assays

Dose response assays of crude lipopeptide extracts against *P. acuta* with 24 h and 72 h exposures were performed. The effective concentrations of molluscicidal activity fell into a narrow range for all seven lipopeptide extracts tested (Figure 5.12). At lower concentrations, no mortality was recorded, while 100% mortality was observed at the higher concentrations, with both of these points lying between 10 and 20 $\mu g.mL^{-1}$ for the most potent extracts and between 20 and 55 $\mu g.mL^{-1}$ for the weaker of the extracts tested. Control tank mortality for these assays comprised 22 tanks containing 6 snails each for a total population size of 132 snails. The observed mortality count was zero.

The visible response to increasing concentrations of crude lipopeptide extract was similar for all those tested. Responses to exposure were visible shortly after initial exposure to the crude extracts, this included snails losing attachment to the sides of the assay tank, snail bodies either extending from or retracting into the shell, and sinking or floating of the snail. Observed behaviour early in the assay after approximately 1 h was similar to that observed on transfer from the assay tanks to observation tanks. In some cases, where exposure had produced a visible change in behaviour, some snails were found to have crawled above the treated water.

For the higher mortality rate doses, a common observation was all 6 snails either sunk to the bottom of the tank, or floated either with the snail body retracted into the shell, or extended outside the shell, with no apparent movement or attachment to the assay tank walls. These observations were linked to complete mortality at higher concentrations, but at lower concentrations recovery was a common occurrence, even while visually indistinguishable just before transfer to the recovery tanks. Visible recovery was usually quick (within hours). However, some snails were observed to remain retracted into the shell for a number of days before normal behaviour was observed on the final day of observation. The strength of the effect and the rate of recovery was observed to follow the increase in crude lipopeptide concentrations.

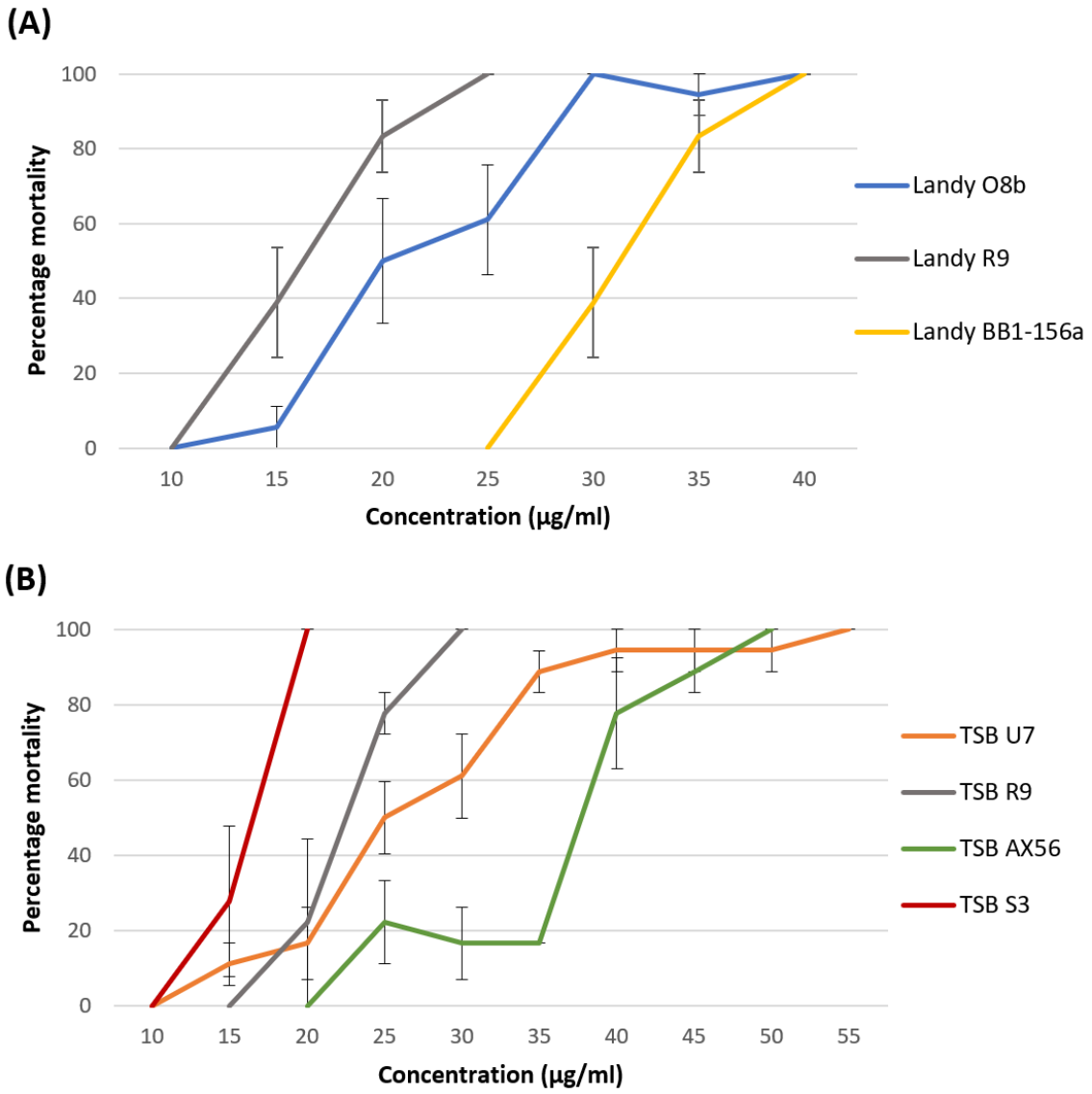

Figure 5.12. Mortality percentages after exposure to crude lipopeptide extracts for 24 hours: (A) Landy medium and (B) TSB media lipopeptide extracts. Each data point is the pooled mortality of 18 snails. Error bars represent the standard error

The data shown in Figure 5.13 was similar to that of Figure 5.12. However, the measurable levels of activity were shifted towards lower concentrations of crude lipopeptide extracts. An observation was that the concentrations of crude lipopeptide extract that had an observable effect on the snails (i.e. causing them to lose their attachment to the sides of the tank, extending from or retreating into the shell, or sinking and floating), these concentrations were associated with high levels of mortality when exposure was increased to 72 h. In general, exposure for 72 h resulted in a narrower range of concentrations within which 0 and 100% mortality was observed. Generally, snails that appeared dead during the 72 h exposure did not recover during

the observation period, unlike that of the 24 h exposure assays. Data for the TSB extract of isolate R9 was limited to two concentrations tested as insufficient material was available to complete these tests. Control tank mortality for these assays comprised 15 tanks containing 6 snails each for a total population size of 90 snails, in which the observed mortality count was zero.

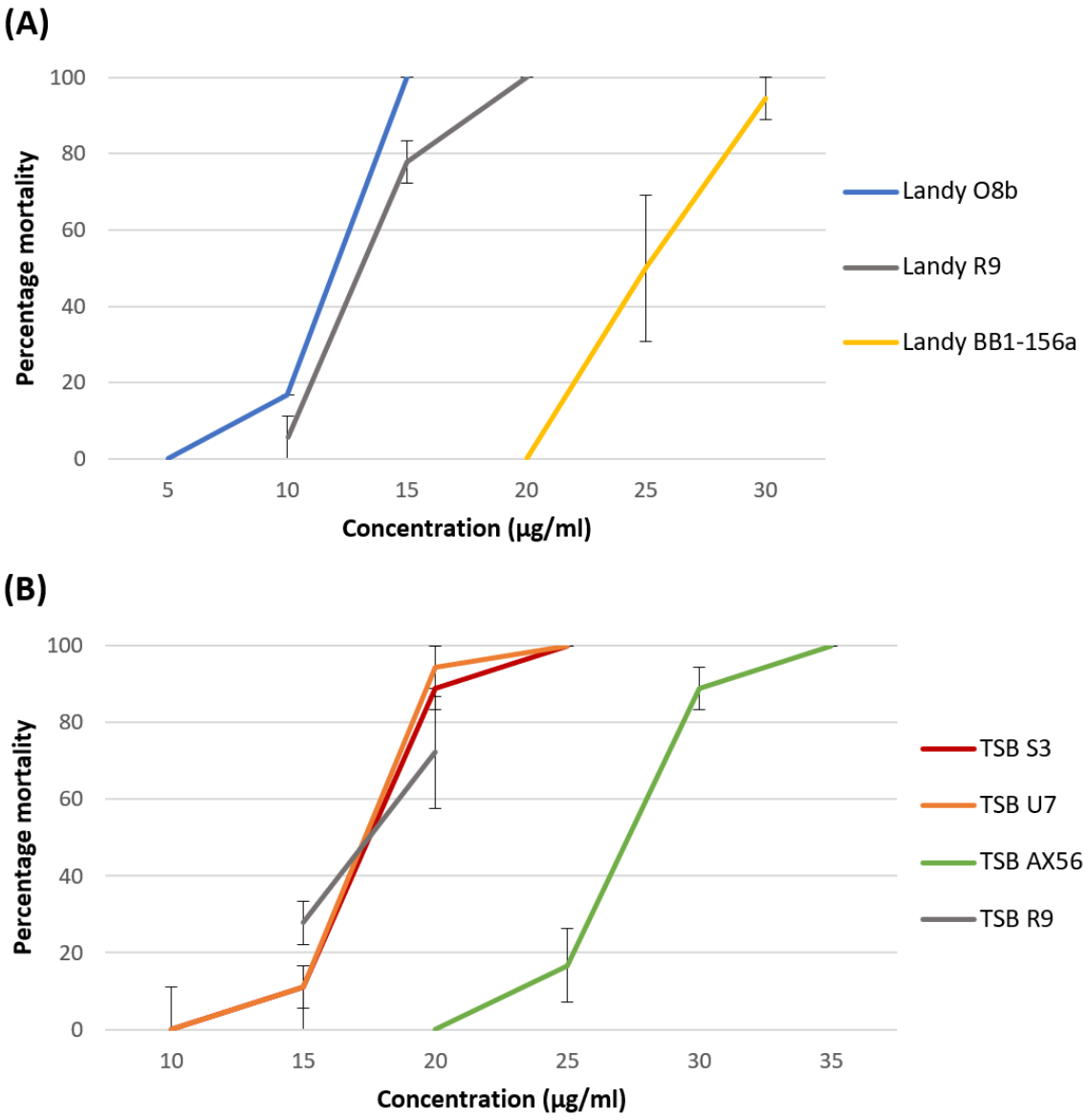

Figure 5.13. Mortality percentages after exposure to crude lipopeptide extracts for 72 hours: (A) Landy medium and (B) TSB media lipopeptide extracts. Each data point is the pooled mortality of 18 snails. Error bars represent the standard error

Tables 5.2 and 5.3 show calculated value for the dose response curve for *P. acuta* exposed to crude lipopeptide extracts for 24 h and 72 h, respectively. TSB R9, 72 h exposure was omitted due to insufficient data points for statistical analysis. Measured lethal concentration (LC) values varied between crude lipopeptide extracts, with the strongest extracts being that of TSB extract from isolate S3 for 24 h exposure with an LC_{50} of 16.37 µg.mL^{-1} (95% CI 15.10-17.69) and Landy medium extract from isolate O8b for 72 h exposure with and LC_{50} of 9.39 µg.mL^{-1} (95% CI 10.72-13.15). The weakest extract tested was the TSB extract from isolate AX56 for 24 h exposure with an LC_{50} of 26.16 µg.mL^{-1} (95% CI 30.45-42.14) and Landy medium extract from isolate BB1-156a for 72 h exposure with an LC_{50} of 25.0 µg.mL^{-1} (95% CI 23.49-26.51). A similar pattern was observed when comparing LC_{10}, LC_{90} and LC_{95} values.

Table 5.2. Dose response of *Physella acuta* exposed to crude lipopeptide extracts for 24 hours

Extract	LC_{10} (µg.mL^{-1})	LC_{50} (µg.mL^{-1})	LC_{90} (µg.mL^{-1})	LC_{95} (µg.mL^{-1})
Landy O8b	14.12 (10.04-16.61)	21.77 (19.77-23.75)	29.42 (26.97-33.43)	31.59 (28.76-36.43)
Landy R9	12.42 (9.24-14.08)	16.45 (14.96-17.93)	20.48 (18.83-23.63)	21.62 (19.74-25.43)
Landy BB1-156a	27.53 (24.45-29.09)	31.17 (29.74-32.59)	34.81 (33.26-37.86)	35.84 (34.08-39.53)
TSB R9	18.75 (15.66-20.33)	22.50 (21.06-23.94)	26.25 (24.67-29.34)	27.31 (25.51-31.05)
TSB S3	13.51 (10.67-14.84)	16.37 (15.10-17.69)	19.23 (17.88-22.17)	20.05 (18.51-23.60)
TSB U7	14.83 (9.76-18.21)	27.10 (24.56-29.56)	39.38 (36.19-44.07)	42.86 (39.15-48.52)
TSB AX56	26.42 (9.92-31.72)	36.16 (30.45-42.14)	45.91 (40.51-63.05)	48.68 (42.54-69.79)

Numbers in brackets are 95% CI intervals. MedCalc® Statistical Software version 22.006 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2023)

Table 5.3. Dose response of *P. acuta* exposed to crude lipopeptide extracts for 72 hours

Extract	LC_{10} (µg.mL^{-1})	LC_{50} (µg.mL^{-1})	LC_{90} (µg.mL^{-1})	LC_{95} (µg.mL^{-1})
Landy O8b	9.39 (7.04-10.59)	11.89 (10.72-13.15)	14.39 (13.13-17.00)	15.09 (13.69-18.20)
Landy R9	10.68 (7.84-11.99)	13.37 (12.08-14.60)	16.06 (14.80-18.71)	16.83 (15.41-20.04)
Landy BB1-156a	20.98 (17.58-22.66)	25.00 (23.49-26.51)	29.02 (27.34-32.42)	30.16 (28.23-34.29)
TSB S3	14.88 (12.42-16.14)	17.50 (16.27-18.73)	20.12 (18.86-22.58)	20.86 (19.46-23.81)
TSB U7	14.89 (12.81-16.03)	17.17 (16.04-18.39)	19.45 (18.26-21.75)	20.09 (18.78-22.81)
TSB AX56	24.32 (21.61-25.66)	27.21 (25.93-28.51)	30.11 (28.76-32.85)	30.93 (29.42-34.23)

Numbers in brackets are 95% CI intervals. MedCalc® Statistical Software version 22.006 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2023)

Measured LC values decreased with increased exposure time; however, the observed changes were different in magnitude between extracts. For example, the extract from isolate S3 in TSB broth showed very little increase in LC value with an increase in exposure time, while isolate O8b Landy medium extract and U7 TSB extract both showed a larger proportional change in activity with increased exposure time (with an LC_{90}/LC_{95} roughly half of 24 h exposure).

5.4 Discussion

This chapter set out to characterise the component lipopeptides associated with crude extracts from selected *Bacillus* spp. and measure the molluscicidal activity of these crude extracts in dose response assays. Surfactin was the only constant lipopeptide present between each isolate's crude lipopeptide extracts from both TSB and Landy medium. While other lipopeptides were detected, the extracts from three isolates (U7, AX56, and O8b) were dominated by surfactin isoforms. This suggests that, if the molluscicidal component of these extracts was indeed a lipopeptide, surfactin was therefore the active molluscicidal component. In the extracts of the remaining three isolates (S3, R9 and BB1-156a), surfactin was also a significant component. However, the presence of other lipopeptides in large proportions — bacillomycin D for isolate S3, iturin for isolate BB1-156a, and bacillomycin L for isolate R9 —suggest that these compounds were also contributing to the observed molluscicidal activity. It was interesting that these three isolates were strongly-performing extracts, which suggested that there was either a molluscicidal role for these lipopeptides, or a synergistic effect with surfactin. Synergistic effects have been reported with a variety of lipopeptide combinations

(Pathak and Keharia, 2014). Assays with pure lipopeptide standards would be required to fully elucidate the role of individual lipopeptide families for their molluscicidal activity.

While mass spectra peak area is only a crude indicator of relative concentrations of lipopeptide in extracts, this did give some indications as to relative lipopeptide proportions. Based on peak area, surfactin was the most abundant lipopeptide for most extracts. Exceptions to this were found in the TSB S3 extract which had a higher percentage of bacillomycin D peaks; the Landy medium BB1-156a extract, which had a higher percentage of iturin peaks; and extracts from isolate R9, which had significant levels of bacillomycin L (Particularly in the Landy medium extract). Peak area also does not take into account any non-lipopeptide constituents of the crude lipopeptide extracts. For example: Isolate O8b Landy lipopeptide extract was predominantly surfactin in composition, and had a strongly molluscicidal effect with an LC_{90} of 29.42 $\mu g.mL^{-1}$, for the 24 h exposure (95% CI 26.97-33.43). In comparison the TSB crude lipopeptide extract of O8b, which was also predominantly surfactin, did not show a strong molluscicidal effect at 100 $\mu g.mL^{-1}$ for 24 h (Chapter 4). Similarly, isolate AX56 had a very similar lipopeptide profiles between Landy medium and TSB extracts, but also the two extracts had very different molluscicidal activity: The LC_{90} of AX56 TSB extract was 45.91 $\mu g.mL^{-1}$ (95% CI 40.51-63.05) with a 24 h exposure, compared to no strong molluscicidal activity at 100 $\mu g.mL^{-1}$ after 24 h with the Landy medium lipopeptide extract (Chapter 4). These results for isolates O8b and AX56 strongly suggest the presence of non-lipopeptide components in the crude extracts. While the lipopeptide profiles may be similar, the concentrations of active compounds were significantly different. It is possible that these non-lipopeptide components were incidental contaminants, simply adding mass to the extract and thus reducing the concentration of lipopeptides. However, it is also possible that a molluscicidal compound was co-extracted with the lipopeptide extraction technique but was not identifiable by the lipopeptide UPLC-ESI-MS protocol used here. Elucidation of either possibility would require either confirmatory snail bioassays using pure lipopeptide compounds; an accurate measurement of lipopeptide concentrations in crude extracts; or purification of crude lipopeptide extracts to a higher level of purity.

While no reports exist that identify lipopeptides as molluscicidal in nature, a wide range of organisms (i.e., fungi, bacteria, insects) are sensitive to surfactin, which increases the likelihood that surfactin has more molluscicidal properties than other lipopeptide variants,

which appear to have much narrower ranges of activity. A different avenue of aquatic snail biocontrol has revolved around the use of plant extracts, and numerous plant species have been identified with molluscicidal activity such as *Sapindus mukorossi* (Gaertn., Soapnut) extracts against *Pomacea canaliculata* (Lamarck, 1819, Golden apple snails) and *Achyranthes aspera* (Linnaeus, 1753) against *Biomphalaria pfeifferi* (Krauss, 1848) and *Radix natalensis* (Krauss, 1848) (Huang *et al.*, 2003; Mandefro *et al.*, 2017). A general trend in this research is the identification of saponins as molluscicides. While direct comparison between different snails' species and assays methodologies is difficult, crude lipopeptide extracts compare favourably to the saponins for the LC_{50} values reported. If the mode of action of saponins is due to their surfactant properties, as has been suggested by the literature, it would stand to reason that Surfactin, as a powerful biological surfactant, would have similar molluscicidal effects.

This chapter identified surfactin as a strong candidate for the molluscicidal actions of the extracts of the six *Bacillus* isolates studied. Therefore, future work to further evaluate surfactin as a molluscicide will involve bioassays against snails using purified surfactin.

5.5 References

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CHAPTER SIX: EVALUATION OF SURFACTIN AS A MOLLUSCICIDE AND SENSITIVITY OF ZEBRAFISH EMBRYOS TO LIPOPEPTIDES

Abstract

Previous research in this project identified surfactin as a potential molluscicidal metabolite present in cell-free culture supernatant of a number of isolates of *Bacillus* spp.. This chapter aims to evaluate whether pure surfactin has molluscicidal properties against two aquatic snail species, *Physella acuta* (Draparnaud, 1805), which was used in initial screening, and secondly against *Pseudosuccinea columella* (Say, 1817), a host snail of *Fasciola* liver fluke. The molluscicidal efficacy of crude lipopeptide extracts and pure surfactin were assessed against a test fish species, *Danio rerio* (F. Hamilton, 1822), as a first step in evaluating ecotoxicity. Commercially-available pure surfactin was found to be molluscicidal against *P. acuta* and *P. columella* with LC_{50} values of 10.04 $\mu g.mL^{-1}$ and 16.58 $\mu g.mL^{-1}$ respectively and LC_{90} of 12.29 $\mu g.mL^{-1}$ and 19.15 $\mu g.mL^{-1}$ respectively, with a 24 h contact time. Crude lipopeptide extracts demonstrated lethal effects on 24 hpf (hours post fertilisation) *D. rerio* embryos with an LC_{50} of between 10.19 and 44.93 $\mu g.mL^{-1}$ with a 24 h contact time. *Danio rerio* exposure to pure surfactin exposure was found to be lethal, with LC_{50} and LC_{90} values of 7.96 and 11.45 $\mu g.mL^{-1}$ respectively, with a 24 h exposure period. In comparison to 24 hpf embryos, 24 hpf eggs are still encased in the chorion, as well as 96 hpf embryos showed lower sensitivity to surfactin with LC_{50} values of 13.96 and 12.26 $\mu g.mL^{-1}$ and LC_{90} values of 17.61 and 16.87 $\mu g.mL^{-1}$, respectively, with 24 h contact times. This is the first report of surfactin having molluscicidal characteristics, but it does raise some ecotoxicological concerns for its potential use as a molluscicide.

6.1 Introduction

Surfactin is a powerful amphiphilic biosurfactant produced by a number of *Bacillus* spp. that has been evaluated for its usefulness in a number of fields including medical, agricultural, food, and petrochemical industries (Zhen *et al.*, 2023, Seydlová and Svobodová 2008, Ongena and Jacques 2007). Surfactin has antimicrobial characteristics which have been applied in plant disease biocontrol, as well as activity against a number of pest insect species such as aphids,

mosquitos and flies (Geetha *et al.*, 2010; Denoirjean *et al.*, 2021, Ngalimat *et al.*, 2021, Ramesar and Hunter, 2023). In a biocontrol capacity, one avenue that has not been explored is the effect of surfactin on aquatic snail pests. Niclosamide, the standard molluscicidal chemical used in aquatic snail control, is highly toxic to fish species (Sokolow *et al.*, 2016, Ross *et al.*, 2017, Zheng *et al.*, 2021).

Surfactin is considered to be less toxic than synthetic surfactants with a higher degree of biodegradability. However, its environmental effects on aquatic species have not been determined in the situation where it is used as a biopesticide in aquatic environments (Deravel *et al.*, 2014, De Oliveira *et al.*, 2017, Fei *et al.*, 2019). Its mammalian toxicity is particularly low, with a no-observed-adverse-effect level (NOAEL) of 500 $mg.Kg^{-1}$ when fed to rats (Hwang *et al.*, 2009). Evaluation of ecotoxicity is an essential step before any pest control agent can be registered and while some aquatic species have been evaluated for sensitivity to surfactin, these results are varied, from extremely sensitive *Anopheles stephensi* (Liston, 1901) to relatively unaffected *Daphnia magna* (Straus. 1820) (Geetha *et al.*, 2010; De Oliveira *et al*. 2017). Research into the impacts of surfactin on fish species has generally revolved around stimulation of growth and other positive effects of this compound, suggesting it may be tolerated well in aquatic environments (Zhai *et al.*, 2016; Wang *et al.*, 2021).

Surfactin was identified as a candidate molluscicide from crude lipopeptide extracts in previous work within this research project (Chapter 5). This chapter aimed firstly to evaluate the activity of pure surfactin as a molluscicide against a proxy aquatic snail, *P. acuta,* and the liver fluke host snail, *P. columella*. Secondly, molluscicidal crude lipopeptide extracts and pure surfactin were assayed against zebrafish (*D. rerio*) embryos to investigate the potential ecotoxicological impacts that this compound may have on non-target aquatic species.

6.2 Materials and Methods

6.2.1 Surfactin standard preparation

A surfactin standard was prepared by dissolving a 50 mg sample of surfactin (Product code S3523, CAS no. 24730-31-2, ≥98.0% HPLC, Sigma-Aldrich, USA) in 10mM phosphate buffer (pH 7.0) and making the solution up to a final volume of 50 mL in a volumetric flask for a

stock concentration of 1 mg.mL^{-1}. This surfactin stock solution was subdivided and stored at -20°C until needed for exposure assays.

6.2.2.1 Physella acuta 24 h surfactin exposure assay

Measurement of the surfactin dose response curve against *P. acuta* with 24 h exposure time was performed using the protocol documented in Chapter 5.2.3.1. Surfactin was tested at concentrations in 2.5 µg.mL^{-1} increments from 5 to 17.5 µg.mL^{-1}, aiming for a range of mortality between 0 and 100%.

Surfactin assay suspensions were prepared in sterile 250 mL glass beakers; 35 mL of aquarium tank water was transferred into the glass beaker and then a volume of surfactin in 10 mM phosphate buffer (pH 7.0) was added, to bring the new total volume to the target concentration. Assays were performed in 50 mL plastic sample jars with perforated lids. Six *P. acuta* snails between 8.0 to 15.0 mm in size were placed in each jar. The water in each bioassay jar was drained and replaced with 10 mL of the appropriate surfactin preparation. Assay tanks were stored under ambient laboratory conditions for 24 h of exposure before transfer of the snails into 500 mL PET assay jars containing 400 mL aquarium tank water. The snails were observed for 72 h, assessing their health status. A total of 9 replicates were performed for each concentration tested. Control tanks were prepared in an identical fashion but without the addition of any surfactin and were run in parallel.

6.2.2.2 Physella acuta 72 h surfactin exposure assay

Measurement of the surfactin dose response curve against *P. acuta* with 72 h exposure time was performed using the protocol documented previously in Chapter 5.2.3.2. Surfactin was tested in 2.5 µg.mL^{-1} increments from 5 to 12.5 µg.mL^{-1}, targeting a range of mortality between 0 and 100%. Surfactin concentrations were prepared in sterile 500 mL glass beakers; 325 mL of aquarium tank water was transferred to the glass beaker and then a calculated volume of surfactin standard in 10 mM phosphate buffer (pH 7.0) was added, to bring the new total volume to the target concentration.

Assays were performed in 500 mL PET plastic jars with perforated lids. Six *P. acuta* snails between 8.0 to 15.0 mm in size were placed in each jar. Jars were emptied of excess water and 100 mL of the appropriate surfactin preparation was added. Assay tanks (500 mL) were stored under ambient laboratory conditions for 72 h of exposure with a small supply of lettuce (approximately 5 cm^2) provided constantly. Once every 24 h during the exposure period, snails that had climbed above the water level were returned into the water either by gentle swirling of the assay tank, or by hand. After 72 h of exposure the amended tank water was removed, retaining all snails, but discarding any remaining lettuce. Assay tanks were then topped up with approximately 400 mL aquarium tank water. Snails were provided with a supply of lettuce and observed for 72 h, assessing their health status. A total of 3 replicates, each of which contained 6 snails were performed for each concentration interval. Control tanks were prepared in an identical fashion but without any addition of a lipopeptide solution.

6.2.2.3 Pseudosuccinea columella assay 24 h surfactin exposure assay

Measurement of the surfactin dose response curve against *P. columella* was performed using the same protocol as used in Section 6.2.2.1. Due to the larger size of the *P. columella* snails, only three snails per tank were included in each replicate. Snails of 15.0 to 20.0 mm in length were chosen for these assays. A total of 7 replicates, each of which contained 3 snails were performed for each concentration tested. Surfactin concentrations were tested in 2.5 $\mu g.mL^{-1}$ increments from 10 $\mu g.mL^{-1}$ to 22.5 $\mu g.mL^{-1}$. Control tanks containing a final concentration of 1 mM pH 7.0 phosphate buffer as well as surfactin and phosphate buffer free controls were run in parallel.

6.2.2.4 Assessments for mortality

Mortality was assessed by observation of snails post exposure during the recovery period. Snails were assessed by observation of movement and feeding behaviour. Lack of movement, inability to adhere to the tank walls and protrusion of the snail body from the shell, or retraction within the shell were indications of mortality with consistent lack of movement on the final day of recovery indicating mortality.

6.2.3 Zebrafish lipopeptide exposure assays

6.2.3.1 Zebrafish embryo preparation

All zebrafish studies were carried out with ethics approval (AREC/00005213/2023) being granted for the project entitled, “Establishment of Zebrafish models for the study of human disease and toxicology”, attached in Appendix J. *Danio rerio* were sourced from the Zebrafish unit, UKZN, Pietermaritzburg. Fertilized eggs were collected soon after spawning and were washed multiple times with system water to remove any debris and dead eggs. Washed eggs were incubated at 28°C for 24 h. Dead eggs were removed, and the remaining eggs were washed with aquarium water before being treated with 5 mL Pronase in E3 medium (5 mM NaCl, 0.17 mM KCL, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ at pH 7.2) at 1 $mg.mL^{-1}$ for 45 minutes to remove the chorion. After incubation these embryos were washed with aquarium water and incubated at 28°C for 1 h. Embryos were then inspected, and any deformed or dead embryos removed. Examples of embryos just after fertilisation and cleaning and at 24 hpf before and after chorion removal are shown in Figure 6.1.

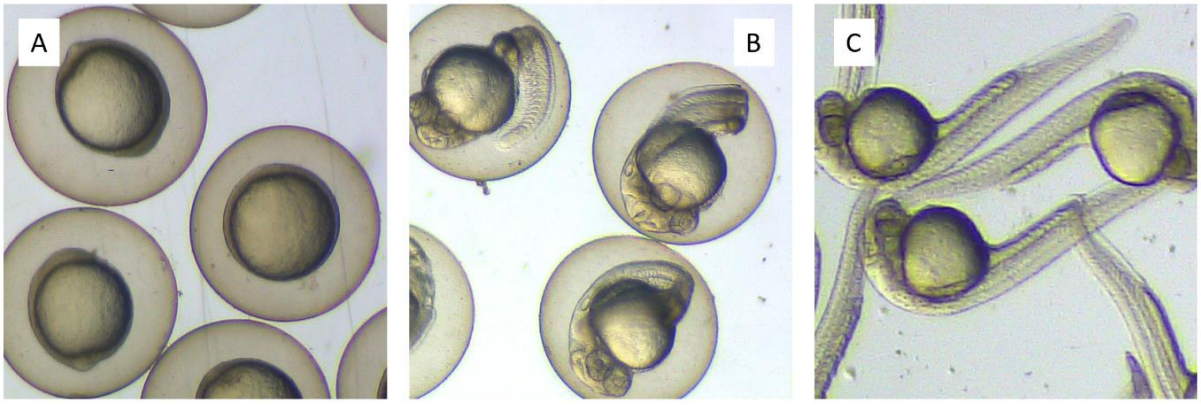

Figure 6.1. *Danio rerio* embryos shortly after fertilisation (A) and 24 hpf before (B) and after chorion removal (C)

6.2.3.2 Crude lipopeptide exposure: Zebrafish embryos 24 hpf

To test the response of zebrafish embryos to the crude lipopeptide extracts, fresh extracts of lipopeptide in 10 mM phosphate buffer (pH 7.0) were produced, as documented in Chapter 4, Sections 4.2.5.1 and 4.2.6.1. Assays were performed with the same extracts tested vs *P. acuta* snails in Chapter 5, as per Section 5.2.3.1. Embryos were prepared as outlined in Section

6.2.3.1. Ten healthy embryos at 24 hpf were placed in each well of a 24-well culture plate. Each well had the aquarium water removed and replace with 1 mL E3 amended with appropriate concentrations of crude lipopeptide extract.

An iterative approach was adopted, whereby each assay was performed for a concentration interval, the results of which informed the concentration intervals for the next round of bioassays. Concentration intervals targeted were 2.5 $\mu g.mL^{-1}$ incremental steps between 5 and 37.5 $\mu g.mL^{-1}$ for all extracts except that of Landy medium BB1-156a, which was tested in 5 $\mu g.mL^{-1}$ incremental steps between 30 and 65 $\mu g.mL^{-1}$. Extracts concentrations were selected targeting a range of mortality between 0 and 100%. Embryos were exposed for 24 h incubated at 28°C before washing three times with sterile E3 medium and a further 24 h incubation with E3 to allow recovery of partially affected embryos. Observations were made using a dissecting microscope (10x magnification) after 24 h of incubation and after the recovery period. Mortality was the primary endpoint; however, any changes in morphology such as oedemata, blood congestion, spinal and ocular malformations, impaired pigment etc. were noted (von Hellfeld *et al.*, 2020). Each extract concentration was tested in triplicate. Control wells with pure E3 media and buffer controls with a final concentration of 1 mM (pH 7.0) phosphate buffer were also included.

6.2.3.3 Surfactin exposure assays: Zebrafish embryos 24 hpf

Zebrafish embryos (24 hpf) were exposed to the surfactin standard to measure their dose response to pure surfactin. Surfactin concentrations were tested in 2.5 $\mu g.mL^{-1}$ intervals between 2.5 and 17.5 $\mu g.mL^{-1}$. These assays were performed as outlined in Section 6.2.3.2. However, 6 replicates per concentrations interval were performed.

6.2.3.4 Surfactin exposure assays: Zebrafish embryos 96 hpf

A comparison of 24 hpf embryos vs older 96 hpf embryos was performed with the surfactin standard. Surfactin concentrations were tested in 2.5 $\mu g.mL^{-1}$ intervals between 7.5 and 20.0 $\mu g.mL^{-1}$ mg mL^{-1}. Embryos were prepared as outlined in Section 6.2.3.2. However, after chorion removal, all embryos were returned to small plastic tanks containing 500mL system water and incubated at 28C for 3 days, refreshing the water and removing any dead embryos

daily during this time. After 96 hpf, 24 well culture plates were prepared in the same manner as for the 24 hpf assays performed in Section 6.2.3.2. Six replicates per concentration interval were performed, targeting a range of mortality between 0 and 100%

6.2.3.5 Surfactin exposure assays: Zebrafish eggs 24 hpf

To determine the protective effects of the chorion upon exposure to surfactin (as opposed to the assays on free embryos conducted above) at 24 hpf, an egg assay was performed, targeting a range of mortality between 0 and 100%. Surfactin concentration was tested in 2.5 $\mu g.mL^{-1}$ intervals between 7.5 and 22.5 $\mu g.mL^{-1}$. These assays were performed as outlined in Section 6.2.3.2; however, no pronase treatment was used to remove the chorion and instead 24 hpf intact eggs were used for this assay. Six replicates per concentrations interval were performed.

6.2.4 Statistical analysis

Dose response curve calculations were performed using MedCalc® Statistical Software version 22.006 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2023). A probit regression analysis was performed using a grouped data format. Replicates were pooled, and due to low mortality rates in control tanks, no baseline mortality adjustment was performed.

6.3 Results

6.3.1 Surfactin vs snail assays

The responses of *P. acuta* and *P. columella* exposed to pure surfactin at various concentrations is shown in Figure 6.2. For both tested snail species, a high level of mortality was observed at low concentrations of surfactin, with complete mortality observed at doses of 17.5 and 22.5 $\mu g.mL^{-1}$ respectively. It was apparent that increasing the exposure time for *P. acuta* from 24 to 72 h did not greatly change the observed mortality rates. No 72 h assay was performed with *P. columella* due to the low number of suitable adult snails produced as well as the greater amount of surfactin standard required for these assays. The baseline mortality rates for the 24 h (22 replicates x 6 snails) and 72 h (15 replicates x 6 snails) controls was 0% mortality for *P. acuta*. In the 24 h *P. acuta* assay, for each concentration the mortality percentage was for 54 snails

from 9 replicates of 6 snails per tank. For the 72 h *P. acuta* assay each data point was comprised of the sum mortality of 18 snails from 3 replicates of 6 snail each.

For *P. columella* a baseline mortality of 2.2% from a total population of 45 snails (15 replicates x 3 snails) was established over the course of the assay. Phosphate buffer controls (1 mM, pH 7.0) produced a mortality rate of 0% from 21 *P. columella* (7 replicates of 3 snails each). Each data point for the 24 h *P. columella* assay was comprised of 21 snails (7 replicates x 3 snails).

The visible response of *P. acuta* and *P. columella* to increasing concentrations of surfactin was similar to that of *P. acuta* when exposed to crude lipopeptides (Chapter 5). Responses to exposure were visible shortly (within 15 minutes) after initial exposure to surfactin proportionate to the surfactin concentration tested; these included snails losing attachment to the sides of the assay tank, snail bodies either extending from or retreating into the shell, and sinking or floating of the snail (Figure 6.3). Similar behavioural observations were made when snails were transferred from the assay tanks to observation tanks. In some cases, where exposure had produced a visible change in behaviour, some snails were found to have crawled above the treated water.

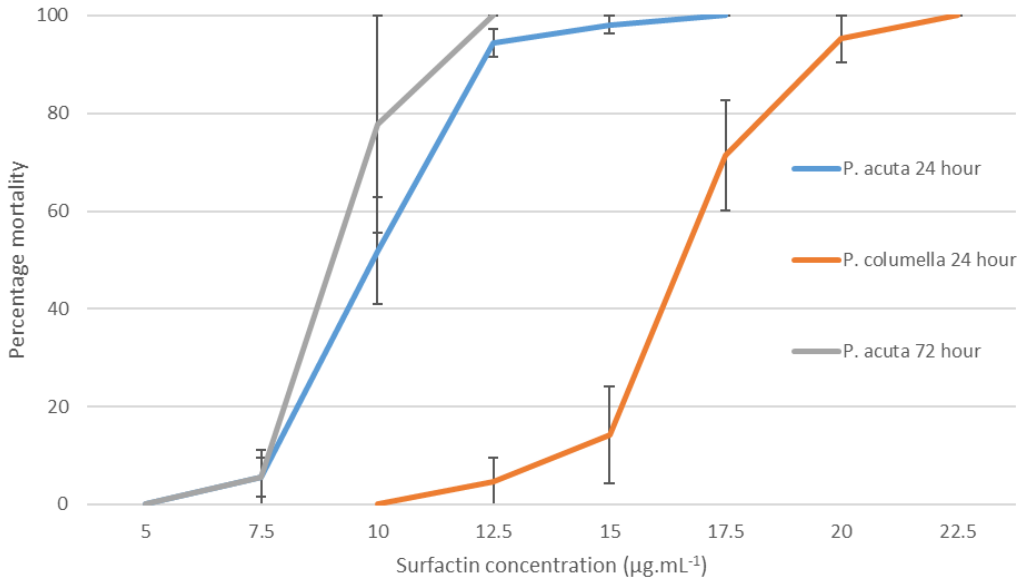

Figure 6.2. Mortality percentages for *P. acuta* and *P. columella* after exposure to pure surfactin

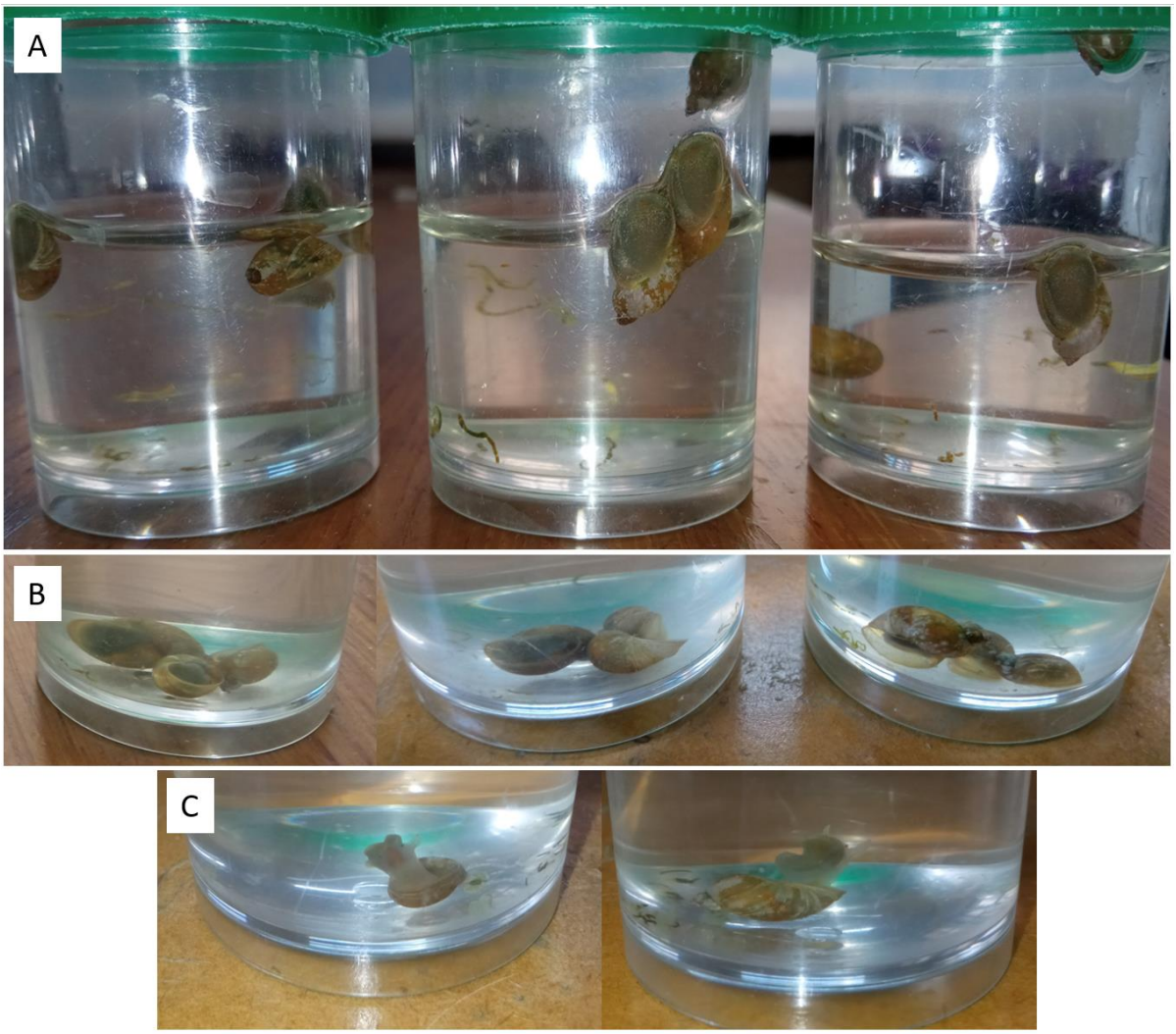

Figure 6.3. Typical behaviour of *P. columella* after 24-hour exposure assays: (A) Surfactin free control; (B) Surfactin at 17.5 µg.mL^{-1} showing snails retracted into their shells; (C) Surfactin at 12.5 µg.mL^{-1} showing the snail body extended out of its shell

At higher doses, a common observation was that all snails either sunk to the bottom of the tank (Figure 6.3 -B), or floated, either with the snail body retracted into the shell, or extended flaccidly outside the shell, with no apparent movement or attachment to the assay tank walls (Figure 6.3 - C). These observations were linked to complete mortality at higher concentrations. However, at lower concentrations, corresponding to the calculated LC_{50} or higher, recovery was a common occurrence, even while being visually indistinguishable to the treatments with higher concentrations just before transfer to the recovery tanks. Visible recovery was usually quick (within hours). However, some snails were observed to remain retracted into the shell for a number of days before normal behaviour was observed on the final day of observation.

The strength of the effect was observed to follow the increase in surfactin concentrations, with lower level of recovery as concentration increased.

No correction for mortality was necessary when dose responses to 24 h and 72 h exposure to surfactin were determined (Table 6.1). LC values for *P. acuta* are very similar between the 24 h and 72 h exposure times with LC_{50} values between 9.19 and 10.04 $\mu g.mL^{-1}$ and LC_{90} values between 10.53 and 12.29 $\mu g.mL^{-1}$ being calculated. *Pseudosuccinea columella* appeared to be less sensitive to surfactin exposure, with LC_{90} values (24 h) almost double the concentration for *P. acuta*.

Table 6.1. Dose response of *P. acuta* and *P. columella* exposed to surfactin for 24 and 72 hours

Surfactin Exposure/Species	**LC_{10} ($\mu g.mL^{-1}$)**	**LC_{50} ($\mu g.mL^{-1}$)**	**LC_{90} ($\mu g.mL^{-1}$)**	**LC_{95} ($\mu g.mL^{-1}$)**
24 h/ *P. acuta*	7.79 (7.02-8.34)	10.04 (9.61-10.47)	12.29 (11.74-13.06)	12.93 (12.29-13.84)
72 h/ *P. acuta*	7.84 (6.44-8.50)	9.19 (8.54-9.80)	10.53 (9.90-11.85)	10.91 (10.20-12.51)
24 h/ *P. columella*	14.01 (12.53-14.91)	16.58 (15.83-17.34)	19.15 (18.26-20.64)	19.88 (18.86-21.67)

Numbers in brackets are 95% CI intervals. MedCalc® Statistical Software version 22.006 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2023)

6.3.2 Crude lipopeptide extracts and surfactin vs Zebrafish embryo assays

Exposure of 24 hpf zebrafish embryos to crude lipopeptide extracts is shown in Figure 6.4 A and B for extracts from TSB and Landy media respectively. Each data point is the mean mortality with 3 replicates per extract per concentration for a total of 30 embryos. Results showed a narrow concentration range between complete mortality and no observable effects. Controls for *D. rerio* embryos shows a base line mortality rate of 3.55% for the E3 control (n = 11/310) and 3.75% mortality for the 1mM Phosphate buffer control (n = 6/160). As the control mortality was lower than 5%, no correction for baseline mortality was performed.

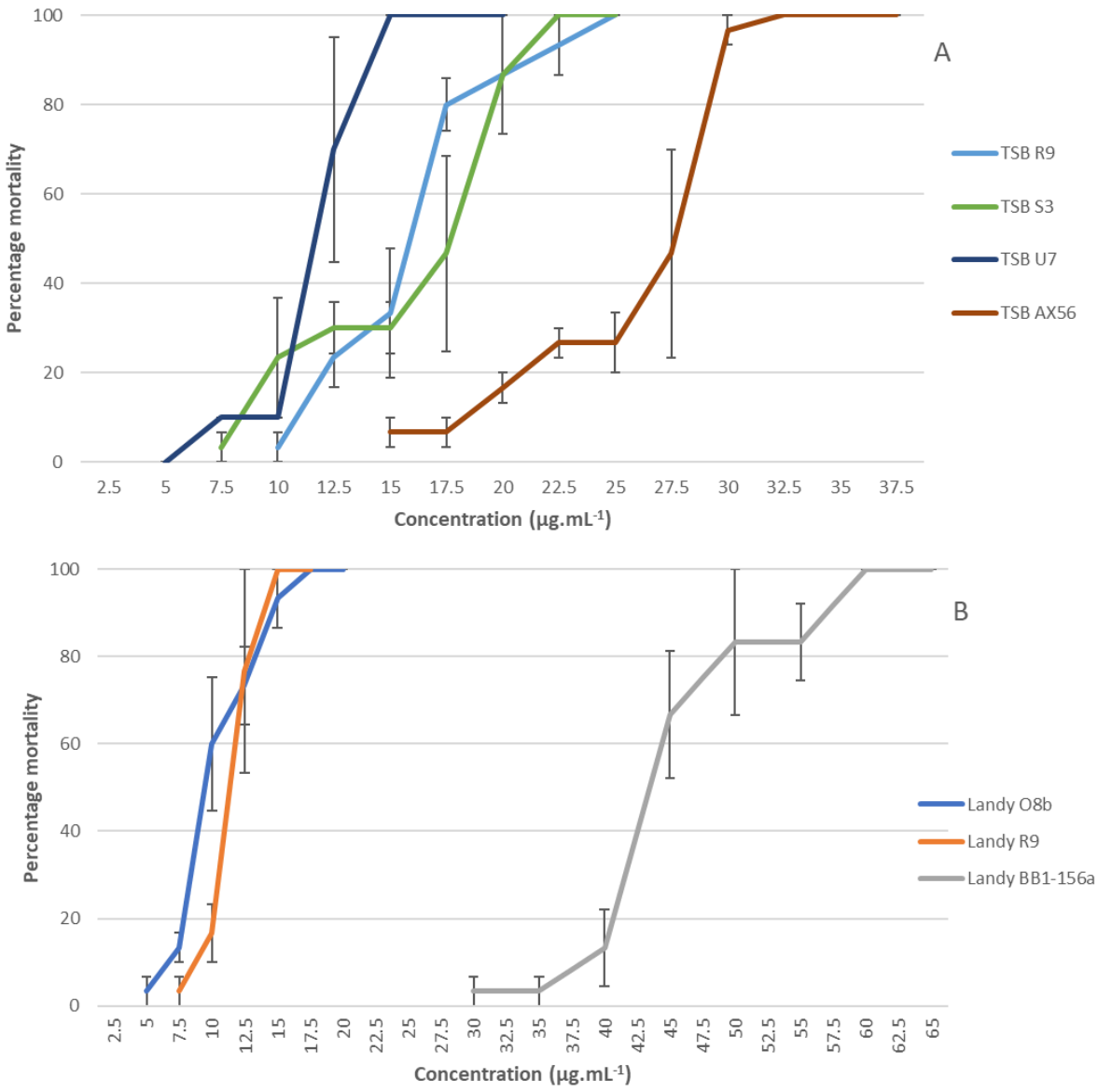

Figure 6.4. Mortality percentages for *Danio rerio* (24 hpf embryos) after exposure to crude lipopeptide extracts derived from *B. velezensis* (R9, S3, AX56) and *B. amyloliquefaciens* (U7) grown in TSB (A) and *B. velezensis* (R9, BB1-156a) and *B. subtilis* (O8b) isolates grown in Landy Media (B)

The best performing extracts (TSB R9, TSB S3, TSB U7, Landy O8b and Landy R9) have a narrow range of concentrations between low mortality and 100% mortality typically between 5 µg.mL^{-1} and 22.5 µg.mL^{-1}. TSB AX56 and Landy BB1-156a stand out as being significantly less active, with a range of active concentrations of 15 to 32.5 µg.mL^{-1} and 35 to 60 µg.mL^{-1}, respectively. Extracts TSB U7, and Landy O8b and R9 stand out as the best performers with

LC_{90} values at ~ 14 µg.mL^{-1} (24 h). In contrast, Landy BB1-156a has the worst performance with an LC_{90} of 53.62 µg.mL^{-1}.

Mortality at higher concentrations resulted in significant changes to the embryo morphology, with the embryos shrinking significantly and turning black in colour compared to the controls (Figure 6.5) . As concentrations decreased a common observation was a slight discoloration for surviving individuals (Figure 6.6). However, this typically was temporary and normal colouration resumed after 24 h (Figure 6.6 A /B and C /D). While some abnormalities were observed (Figure 6.6 E /F), such as bent spines and malformed eyes, there was no consistent pattern associated with a specific concentration or a specific extract. The appearance of such malformations appeared at a low frequency in control replicates as well as test wells, with no discernible pattern. Embryos observed to be alive after the initial exposure period were generally alive after the 24-observation period. No observable differences were noted between the E3 control and 1 mM phosphate buffer controls, as well as the sub-lethal concentrations of crude lipopeptides.

Table 6.2. Lethal concentration (LC) values for assays of *Danio rerio* embryo (24 hpf) exposure to crude lipopeptide extract

Extract / Isolate	**LC_{10} (µg.mL^{-1})**	**LC_{50} (µg.mL^{-1})**	**LC_{90} (µg.mL^{-1})**	**LC_{95} (µg.mL^{-1})**
Landy O8b	6.44 (4.96-7.44)	10.19 (9.43-10.93)	13.94 (12.98-15.33)	15.00 (13.89-16.67)
Landy R9	9.17 (8.07-9.86)	11.29 (10.72-11.85)	13.40 (12.71-14.50)	14.00 (13.21-15.32)
Landy BB1-156a	36.24 (33.27-38.36)	44.93 (43.33-46.52)	53.62 (51.54-56.53)	56.08 (53.67-59.56)
TSB R9	11.06 (9.34-12.25)	15.75 (14.89-16.58)	20.43 (19.33-22.01)	21.76 (20.47-23.67)
TSB S3	9.61 (4.68-12.03)	15.60 (13.51-17.61)	21.58 (19.23-26.30)	23.27 (20.56-29.06)
TSB U7	8.81 (7.63-9.58)	11.39 (10.77-12.02)	13.98 (13.21-15.16)	14.72 (13.83-16.12)
TSB AX56	19.29 (14.65-21.73)	25.42 (23.29-27.50)	31.54 (29.15-36.03)	33.28 (30.54-38.72)

Numbers in brackets are 95% CI intervals. MedCalc® Statistical Software version 22.006 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2023)

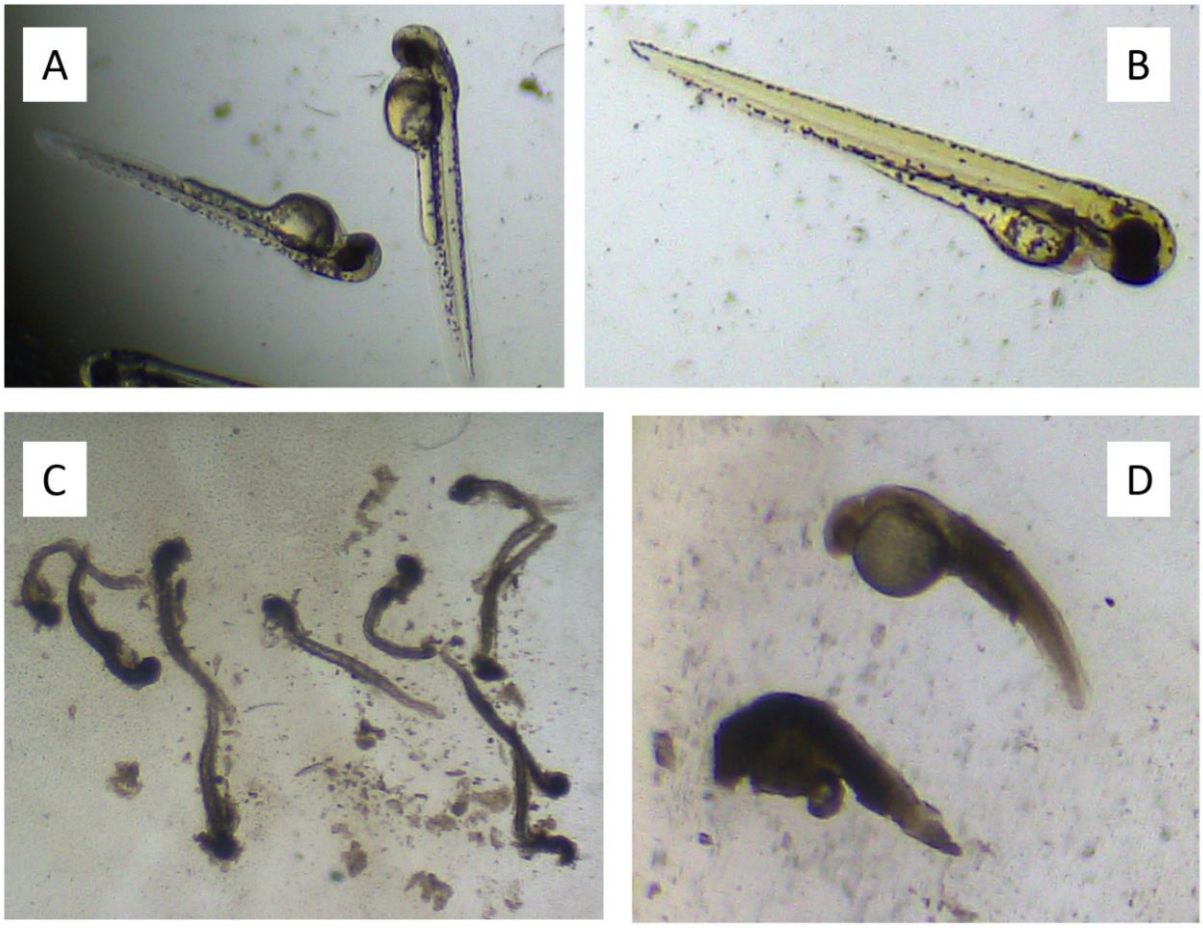

Figure 6.5. Typical appearance of *Danio rerio* embryos after exposure to crude lipopeptide extracts and E3 media controls: (A) E3 control embryos 48 hpf after 24 hours; (B) E3 control embryos 72 hpf after 24-hour recovery; (C) Landy media, Isolate R9 (15 µg.mL^{-1}) treated embryos showing complete mortality after exposure; (D) TSB media, U7 extract (15 µg.mL^{-1}) treated embryos showing complete mortality after exposure

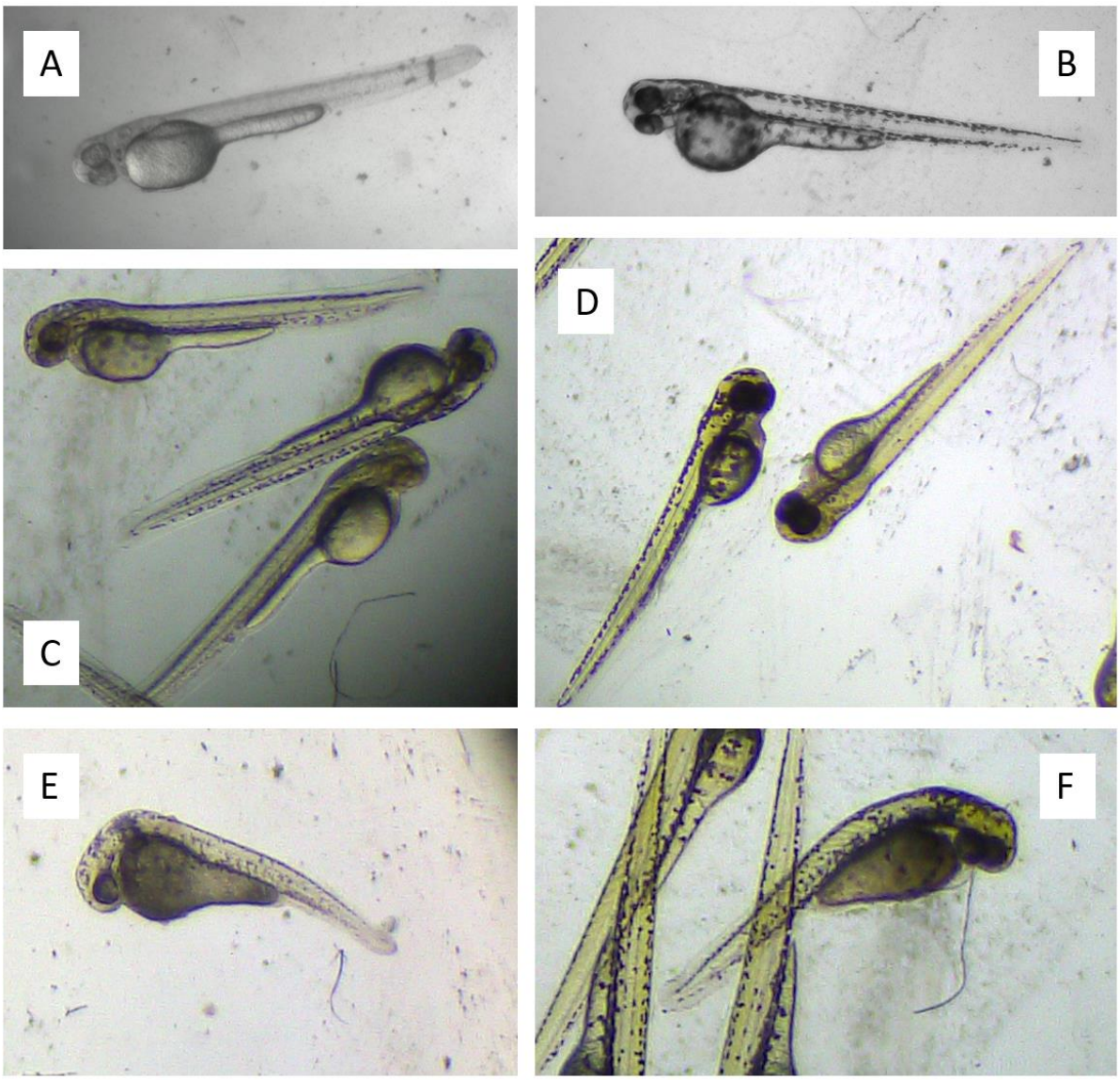

Figure 6.6. Typical observations of pale embryos and deformations of embryos of *Danio rerio*: (A / B) Landy media, isolate O8b (15 µg.mL^{-1}) after 24- hour exposure (A) and after 24-hour recovery (B); (C / D) Landy media, isolate R9 (7.5 µg.mL^{-1}) after 24- hour exposure (C) and after 24-hour recovery (D); (E / F) Landy media, isolate R9 (12.5 µg.mL^{-1}) after 24- hour exposure (E) and after 24-hour recovery (F)

Exposure of 24 hpf zebrafish embryos to the pure surfactin standard (Figure 6.7) resulted in similar results to the best-performing crude lipopeptides, as in Figure 6.4. In comparison, the 24 hpf eggs and 96 hpf embryos were susceptible to a similar range of concentrations but were slightly less sensitive than the 24 hpf embryos. Each data point was the mean of 6 replicates (n=60). Controls for *D. rerio* 24 hpf eggs showed a baseline mortality rate of 3% for the E3 control (n = 3/100) and 1.67% mortality for the 1mM Phosphate buffer control (n = 1/60).

Controls for *D. rerio* 96 hpf eggs had a baseline mortality rate of 1.25% for the E3 control (n = 1/80) and 0% mortality for the 1mM Phosphate buffer control (n = 0/60). Control mortality rates are similar to that of 24 hpf embryos, and as this was lower than 5%, no correction for baseline mortality was performed. Levels of mortality presented in Figure 6.8 and 6.9 for 24 hpf eggs and 96 hpf embryos were similar to those affecting 24 hpf embryos, with coagulation of the embryo structures being a common effect, which was directly proportional to increasing surfactin concentrations. With 24 hpf embryos, the embryos either died within the egg structure, or the embryos that hatched during the exposure process were not noted as harmed.

The LC values for pure surfactin are similar in scale to those of the crude lipopeptide extracts but showed an effect at lower concentrations (Table 6.3). The 72 hpf eggs appeared to be more tolerant of surfactin than the 24 hpf embryos; 96 hpf embryos show a similarly enhanced survival rate at concentrations lethal for 24 hpf embryos.

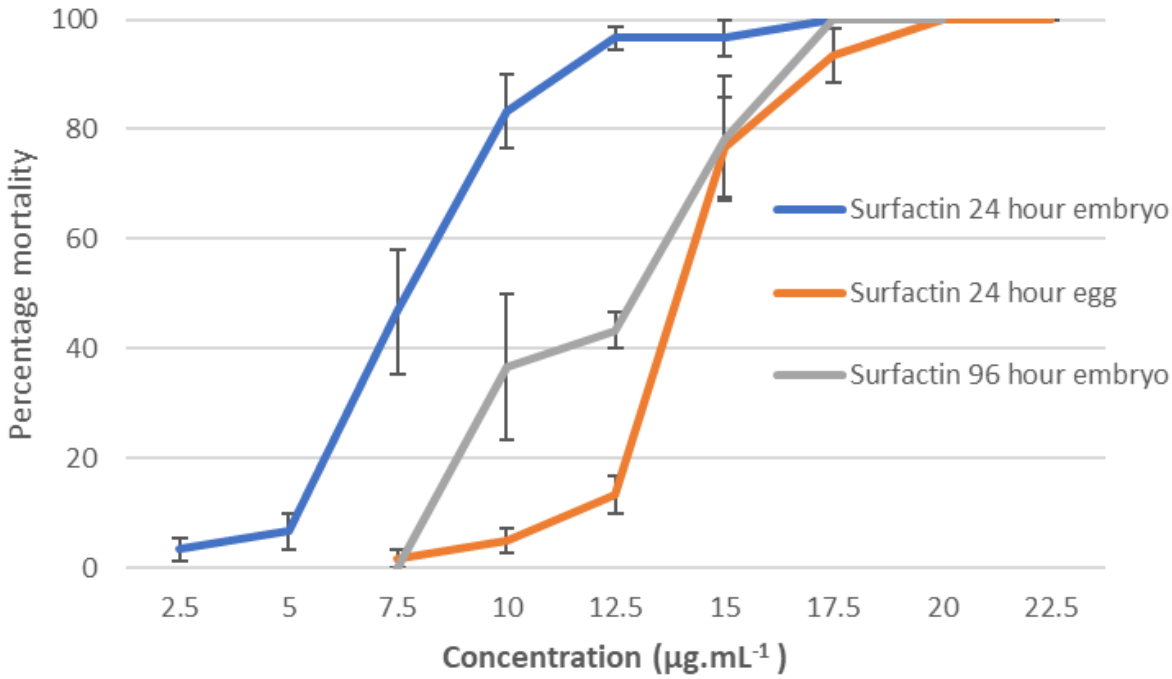

Figure 6.7. Mortality percentages for *D. rerio* after exposure to pure surfactin

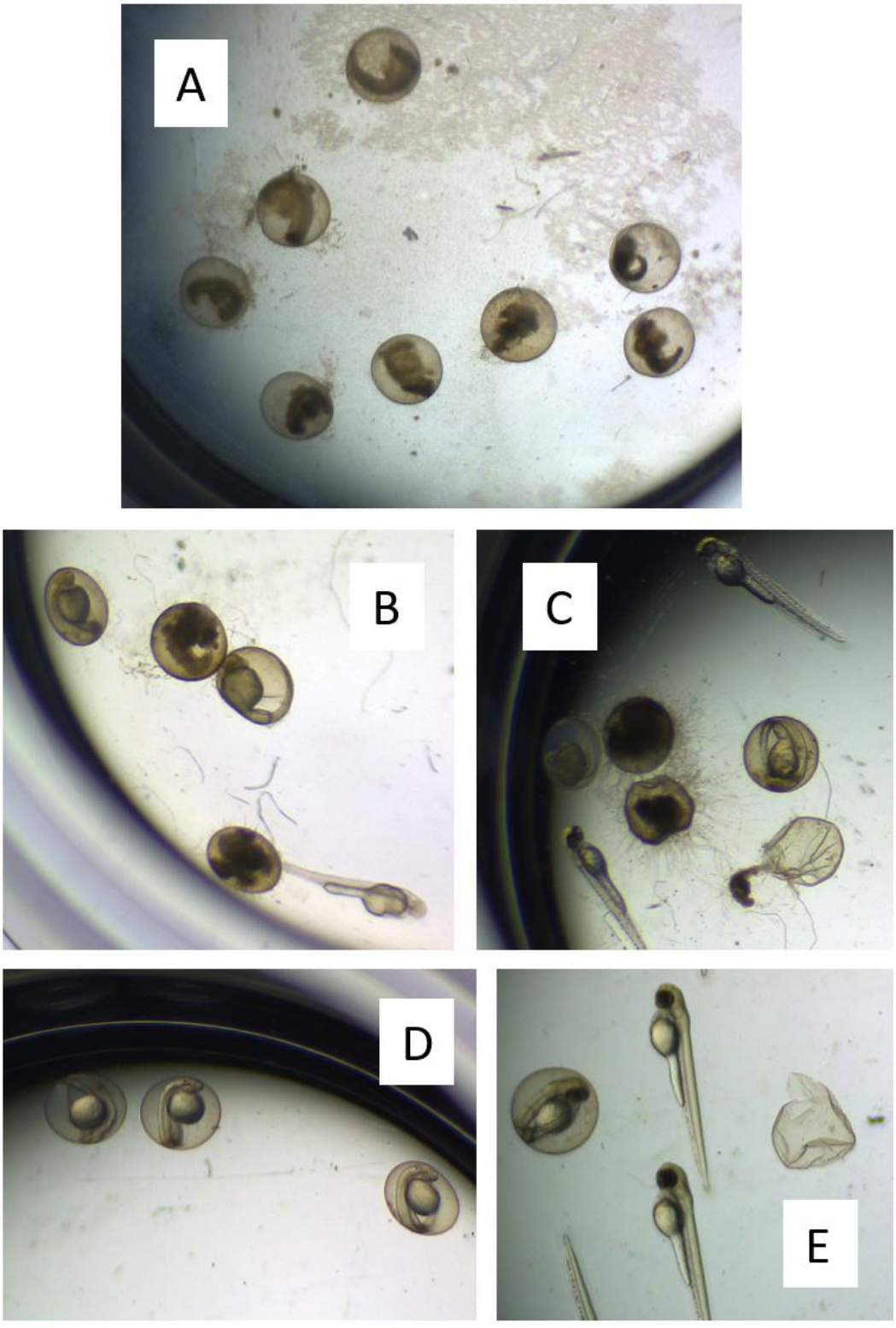

Figure 6.8. Typical observations of 24 hpf egg assayed vs surfactin: (A) Surfactin 22.5 µg.mL^{-1} 100% mortality of the embryos within eggs; (B / C) Surfactin 17.5 µg.mL^{-1} varied mortalities within eggs after 24- hour exposure (B) and after 24-hour recovery (C); (D / E) Surfactin µg.mL^{-1} indistinguishable from untreated controls (data not shown) after 24- hour exposure (D) and after 24-hour recovery (E)

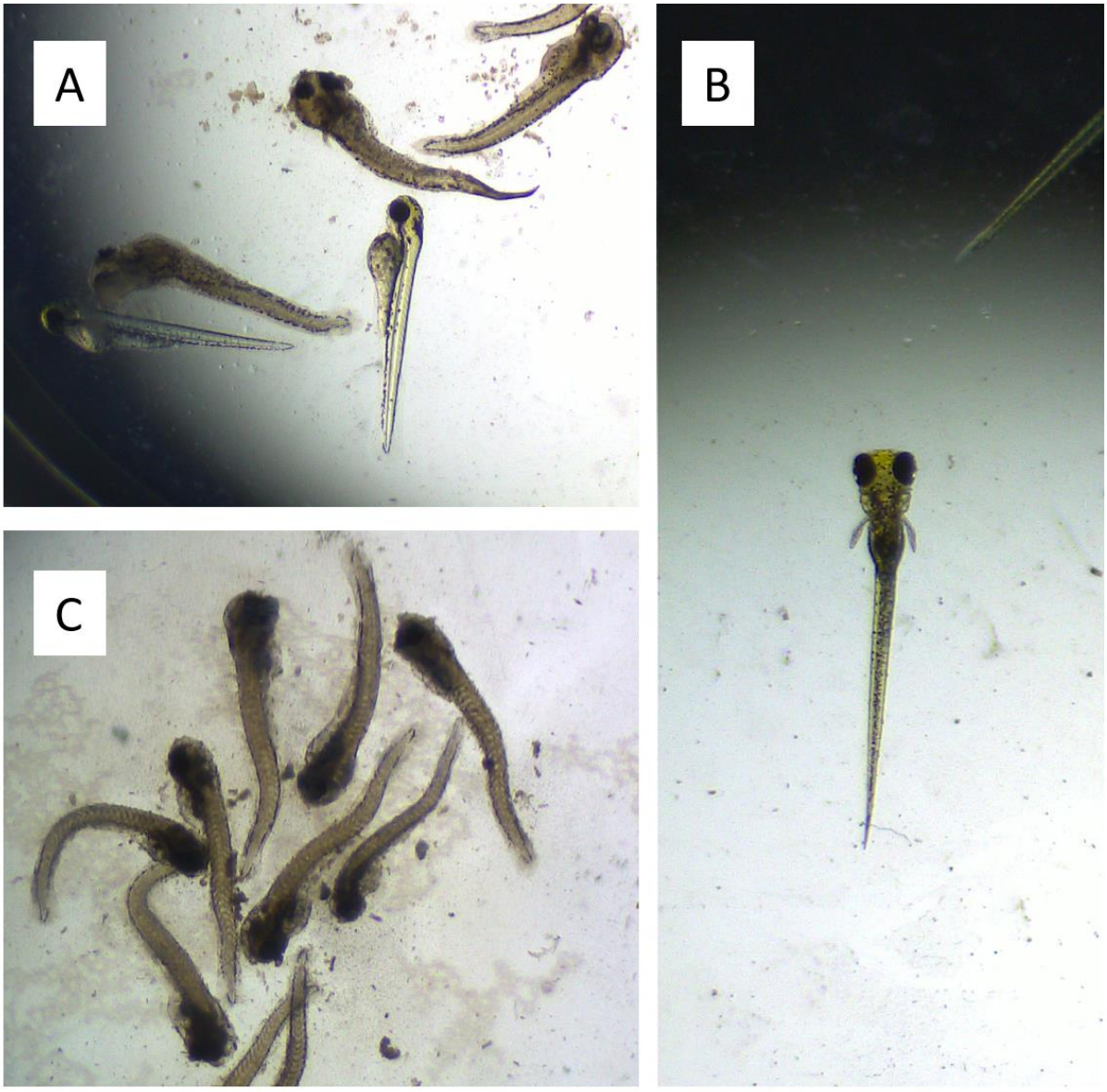

Figure 6.9. Typical observations of 96 hpf embryos assayed vs surfactin: (A /B) Surfactin at 12.5 µg.mL^{-1} caused some mortality of embryos after 24 hours exposure (A) and 24 hours recovery (B); (C) Surfactin 20 µg.mL^{-1} caused complete mortality

Table 6.3. Lethal concentration (LC) values for assays of *D. rerio* exposure to surfactin at embryo (24/96 hpf) and egg stages (24 hpf chorion intact)

Surfactin Assay	LC_{10} (µg.mL^{-1})	LC_{50} (µg.mL^{-1})	LC_{90} (µg.mL^{-1})	LC_{95} (µg.mL^{-1})
24 hpf embryo	4.47 (1.86-5.86)	7.96 (6.74-9.15)	11.45 (10.09-13.98)	12.44 (10.88-15.50)
96 hpf embryo	8.67 (4.19-10.47)	12.26 (10.47-13.95)	15.85 (14.12-20.04)	16.87 (14.91-22.02)
24 hpf egg	11.12 (8.77-12.32)	13.96 (12.87-15.06)	16.81 (15.60-19.15)	17.61 (16.24-20.44

Numbers in brackets are 95% CI intervals. MedCalc® Statistical Software version 22.006 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2023)

6.4 Discussion

Experiments were run to measure the molluscicidal activity of lipopeptide extracts and a surfactin standard against *P. acuta* and *P. columella* snails. In addition, potential ecotoxicological effects of surfactin were assessed by zebrafish embryo exposure assays. From the results of the surfactin assays against *P. acuta* and *P. columella*, it is apparent that this lipopeptide possesses molluscicidal properties. Control assays containing a final concentration of 1 mM phosphate buffer for *P. columella* (this chapter) and in Chapter 4 (Section 4.3.1) for *P. acuta*, demonstrated no molluscicidal effects. As the surfactin standard was dissolved in phosphate buffer, the prepared tank waster/surfactin solutions contained buffer, however at no point was the effective phosphate buffer concentration above 0.025 mM for any assay against *P. columella* or *P. acuta*.

In both cases, exposure of snails to these concentrations of phosphate buffer was indistinguishable from aquarium water controls. The calculated LC values for surfactin vs *P. acuta* with 24 h and 72 h exposure times, were comparable with the concentrations of molluscicidal crude lipopeptide extracts, as determined in Chapter 5 (Section 5.3.2), with LC_{90} values of 12.29 and 10.53 µg.mL^{-1}, respectively. The effects of surfactin against *P. columella* were measured and while this snail species appears to be more resistant to surfactin than *P. acuta*, a strong molluscicidal effect with an LC_{90} of less than 20 µg.mL^{-1} was observed with 24 h exposure. Observations of snail behaviour during pure surfactin assays was consistent with that observed with *P. acuta* snails exposed to crude lipopeptide extracts during and after exposure (Chapter 5 Section 5.3.2). To date, the present study is the first report of a molluscicidal effect of surfactin against a snail species. Molluscicidal activity at a relatively

low concentration suggest that surfactin may be a useful compound for use in aquatic snail control.

If surfactin is to be utilised as a chemical control method targeting aquatic snails, then research into non-target effects is required. For this reason, the screening of *D. rerio* embryos exposed to surfactin was undertaken as an indicator of the ecotoxicological safety of surfactin. Molluscicidal crude lipopeptides were assayed against 24 hpf zebrafish embryos and demonstrated that these extracts are lethal at low concentrations, with LC_{50} values between 10.19 and 44.93 $\mu g.mL^{-1}$ and LC_{90} values between 13.4 and 53.62 $\mu g.mL^{-1}$. Overall, the crude lipopeptides show similar relative activity patterns against *D. rerio* and *P. acuta* snails. As these extracts were crude, unquantified mixtures of lipopeptides, the role of other lipopeptides in observed zebrafish mortality and any synergistic or antagonist effects required further evaluation outside the scope of this study.

Danio rerio embryos assays with pure surfactin confirm that this lipopeptide is at least partially responsible for the observed embryo mortality. Surfactin assays targeting 24 hpf *D. rerio* eggs with intact chorions, suggest that there is at least some protective effect of the egg structure to surfactin, with almost double the concentration required for a lethal effect to be observed compared to chorion-free 24 hpf embryos. Older *Danio rerio* embryos at 96 hpf also show a higher degree of resistance to surfactin than 24 hpf embryos, with similar LC values to that of 24 hpf egg assays.

Danio rerio is a widely used model species for aquatic toxicity, and other researchers have presented findings of surfactin activity against *D. rerio.* However, the present study is the first using 24 hpf embryos. Previous published works have focussed on more mature fish and the protective, growth promotional aspects of this compound (Zhai *et al.*, 2016, Wang *et al.*, 2021). Fei *et al.* (2019) evaluated surfactin against juvenile *D. rerio* (specific age not stated) and found LC_{50} values greater than 1000 $\mu g.mL^{-1}$ with a 96 h contact time. Wang *et al.* (2021) report no mortality at a concentration of 1000 $\mu g.mL^{-1}$ for 72 hpf *D. rerio* embryos with a 6 h contact time, while the concentration range with protective effects against inflammation, oxidative stress, and hepatic injury were reported at concentrations between 20 and 60 $\mu g.mL^{-1}$. While the age of the embryos and the exposure times in the present study and that of Wang *et al*.

(2021) and Fei *et al.* (2019) are different, it is difficult to reconcile the extreme differences in sensitivities noted.

While this is the first report of molluscicidal activity for surfactin, a number of other aquatic species are sensitive to surfactin. These range from a high degree of sensitivity, such as *A. stephensi* mosquito larvae with reported LC_{50} values in the 2 $\mu g.mL^{-1}$ range for a crude surfactin extract (Geetha *et al.*, 2010), to non-sensitive in the case of *D. magna* with an EC_{50} (Effective concentration) of 170.1 $\mu g.mL^{-1}$ to achieve immobilisation (De Oliveira *et al.*, 2017). The wide range of surfactin concentrations that induce a response from different species makes ecotoxicological investigations more complicated. Any determination would require multispecies analysis to determine the full extent of any ecological implications in a given aquatic habitat.

A direct comparison between the LC values determined in this study for pure surfactin against *P. acuta* and *P. columella* are comparable to those reported in the literature for plant-based molluscicides tested against a variety of aquatic snail species (Zheng *et al.*, 2021). The reported LC_{100} for niclosamide is 1 $mg.L^{-1}$, which has an advantage over surfactin in terms of concentrations required. However, this is only one metric of comparison for the utility of a molluscicide (Zheng *et al.*, 2021). Direct comparisons between compounds are complicated, based on the differences in assay methodology, compound purity, and the varieties of snail species used in the assays.

While we cannot confirm a mechanism by which surfactin is molluscicidal, surfactin's association with perforation of cell membranes and pore formation are typically cited as being responsible for its antimicrobial effects (Chen *et al.*, 2022). The surfactant properties of surfactin may have altered surface tension which may have caused the snails to lose adhesion to the sides of the tank, this may have prevented escape at higher concentrations and ensured lethal exposure. With plant molluscicides, there is a large diversity of compounds represented. However, the snail outer membrane is a likely candidate for the site of action (WHO, 1983). Indeed, the narrow range of concentrations eliciting either complete mortality or no observable effect is similar between surfactin and that previously reported for plant molluscicidal compounds (WHO, 1983).

The finding of molluscicidal activity at low concentration of surfactin suggests that this compound may be useful to replace or augment synthetic molluscicides, and the fact that it is generally less toxic, and biodegradable are advantages. However, further work testing surfactin against other target snail species to establish each species-specific level of sensitivity is required. Non-target effects may be a concern, as we found sensitivity for *D. rerio* embryos, although this is in conflict with the reported LC_{50} values from other studies. This area requires further studies, which may be specifically targeted against sensitive species in specific aquatic environments and locations.

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CHAPTER SEVEN: GENERAL OVERVIEW AND CONCLUSIONS

This research aimed to identify aerobic, endospore-forming bacteria able to cause mortality in the aquatic snail populations that act as the intermediate hosts of *Fasciola* liver flukes in South Africa. *Fasciola* liver flukes are a worldwide concern to livestock, and fascioliasis is considered a neglected tropical disease in humans (Mas-Coma *et al.,* 2009; Mas-Coma *et al.,* 2018). Targeting the snails with a biocontrol agent could reduce reliance on chemical molluscicides and provide an additional tool in mitigating the damage caused by the fluke parasites (King and Bertsch 2015; Sokolow *et al.,* 2018). In addition, such a biological control agent may have potential use to help control a number of other aquatic snail species that are intermediate hosts for diseases such as schistosomiasis, or manage invasive snail species that displace indigenous snail populations, damage the local environment, and pose a threat to crops (Appleton and Miranda, 2015; King and Bertsch 2015; Yang *et al.,* 2018).

The objectives for this study can be summarised as follows: -

1) Isolate aerobic endospore-forming bacteria from aquatic environments,
2) Screen bacterial isolates for molluscicidal potential against liver fluke host snails,
3) Characterise isolates and measure their biocontrol potential,
4) Determine the mode of action of molluscicidal isolates.

Findings from this study

The original plan of this research was to screen aerobic endospore-forming bacteria on host snails of liver fluke in South Africa. To this end, a breeding population of *Pseudosuccinea columella* (Say, 1817) was sourced from Dr J.A. van Wyk, and wild populations of *Radix natalensis* (Krauss, 1848) and *P. columella* were collected. While a breeding population of *P. columella* was successfully maintained over the course of this study, the growth of this population was insufficient for broad screening of bacterial isolates. *Radix natalensis* was not successfully bred in captivity. To facilitate the initial screening of bacterial isolates, a large population of adult snails was required, and to this end, a proxy aquatic snail species was chosen with similar ecology to the originally targeted snails. The chosen species, *Physella acuta* (Draparnaud, 1805), was easily reared under laboratory conditions, and offered a fast

growth rate to adulthood and prolific egg laying. Therefore, *P. acuta* was used for all screening of aerobic endospore-forming bacterial isolates.

The initial screening of aerobic endospore-forming bacteria saw a total of 1,180 isolates tested. No obvious pathogenic capabilities were identified from these isolates. With the absence of any strong molluscicidal isolates and with the initial screening process not geared to detect weaker molluscicidal potential, it was decided to re-evaluate any isolates that were associated with snail death during the initial screening. This group of 124 isolates was rescreened against snails directly in the form of bacterial applications, and by putting cell-free spent culture supernatant into snail tanks. In this follow-up assay, no isolates with strong molluscicidal activity were found. As such, the initial premise of screening bacterial isolates against snails did not yield the desired results. Instead, it was found that a large number of isolates screened produced molluscicidal compound/s in cell-free spent culture supernatant. This indicated that, although a snail pathogen was not found from among the pool of isolates, the production of metabolite/s with molluscicidal activity had been identified. The isolates which had the best performing cell-free spent culture supernatant were identified as *Bacillus velezensis* (Ruiz-García *et al.,* 2005) and closely related *Bacillus subtilis* (Ehrenberg, 1835) and *Bacillus amyloliquefaciens* (Priest *et al.,* 1987).

The focus of this research project then shifted from the identification of a direct snail biocontrol agents to pursuing the characterisation of the nature of these molluscicidal metabolites. Extraction and identification of active fractions using mass spectrometry identified a number of lipopeptides, including iturin, fengycin, and bacillomycin-D and -L, and common to all extracts, surfactin. Surfactin is a biosurfactant identified as being active against a range of bacterial, fungal and insect pest species, but activity against molluscs had not yet been described in the published literature (Ongena and Jacques, 2008; Geetha *et al.,* 2012; Denoirjean *et al.,* 2021; Koim-Puchowska *et al.,* 2023). A pure commercial surfactin standard was sourced, and when evaluated for molluscicidal activity, it was confirmed as being molluscicidal against *P. acuta* and *P. columella.* This is the first report of molluscicidal properties associated with surfactin.

For this research a number of findings and determinations were made:

1) Screening on the host snails of *Fasciola* proved more difficult than expected.
2) Snail pathogens appear rare amongst aerobic endospore-forming bacteria, or do not present activity in a way detectable by the screening methodology used.
3) *Physella acuta* snails are amendable for growth under laboratory conditions and offer an easily accessible source of snails for any future aquatic snail research.
4) The production of molluscicidal metabolites from aerobic endospore-forming bacteria when grown in artificial culture media appears common to *B. velezensis* and other members of the *B. subtilis* group.
5) Crude lipopeptides extracts of these bacteria have molluscicidal activity against *P. acuta.*
6) Surfactin, in particular, was molluscicidal against both *P. acuta* and *P. columella*.

Future bacterial screening assays

This study had good reasons to investigate endospore-formers for molluscicidal abilities and has uncovered a mode of action that these species may offer to snail biocontrol in the form of lipopeptides. While this study did not find any isolates with direct molluscicidal activity, it is still possible that a future broad screening endeavour could identity such an organism, as has been found previously in a *Paenibacillus* sp. (Duval *et al.*, 2015). Without identifying bacterial species with direct molluscicidal activity, it is difficult to focus any future screening on a narrower range of *Bacillaceae* species. From the beginning of this research, a broad screening strategy was adopted with the intention of detecting *Bacillaceae* species with molluscicidal modes of action. When designing a screening protocol, the opposing pulls of high throughput vs high sensitivity are difficult to balance. There may be an advantage to increasing sensitivity for weak molluscicidal isolate detection, if only to allow a more targeted focus on species identified as having potential. The assay performed in the present study is simple enough to reproduce on a much larger scale, allowing a brute force approach by increasing the scale of snail rearing and isolate testing.

It may be that screening targeted directly on the *Fasciola* host snail species may be a better strategy than using a proxy species. However, rearing of these *Fasciola* intermediate host snails in captivity proved difficult in this study, but it may be achievable elsewhere, or with wild

populations if these are available. Other options are to target snails that are already infected with flukes, as this may offer a weaker snail that is more easily attacked by a pathogenic bacterial strain (Sorensen and Minchella, 2011). Alternatively, targeting other snail life stages may offer a more promising avenue for future work, either in targeting the eggs or the juvenile snails. This could also allow for higher-throughput assays that do not require adult snails. It may also be that pathogens need assisted entry into the snail for a strong effect to be observed, in which case a snail food bait formulated with the bacteria to be tested could be developed further. A dry formation of endospores, cells, and cell debris incorporated into a food pellet would have avoided the problems with premature endospore germination experienced in this study.

Future for surfactin and lipopeptide research

This research work established the required concentration of surfactin for a lethal effect, with short term exposure, for two aquatic snail species. However, the question of how to achieve these levels in an aquatic environment is unanswered. Broadly two options exist: Either dosing a water body with live bacteria for surfactin production *in situ,* or by direct dosing of surfactin itself.

Is it possible for a bacterial isolate to produce surfactin *in situ* to have a molluscicidal effect on adult snails or any other life stage? And, if so, under what conditions is this possible? Initial laboratory-scale assays performed in this study suggest that this may be difficult to achieve. Ultimately, screening for bacterial isolates capable of surfactin production in the field would be significantly different from the screening performed in this study, specifically targeting production of lipopeptides in aquatic environments. Production of surfactin under laboratory conditions on artificial media may select for good producers under these defined conditions, but does not necessarily translate to good production of surfactin in aquatic ecosystems. While laboratory production of surfactin may identify certain strains and isolates of *Bacillus*, it may be that these isolates are not competitive in the natural, lower nutrient settings of a water body. Furthermore, flowing water sources may not allow for adequate surfactin concentrations and contact times required for snail mortality, and thus may be unsuitable for surfactin to act as a molluscicide. However, there are many situations on dairy and beef farms with small static bodies of water where achieving the desired concentrations of surfactin *in situ* may be effective.

Another avenue is the application of surfactin as direct dosing of small, isolated water bodies to the required concentrations. This treatment methodology would have more in common with chemical molluscicides but may offer advantages in biodegradability and non-target effects compared to synthetic molluscicides (De Oliveira *et al.,* 2016). Any use of surfactin in this manner would require a significant decrease in production cost. However, significant work is being done on this front, and this may allow cost effective use (Dhanarajan and Sen, 2014; Zhou *et al.,* 2023). If surfactin can be encapsulated to prevent dispersion into a water body, can ingestion result in lethal effects to snails?

Ultimately, an important consideration is whether surfactin is suitable for use in the field for snail control. Field-based research assessing efficacy would be required alongside further investigations into ecotoxicity and non-target effects. While surfactin is generally seen as non-toxic, this research has demonstrated that zebra fish embryos were sensitive to surfactin. Hower, surfactin has advantages over synthetic chemical molluscicides in the form of biodegradability, which may offer an acceptable compromise (De Oliveira *et al.,* 2016). There are also broader implications to the use of surfactin as a molluscicide. The effects of long-term exposure of target aquatic snails to surfactin or other lipopeptides at sub-lethal concentrations are unknown, as well as effects on feeding behaviour and breeding after exposure. If long term exposure at low concentrations does have lethal effects on snail populations, this may allow *in situ* production to be a viable option. The determination of effective concentrations of lipopeptides on other snail life stages such as eggs or juveniles may also offer an avenue of control, possibly at concentrations that are easier to achieve than required for adult snails. There are further questions of whether snails infected with fluke are more sensitive to surfactin, or even other lipopeptides. With the identification of surfactin as molluscicidal, a number of research questions are implied: Are other lipopeptides also molluscicidal? And if so, are they more or less active against target snails and are they more or less active against non-target aquatic species? Can other lipopeptides work synergistically with surfactin to increase snail sensitivity? There is also room to investigate the opportunity for synergistic biocontrol of other pest species, such as mosquitoes (Maget-Dana *et al.,* 1992; Romero *et al.,* 2007; Geetha *et al.,* 2012; Deravel *et al.,* 2014).

Conclusions

The primary goal of this thesis was to identify bacterial isolates which could be used to control aquatic snail populations. While this research did not find an isolate able to cause significant adult snail mortality in a simulated aquatic environment, it did uncover the potential use of surfactin as a molluscicide. This research constitutes a first step in developing a biological-based approach to snail control and opens up a number of research avenues for further investigation related to lipopeptides and their implementation in aquatic snail biocontrol.

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APPENDIX A

Table of details of soil and snail sampling sites within the KwaZulu-Natal and Free State provinces of South Africa

Sample Code	Site Description	Sample Description	GPS Location		Sampling Date
			Latitude	Longitude	
A0	KwaZulu-Natal, Pietermaritzburg, UKZN Disease Garden	Ant nest soil	-29.6266	30.40409	12/02/2018
A	KwaZulu-Natal, Pietermaritzburg, Foxhillspruit Canal River	Bank	-29.6291	30.38864	05/03/2018
B	KwaZulu-Natal, Pietermaritzburg, Foxhillspruit Canal River	Bottom (rocky)	-29.628	30.38956	05/03/2018
C	KwaZulu-Natal, Pietermaritzburg, UKZN Disease Garden	Ants nest soil 2	-29.6266	30.40409	26/03/2018
D	KwaZulu-Natal, Pietermaritzburg, UKZN Disease Garden	Cabbage patch soil	-29.6266	30.40409	26/03/2018
E	KwaZulu-Natal, Pietermaritzburg, Woodgrove Retirement Village	Stream bank soil	-29.5906	30.36804	3/04/2018
F	KwaZulu-Natal, Pietermaritzburg, Natal Canoe Club Campsdrift	Upper bank soil	-29.6223	30.37679	3/04/2018
G	KwaZulu-Natal, Pietermaritzburg, Natal Canoe Club Campsdrift	Lower bank soil	-29.6223	30.37679	3/04/2018
H	KwaZulu-Natal, Pietermaritzburg, Botanical Garden	Silt pond stream soil	-29.6071	30.34442	16/04/2018
I	KwaZulu-Natal, Pietermaritzburg, Botanical Garden	Silt pond soil	-29.6071	30.34442	16/04/2018
J	KwaZulu-Natal, Pietermaritzburg, Botanical Garden	River bank soil	-29.6066	30.34466	16/04/2018
K	KwaZulu-Natal, Pietermaritzburg, Botanical Garden	River mud flats soil	-29.6066	30.34466	16/04/2018
L	KwaZulu-Natal, Pietermaritzburg, Botanical Garden	Wetland bank soil	-29.6064	30.34479	30/04/2018
M	KwaZulu-Natal, Pietermaritzburg, Botanical Garden	Duck pond soil	-29.6061	30.34455	30/04/2018
N	KwaZulu-Natal, Pietermaritzburg, Botanical Garden	Forest seepage pool soil	-29.6052	30.34541	30/04/2018
O	KwaZulu-Natal, Pietermaritzburg, Botanical Garden	Weir flats soil	-29.6047	30.34738	30/04/2018
P	KwaZulu-Natal, Pietermaritzburg, Botanical Garden	Lily pond soil	-29.6047	30.34844	30/04/2018
Q	KwaZulu-Natal, Pietermaritzburg, Botanical Garden	Stream soil	-29.605	30.34863	30/04/2018
R	KwaZulu-Natal, Pietermaritzburg, Msunduzi River	Mudflats soil	-29.6286	30.36764	14/05/2018
S	KwaZulu-Natal, Pietermaritzburg, Patricia Road	Flats soil	-29.5664	30.36585	14/05/2018
T	KwaZulu-Natal, Pietermaritzburg, Patricia Road	Bank soil	-29.5664	30.36585	14/05/2018
U	KwaZulu-Natal, Pietermaritzburg, Hatcheries Park, Chase Valley	Stream soil	-29.5698	30.36634	14/05/2018
V	KwaZulu-Natal, Pietermaritzburg, Athlone	Weir soil	-29.5887	30.35171	16/07/2018

Cont. Table of details of soil and snail sampling sites within the KwaZulu-Natal and Free State provinces of South Africa

Sample Code	Site Description	Sample Description	GPS Location		Sampling Date
			Latitude	Longitude	
W	KwaZulu-Natal, Pietermaritzburg, Athlone	Footbridge soil	-29.5887	30.35171	16/07/2018
X	Free State, Clarens	Dry stream bed (loam) soil A	-28.5157	28.41192	04/02/2019
Y	Free State, Clarens	Dry streambed (clay-sand) soil	-28.5165	28.40897	04/02/2019
Z	Free State, Clarens	Dry stream bed (loam) soil B	-28.5165	28.40911	12/02/2019
AA	Free State, Clarens, Van Reenan Street	Dry riverbank	-28.5155	28.41307	12/02/2019
AB	Free State, Montrose Shell Ultra City	Duck Pond A	-28.3568	29.28477	18/02/2019
AC	Free State, Montrose Shell Ultra City	Duck Pond B	-28.3565	29.28466	18/02/2019
AD	KwaZulu-Natal, Pietermaritzburg, Warwick Road	Streambed soil	-29.5632	30.34199	13/03/2019
AE	KwaZulu-Natal, Pietermaritzburg, Warwick Road	Causeway soil	-29.563	30.34143	13/03/2019
AF	KwaZulu-Natal, Pietermaritzburg, Warwick Road	Stream 2 A	-29.5628	30.34152	13/03/2019
AG	KwaZulu-Natal, Pietermaritzburg, Warwick Road	Stream 2 B	-29.5628	30.34152	13/03/2019
AH	KwaZulu-Natal, Pietermaritzburg, UKZN Botanical Garden	Land snail carcass	-29.6245	30.40365	15/04/2019
AI	KwaZulu-Natal, Pietermaritzburg, UKZN Botanical Garden	Land snail carcass freshly dead	-29.6245	30.40365	15/04/2019
AJ	KwaZulu-Natal, Pietermaritzburg, Hillside Road	Garden stream A	-29.6054	30.3502	20/05/2019
AK	KwaZulu-Natal, Pietermaritzburg, Hillside Road	Garden stream B	-29.6054	30.3502	20/05/2019
Al	KwaZulu-Natal, Pietermaritzburg, Hillside Road	Riverbank soil A	-29.6067	30.35001	20/05/2019
AM	KwaZulu-Natal, Pietermaritzburg, Hillside Road	Riverbank soil B	-29.6067	30.35001	20/05/2019
AN	KwaZulu-Natal, Pietermaritzburg, Wylie Park	Waterlogged 'marsh'	-29.5883	30.35017	22/07/2019
AO	KwaZulu-Natal, Pietermaritzburg, Wylie Park	Stream A	-29.5881	30.34758	22/07/2019
AP	KwaZulu-Natal, Pietermaritzburg, Wylie Park	Stream B	-29.5881	30.34758	22/07/2019
AQ	KwaZulu-Natal, Durban, Paradise Valley	River sediment	-29.8322	30.8922	2207/2019
AR	KwaZulu-Natal, Nottingham Road, Strathdean Farm dam	Dead snails (8)	-29.3578	30.02327	26/08/2019
AS	KwaZulu-Natal, Nottingham Road, Strathdean Farm dam	Sample 1	-29.3579	30.02334	08/09/2019
AT	KwaZulu-Natal, Nottingham Road, Strathdean Farm dam	Sample 2	-29.358	30.02403	15/09/2019
AU	KwaZulu-Natal, Nottingham Road, Strathdean Farm dam	Sample 3	-29.3579	30.02426	22/10/2019
AV	KwaZulu-Natal, Nottingham Road, Strathdean Farm dam	Sample 4	-29.3577	30.02439	03/11/2019

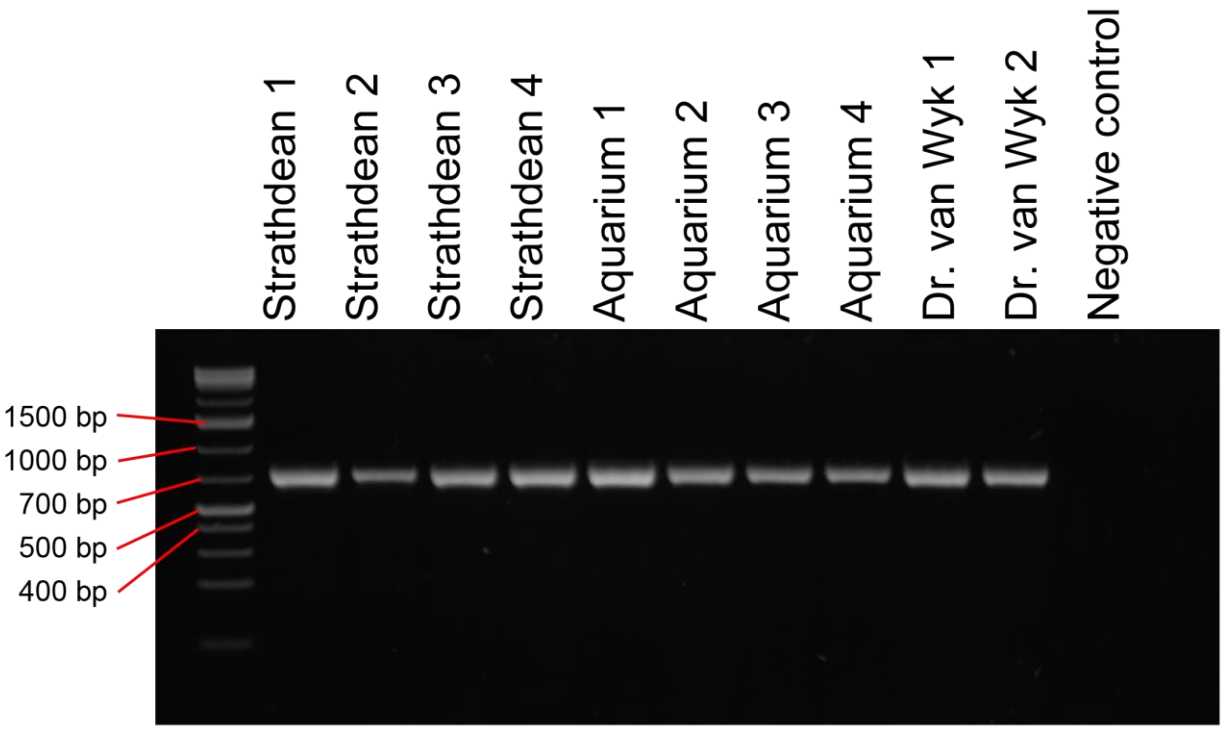
Cont. Table of details of soil and snail sampling sites within the KwaZulu-Natal and Free State provinces of South Africa

Sample Code	Site Description	Sample Description	GPS Location		Sampling Date
			Latitude	Longitude	
AW	KwaZulu-Natal, Nottingham Road, Strathdean Farm dam	Sample 5	-29.3574	30.02386	03/11/2019
AX	KwaZulu-Natal, Nottingham Road, Strathdean Farm dam	Sample 6	-29.3574	30.02366	24/11/2019
AY	KwaZulu-Natal, Nottingham Road, Strathdean Farm dam	Sample 7	-29.3576	30.02321	24/11/2019
AZ	KwaZulu-Natal, Durban, Paradise Valley	Riverbank	-29.8319	30.89218	16/03/2020
BA1	KwaZulu-Natal, Zenzani, Hopewell Farm	Wetland, heavy vegetation 1	-29.4142	30.00448	10/09/2020
BA2	KwaZulu-Natal, Zenzani, Hopewell Farm	Wetland, heavy vegetation 2	-29.4142	30.00448	10/09/2020
BB1	KwaZulu-Natal, Zenzani, Hopewell Farm	Drainage ditch 1	-29.4136	30.01106	12/10/2020
BB2	KwaZulu-Natal, Zenzani, Hopewell Farm	Drainage ditch 2	-29.4136	30.01106	12/10/2020
BC1	KwaZulu-Natal, Zenzani, Hopewell Farm	Large dam 1	-29.4138	30.00918	26/10/2020
BC2	KwaZulu-Natal, Zenzani, Hopewell Farm	Large dam 2	-29.4124	30.00764	26/10/2020
BD1	KwaZulu-Natal, Zenzani, Hopewell Farm	Small pond 1	-29.4175	30.00066	9/11/2020
BD2	KwaZulu-Natal, Zenzani, Hopewell Farm	Small pond 2	-29.4175	30.00066	9/11/2020
BC8	KwaZulu-Natal, Zenzani, Hopewell Farm	Large dam 3	-29.4127	30.01164	7/12/2020
BA3	KwaZulu-Natal, Zenzani, Hopewell Farm	Wetland, heavy vegetation	-29.4131	30.0104	7/12/2020

APPENDIX B

Gel electrophoresis image of COX-1 PCR amplicons for snail identification showing the ~700bp product

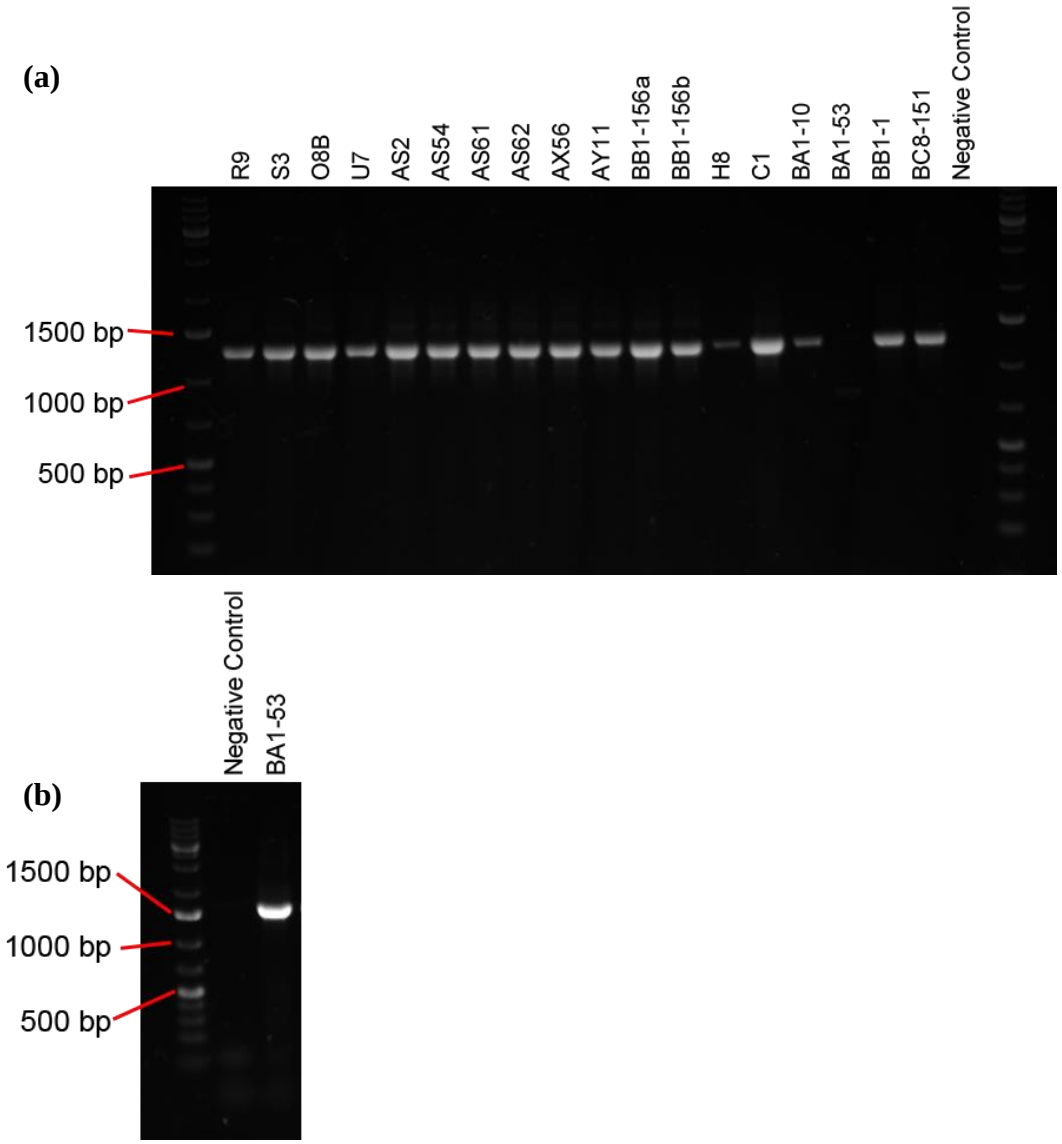
Amplified product (2-5 µL) was visualised on agarose gel (1% w/v) made up with sodium boric acid buffer (SB) (Brody and Kern, 2004), stained using SYBR Safe (Thermo Fisher Scientific, Waltham, Massachusetts, United States) at 0.5x concentration (0.5 µL per 10 mL gel volume). Product size was visually estimated using a GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Gels were run for 80 minutes at 80 V before visualising and image capture using a SynGene G:Box and Syngene GeneSnap software (version 7.09) (Syngene, Cambridge, England).



APPENDIX C

Gel electrophoresis image of 16S rDNA amplicons for snail identification showing the ~1400 bp product, where (a) shows the amplicons for primers BacF / R1378, and (b) shows the amplicon for primers fD1 / rP2 for isolate BA1-53

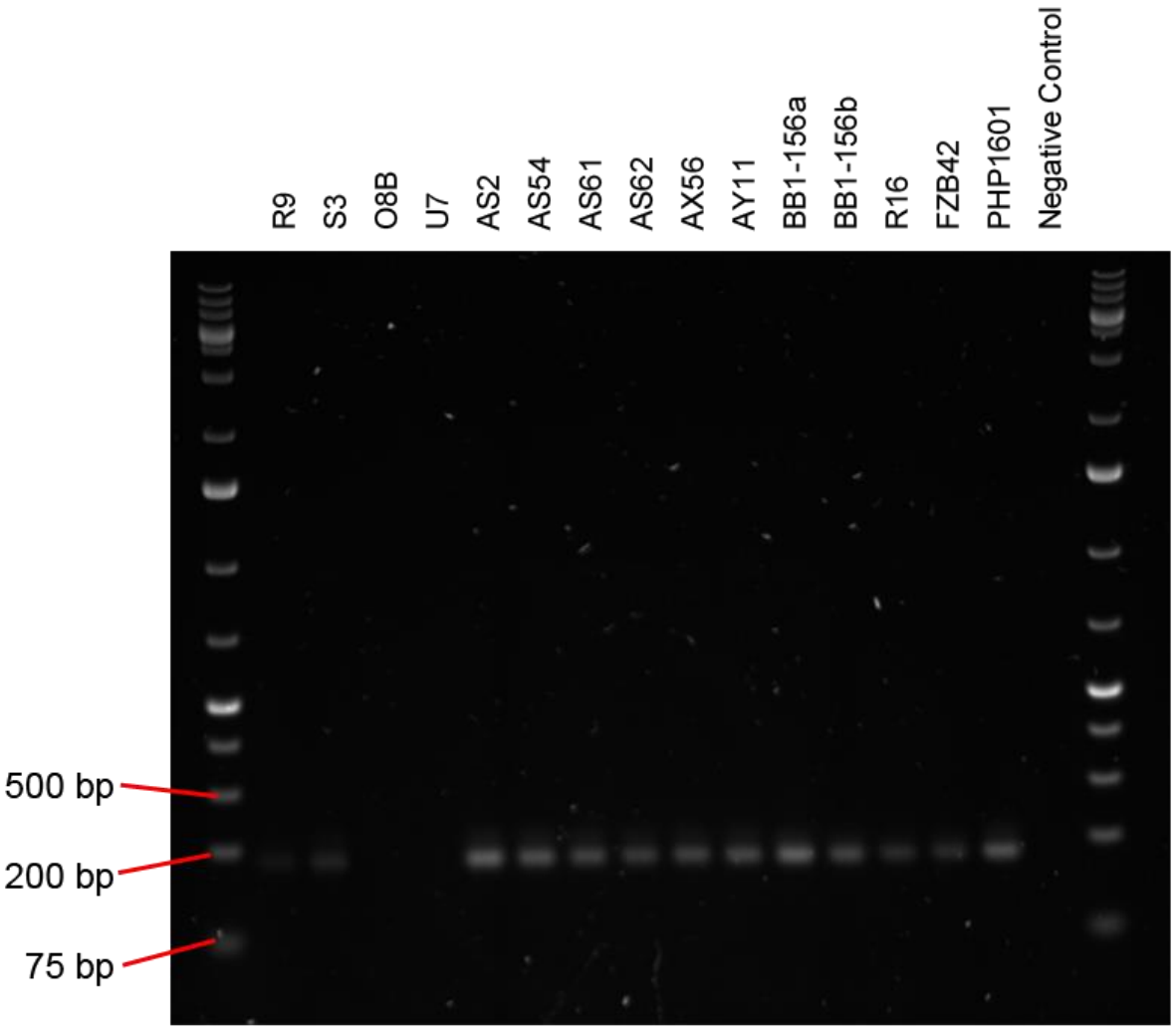
Amplified product (2-5 µL) was visualised on agarose gel (1% w/v) made up with sodium boric acid buffer (SB) (Brody and Kern, 2004), stained using SYBR Safe (Thermo Fisher Scientific, Waltham, Massachusetts, United States) at 0.5x concentration (0.5 µL per 10 mL gel volume). Product size was visually estimated using a GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Gels were run for 80 minutes at 80 V before visualising and image capture using a SynGene G:Box and Syngene GeneSnap software (version 7.09) (Syngene, Cambridge, England).



APPENDIX D

Gel electrophoresis image of *Bacillus velezensis* endpoint PCR amplicons for snail identification showing the ~180 bp product

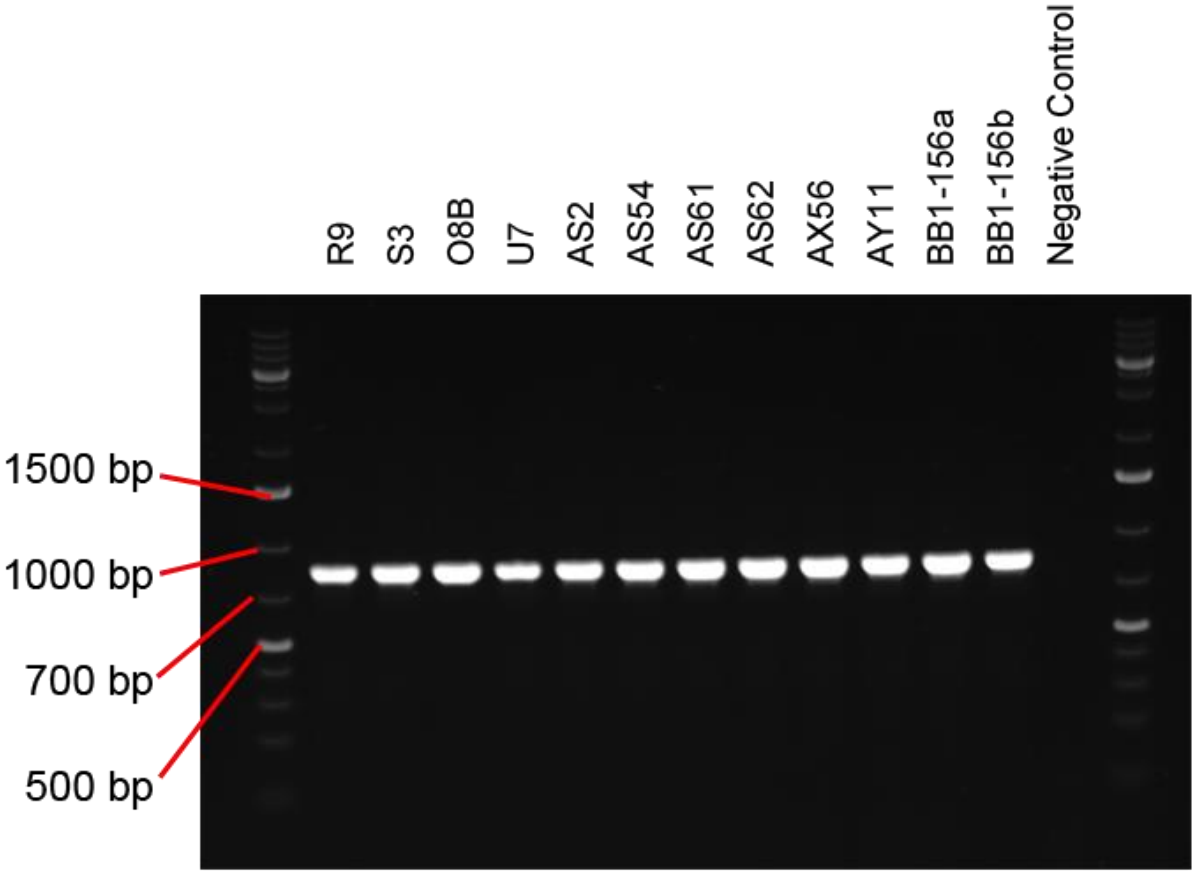
Amplified product (2-5 µL) was visualised on agarose gel (1% w/v) made up with sodium boric acid buffer (SB) (Brody and Kern, 2004), stained using SYBR Safe (Thermo Fisher Scientific, Waltham, Massachusetts, United States) at 0.5x concentration (0.5 µL per 10 mL gel volume). Product size was visually estimated using a GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Gels were run for 80 minutes at 80 V before visualising and image capture using a SynGene G:Box and Syngene GeneSnap software (version 7.09) (Syngene, Cambridge, England).



APPENDIX E

Gel electrophoresis image of *rpoB* amplicons for snail identification showing the ~900 bp product

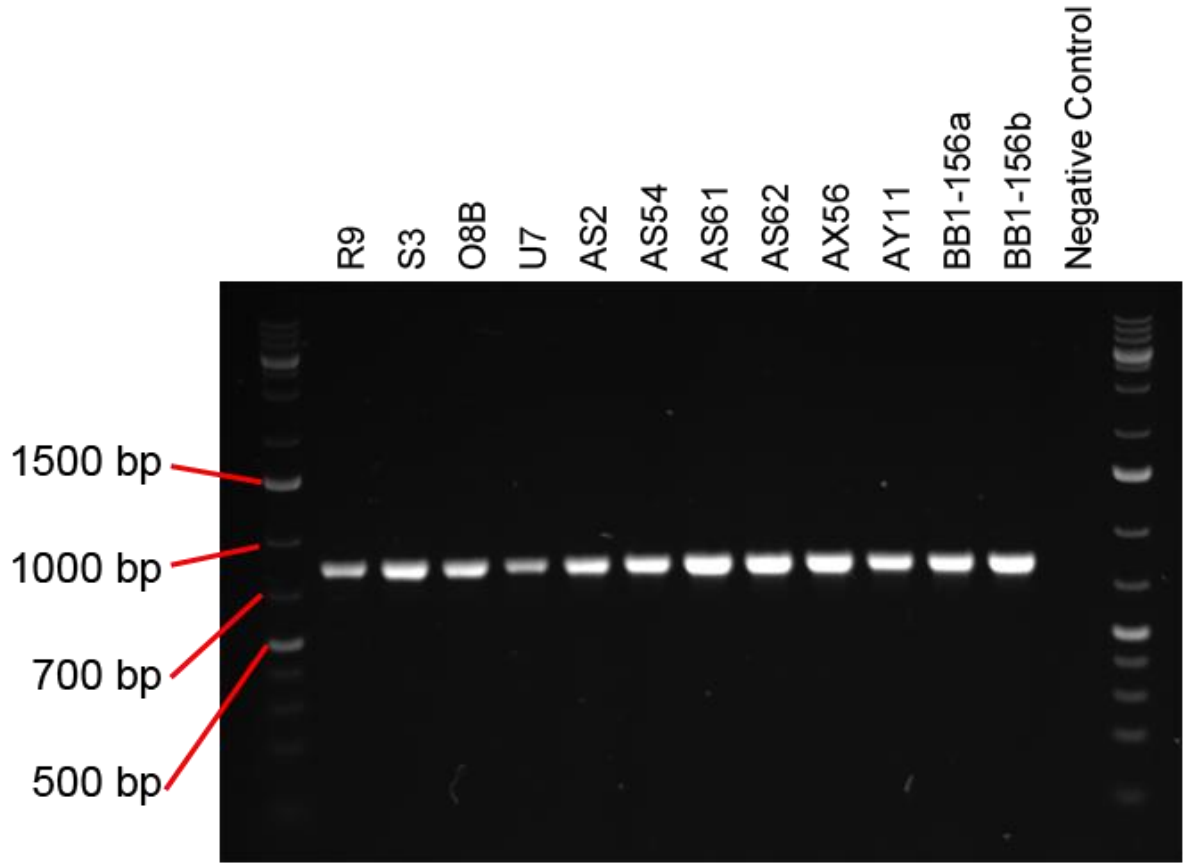
Amplified product (2-5 µL) was visualised on agarose gel (1% w/v) made up with sodium boric acid buffer (SB) (Brody and Kern, 2004), stained using SYBR Safe (Thermo Fisher Scientific, Waltham, Massachusetts, United States) at 0.5x concentration (0.5 µL per 10 mL gel volume). Product size was visually estimated using a GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Gels were run for 80 minutes at 80 V before visualising and image capture using a SynGene G:Box and Syngene GeneSnap software (version 7.09) (Syngene, Cambridge, England).



APPENDIX F

Gel electrophoresis image of *dnaJ* amplicons for snail identification showing the ~900 bp product

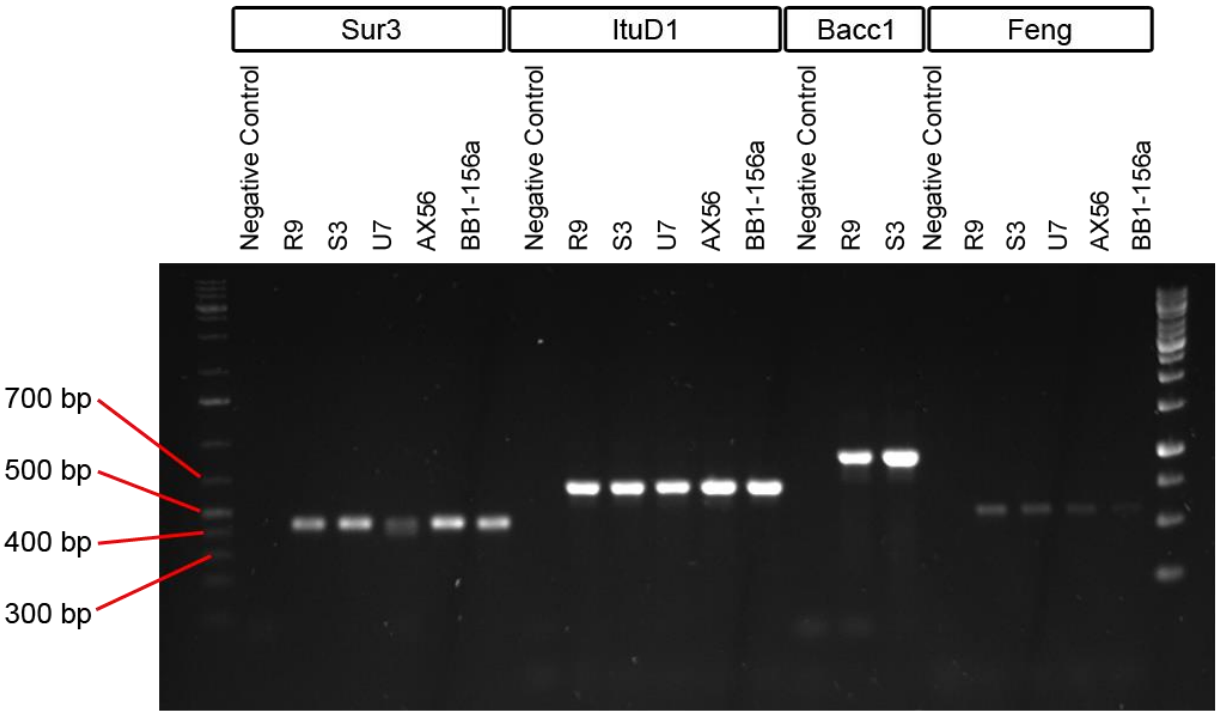
Amplified product (2-5 µL) was visualised on agarose gel (1% w/v) made up with sodium boric acid buffer (SB) (Brody and Kern, 2004), stained using SYBR Safe (Thermo Fisher Scientific, Waltham, Massachusetts, United States) at 0.5x concentration (0.5 µL per 10 mL gel volume). Product size was visually estimated using a GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Gels were run for 80 minutes at 80 V before visualising and image capture using a SynGene G:Box and Syngene GeneSnap software (version 7.09) (Syngene, Cambridge, England).



APPENDIX G

Gel electrophoresis image of lipopeptide gene marker PCR amplicons for snail identification showing the product sizes for the following primers: Surfactin SUR3 (~ 450 bp), iturin ITUD (~650 bp), bacillomycin BACC1 (~900 bp), and fengycin FENG (~550 bp)

Amplified product (2-5 µL) was visualised on agarose gel (1% w/v) made up with sodium boric acid buffer (SB) (Brody and Kern, 2004), stained using SYBR Safe (Thermo Fisher Scientific, Waltham, Massachusetts, United States) at 0.5x concentration (0.5 µL per 10 mL gel volume). Product size was visually estimated using a GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Gels were run for 80 minutes at 80 V before visualising and image capture using a SynGene G:Box and Syngene GeneSnap software (version 7.09) (Syngene, Cambridge, England).



APPENDIX H

Table of snail mortality (%) from assays using cell-free culture supernatant from tryptic soya broth and Landy medium, respectively, after 24 h exposure

Isolate	Broth % (v/v)	TSB Broth	Landy Broth
S3	10	100	100
	5	100	100
	2.5	91.7	0
R9	10	100	100
	5	50	0
	2.5	16.7	0
O8b	10	100	100
	5	33.3	100
	2.5	0	0
U7	10	100	17.7
	5	100	0
	2.5	100	0
AX56	10	100	0
	5	100	0
	2.5	100	0
BB1-156a	10	91.7	100
	5	41.7	0
	2.5	0	0

APPENDIX I

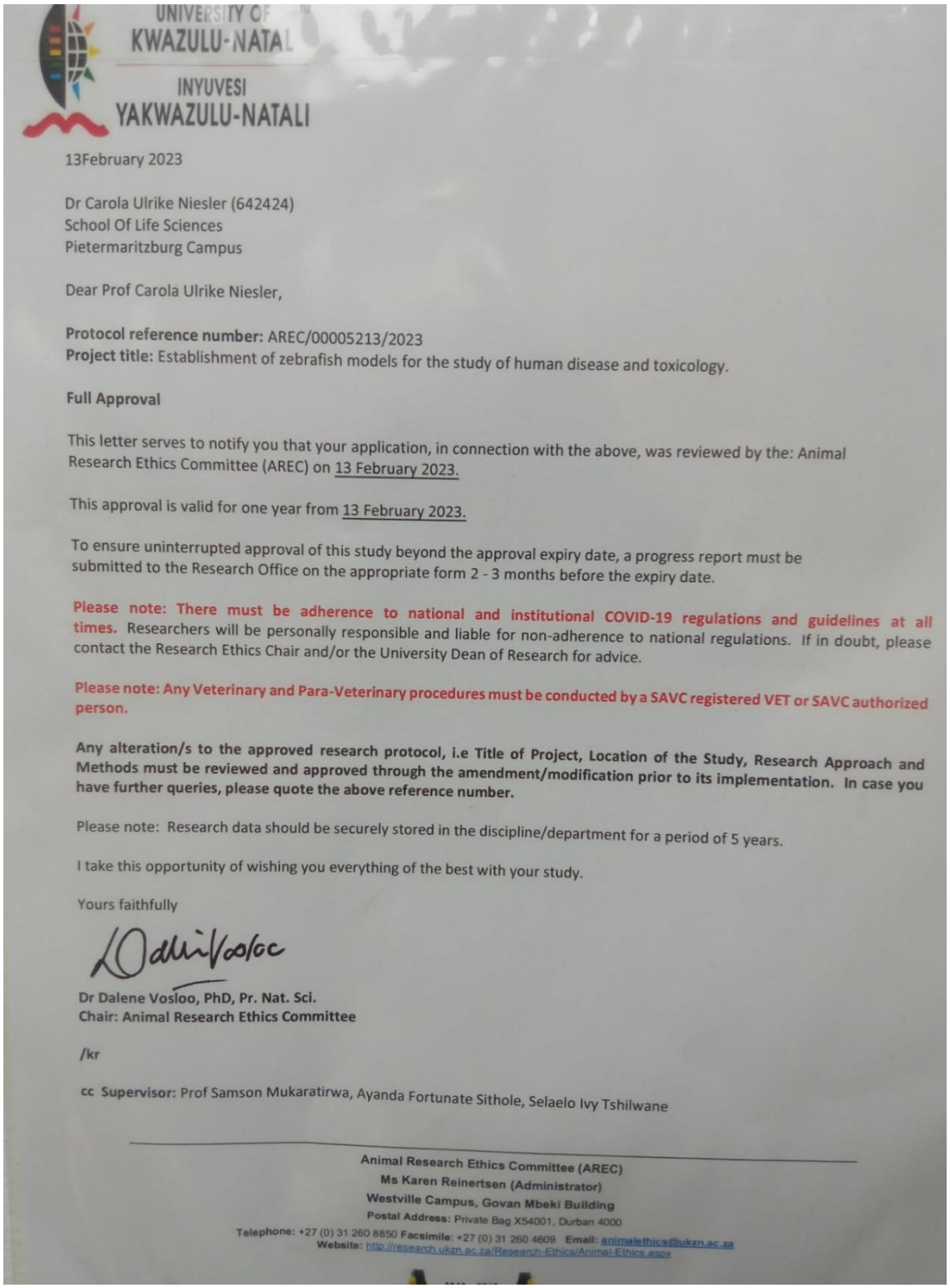
Table of snail mortality (%) from assays using crude lipopeptide extracts from tryptic soya broth and Landy medium, respectively, after 24 h exposure

Isolate	Extract (µg.mL^{-1})	TSB Extract	Landy Extract
S3	200	NT	NT
	100	100	100
	50	100	33.3
	25	33.3	0
R9	200	NT	NT
	100	100	100
	50	100	100
	25	16.7	0
O8b	200	100	NT
	100	41.7	100
	50	0	83.3
	25	0	0
U7	200	NT	100
	100	100	83.3
	50	100	16.7
	25	41.7	0
AX56	200	NT	100
	100	100	16.7
	50	100	0
	25	83.3	0
BB1-156a	200	NT	NT
	100	100	100
	50	8.3	0

NT – not tested

APPENDIX J

Copy of the Animal Ethics Research Committee approval for the "Establishment of Zebrafish models for the study of human disease and toxicology" (AREC/00005213/2023) for zebrafish research studies conducted at the University of KwaZulu-Natal



UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

13February 2023

Dr Carola Ulrike Niesler (642424)
School Of Life Sciences
Pietermaritzburg Campus

Dear Prof Carola Ulrike Niesler,

Protocol reference number: AREC/00005213/2023
Project title: Establishment of zebrafish models for the study of human disease and toxicology.

Full Approval

This letter serves to notify you that your application, in connection with the above, was reviewed by the: Animal Research Ethics Committee (AREC) on 13 February 2023.

This approval is valid for one year from 13 February 2023.

To ensure uninterrupted approval of this study beyond the approval expiry date, a progress report must be submitted to the Research Office on the appropriate form 2 - 3 months before the expiry date.

Please note: There must be adherence to national and institutional COVID-19 regulations and guidelines at all times. Researchers will be personally responsible and liable for non-adherence to national regulations. If in doubt, please contact the Research Ethics Chair and/or the University Dean of Research for advice.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

Dr Dalene Vosloo, PhD, Pr. Nat. Sci.
Chair: Animal Research Ethics Committee

/kr

cc Supervisor: Prof Samson Mukaratirwa, Ayanda Fortunate Sithole, Selaelo Ivy Tshilwane

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