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Evaluation of the larvicidal potential of *Bacillus velezensis* strain PHP1601 as a viable biological control agent against selected fly species

By

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in Microbiology

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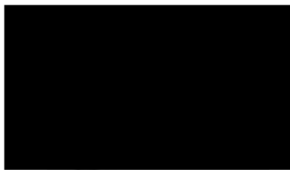
PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Microbiology, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg Campus, South Africa. This research project was financially supported by Andermatt Plant Health Products (APHP).

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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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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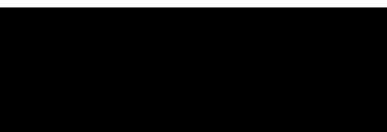

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DECLARATION 2: PUBLICATION

My role in each paper is indicated. The * indicates corresponding author.

Chapter 2

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DISSERTATION ABSTRACT

Flies are one of the most abundant and prevalent insect pests posing a growing threat to various sectors of the economy. In response to this, a study was undertaken to evaluate *Bacillus* spp. strain PHP1601 as a candidate biocontrol agent against *Lucilia cuprina* larvae as a proxy for fly species of biocontrol significance. The identity of PHP1601 was confirmed as *B. velezensis* using MLSA and species-specific PCR. Bioassays demonstrated a larvicidal effect of cell, endospore ($10^2 - 10^{10}$ cells/endospores g^{-1}) and cell-free supernatant (1 – 30% v w^{-1}) treatments on second instar larvae of *L. cuprina*. Studies were directed to the larvicidal effect of extracellular compounds, namely lipopeptides. Crude lipopeptide extract (CLP) was acquired using organic extraction from Landy broth. Bioassays with CLP extract (5 – 1000 µg g^{-1}) resulted in a dose-dependent larvicidal response. Lipopeptides in the CLP extract were purified by TLC and characterised using UPLC ESI-TOF MS. This indicated the presence of iturin, fengycin and surfactin homologues of which, the purified surfactin fraction (Rf 0.91) was the most larvicidal. Bioassays were repeated with commercial surfactin, confirming its larvicidal potency, exhibiting an LC_{50} of 9.87 µg g^{-1} at 240 h. Larvae scent choice tests using TSB and MG bioassay medium fermented by PHP1601 showed that resulting VOCs were attractive to fly larvae, which was considered a viable trait of a fly biocontrol agent. CG-MS of the VOCs produced indicated that ketones were the dominant VOC class and, presumably, the major contributor to this larvae attraction effect. Field performance evaluation using pig manure trials demonstrated successful inhibition of several fly species of agricultural and veterinary importance using endospore treatments (10^5 and 10^{10} endospores g^{-1}) of PHP1601. qPCR and REP-PCR fingerprinting confirmed that PHP1601 could grow in the manure slurries and was amiable to recovery and monitoring. Zebrafish embryo toxicity bioassays of the CLP produced by PHP1601 indicated that they achieved an LC_{50} of 22.77 µg $ml^{-1,}$ which characterised these metabolites as slightly toxic. Genome mining detected no genes associated with pathogenicity or virulence and presented no apparent pathogenic threat. The investigation demonstrated that *B. velezensis* PHP1601 is a viable fly biocontrol candidate and constitutes the first report of a *B. velezensis* antagonist of Brachycera flies.

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TABLE OF CONTENTS

PREFACE ii

DECLARATION 1: PLAGIARISM iii

DECLARATION 2: PUBLICATION...iv

DISSERTATION ABSTRACT v

ACKNOWLEDGMENTS...vi

TABLE OF CONTENTS..vii

LIST OF TABLES xiii

LIST OF FIGURES...xiv

LIST OF ABBREVIATIONS xvi

INTRODUCTION..1

1. CHAPTER ONE: LITERATURE REVIEW 6

1.1 Introduction 6

1.2 Overview of the progression of biocontrol 7

1.3 Genus *Bacillus* (Bacillales: Bacillaceae)..9

1.3.1 Endospore formation 11

1.3.2 Germination of endospores...13

1.4 Biocontrol applications associated with the *B. cereus sensu lato* group......................14

1.4.1 Concerns regarding the longevity of *B. thuringiensis* treatments 16

1.5 Biocontrol applications associated with the *B. subtilis sensu lato* group.....................17

1.5.1 Suppression of plant-associated microbial pathogens 17

1.5.2 Antagonism of insect pests 18

1.6 The role of *Bacillus*-associated lipopeptides in biocontrol applications......................20

1.7 Entomopathogenic microorganisms 22

1.7.1 Entomopathogenic bacteria: *Pseudomonas* spp. 23

1.7.2 Entomopathogenic fungi: *Beauveria bassiana* 24

1.8 Comparison of microbial and chemical insecticides....26
1.9 Criteria for evaluating microbial biocontrol candidates....29
1.10 Establishing a biocontrol candidate....30
1.11 Target for biocontrol: Members of true flies, Diptera....33
1.11.1 Fly life cycle....34
1.11.2 The need for control of flies....35
1.11.2.1 Blowflies, threats to livestock farms and humans....35
1.11 Conclusion....37
1.12 References....37
2. CHAPTER TWO: FIRST REPORTED INCIDENCE OF *BACILLUS VELEZENSIS* EXHIBITING EFFECTIVE ANTAGONISM AGAINST A BLOWFLY SPECIES, *LUCILIA CUPRINA*....61
2.1 Abstract....61
2.2 Introduction....62
2.3 Methods and materials....63
2.3.1 Bacterial culture....63
2.3.2 DNA extraction....63
2.3.3 MLSA and species-specific PCR identification of PHP1601....63
2.3.4 Capture and rearing of blowflies....64
2.3.5 Identification of captured flies and assessment of population purity....65
2.3.6 Growth of PHP1601 in MG agar....65
2.3.7 Larvicidal trials evaluating PHP 1601 vegetative cell and endospore treatments....66
2.3.8 Larvicidal effect of cell-free supernatant (CFS) of PHP1601....66
2.3.9 Lipopeptide biosurfactant production and assessment....67
2.3.10 Larvicidal effect of crude lipopeptides....67
2.3.11 Statistical analysis....67
2.4 Results....68
2.4.1 Identification of PHP1601....68

2.4.2 Characterisation and identification of captured flies 69

2.4.3 Larvicidal effect of viable culture of PHP1601 70

2.4.4 Larvicidal effect of PHP1601 CFS 73

2.4.5 Larvicidal effect of CLP extracts on *L. cuprina* larvae 74

2.5 Discussion 77

2.6 References 80

3. CHAPTER THREE: ELUCIDATION OF LIPOPEPTIDE BIOSURFACTANTS RESPONSIBLE FOR THE LARVICIDAL ACTIVITY OF *BACILLUS VELEZENSIS* PHP1601 TOWARDS *LUCILIA CUPRINA* LARVAE 85

3.1 Abstract 85

3.2 Introduction 86

3.3 Methods and materials 87

3.3.1 Bacterial culture 87

3.3.2 Production and extraction of lipopeptide biosurfactants 87

3.3.3 Detection and characterisation of lipopeptide biosurfactants 87

3.3.4 Purification of lipopeptide biosurfactants 88

3.3.5 Fly population 89

3.3.6 The larvicidal effect of lipopeptide fractions 89

3.3.7 Larvicidal effect of surfactin standard 89

3.3.8 Statistical analysis 90

3.4 Results 90

3.4.1 Characterisation of lipopeptide biosurfactants 90

3.4.2 TLC fractionation and purification of lipopeptide biosurfactants from CLP extracts 91

3.4.3 Larvicidal assays with TLC purified lipopeptide biosurfactants 95

3.4.4 Larvicidal effect of surfactin standard 96

3.4.5 Influence of lipopeptide biosurfactants on larvae behaviour and physiology 98

3.5 Discussion 99

3.6 References 102

4. CHAPTER FOUR: OLFACTORY RESPONSE OF *LUCILIA CUPRINA* LARVAE TO VOLATILE ORGANIC COMPOUNDS PRODUCED BY *BACILLUS VELEZENSIS* PHP1601 107

4.1 Abstract 107

4.2 Introduction 108

4.3 Methods and Materials 109

4.3.1 Bacterial culture 109

4.3.2 Fly population 110

4.3.3 Production of PHP1601 scent cues 110

4.3.4 Scent choice tests 111

4.3.5 Scent profiling of PHP1601 112

4.3.6 Statistical analysis 114

4.4 Results 114

4.4.1 Olfactory choice bioassays of VOCs from fermented TSA 114

4.4.2 Olfactory choice bioassays of VOCs from fermented MG bioassay medium 117

4.4.3 Preference of larvae towards the scent of PHP1601 cultured on pig manure 118

4.4.4 Identification of VOC produced by PHP1601 119

4.5 Discussion 122

4.6 References 125

5. CHAPTER FIVE: MANURE TRIAL EVALUATION OF *BACILLUS VELEZENSIS* PHP1601 AS A VIABLE BIOCONTROL CANDIDATE AGAINST NUISANCE FLY SPECIES AT AN OPERATIONAL PIGGERY 129

5.1 Abstract 129

5.2 Introduction 130

5.3 Methods and materials 131

5.3.1 Bacterial strains 131

5.3.2 Trial design 132

5.3.3 Manure trial 132

5.3.4 Identification of captured flies 133

5.3.5 Detection and quantification of *B. velezensis* in pig manure slurries by qPCR .. 133

5.3.6 Statistical analysis 135

5.4 Results 136

5.4.1 Influence of treatments on fly oviposition 136

5.4.2 Manure biocontrol trial 1 136

5.4.3 Manure biocontrol trial 2 139

5.4.4 Identification of the host range of flies antagonised by *B. velezensis* PHP1601 . 141

5.4.5 Growth of *B. velezensis* PHP1601 in pig manure slurries 141

5.5 Discussion 145

5.6 References 148

6. CHAPTER SIX: TOXICOLOGICAL ASSESSMENT OF *BACILLUS VELEZENSIS* PHP1601 BASED ON ZEBRAFISH EMBRYO TOXICITY ASSAYS AND GENOME MINING 151

6.1 Abstract 151

6.2 Introduction 152

6.3 Methods and materials 153

6.3.1 Bacterial culture 153

6.3.2 Lipopeptide biosurfactant production 154

6.3.3 Identification and quantification of lipopeptide biosurfactants produced by PHP1601 154

6.3.4 Zebrafish embryo toxicity assays 155

6.3.5 DNA extraction 156

6.3.6 Whole genome sequencing and assembly 156

6.3.7 Bioinformatic assessments 157

6.3.8 Statistical analysis 157

6.4 Results 158

6.4.1 Quantification and identification of lipopeptide biosurfactants produced by PHP1601 158

6.4.2 ZET bioassays 159

6.4.3 Growth of PHP1601 in freshwater media 161

6.4.4 Genome description 163

6.4.5 Average nucleotide identity (ANI) facilitated species clustering of PHP1601 ... 163

6.4.6 Genome mining for toxin production and pathogenicity 165

6.4.7 Genome mining for AMR genes using CARD 166

6.4.8 Genome screening for metabolite production using AntiSMASH 169

6.5 Discussion 169

6.6 References 173

7. CHAPTER SEVEN: GENERAL OVERVIEW AND CONCLUSION 180

7.1 Summary of findings 180

7.2 Contributions of the study and recommendations for future assessments of fly biocontrol *B. subtilis sensu lato* candidates 184

7.3 Future investigations 188

7.4 Conclusion 191

7.5 References 192

8. APPENDIX 196

Appendix A: First reported incidence of *Bacillus velezensis* exhibiting effective antagonism against a blowfly species, *Lucilia cuprina* 196

Appendix B: Elucidation of lipopeptide biosurfactants responsible for the larvicidal activity of *Bacillus velezensis* PHP1601 towards *Lucilia cuprina* larvae 203

Appendix C: Manure trial evaluation of *Bacillus velezensis* PHP1601 as a viable biocontrol candidate against nuisance fly species at an operational piggery 206

Appendix D: Toxicological assessment of *Bacillus velezensis* PHP1601 based on zebrafish embryo toxicity assays and genome mining 212

LIST OF TABLES

Table 1.1 Example of the *Bacillus subtilis sensu lato* involved in insect biocontrol. 20
Table 3.1 Lipopeptide biosurfactant species detected in the TLC-purified lipopeptide fractions of PHP1601. 94
Table 4.1 Qualitative analysis of volatile organic compounds produced by *B. velezensis* PHP1601 121
Table 5.1 Population dynamics of *B. velezensis* over the course of the manure trial............143
Table 6.1 Virulence factors detected in the genome of *B. velezensis* PHP1601....................166
Table 6.2 AMR gene profile of PHP1601 determined by CARD analysis 168
Table 6.3 Gene clusters identified for secondary metabolite production in the genome of *B. velezensis* PHP1601 by AntiSMASH genome mining 169

LIST OF FIGURES

Fig. 1.1 Summarised process of endospore formation of representative of *Bacillus* spp. 13
Fig. 1.2 Lytic mode of action of *B. thuringiensis* Cry protein...15
Fig. 1.3 Insecticidal cellular death signalling mode of action of *B. thuringiensis*.................16
Fig. 1.4 Representative isoforms of main lipopeptide families; surfactin, iturin and fengycin. 22
Fig. 1.5 Diseased mummified silkworms.. 25
Fig. 2.1 Maximum likelihood phylogenetic tree inferring the evolutionary relationships of PHP1601 and selected reference sequences from NCBI GenBank based on MLSA of partial 16S rRNA, *dna*J, *gyr*A and *rpo*B gene sequences. 69
Fig. 2.2 The larvicidal effect of cell (a) and endospore (b) treatments of PHP1601 on *L. cuprina* larvae over a 240-h assay.. 72
Fig. 2.3 Larvicidal effect of cell-free supernatant of PHP1601 on *L. cuprina* larvae over a 480-h assay...74
Fig. 2.4 Larvicidal effect of crude lipopeptide extracts from PHP1601 on *L. cuprina* larvae over a 480-h assay...76
Fig. 3.1 UPLC chromatogram of eluted compounds in the CLP of PHP1601. The compounds present in the elution peaks were characterised by ESI-TOF MS.. 91
Fig. 3.2 Thin layer chromatography of CLP extract of *Bacillus velezensis* PHP1601. 93
Fig. 3.3 Larvicidal effect of TLC purified lipopeptide fractions from PHP1601 on larvae of *L. cuprina*. 96
Fig. 3.4 Larvicidal effect of commercial surfactin on larvae of *L. cuprina*...........................98
Fig. 4.1 Larvae preference (mean ± SE) towards VOCs produced by PHP1601 fermented TSA only (a), uninoculated TSA only (b), endospore biomass (c), cell biomass (d) and comparatively between fermented TSA and the TSA control (e).......................................116
Fig. 4.2 Larvae preference (mean ± SE) towards VOC produced by PHP1601 from the fermentation of the MG agar bioassay medium..118
Fig. 4.3 Larvae preference (mean ± SE) towards VOC produced by PHP1601 from the fermentation of pig manure...119
Fig. 5.1 Effect of *B. velezensis* PHP1601 endospore treatments on fly emergence trends over a 24-d period (a), and averaged total number of flies counted during the primary fly emergence event (b). 138

Fig. 5.2 Effect of *B. velezensis* PHP1601 endospore treatments of on fly emergence trends over a 33-d period (a), and averaged total number of flies counted during each fly emergence event (b). Error bars represent the SE of the mean. 140
Fig. 5.3 REP-PCR genomic fingerprints of representative *B. velezensis* colonies isolated from a 10^5 endospore g^{-1} manure treatment at 0, 16 and 33 d... 144
Fig. 6.1 Representative RP HPLC chromatogram of lipopeptide biosurfactant compounds present in the CLP of PHP1601. 159
Fig. 6.2 Mortality (%) of zebrafish exposed to CLP extracts of PHP1601 for 96 h.. 160
Fig. 6.3 Zebrafish embryos exposed to CLP extracts of PHP601 for 24 h.. 161
Fig. 6.4 Survival of *B. velezensis* PHP1601 in river water and artificial freshwater. Autoclave killed cells (Ac) were included as dead cell controls.. 162
Fig. 6.5 Dendogram of whole genome ANI similarity of *B. velezensis* PHP1601 compared to representative *Bacillus* spp. 164

LIST OF ABBREVIATIONS

Ac	-	Autoclave killed
AEFB	-	Aerobic endospore-forming bacteria
ANOVA	-	Analysis of variance
BCA	-	Biocontrol agent
CFS	-	Cell-free supernatant
CLP	-	Crude lipopeptide
DNA	-	Deoxyribose nucleic acid
EDTA	-	Ethylenediaminetetraacetic acid
ESI-TOF	-	Electron spray ionisation time of flight
G+C	-	Guanine and cytosine
HSD	-	Honesty significant different
IPM	-	Integrated pest management
KW	-	Kruskal Wallis ANOVA
LC	-	Lethal concentration
LT	-	Lethal time
MG agar	-	Maggot growth agar
MLSA	-	Multiple locus sequence analysis
MS	-	Mass spectroscopy
NRPS	-	Non ribosomal peptide synthease
OD_{600nm}	-	Optical density at 600 nm
PBS	-	Phosphate buffered saline
PCR	-	Polymerase chain reaction

PKS	-	Polyketide synthetase
Rf	-	Retention factor
REP	-	Repetitive extragenic palindromic
RP-HPLC	-	Reverse-phase high-performance liquid chromatography
16S rRNA	-	16S ribosomal RNA gene
SG	-	Schaeffer's broth
TLC	-	Thin layer chromatography
TSA	-	Trypticase soy agar
TSB	-	Trypticase soy broth
UPLC	-	Ultra performance liquid chromatography
UV	-	Ultraviolet
VOC	-	Volatile organic compound

INTRODUCTION

Over the years, there has been a continuous need to control unwanted vegetation, animals, insects, microbes, and diseases. This has brought about the rise of control agents to kill, inhibit, or ward-off unwanted organisms. Flies (order: Diptera) are one of the most abundant and prevalent insect pests that pose a growing threat to various sectors of the economy (Alam et al. 2020; Wangithi et al. 2021; Diller et al. 2023). They possess rapid growth rates and have relatively short development times, which promote their ability to cause infestations. This has contributed to substantial economic losses by increasing food security threats and promoting disease transmission (Alam et al. 2020; Kuye 2020; Wangithi et al. 2021).

Fly species commonly recognised as threats to agriculture and health sectors include *Musca domestica* (common house fly; Diptera: Muscidae) and *Lucilia cuprina* (Australian blowfly; Diptera: Calliphoridae) (Wang et al. 2013; Alam et al. 2020; Wangithi et al. 2021). The common house fly, *M. domestica*, readily develops resistance to many of the pesticides used to control them, which hinders the development of effective new pesticides (Scott et al. 2000; Alam et al. 2020). It thrives in areas of human habitation where they are attracted to refuse, faeces, or other malodorous sources. Over 130 pathogens, including potentially life-threatening diseases such as bacterial dysentery caused by *Shigella dysenteriae* (Enterobacterales: Enterobacteriaceae) can be spread by fly transmission. *Lucilia cuprina* is recognised globally as a pest where flies lay their eggs on the skin and tissue of animals and humans, allowing the larvae to burrow into the host tissue, causing myiasis or “flystrike” in animals (Limsopatham et al. 2017). Consequently, it is the leading cause of flystrike in Australia, New Zealand, and South Africa (Limsopatham et al. 2017).

Conventional nuisance and veterinary fly control measures have relied extensively on chemical insecticides such as dichloro-diphenyl-trichloroethane, deltamethrin and malathion (Wang et al. 2013; Hassan et al. 2021). An over reliance, and possible misuse, of chemical control approaches has led to a rise in insect resistance that has resulted in a reduction or loss of pesticide efficacy (Ndlela et al. 2016; Kim et al. 2018; Muriithi et al. 2019). This has resulted in the use of higher dosages and an increase in the frequency of pesticide applications, which promotes further development of pesticide resistance in insect populations. Many chemical insecticides harm ecosystem health because they are toxic to non-target organisms and recalcitrant to degradation. In response to this, biological control

approaches that use pre-existing predators, or microorganisms, to control pests have been considered a promising alternative (Barratt et al. 2018). Increasingly, bacterial-based biocontrol has gained attention as a competitive and viable alternative to chemical pesticides for controlling various insect pests, including flies. Members of the *Bacillus cereus sensu lato* group, particularly strains of *Bacillus thuringiensis*, have gained prominence due to the plethora of insecticidal toxins these organisms produce. These organisms, or toxins derived from them, have been used to control fly species such as *Anastrepha fraterculus* (Diptera: Tephritidae) and *Simulium* spp. (Diptera: Simuliidae) (Iburg et al. 2015; Martins et al. 2018). However, reports indicating the development of insect resistance to *B. thuringiensis* related toxins suggest that this control strategy cannot be relied on indefinitely and that there is an ongoing need for alternative control measures to be developed (Zhou et al. 2020; Qin et al. 2021).

Members of the *B. subtilis sensu lato* group, including strains of *B. subtilis*, *B. amyloliquefaciens* and *B. velezensis*, are recognised for their plant growth promotion traits and antagonism of plant-associated microbial pathogens (Samaras et al. 2021). Whereas, insect biocontrol applications associated with this group of organisms appear limited and relatively unexplored. Several studies have shown that members of this group are effective in controlling the larvae of mosquito spp., such as *Anopheles stephensi* (Diptera, Nematocera: Culicidae), and leafworm, *Spodoptera littoralis* (Lepidoptera: Noctuidae) (Geetha et al. 2012; Ghribi et al. 2012); however, biocontrol investigations on flies of the Dipteral suborder Brachycera have not been documented to-date.

With these considerations in mind, a study was initiated with the aim to evaluate the biocontrol efficacy of *B. velezensis* strain PHP1601 as an antagonist against selected fly species of the sub order Brachycera. This strain had previously been isolated from fly larvae cadavers, and it was hypothesised that it had caused their demise. The main objectives of the investigations are summarised as follows:

1. To capture, rear and identify a population of flies for biological control studies.
2. To confirm the taxonomic affiliation of PHP1601.
3. To determine the larvicidal effect of a range of PHP1601 treatments.
4. To characterise the bioactive biosurfactant compounds associated with PHP1601 and determine their impacts on fly larvae antagonism.
5. To assess the impact of volatile organic compounds produced by PHP1601 on fly

larvae behaviour.

6. To determine the biocontrol effectiveness of PHP1601 in manure trials run under naturally prevailing conditions.
7. To assess the levels of aquatic risk associated with lipopeptide biosurfactants by PHP1601 through zebrafish embryo toxicity assays and genome mining.

The thesis has been divided into seven chapters. Chapter 1 comprises a literature review providing an overview of the trends in insect biocontrol, the need for control of flies, previous and current chemical and biological control strategies, and an overview of the *B. subtilis sensu lato* group and their role in biological control. Chapters 2 to 6 address the specific objectives of the study, presented as independent chapters for scientific publications. Chapter 7 summarises the research conducted and presents a route for future research avenues.

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CHAPTER ONE: LITERATURE REVIEW

1.1 Introduction

Biocontrol is defined as managing or controlling vegetation, animals, insects and microbes through the use of their naturally occurring predators or antagonists (Barratt et al. 2018). In recent years, adoption of biocontrol practices and products as an alternative to chemical control options have increased because they are eco-friendly and generally considered safe to use with low- to non-toxic effects on non-target species (Barratt et al. 2018; Galli et al. 2024). With increased pest resistance to chemical control agents being reported, biocontrol alternatives are a promising approach to maintain pest control.

Specifically, the biocontrol of insects refers to the application of other organisms, such as parasitic wasps, or entomopathogenic microorganisms, to suppress or eradicate the desired insect pest. Certain fly species are considered to be pests of significance due to their role as vectors of disease and the threats they pose to agriculture (Limsopatham et al. 2017; Alam et al. 2020; Wangithi et al. 2021). Examples include green bottle blowflies (Diptera: Calliphoridae; *Lucilia cuprina*), the causative agent of cattle myiasis, tsetse flies (Diptera: Glossinidae; *Glossina morsitans*), the vector of sleeping sickness, and, fruit flies (Diptera: Tephritidae; *Dacus* spp.) as horticultural threats (Kuria and Oyedeji 2020; Singh et al. 2020; Soundararajan 2020; Freitas et al. 2021). Chemical control agents such as deltamethrin or malathion have become ineffective against many fly pests due to the increase in pesticide resistance within their populations (Muriithi et al. 2019). These chemicals also are genotoxic agents that are detrimental to the ecosystem. Consequently, the biocontrol of flies has been considered as a viable means of addressing this issue.

Bacillus thuringiensis (Bacillales: Bacillaceae) is a well-known example of a bacterial species that has been harnessed and successfully commercialised as an insecticidal biocontrol agent (Wang et al. 2020a). It is a member of the *B. cereus sensu lato* group of *Bacillus* spp., which has undergone extensive biocontrol investigations (Dunlap et al. 2016; Dunlap 2019; Valtierra-de-Luis et al. 2020; Wang et al. 2020a; Sujayanand et al. 2024). Another well-known *Bacillus* spp.

group is the *B. subtilis sensu lato* group, which contains representatives known for producing bioactive compounds with broad biotechnological applications (Dunlap et al. 2016; Dunlap 2019). Insect biocontrol studies of these species are limited and relatively unexplored. Hence, this presents an opportunity for the potential of new microbial biocontrol strains to be assessed.

The current study focuses on the evaluation of *B. velezensis* strain PHP1601, a *B. subtilis sensu lato* group member, as a potential biocontrol candidate against fly species. A literature review was conducted to explore various aspects of biocontrol, focusing on the biocontrol of flies with members of the *B. subtilis sensu lato* group. To this end, the following topics were covered: an overview of the history of biocontrol; a comparison of the viability of chemical and microbial control agents; the process of evaluating microbial biocontrol agents; the role of the *B. subtilis sensu lato* group in the field of biocontrol; and the need for the biocontrol of fly species.

1.2 Overview of the progression of biocontrol

A biological control agent facilitates biological control, i.e., it is the physical entity that antagonises or kills the target pest (Barratt et al. 2018; Vlajkov et al. 2022). This is a fundamental process of ensuring balance in nature that humans intentionally, or unintentionally, adopt to control pests, diseases, and their associated side effects (Barratt et al. 2018; Galli at al. 2024).

The first major successful application of biocontrol occurred in the late 1880s that demonstrated the application of the parasitic fly, *Cryptochaetum iceryae* (Williston) (Diptera: Cryptochetidae), and the vedalia beetle, *Novius cardinalis* (Mulsant) (Coleoptera: Coccinellidae), to eliminate cottony-cushion scale, *Icerya purchasi* (Maskell) (Hemiptera: Monophlebidae), as crucial insect threat to citrus orchards in the United States of America (USA) (Caltagirone 1981Kairo and Murphy 1995; Barratt et al. 2018). What made this a novel application of biocontrol was that it utilised multiple enemies of *I. purchasi* to control the outbreak effectively. *Novius cardinalis* was more effective at decreasing the population of *I. purchasi* during Summer and Fall, whilst *C. iceryae* was shown to be more effective during Winter and early Spring (Quezada and DeBach 1973).

From the early 1940s to the 1960s, swarms of Rhodes grass mealybug, *Antonina graminis*

(Maskell) (Hemiptera: Pseudococcidae), spread rapidly throughout the state of Texas (USA) consuming and destroying vast expanses (~ 155 400 km^2) of forage grass, including Rhodes grass and other grass species (Hagen et al. 1976; Caltagirone 1981). Subsequently, the parasitic wasp, *Neodusmetia sangwani* (Rao) (Hymenoptera: Encyrtidae), was introduced as a known predator of the mealybug to successfully control its outbreak (Caltagirone 1981; Hagen et al. 1976; Shylesha and Mani 2016). The wasps' parasitoid mechanism involved laying its eggs inside the insect so that when they hatched, the larvae would feed on the mealybug (Caltagirone 1981; Potter and Braman 2003; Shylesha and Mani 2016). This effectively curbed the spread and damage caused by *A. graminis* (Batista et al. 2018).

These milestones showed that naturally occurring parasitoids could be used as viable pest-control agents. However, biocontrol is not limited to macro-organisms as microbial-based biocontrol applications have also been harnessed. The concept of microbial biocontrol was introduced as early as the 1800s and is widely attributed to the work of Agostino Bassi and Louis Pasteur, based on their research on insect pathology and microbial interactions (Lord 2005). Specifically, Bassi showed that the mortality of silkworms and other insect species could be induced by artificially inoculating them with the fungus, *Beauveria bassiana* (Hypocreales: Cordycipitaceae) (Lord 2005). Formally, the first proposal of microbial biocontrol was made in 1873 (Lord 2005).

Among the first successful microbial biocontrol agents used were *Bacillus popilliae* and *B. lentimorbis* to control the Japanese beetle, *Popillia japonica* (Newman) (Coleoptera: Scarabaeidae) (Shanovich et al. 2019). In the early 1900s, *P. japonica* posed a significant threat to agriculture and spread across the eastern states of the USA (Shanovich et al. 2019). In 1921, *P. japonica* was found to be highly susceptible to a previously undocumented disease, which was subsequently found to be caused by the entomopathogenic bacteria *B. popilliae* and *B. lentimorbis* (Steinhaus 1957; Steinhaus 1975; Lord 2005; Shanovich et al. 2019). By the mid-1940s, state and federal agencies embarked on rapidly producing and commercialising *B. popilliae* endospores as a viable microbial biocontrol agent against *P. japonica* (Steinhaus 1957; Steinhaus 1975; Lord 2005).

Viruses have also been considered as candidates for biocontrol. Insect baculoviruses are known to infect insects and are viable biocontrol agents because they are non-pathogenic to vertebrates and plants whilst being virulent towards their insect hosts (Lord 2005; Popham et al. 2016). In

the 1940s, baculoviruses were found to be effective against a range of insects, but it was only in 1970 that the first registered baculovirus product, VironH, a nuclear polyhedrosis virus was released (Steinhaus 1975; Lord 2005; Knox et al. 2015; Popham et al. 2016).

The examples mentioned above have influenced the development of the modern field of biocontrol. Effective microbial biocontrol agents should be host-specific, effective in the environment where the pest is active, and non-pathogenic to non-target organisms. Some of the well-known microbial biocontrol agents include *Bacillus* spp., *Pseudomonas* spp. (Pseudomonadales: Pseudomonadaceae) and *Trichoderma* spp. (Hypocreales: Hypocreaceae) (McSpadden and Driks 2004; Berg et al. 2017; Khasa 2017; Wang et al. 2020a). Strains of *B. thuringiensis* are virulent entomopathogens that produce insecticidal crystalline toxins (Pohare et al. 2021). The genes coding these toxins have been used to develop genetically modified plants that express these toxins as a further insect control measure in agricultural crops (Pohare et al. 2021). Strains of *B. velezensis* and *B. amyloliquefaciens* have been used as antagonists of microbial plant pathogens through the production of bioactive compounds such as lipopeptides (Casals et al. 2021; Mácha et al. 2021). *Trichoderma* spp. has been used worldwide as a fungal antagonist that acts through mycoparasitism (Mohamed et al. 2020). *Pseudomonas* spp. are involved in the biocontrol of fungal phytopathogens by producing surface-active rhamnolipids. Many of these species also provide other beneficial traits to the environment it is administered in, such as functioning as bio-fertilisers (*Trichoderma* spp.) or acting as plant growth-promoting bacteria (*B. velezensis* and related spp.) (Mohamed et al. 2020; Pohare et al. 2021). As a result, biocontrol options are considered viable avenues for sustainable pest control and warrant consideration in using or developing control strategies against new or existing pests.

1.3 Genus *Bacillus* (Bacillales: Bacillaceae)

Members of the genus *Bacillus* belong to the family Bacillaceae (phylum: Firmicutes) and include representatives that are Gram-positive, soil ubiquitous, rod-shaped, aerobic endospore-forming bacteria (AEFB) with low guanine-cytosine% contents (Amin et al. 2015; Mandic-Mulec et al. 2016; Rocha et al. 2023). *Bacillus* is the most species-rich and well-researched genus of the *Bacillaceae* and is recognised for the role members play in various biocontrol applications (Mácha et al. 2021; Pohare et al. 2021). The genus *Bacillus* was first proposed in

1872 by Cohn, who classified the reference species, *B. subtilis*, as an aerobic, rod-shaped, endospore-forming organoheterotroph (Cohn 1962). Since then, the standard phenotypic classification of *Bacillus* spp. has changed and relies heavily on genomic and phylogenetic analysis to distinguish them at the species level (Mandic-Mulec et al. 2016). Due to the phylogenetic and phenotypic similarity amongst many representatives of the *Bacillus* genus, closely related species have been assigned to senu lato groupings, namely the *B. cereus* and the *B. subtilis sensu lato* groups (Mandic-Mulec et al. 2016; Rocha et al. 2023).

The *B. cereus sensu lato* group are comprised of closely related Gram-positive bacteria that are wider than 1 µm, produce ellipsoidal endospores, have swollen sporangia and are generally mesophilic and neutrophilic (Fritze 2007; Wang et al. 2023). This group includes heterotrophic and pathogenic representatives that are commonly soil-borne (Rooney et al. 2009; Cote et al. 2024). The distinguishing factor that segregates this group of endospore formers from the rest of the genus is their inability to produce acid from mannitol and their ability to produce lecithinase (Fritze 2007). This group is comprised of strains of *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides* and *B. pseudomycoides* (Fritze 2007; Rooney et al. 2009; Mandic-Mulec et al. 2016; Wang et al. 2023). Members of this group are difficult to distinguish due to a high degree of phenotypic similarity and genomic relatedness (Mandic-Mulec et al. 2016; Wang et al; 2023). For example, the *B. anthracis* pOX-1 and pOX-2 pathogenic plasmids are found in some strains of *B. cereus* and *B. thuringiensis* (Ehling-Schulz et al. 2006; Mandic-Mulec et al. 2016).

Members of the *Bacillus subtilis* senu lato group comprise Gram-positive AEFB that, generally, are found as environmental strains that are non-pathogenic (Fritze 2007; Mandic-Mulec et al. 2016; Vlajkov et al. 2022). They are characterised as being less than 1 µm wide, having ellipsoidal endospores, which may or may not lead to swollen sporangia, and being mesophilic and neutrophilic to alkalitolerant (Fritze 2007). Species within this group are highly related and can be difficult to distinguish phenotypically and phylogenetically; consequently, a reliance on phenotypic testing and 16S rRNA gene sequence analysis can lead to strains being misidentified (Fritze 2007; Mandic-Mulec et al. 2016). Members of the *B. subtilis sensu lato* group includes strains of; *B. subtilis*, *B. velezensis*, *B. amyloliquefaciens*, *B. pumilus*, *B. licheniformis*, *B. mojavensis*, *B. endophyticus*, *B. valismortis*, *B. sonorensis*, *B. atrophaeus*, *B. aquimaris*, *B.*

carboniphilus, *B. sporothermodurans* and *B. oleronius* (Mandic-Mulec et al. 2016; Rocha et al. 2023).

1.3.1 Endospore formation

The most important distinguishing feature of the members of the genus *Bacillus* is their ability to form endospores that are highly resistant to heat, desiccation, radiation and chemicals (Sella et al. 2015; Kalinowski et al. 2018; Zhao et al. 2023). Endospores have been viewed as a favourable trait of biocontrol agents because they offer a longer shelf life of the product, tolerate various processes in formulation production, and are resistant to abiotic factors (Yánez-Mendizabal et al. 2012; Gotor-Vila et al. 2017; Zhao et al. 2023). These structures allow *Bacillus* spp. to enter a state of dormancy when unfavourable conditions arise and revert to their vegetative state once favourable conditions return (Egan et al. 2017; Gotor-Vila et al. 2017; Rodríguez et al. 2024).

The process of endospore formation is highly regulated by a variety of genes responding to a wide range of environmental stimuli (Driks 2002; McKenney et al. 2013; Rodríguez et al. 2024). However, one of the most significant inducers of endospore formation is nutrient depletion (Driks 2002; McKenney et al. 2013). The process of sporulation is summarised in Fig. 1.1. The first stage of sporulation takes approximately 8 h. This process involves receiving sufficient stress stimuli that induce the phosphorylation of the transcriptional master regulator, SpoOA, by phosphotransferases SpoOF and SpoOB (Losick et al. 2003; Rodríguez et al. 2024). This is regulated by five auto-phosphorylating histidine kinases (KinA-KinE) (Tan and Ramamurt 2013). The phosphorylated SpoOA regulator (SpoOA-P) can now induce the expression of approximately 121 genes involved in endospore formation (Driks 2002; McKenney et al. 2013; Rodríguez et al. 2024).

In the early stages of sporulation, the cell makes a copy of its DNA and expresses the RacA protein (Tan and Ramamurt 2013). This protein binds to guanine-cytosine-rich repeats in the copied DNA and anchors it to either side of the cell (Tan and Ramamurt 2013). One site is for establishing the region development of the forespore and the other for the sustained functioning of the mother cell to continue the process of sporulation (Losick et al. 2003; Piggot and Hilbert 2004; Tan and Ramamurt 2013).

After the DNA has been segregated, asymmetric cell division encloses the DNA at the forespore region in the septum (Fig. 1.1). This is dependent on two factors: (1) an increase in levels of the cell division protein, FtsZ and (2) the production of the SpoIIE protein (Piggot and Hilbert 2004; Tan and Ramamurt 2013). Sigma factor F is produced under the regulation of SpoOA-P and influences pre-spore formation. However, the exact mode of action of this factor is not well understood (Tan and Ramamurt 2013). FtsZ facilitates the formation of ring-like structures at the polar regions of the cell for the formation of a membrane around the developing endospore (Piggot and Hilbert 2004). Subsequently, through the process of engulfment, the pre-spore is surrounded by an additional membrane layer. This results in the formation of the forespore.

After forming the forespore, the mother cell expresses peptidoglycan degrading proteins to initiate cell autolysis (Losick et al. 2003) (Fig. 1.1). In conjunction with this, sigma factor K is released and simultaneously, sigma factor G is activated in the forespore (Losick et al. 2003; Tan and Ramamurt 2013). These sigma factors activate gene transcription of proteins involved in building endospore structural components. The forespore becomes the endospore core and is protected by high concentrations of calcium and dipicolinic acid. To allow for maturation of the forespore, the protein SpoVM, produced in the mother cell, tethers morphogenic proteins like SpoIVA to the forespore to form a cortex containing specialised peptidoglycan. These proteins also play a role in the formation of the endospore coat (Tan and Ramamurt 2013). Once the endospore is fully matured, it is released into the environment by apoptosis (Tan and Ramamurt 2013).

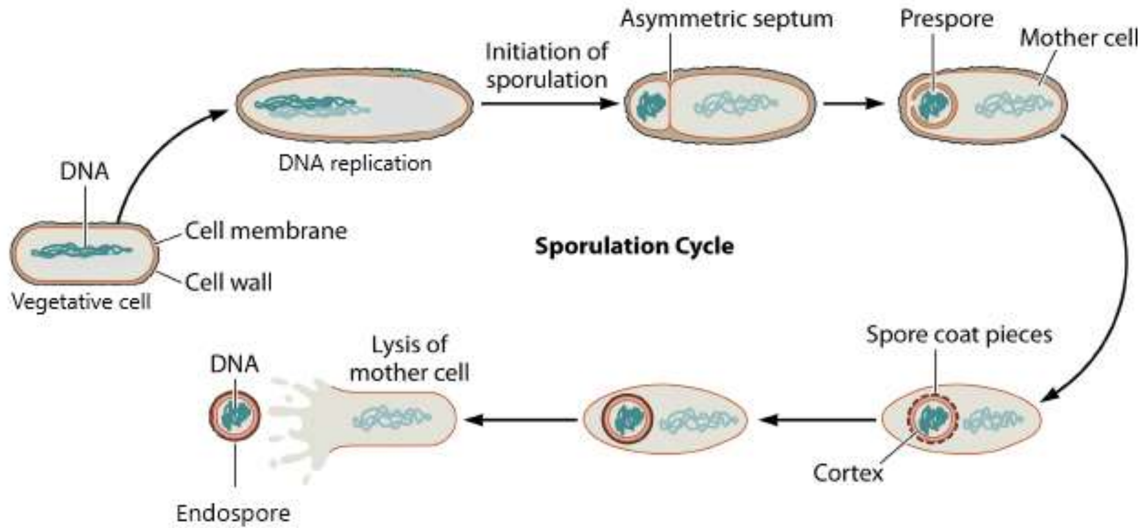

Fig. 1.1 Summarised process of endospore formation of representative of *Bacillus* spp. Diagram adapted from Al-Hinai et al. (2015).

1.3.2 Germination of endospores

An endospore's fundamental structural and biochemical properties protect the dormant cell and allows it to germinate when favourable conditions arise. Germination is triggered by conditions such as nutrient availability and suitable environmental conditions (Korza et al. 2016; Rodríguez et al. 2024). Specifically, germination receptors can be activated by L- amino acids (L-asparagine, alanine, and valine) (Setlow 2014). Receptors and other proteins such as SpoIM and SpoVA mediate the recognition of environmental conditions that stimulate endospore germination. SpoVA makes up protein channels, which chelate with divalent calcium ions (Jalil et al. 2024). These SpoVA channels open when the germination response is triggered, which releases dipicolinic acid and its acid-calcium complexes and their replacement by water (Korza et al. 2016; Jalil et al. 2024). When the endospore reaches a water content of approximately 80%, cortex-lytic enzymes are activated, which digest the endospore coat (Korza et al. 2016). This facilitates the germination of the endospore and the initiation of a vegetative lifestyle of the bacterium (Korza et al. 2016; Jalil et al. 2024).

1.4 Biocontrol applications associated with the *B. cereus sensu lato* group

Strains of *B. thuringiensis* and *B. mycoides* are acknowledged as potential insect biocontrol candidates due to their ability to produce bioactive insecticidal toxins (Wang et al. 2020a; Zhao et al. 2023; Sujayanand et al. 2024). In particular, *B. thuringiensis* strains have been pursued as potentially viable biocontrol agents against various insect pests (Jurat-Fuentes et al. 2021; Gupta et al. 2024). This species is distinguished from others by its ability to form parasporal crystal bodies that contain proteinous endotoxins known as “Cry” and “Cyt” proteins that are responsible for its insecticidal effect (Alahyane et al. 2021; Jurat-Fuentes et al. 2021; Gupta et al. 2024). These endotoxins are also synonymous with the term “Bt toxins” and have demonstrated effective control against a range of insect species belonging to various orders including Diptera, Hymenoptera, Homoptera, Orthoptera, and Mallophaga and even against protozoa and nematodes (Schnepf et al. 1998; van Frankenhuyzen 2017; Zheng et al. 2018; Alahyane et al. 2021; Gupta et al. 2024).

The models of the insecticidal mechanism of *B. thuringiensis* suggest that the main toxin involved is a 3d-Cry protein and can occur by two potential mechanisms (Soberón et al. 2016; Chakrabarty et al. 2022). The first involves the induction of pore formation in the lining of the insect’s midgut, thus inducing death due to intensive cellular leakage (Bravo et al. 2015; Chakrabarty et al. 2022). The second is via a signal transduction pathway that induces the autolysis of cells lining the insect’s midgut (Bravo et al. 2015; Soberón et al. 2016).

The Bt toxin pore formation model is shown in Fig. 1.2. It suggests that an insect dies upon consuming a medium containing crystalline Bt toxin proteins, specifically the 3d-Cry proteins (Jurat-Fuentes et al. 2021; Chakrabarty et al. 2022). Upon ingestion, the toxins are solubilised due to the alkaline pH of the insect’s foregut (Bravo et al. 2015). Afterwards, the midgut proteases digest the Bt proteins, releasing the protease-resistant and active core of the Bt toxins (Bravo et al. 2015). The activated toxins bind to receptors on the brush border membrane vesicles of the midgut epithelial cells (Bravo et al. 2015; Soberón et al. 2016; Jurat-Fuentes et al. 2021). Once bound, the toxins insert themselves into the membrane of the epithelial cells and aggregate to form pores in the membrane (Whalon and Wingerd 2003). The formation of pores creates a diffusion gradient and forces water and ions into the compromised cells (Whalon and Wingerd 2003). This leads to extensive cell lysis, and mortality is reached due to intense damage

to the midgut membrane, which leads to starvation or septicaemia (Whalon and Wingerd 2003).

The cellular death signalling model is depicted in Fig. 1.3. This model suggests that the binding of Bt toxins to receptors on the midgut epithelium induces a cellular signalling cascade responsible for the death of the insect host (Zhang et al. 2005; Melo et al. 2014) (Fig. 1.3). This process is proposed to be initiated by the Cry1 toxin binding to a cadherin receptor BT-R1 located on the midgut epithelium (Zhang et al. 2005; Melo et al. 2014). Once attached, a Mg^{2+} dependent cellular signalling pathway is activated that is associated with the initiation of an autolysis process. It has been proposed that these processes are linked with the opening of divalent ion channels in the cell membrane to allow the influx of water and ions into the cell (Melo et al. 2014). This results in increased water in the cell and its autolysis (Melo et al. 2014). After the midgut has been sufficiently compromised, the death of the host is achieved (Zhang et al. 2005; Melo et al. 2014). This model is gaining more attention; however, it is not completely elucidated (Melo et al. 2014).

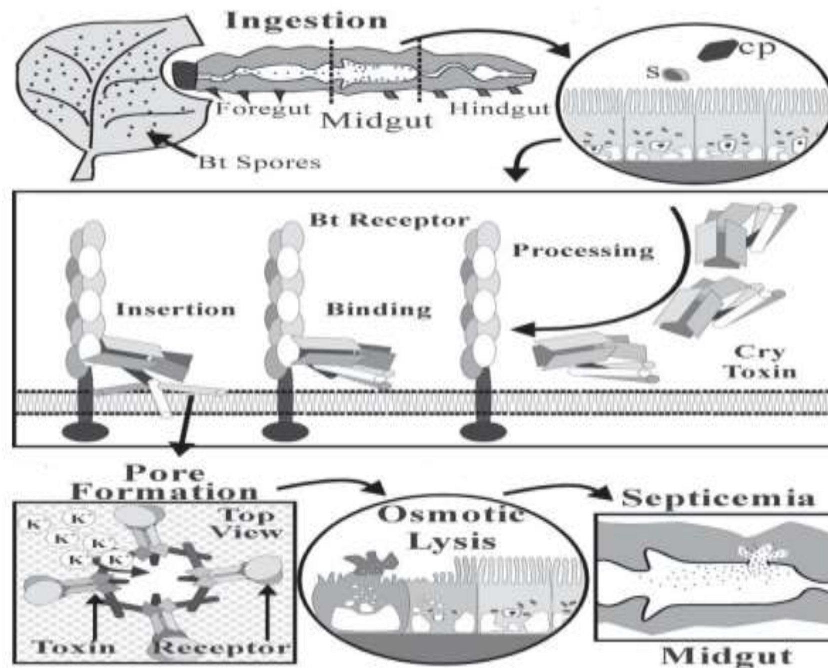

Fig. 1.2 Lytic mode of action of *B. thuringiensis* Cry protein. Image was adapted from Whalon and Wingerd (2003).

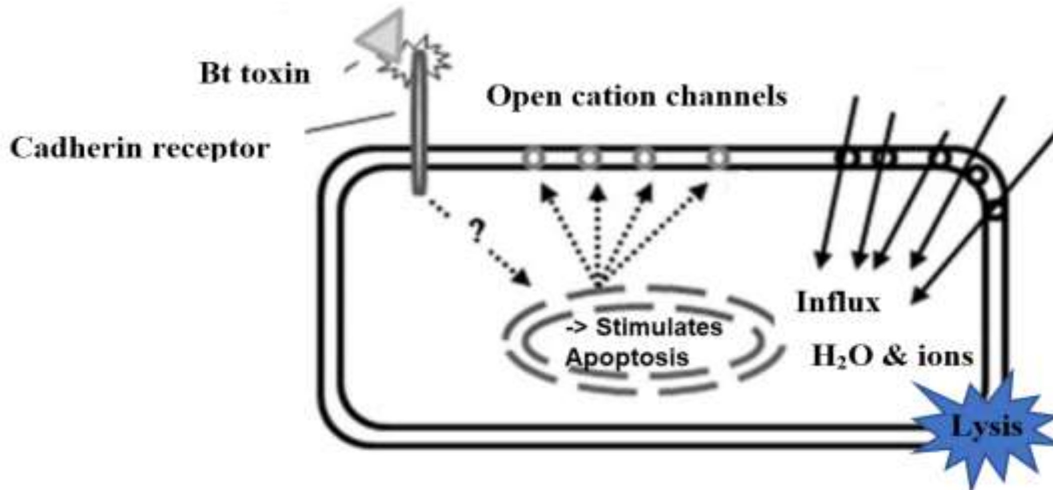

Fig. 1.3 Insecticidal cellular death signalling mode of action of *B. thuringiensis.* The image was adapted from Melo et al. (2016).

1.4.1 Concerns regarding the longevity of *B. thuringiensis* treatments

The bioinsecticide market is heavily reliant on *B. thuringiensis* products, which is attributed to its potency and effectiveness in controlling various insect pests. Its market share accounts for approximately 90 - 97% of the market (Liang et al. 2015; Chandarana et al. 2022; Galli et al. 202). However, concerns about the intensive and extensive application of *B. thuringiensis* related products, are confirmed by the rise of insect resistance, as seen with chemical pesticides (Ma et al. 2021; Chakrabarty et al. 2022; Xiao et al. 2024). These concerns have stimulated research for suitable alternatives that would not be ineffective due to insect cross-resistance to analogues of Bt toxins or other treatments (Liang et al. 2015; Ma et al. 2021; Xiao et al. 2024).

Insects have developed, or acquired, a range of different mechanisms to counter the effects of Bt toxins. Insects of the order Lepidoptera, such as *Trichoplusia ni* (Hubner) (family: Noctuidae), *Helicoverpa* spp. (Hardwick), *Pectinophora gossypiella* (Busck) (family: Gelechiidae) and *Plutella xylostella* (Linnaeus) (family: Plutellidae) have developed resistance to the *B. thuringiensis* Cry toxins, attributed to the expression of prophenoloxidases which inactivates the toxin or by mutations of their ABC transporters to prevent binging to their midgut (Yang et al. 2019; Li et al. 2023). Specifically, strains of *P. gossypiella* have also been documented to possess a mutant allele in the cells of their midgut that encodes for a cadherin protein that lacks

its transmembrane region, which the Cry1Ac toxin binds to (Wang et al. 2018; Xiao et al. 2024). This mutation conferred a 220-fold increase in resistance to Cry1Ac and a 2.1-fold cross-resistance to Cry2Ab toxins (Wang et al. 2018). Other insects known to have or readily acquire resistance to Bt toxins include *Diatraea saccharalis* (Fabricius) (Lepidoptera: Crambidae), *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) and *Diabrotica barberi* (Smith and Lawerance) (Coleoptera: Chrysomelidae) (Calles-Torrez et al. 2019; Boaventura et al. 2021; Xiao et al. 2024).

1.5 Biocontrol applications associated with the *B. subtilis sensu lato* group

Strains of the *B. subtilis sensu lato* group are important constituents of the soil/rhizosphere microbiome and can possess various plant growth promoting traits such as antagonising plant-associated pathogens, enhancing nutrient mineralisation and uptake, and, stimulating plant growth promotion through the production of growth factors (Shafi et al. 2017; Hashem et al. 2019; Vlajkov et al. 2022; Rocha et al. 2023). Due to their close association with various plant species, they have also been shown to stimulate the induction of systemic plant resistance (Hashem et al. 2019; Vlajkov et al. 2022). Several strains have also been documented to possess the potential to function as new and novel insect biocontrol candidates; however, this has remained a relatively unexplored field.

1.5.1 Suppression of plant-associated microbial pathogens

Members of the *B. subtilis sensu lato* group have been extensively studied and recognised as antagonists to various microbial plant pathogens (Penha et al. 2020; Vlajkov et al. 2022; Bhakat et al. 2023). They have demonstrated effective control mechanisms against significant plant pathogens such as *Acidovorax citrulli* (Burkholderiales: Comamonadaceae), *Fusarium* spp. (Hypocreales: Nectriaceae), *Botrytis cinerea* (Helotiales: Sclerotiniaceae) and *Colletotrichum* spp. (Glomerellales: Glomerellaceae) (Khan et al. 2021; Khedher et al. 2021; Arroyave-Toroet et al. 2017; Yang et al. 2024). Fungal pathogens account for 8 – 40% of worldwide crop losses (Khan et al. 2021). For example, in 1989, 90% of watermelon yield losses in the USA were attributed to bacterial *Acidovorax* infections (Klomchit et al. 2021).

A major control mechanism associated with members of this group is attributed to the production and release of amphiphilic biosurfactant lipopeptide compounds (Penha et al. 2020; Bhakat et al. 2023). These lipopeptides have been characterised based on their chemical structure into three main families, viz. fengycin, iturin and surfactin (Penha et al. 2020; Bhakat et al. 2023). The mechanism(s) through which these lipopeptides elicit pathogen antagonism relies on their ability to interact with and disrupt biological membranes and inhibit crucial enzymes (Penha et al. 2020; Khan et al. 2021). Fengycin is produced by *B. amyloliquefaciens*, *B. velezensis* and *B. subtilis* and is effective against many fungal pathogens, including *Magnaporthe* spp. (Magnaporthales: Magnaporthaceae), *Plasmodiophora brassicae* (Plasmodiophorida: Plasmodiophoridae), *Botryosphaeria dothidea* (Botryosphaeriales: Botryosphaeriaceae) and various *Fusarium* spp. (Hypocreales: Nectriaceae) (Khan et al. 2021). Iturins are also produced by these organisms and have also been shown to be active against fungal pathogens such as *Monilinia* spp. (Helotiales: Sclerotiniaceae), *Botrytis* spp. (Helotiales: Sclerotiniaceae) and *Fusarium* spp. (Penha et al. 2020; Wang et al. 2020b; Khan et al. 2021; Heo et al. 2024). Iturin has also been shown to be active against bacterial pathogens such as *Pectobacterium carotovorum* (Enterobacterales: Pectobacteriaceae). Surfactin is produced by numerous members of the *B. subtilis sensu lato* and has mainly been shown to exhibit antibacterial properties against various plant-pathogens, including *Pectobacterium carotovorum*, *Acidovorax citrulli* (Burkholderiales: Comamonadaceae) and *Xanthomonas campestris* (Xanthomonadales: Xanthomonadaceae) (Penha et al. 2020; Gu et al. 2021; Khan et al. 2021; Mácha et al. 2021). It is also reported to show some level of antagonism towards fungal pathogens such as *Colletotrichum gloeosporiodes* (Glomerellales: Glomerellaceae) and *Fusarium* spp. (Bhakat et al. 2023). In recent years, one of the most studied biocontrol agents of the *B. subtilis sensu lato* is *B. velezensis* FZB42^{T}, which produces a range of bioactive lipopeptide compounds and demonstrates antagonism towards a wide range of plant pathogens (Mácha et al. 2021).

1.5.2 Antagonism of insect pests

Members of *B. subtilis sensu lato* group have shown antagonism towards a range of insect species (Table 1.1); with much of the research focus being aimed at controlling different types of mosquitoes such as *Aedes aegypti* (Linnaeus) (Diptera, Nematocera: Culicidae), *Anopheles*

stephensi (Liston) (Diptera, Nematocera: Culicidae) and *Culex quinquefasciatus* (Say) (Diptera, Nematocera: Culicidae) (Das and Mukherjee 2006; Geetha and Manonmani 2010; Manonmani et al. 2011; Bhuvaneswari et al. 2015; Falqueto et al. 2021). Currently, few reports of the biocontrol of Brachycera flies using members of the *B. subtilis* group have been documented (Rocha et al. 2023).

The mechanism of action through which members of this group are thought to achieve insect biocontrol is associated with the production and activity of lipopeptide biosurfactants (Das and Mukherjee 2006; Geetha and Manonmani 2010; Manonmani et al. 2011; Ghribi et al. 2012). These compounds exhibit an insecticidal response when ingested by the insect or when it is applied topically (Geetha et al. 2011; Ghribi et al. 2012). This suggests that these compounds may be capable of penetrating the insect chitin exoskeleton and execute its insecticidal effect. Mnif et al. (2013) proposed that crude lipopeptide extracts from *B. subtilis* SPB1 caused severe damage to cellular and tissue of *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae) larvae, thereby compromising the intestinal tract, which could have contributed to extensive cell leakage and, ultimately, the death of the larvae (Mnif et al. 2013).

Current gaps in the literature include; determining the scope and effectiveness of *B. subtilis sensu lato* members in their ability to antagonise insect species from different orders such as the Brachycera; determining the specific lipopeptide responsible for the observed insecticidal effect; performing field trial assessments to determine of biocontrol effectiveness of strains under naturally prevailing conditions; and, to further elucidate the insecticidal mechanism of action.

Table 1.1 Example of the *Bacillus subtilis sensu lato* involved in insect biocontrol.

Strains	Pest	Developmental stage of pest	Treatment	Reference
B. subtilis	*A. aegypti*	Mosquito larvae	Crude lipopeptide exposure	Falqueto et al. 2021
B. subtilis PTB185 and *B. pumilus* PTB180	*Aulacorthum solani*, *Aphis gossypii*	Aphid nymphs	Cell exposure	Kahia et al. 2021
B. subtilis SPB1	*E. ceratoniae*	Moth larvae	Crude lipopeptide extracts	Mnif et al. 2013
B. subtilis SBP1	*Spodoptera littoralis*	Moth larvae	Crude lipopeptide extracts	Ghribi et al. 2012
B. amyloliquefaciens	*A. stephensi*, *C. quinquefasciatus*	Mosquito larvae and pupa	Crude lipopeptide extracts	Geetha et al. 2011
B. subtilis VCRC B471	*A. stephensi*	Mosquito larvae and pupa	Crude lipopeptide extract	Geetha and Manonmani 2010

1.6 The role of *Bacillus*-associated lipopeptides in biocontrol applications

Bacillus-associated biosurfactants are secondary metabolites characterised as amphiphilic surface-active compounds (Rodríguez-López et al. 2021; Etemadzadeh et al. 2023). Structurally, lipopeptides have a cyclic hydrophilic amino acid head and a polar hydrophobic fatty acid tail (Zhu et al. 2021; Wang et al. 2024). This structural arrangement allows them to interact with and destabilise cellular membranes. Lipopeptides are effective antagonists of plant pathogens, and there is some evidence that they have a potential for use in insect biocontrol applications (Mnif et al. 2013; Rangarajan and Clarke 2015; Fazle and Baek 2020). These compounds have gained attention in the field of biocontrol because they are biodegradable and environmentally friendly (Rangarajan and Clarke 2015; Wang et al. 2024).

Lipopeptide biosurfactants produced by *Bacillus* spp. are classified into three main families, surfactins, iturins and fengycins (Fig. 1.4) (Penha et al. 2020; Gutiérrez-Chávez et al. 2021). These lipopeptides are commonly produced by strains of *B. amyloliquefaciens*, *B. velezensis* and *B. subtilis* (Adeniji et al. 2019; Lin et al. 2020; Ma et al. 2020; Wan et al. 2021). The ability of a strain to produce a lipopeptide, or a range of lipopeptides, is dependent on the lipopeptide operons it contains; for example, surfactin operon containing the *srf*A, *srf*B, *srf*C and *srf*D genes (Hussein and Fahim 2017; Wang et al. 2024). Lipopeptide biosurfactant synthesis is a complex process involving many large modular enzymes. In summary, lipopeptide production is

performed primarily by a range of non-ribosomal peptide synthetases (NRPS) and polyketide synthetases NRPS hybrids (PKS-NRPS) (Shaligram and Singhal 2010; Gutiérrez-Chávez et al. 2021; Ruiz et al. 2024). These NRPS have at least three catalytic domains for (1) adenylation, (2) a peptidyl carrier site and (3) a region to link peptides by condensation reactions (Gutiérrez-Chávez et al. 2021; Ruiz et al. 2024). The order in which amino acids are initially joined to the fatty acid chain or added to each other in the peptide ring is dictated by the type of NRPS and PKS-NRPS the strain encodes. One of the enzymatic sites of the NRPS facilitates the production of a fatty acid chain (Shaligram and Singhal 2010). The protein carrier site binds amino acids and promotes the development of the amino acid chain. The condensation site facilitates the cyclisation of the peptide chain and attaches it to the fatty acid chain (Shaligram and Singhal 2010; Wang et al. 2024).

Lipopeptide biosurfactants are differentiated from each other by their molecular weight and composition of amino acids in their peptide ring. Surfactins are one of the most potent lipopeptide biosurfactants (Raaijmakers et al. 2010; Santos et al. 2019; Ruiz et al. 2024). They are characterised by having a 13 – 15 carbon β-hydroxy fatty acid chain that is linked to a lactone ring containing seven amino acids. The amino acids commonly associated with surfactins are aspartic acid, glutamic acid, valine and leucine (Santos et al. 2019). Fengycins are higher molecular weight lipopeptides characterised by having 14 – 18 carbon β-hydroxy fatty acid chain that is linked to a lactone ring containing 10 amino acids (Gutiérrez-Chávez et al. 2021). These amino acids include isoleucine, tyrosine, glutamine, proline and alanine that are arranged differently among various isoforms. Iturins are a similar class of lipopeptides; however, they contain a 14 – 15 carbon β-hydroxy fatty acid chain that is linked to a heptapeptide ring (Zhou et al. 2020). This ring structure is composed of different arrangements of asparagine, tyrosine, glutamine, proline and serine (Gutiérrez-Chávez et al. 2021). Previously, mycosubtilin, bacillopeptin and bacillomycin were recognised as distinct lipopeptide classes, however due to recent reclassification, they have been assigned as different types of iturins (Guez et al. 2021; Ruiz et al. 2024; Wang et al. 2024).

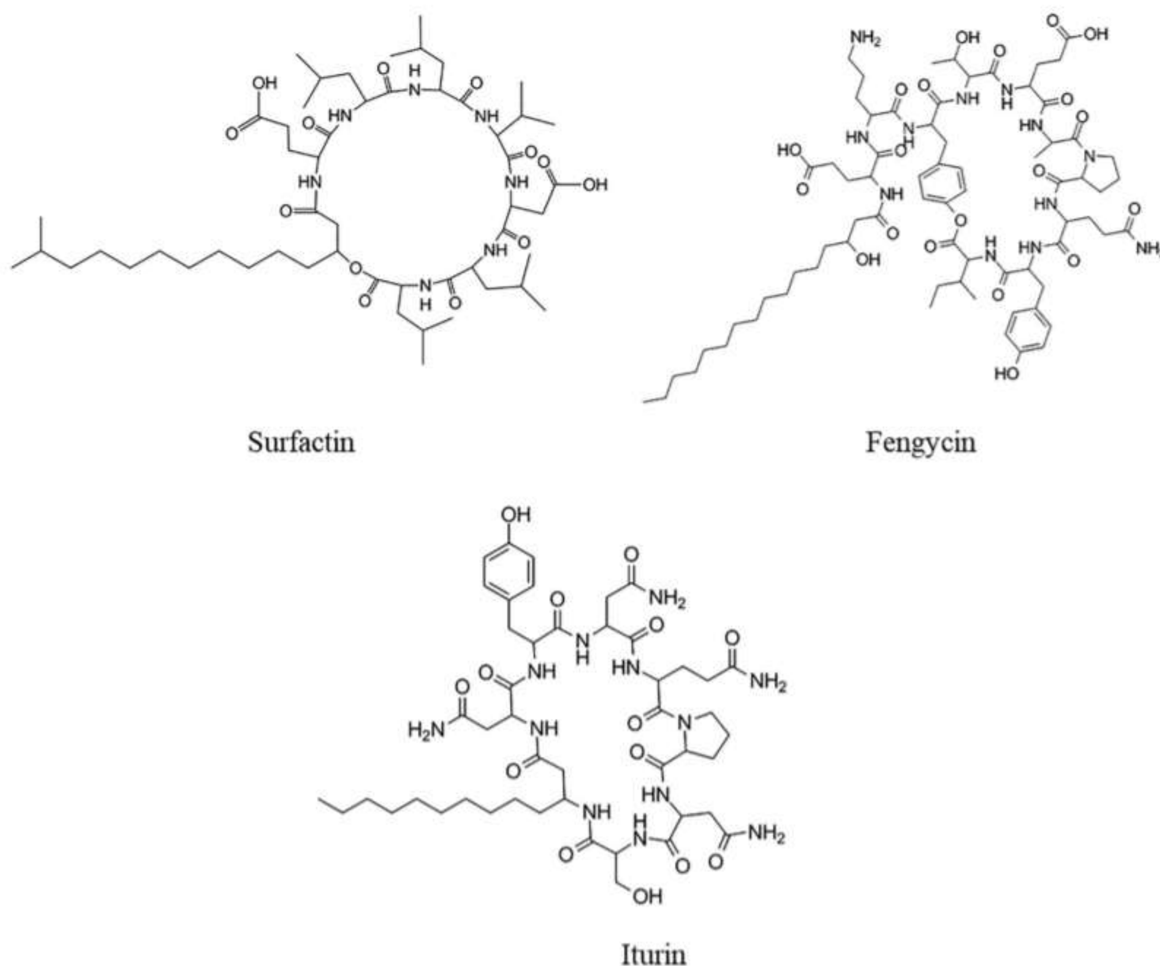

Fig. 1.4 Representative isoforms of main lipopeptide families; surfactin, iturin and fengycin. The image was adapted from Gutiérrez-Chávez et al. (2021).

1.7 Entomopathogenic microorganisms

In the current biocontrol market, *B. thuringiensis* strains have become the preferred candidates for insect biocontrol applications (Ma et al. 2021; Li et al. 2023). However, this species is not the only one that has been considered for insect biocontrol. For example, *Pseudomonas* spp. and *Beauveria bassiana* strains show promise as effective insecticidal candidates against a range of insect pests (Mascarin and Jaronski 2016; Suganthi et al. 2017). These species have different insecticidal modes of action compared to *B. thuringiensis*; they, therefore, can provide crucial information about how insect pathogenicity varies among different species, target sites of infection, the infection process, and which bioactive metabolites are antagonistic towards insects.

1.7.1 Entomopathogenic bacteria: *Pseudomonas* spp.

The genus *Pseudomonas* encompasses a group of aerobic, flagellated Gram-negative rod-shaped bacteria within the Pseudomonadaceae (class: Gammaproteobacteria) (Pachori et al. 2019; Medić et al. 2020; Teoh et al. 2021). Representatives of this genus are regarded as opportunistic pathogens that can cause gastrointestinal, urinary tract and bloodstream infections; others are known for their biotechnological applications such as the biodegradation of hydrocarbon compounds such as n-alkanes and polycyclic aromatic compounds (Pachori et al. 2019; Medić et al. 2020). Members of the *Pseudomonas* are also recognised for their ability to antagonise a range of insect species, including *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), *Helopeltis* spp. (Hemiptera: Miridae) and *Aedes albopictus* (Skuse) (Diptera: Culicidae) (Mostakim et al. 2012; Suganthi et al. 2017; Hamze et al. 2023).

Representative entomopathogenic *Pseudomonas* spp. includes strains of *P. putida*, *P. aeruginosa*, *P. chlororaphis*, *P. fuorescens* and *Pseudomonas protegens* (Teoh et al. 2021; Raio 2024). *Pseudomonas aeruginosa* is recognised to be one of the most pathogenic insecticidal species within the genus and has shown acute antagonism towards strains of *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae), *Bombyx mori* (Linnaeus) (Lepidoptera: Bombycidae), *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), and *Adalia bipunctata* (Linnaeus) (Coleoptera: Coccinellidae) (Mostakim et al. 2012; Teoh et al. 2021; Sundar et al. 2021). The mechanism of its pathogenicity towards insects is multifocal and depends on factors such as the species or strain of *Pseudomonas*, type of insect and host immunity.

The entomopathogenic mechanism of *Pseudomonas* spp. is not fully elucidated; however, current models suggest that the insecticidal effects vary amongst species but depend on the production of extracellular bioactive metabolites (Teoh et al. 2021; Raio 2024). When consumed by the insect, *Pseudomonas* sp. release a range of different extracellular virulence compounds (Teoh et al. 2021). One of these is the quorum-sensing regulator, Rh1R, which suppresses the immune system and antibacterial defence systems of the insect (Teoh et al. 2021). Some strains have been reported to release a metalloprotease, AprA, that degrades antimicrobial peptides released by the host immune system (Teoh et al. 2021). This allows the strain to invade the intestinal barrier and colonise the anterior midgut and haemolymph. Strains of *P. entomophila*

produce monalysin, a β-type pore-forming toxin that causes severe damage and destruction of the midgut (Teoh et al. 2021). Other extracellular compounds associated with *Pseudomonas* spp. virulence includes extracellular proteinases such as LasB, LasA, the PIP47Aa insecticidal protein, exotoxin A, cyclic lipopeptides and rhamnolipids, chitinase and hydrogen cyanide (Teoh et al. 2021). Colonisation of insect gut by *Pseudomonas* spp. promotes further pathogenicity, which can lead to the development of bacteraemia and the eventual death of the host (Teoh et al. 2021; Raio 2024).

Although entomopathogenic *Pseudomonas* spp. express a variety of insecticidal compounds, their application as biocontrol agents have been limited and not readily adopted in IPM practices (Stockwell and Stack 2007). Reasons that have hindered their integration into the biocontrol market include that some phytopathogenic strains are opportunistic human pathogens; and since they are non-sporulating they are more prone to viability loss in formulation processes and, consequently, have relatively short shelf lives (Stockwell and Stack 2007).

1.7.2 Entomopathogenic fungi: *Beauveria bassiana*

Beauveria bassiana is a naturally occurring soil fungus and a highly virulent insect pathogen that causes white muscardine disease (Fig. 1.5) (Shanmugam and Seethapathy 2017; Wang et al. 2017; Bhattacharyya et al. 2022). Strains of *B. bassiana* have shown success in various integrated pest management strategies against different insect and arachnid pests in the agriculture, forestry, and veterinary sectors (Mascarin and Jaronski 2016; Koller et al. 2023). Additionally, commercial formulations of *B. bassiana* are economic to produce at scale, are stable and prevents domestication or loss in virulence of the biocontrol agent (Sharma et al. 2023). These attributes have contributed to its success as an insect biocontrol agent.

The mechanism of action of *B. bassiana* has been extensively studied and is an example of entomo-parasitism. This mode of action relies on its conidia (asexual propagules) spores to initiate the infection process (Bhattacharyya et al. 2022). Infection begins when the conidia get attached to the cuticle of the insect and start to germinate (Boucias et al. 1998; Bhattacharyya et al. 2022). Germination can be catalysed by levels of high relative air humidity which promotes infection (Boucias et al. 1998; Feng et al. 2008). Germ tubes develop from the conidia during germination, which penetrates the insects' cuticle and invades into its haemocoel (Boucias et al.

1998; Feng et al. 2008). Within the host, the fungus hyphal bodies and mycelia grow by using the hosts blood and tissue as its source of nourishment (Boucias et al. 1998; Sharma et al. 2023). This process eventually leads to the death of the host and its mummification in the fungal mass (Fig. 1.5) (Boucias et al. 1998; Sharma et al. 2023).

Several factors have contributed to the success of *B. bassiana* as a biocontrol agent. As a spore-forming fungi it is considered an industrially viable biocontrol candidate because it can be produced in bulk as a dry spore formulation, having a longer shelf life and being able to be applied or dispersed into a target area in different ways (Blanford et al. 2012; Nyadar et al. 2017; Sharma et al. 2023). Also, during its infection process, it can still be transmitted to other insects that make contact with its conidia or fungal mass (Hollingsworth et al. 2020; Koller et al. 2023). By using infected insects as "carriers", the biocontrol range and effectiveness increased, especially when insect or pest pressure is high.

Fig. 1.5 Diseased mummified silkworms. The image was adapted from Wang et al. (2017). The specimen on the left represents an infected silkworm from the control group, whereas the specimen on the right represents the mummified silkworm recovered from a *B. bassiana* treatment.

1.8 Comparison of microbial and chemical insecticides

Two main routes have been taken to control insect pests, i.e. using chemical or biological control agents. Chemical control agents comprise chemically synthesised insecticidal compounds to target and control specific insect pests, while biocontrol agents use their pre-existing predators or entomopathogenic microorganisms to facilitate insect control (Alaux et al. 2010; Rani et al. 2021; Fernandes et al. 2023). Both control mechanisms have been used reliably and have significantly contributed towards promoting higher agriculture yields, crop quality, food security and disease and pest management (Li et al. 2009; Labaude and Griffin 2018; Sharma et al. 2023). However, both are not equal with respect to their sustainable use, effectiveness, host range, dosages, as well as their environmental and ecological impacts (Li et al. 2009; Labaude and Griffin 2018; Sharma et al. 2023). As such, their viability in the context of insect control needed to be assessed.

With a constant increase in the human population, the estimated global population size is expected to exceed 10 billion by 2100 (Nawaz et al. 2016). This will be accompanied by increasing demands for food, land and refuse removal. As such, there will be a growing need to control targeted pests in order to prevent the spread of disease, ensure agricultural success and promote food security (Nawaz et al. 2016). Historically, chemical pesticides agents were developed and utilised as a non-labour-intensive strategy for controlling weeds, phytopathogens and insect pests (Stapleton 2005; Sadasivaiah et al. 2007; Nawaz et al. 2016). Specifically in the agricultural sector, utilisation of synthetic insecticides were accompanied by a significant increase in crop yield, quality, profit and economic food security (Sadasivaiah et al. 2007; Sharma at al. 2023). Accompanying with an increase in chemical pesticides utilisation, came an increase in research and development of chemical compounds to increase its host specificity, reduce dosages, decrease toxicity and decrease in environmental residue (Sadasivaiah et al. 2007; Sharma at al. 2023).

One of the most widely used insecticide was dichloro-diphenyl-trichloroethane (DDT). DDT entered the market from the 1950s where it was used as a reliable insecticide to ensure crop conservation, control of mosquitoes, the vector of malaria, and to decrease the spread of Typhus fever, via body lice (Harrison 1978; Stapleton 2005; Donets and Sadasivaiah et al. 2007; Khan et al. 2017; Tsygankov 2023). The effectiveness of DDT and its derivatives is attributed to their

neurotoxic mode of action that targets voltage-gated sodium channels (Sadasivaiah et al. 2007; Khan et al. 2017). They cause these channels to open thereby creating a sodium gradient to promote the excitement of cells, which, in turn, leads to lethal muscle spasms and eventually death of the insect (Khan et al. 2017). Due to concerns of its toxicity on non-target organisms, its eligibility was reassessed and in 1972 the United States Environmental Protection Agency issued a cancellation order on DDT due to the mounting evidence of its toxicity and threat to wildlife and the environment.

The most concerning threats were that DDT and similar organic halogenated insecticides had very slow degradability and, therefore can persist in the environment for prolonged periods of time (Alawi et al. 2018; Chen et al. 2023). The half-life of DDT is between 4-30 years in soil and 26-56 days in water (Setyo et al. 2018). This trait registers DDT and synthetic pesticides as persistent organic pollutants (Haque et al. 2017; Setyo et al. 2018; Chen et al. 2023;). Halogenated organic insecticides are also known to be highly disruptive to aquatic ecosystems (Khan et al. 2017; Fernandes et al. 2023). This can result in its bioaccumulation of synthetic pesticides up the food chain, where it can concentrate itself in the tissue and fat of living organisms (Haque et al. 2017; Setyo et al. 2018). Other side effects of synthetic pesticides include; thinning of eggshells and infant mortality in birds, sterility in fish, disruption of the endocrine system in mammals, and, an increase in the risk of cancer and infertility in humans (Turusov et al. 2002; Booij et al. 2016; Haque et al. 2017; Kamata et al. 2020; Fernandes et al. 2023).

Another major concern about the future of chemical pesticides pertain to their effectiveness in response to a rise in insect resistance to various chemical pesticides (Wan et al. 2019; Fernandes et al. 2023). Insect resistance to chemical pesticides has been attributed to mutations in various genes, which can be passed onto their offspring (Traylor et al. 2017). In the context of fly biocontrol, mutations of the cytochrome 450 enzyme have been attributed to a growing resistance to organophosphorus, organochlorine, carbamate, pyrethroid and benzoylurea class pesticides in *L. cuprina* and *Musca domestica* (Linnaeus) (Diptera: Muscidae) flies (Traylor et al. 2017). Mutations that confer resistance to these pesticides are hereditary; consequently, the number of individuals resistant to pesticides increase over time and correlates with an increase in economic loss (Bendele et al. 2023). Anthropogenic factors contributing to increases in insect

resistance to chemical pesticides include; inconsistent applications or dosages, overuse of pesticides, and, an overreliance on the same class of pesticide (Bass and Jones 2018; Bendele et al. 2023).

Microorganisms with biological control potential innately exist in the natural environment and, therefore, do not pose a harmful effect on the environment (Sayed and Behle 2017; Bass and Jones 2018 Singh et al. 2019; Dixon et al. 2023). Hence, microbial-based insecticides are considered as viable environmentally-friendly control alternatives to chemical pesticides. Many of these biocontrol agents show host-specificity and can replicate and remain infective in the environment, which increases the effectiveness of insect control schemes by extending their effective period in the environment (Singh et al. 2019; Dixon et al. 2023). Many of these microorganisms produce extracellular metabolites that are biodegradable and are involved in beneficial secondary processes such as inducing an immune response in plants or functioning as plant growth promoters (Mishra and Arora 2016; Singh et al. 2019; Zou et al. 2022). Microbial biocontrol agents generally show low- to no-toxic effects towards mammals, which allows them to be used for pre- and post-crop harvest insecticidal treatments (Nawaz et al. 2016). Some microbial biocontrol agents have even shown efficacy in controlling certain insects that are resistant to various chemical pesticides (Boyer et al. 2012). As a result of these factors, microbial insecticides have increasingly become an important part of modern pest management practices and are considered as sustainable options for pest control.

Microbial pesticides are gaining new grounds in the biocontrol market; however, their growth is hampered by various challenges that limit their ability to compete with chemical control agents. Such limitations include having a slower onset of pest mortality, limited studies and field evaluations, not all microbial species can be produced as dry formulations, liquid microbial formulations tend to have a short shelf life and viability varies over time, variation in the effectiveness of the microbial strains depending on the prevailing environmental conditions and complex regulatory, and legislative, requirements for commercialisation of a biocontrol product (Nawaz et al. 2016; Arthurs and Dara 2019; Singh et al. 2019; Helepciuc and Todor et al. 2022). These are all factors that need to be addressed for microbial biocontrol agents to become more prominent in the biocontrol market.

1.9 Criteria for evaluating microbial biocontrol candidates

The ability of a microorganism to exhibit significant pest antagonism is one of the many criteria that need to be present for a strain to be selected for further evaluation for use as a biocontrol agent. The evaluation process is extensive and requires information regarding modes of action, safety, effectiveness in the environment and compliance with the legislative and national environmental protection agency approved standards (Goettel et al. 2001; Helepciuc and Todor et al. 2022). The USA Environmental Protection Agency categorises microbial biocontrol agents into three main categories: (1) naturally-occurring entities that have a non-toxic mode of action, (2) microbial entomopathogens, and (3) plant-incorporated protectants created from genetically engineered plant and microorganisms (Ruiu 2018). Most countries are signatories of the Convention on Biological Diversity, which ensures that biological products do not pose a threat to regional biodiversity (Goettel et al. 2001). The North American Microbial Biocontrol Working Group has emphasised several important aspects regarding the use of microbial biopesticides that need to be taken into consideration; these include (Goettel et al. 2001; Mancebo et al. 2011):

- Allergenicity: The biocontrol microorganism or formulation should be scrutinised for potential immune responses by humans and other allergic reactions. If any allergic reactions are found, the product should be reassessed for human use or have the allergic reaction listed as a potential side effect.
- Toxicity: Toxicological studies need to demonstrate that the biocontrol agent must not have any adverse effects on the applicator, vertebrates and the environment.
- Pathogenicity: It should be demonstrated that the biocontrol agent is specific to the target insect pest(s) and not be pathogenic to non-target organisms.
- Depletion of hosts: Studies need to demonstrate that reducing the pest population does not adversely affect non-target organisms, disrupt the food web, threaten the local biodiversity or contribute to the rise of other pest species.
- Competitive displacement: The biocontrol agent must not out-compete the native organisms in the area it is applied. Antagonism should be pest-specific.
- Host range: It is important to know the potential host range of the biocontrol agent and whether the microorganism is an obligate pathogen to a few closely related species or if it

shows broad-spectrum activity.

- Dose-related susceptibility: The susceptibility of a pest to infection is dependent on the dosage or concentration of toxin or biopesticide present. The threshold concentration required for successfully applying a biopesticides under normal conditions needs to be determined. Even if a suitable dosage regimen is in place, it does not necessarily imply that the target pest would be successfully infected. This process is influenced by various environmental factors such as UV exposure, humidity and temperature.
- Persistence and dispersal: The microbial biopesticide must remain viable for a long enough period to elicit its pesticide effect.
- Indigenous vs non-indigenous organisms: Microorganisms isolated from an environment similar to its intended area of application is likely to be more suited to the prevailing conditions and have a higher chance of success than non-indigenous microorganisms.
- Genetically modified microbial control agents: Microbial strains can be genetically modified to increase their infectivity, host range and pathogenicity. Consequently, this can alter the behaviour of the strain in the environment. Genetically modified microorganisms are unique as they have never existed in nature, and therefore their role and impact in nature and the ecosystem are unknown. Due to this, genetically modified strains require more rigorous evaluation to assess their safety and candidacy as biocontrol agents.

These criteria should be considered from the early stages of screening and evaluation of a microbial biocontrol agent to increase the likelihood of a potential candidate being commercialised.

1.10 Establishing a biocontrol candidate

The value of biopesticides has been increasing in the current biocontrol market. In 2020, Biological pesticides represented 6% of the global pesticide market share in 2020, and is expected to double and reach ~ 15 % by 2031, accompanied with the decrease in chemical pesticide utilisation (Smagghe et al. 2023; Somal et al. 2024). In order to identify new products and grow the biopesticide market, a process of screening, evaluating and commercialising a biocontrol agent needs to be followed (Helepciuc and Todor et al. 2022; Gressel 2024). This

process involves screening for applicable strains, characterisation and identification of the biopesticide, elucidating its mode of action, assessing its level of pest antagonism, field trial evaluations and legislative compliance (Helepciuc and Todor et al. 2022; Smagghe et al. 2023).

Isolation and screening of microorganisms for entomopathogenic or other forms of pest control are considered the first step in establishing a biocontrol agent (Kiewnick 2007). This process can be costly and labour-intensive because it involves selective isolation of potential biocontrol strains and/or testing multiple isolates for pest antagonism (James et al. 2018; Köhl et al. 2019). These evaluations are generally done at a small scale under laboratory-controlled conditions to select the most efficient or potent antagonist (James et al. 2018; Javal et al. 2019). The screening process narrows the potential biocontrol candidates to a few strains with the highest chance of success (Kiewnick 2007). This reduces cost in subsequent evaluation steps and makes the evaluation process more sustainable (Kiewnick 2007).

Once the best-performing strains have been selected and the pest antagonistic effect is confirmed, the strains need to be characterised and identified. This information confirms whether the desired biocontrol candidate was isolated and is required when requesting ethical clearance for further studies or safety approval by legislative authorities such as the USA Environmental Protection Agency or Food and Drug Authorities (Kiewnick 2007; Dunlap 2019; Köhl et al. 2019). Some genera of microorganisms, such as the *Bacillus*, have undergone changes in nomenclature and require new testing approaches to accurately determine their identity (Dunlap 2019). Molecular identification approaches such as multiple-locus sequence analysis have been used to reliably identify bacterial species, while fungi have been identified by PCR amplification and sequence analysis of their internal transcribed spacer regions (McKinnon et al. 2017; Shi et al. 2017; Dunlap 2019; Li et al. 2020; Bhadani et al. 2021).

The third stage in the BCA evaluation process involves running bioassay trials to determine and select the most effective strains from the post-screening batch (Kiewnick 2007; Köhl et al. 2019). Information pertaining to insecticidal rates, dosing rates, lethal exposure times, modes of action, bioactive compounds produced, host range and toxicity is also collected for registration purposes (Rajaofera et al. 2018; Ivorra et al. 2019). Many of these investigations are conducted under controlled laboratory conditions (Hmani et al. 2017; Rajaofera et al. 2018; Ivorra et al. 2019). Toxicological studies are carried out to determine whether candidate biocontrol agents

negatively impact non-target indicator organisms such as Zebrafish (Cypriniformes: Cyprinidae, *Danio rerio*), planktonic crustaceans (Cladocera: Daphniidae, *Daphnia magna*), and mice (mammal representative) (Hmani et al. 2017; Ivorra et al. 2019). The biocontrol candidate must also meet the countries' safety standards where it is to be used and sold. Hence, toxicity studies are done in compliance with national and international safety authorities, which can include the Pest Control Board (Africa), USA Environmental Protection Agency and European Union Plant Protected Products regulations (Frederiks and Wesseler 2019; Vekemans and Marchand 2020; Somal et al. 2024).

Field trial evaluation of a candidate biocontrol strain is crucial to determine whether it is antagonistic under real-world conditions (Vurukonda et al. 2018; Besset-Manzoni et al. 2019). Many biocontrol candidates are eliminated from the evaluation process due to their failure to antagonise the target pest in the environment or due to poor or inconsistent results (Besset-Manzoni et al. 2019). This stage can also be used to assess formulation options, application methods and application rates (Derua et al. 2018; Fan et al. 2019; Johnson et al. 2020; Reinbacher et al. 2021; Vimala et al. 2021). These trials can also be used to determine the impact the candidate biocontrol agent has on non-target organisms. Field trial duration can range from a few weeks to several months (Derua et al. 2018; Vurukonda et al. 2018; Johnson et al. 2020; Vimala et al. 2021). Biocontrol candidates or product formulations that perform well in their field trial evaluations can then undergo product registration and approval.

The registration and legislative approval of a biocontrol product is the final stage in the evaluation process (Kiewnick 2007; Arthurs and Dara 2019; Helepciuc and Todor et al. 2022; Somal et al. 2024). In South Africa, the evaluation and approval of biocontrol agents are determined by the Department of Agriculture, Forestry and Fisheries, under the Agricultural Pests Act 36 of 1983, and by the Department of Environmental Affairs under the Environment Conservation Act 73 of 1989 (Klein et al. 2011). In conjunction with this, the product must also comply with the International Plant Protection Convention and International Standards for Phytosanitary Measures developed by the Food and Agriculture Organisation of the United Nations (Klein et al. 2011). Among the main criteria required by these authorities include evidence that the proposed biocontrol agent is not antagonistic to non-target organisms, toxicological studies confirming that it complies with national and international safety standards,

and its environmental impact assessment (Klein et al. 2011). Many countries consider these criteria more vital than the biocontrol effectiveness of the product because that is considered to be a requirement of the company trying to approve it (Kiewnick 2007; Arthurs and Dara 2019; Helepciuc and Todor et al. 2022; Somal et al. 2024). The regulatory process in South Africa, and in other counties, are known to be costly and incur long processing times (Kiewnick 2007; Klein et al. 2011; Arthurs and Dara 2019; Köhl et al. 2019; Somal et al. 2024). This is one of the major hindrances reducing the number of products entering the biocontrol market internationally (Kiewnick 2007; Klein et al. 2011; Arthurs and Dara 2019; Köhl et al. 2019; Helepciuc and Todor et al. 2022). However, once the biocontrol agent or formulation has been approved, it can be marketed and sold as a biocontrol agent (Arthurs and Dara 2019; Helepciuc and Todor et al. 2022; Somal et al. 2024).

1.11 Target for biocontrol: Members of true flies, Diptera.

The order Diptera encompasses all true fly species (Mayhew 2007; Song et al. 2023). This group of flies are characterised by having only a single pair of wings located on the mesothorax used for flight (Irwin et al. 2003). This order is estimated to contain approximately 195 000 documented species (Mayhew 2007; Badii, 2020).

The Diptera is divided into 2 distinct suborders, the Nematocera and Brachycera (Baldacchino et al. 2018; Song et al. 2023). Both suborders contain hematophagous species and species of economic and medical importance. Examples of these flies include sand flies (Nematocera) and tsetse flies (Brachycera) (Ribeiro et al. 2010; Baldacchino et al. 2018). Members of the Nematocera are characterised as having long, thin and highly segmented antennal flagella (Baldacchino et al. 2018; Badii, 2020; Song et al. 2023). These insects include black flies (Diptera: Simulidae), biting midges (Diptera: Ceratopogonidae), mosquitoes (Diptera: Culicidae) and frog feeding flies (Diptera: Corethrellidae) (Ribeiro et al. 2010). Brachycera is the larger of the 2 suborders, encompassing over 100 families and more than 75 000 species (Wiegmann et al. 2003). These species are usually characterised by having wider bodies and shorter antennae (Song et al. 2023). This group includes the commonly associated blowflies, fruit flies, horse flies, flower flies, house flies, green bottle flies, etc. (Wiegmann et al. 2003). Flies of the Brachycera are of interest in this study due to their relatively high abundance within the

Diptera and their medical and agricultural importance.

1.11.1 Fly life cycle

The life cycle of Brachycera flies is important in biocontrol research because it equips scientists with the essential knowledge to disrupt the fly reproductive cycle by targeting certain developmental stages (Bansode et al. 2017). Many Brachycera flies have similar fly developmental cycle, with some flies completing one life cycle within 12 days (Va et al. 2009; Ong et al. 2015; Flatt 2020; Sontowski and van Dam 2020). The life cycle of flies is greatly influenced by the local climate and the prevailing conditions (Ong et al. 2015; Bansode et al. 2017; Flatt 2020; Bansode and More 2024).

Post copulation, the fertilised eggs are laid on a medium that serves as a source of nourishment for the new progeny. The eggs are ~ 0.5 mm in length; during the onset of embryonic development, the embryo develops into a zygote (Va et al. 2009; Ong et al. 2015; Bansode et al. 2017). The zygote stage is characterised by the zygote consuming its egg encasement. When the zygote hatches it is known as a larva. It takes ~ 24 h at 25ºC for an embryo to develop into a larva (Ong et al. 2015: Flatt 2020).

The larval stage is characterised by rapid growth rates and a rapid increase in size due to endoreplication of the larval tissue and the development of organ systems (Va et al. 2009; Bansode et al. 2017: Flatt 2020). As the larvae eat and grow, they transition through three growth stages or instars. Each transition is accompanied by a moulting process where the outer cuticle layer of the larva is removed and replaced with a new one to accommodate their increase in size (Va et al. 2009). After the third instar stage, the larva stops feeding and moves to a suitable site for pupation and metamorphosis. Larvae typically distance themselves from their food source and locate a dark warm site for pupation to avoid predators or competitors. The larva creates a cuticle shell-like structure for pupation called the puparium (Va et al. 2009: Flatt 2020). As the puparium forms, it hardens and progressively becomes darker; eventually developing into a pupa. Under ideal conditions, maturation of the puparium can take 2.5 – 3 days (Ong et al. 2015). Once the puparium has matured, the pupae undergo metamorphosis, and it can take 3.5-4.5 days before the adult flies emerge. Compared to larvae, adult flies are considered to be sophisticated higher organisms, capable of complex behaviours such as courtship, sleep cycles,

memory and flight. (Pandey and Nichols 2011). Male flies are generally larger than female flies and can be distinguished based on size. Female flies are ready to mate within less than 24 h and can lay ~ 100 eggs per day (Flatt 2020). Adult flies can live up to 2 months after they hatch (Pandey and Nichols 2011).

Studies on the biocontrol of flies have primarily targeted the larvae stage for fly antagonism (Prince et al. 2024). Since larvae display high feeding rates, this developmental stage is ideal for introducing a biocontrol agent, or its bioactive metabolites, to their food source. This strategy has shown promise in reducing several types of fly populations, including blowflies, shore flies, blood-sucking black flies, Queensland fruit flies and even mosquitos (Molly 1990; Gough et al. 2005; Vänninen and Koskula 2010; Prince et al. 2024). The pupa stage is not considered a suitable primary biocontrol target because the developing larva is encased in the pupa shell and is protected from the environment (Ong et al. 2015). Adult flies are also harder to target for biocontrol applications because they are very active, are not restricted in their movements which allows them to disperse widely within their immediate habitat (Ong et al. 2015).

1.11.2 The need for control of flies

Flies are present in vast numbers globally and inhabit nearly every biome (Liu et al. 2023). Many fly species are beneficial and play important roles in nature, whereas, others pose significant threats to agriculture, animals, and humans (Desquesnes et al. 2016; Muriithi et al. 2016; Manrakhan et al. 2017; Haran et al. 2019; Muriithi et al. 2019; Murtaza et al. 2021). Due to their close association with human habitation and agricultural practices, there is a need to be able to control fly populations as a matter of health and safety concerns (Manrakhan et al. 2017; Liu et al. 2023). The following example highlights the need for biocontrol strategies to control flies that are considered pests.

1.11.2.1 Blowflies, threats to livestock farms and humans.

Blowflies (Diptera: Calliphoridae) are characterised by having metallic blue, metallic green or black bodies. These flies encompass many genera, most notably *Lucilia* and *Chrysomya*, and are known as vectors of diseases that afflict humans and animals (Sukontason et al. 2007; Bernhardt

et al. 2017; Caleffe et al. 2019; Liu et al. 2023). Amongst these groups of flies, *L. cuprina* has been identified as a pest of veterinary and medical importance due to it being the primary causative agent of flystrike or myiasis (Caleffe et al. 2019; Liu et al. 2023).

Flystrike is a global threat that affects countries with warmer climates, such as South Africa, New Zealand, and Australia (Traylor et al. 2017; Sales et al. 2020; Liu et al. 2023). It is caused when blowflies lay their eggs on the skin of warm-blooded animals, and the emerging larvae burrow into the host's flesh and consume it as they develop (Traylor et al. 2017; Sales et al. 2020). This is known as myiasis, and if left unattended, secondary infections can develop that can lead to anaemia and toxaemia which can be fatal (Leathwick et al. 2019). Cases of myiasis in humans have also been reported and was caused by at least 50 different species (Kuria and Oyedeji 2020). With an increase in global warming, incidences of flystrike are expected to increase (Leathwick et al. 2019).

Conventional control measures for these flies involve the use of organophosphate insecticides such as dicyclanil and cyromazine (Sales et al. 2020). However, the effectiveness of these treatments has declined due to the rise of chemical resistance in blowfly species such as *L. cuprina*, which makes it difficult to control them (Traylor et al. 2017; Sales et al. 2020). Resistance to chemical pesticides has been attributed to mutations of its cytochrome P450 monooxygenase, or other genes, which allow it to chemically alter and deactivate synthetic pesticides (Traylor et al. 2017; Sales et al. 2020).

Some of the currently proposed control measures for these flies include developing fly traps, implementing biocontrol programs, and removing fly breeding sites (Leathwick et al. 2019). Many fly biocontrol formulations have used environmental applications of entomopathogenic fungi, such as *Tolypocladium cylindrosporum* (Hypocreales: Ophiocordycipitaceae) and *Octosporea muscaedomesticae* (Pezizales: Pyronemataceae), or endospore formulations of *B. thuringiensis* (Leathwick et al. 2019). These strains were shown to significantly reduce the number of blowflies by eliciting a lethal response to both flies and larvae (Leathwick et al. 2019). *Bacillus thuringiensis* Bt toxin formulations have been used as coat treatment on livestock and can protect against flystrike for up to 11 weeks (Leathwick et al. 2019). Currently, evidence suggests that biocontrol options could be a viable solution in flystrike control and warrants further investigation (Traylor et al. 2017; Leathwick et al. 2019; Sales et al. 2020; Liu et al.

2023).

1.11 Conclusion

Biological control is considered a more sustainable approach to controlling insect pests than chemical control, especially considering the current rise of insect resistance to chemical pesticides. Members of the *B. subtilis sensu lato* group show promise as biocontrol candidates of various pests ranging from phytopathogens to insects. This is attributed, primarily, to their ability to produce a variety of bioactive lipopeptide biosurfactants. This provides grounds to investigate whether members of the *B. subtilis sensu lato* group, namely strains of *B. velezensis*, can act as antagonists of flies within the suborder Brachycera. Since no biocontrol studies have been conducted to date with members of this *sensu lato* group against fly representatives of this suborder, it presents an avenue for potentially novel research. The current investigation aims to evaluate the potential of *B. velezensis* PHP1601 as a potential candidate for use in the biocontrol of selected fly species.

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CHAPTER TWO: FIRST REPORTED INCIDENCE OF *BACILLUS VELEZENSIS* EXHIBITING EFFECTIVE ANTAGONISM AGAINST A BLOWFLY SPECIES, *LUCILIA CUPRINA*

2.1 Abstract

Bacillus sp. PHP1601 (Bacillales: Bacillaceae), a member of the *B. subtilis sensu lato*, possesses an interesting ability to antagonise fly larvae. Blowflies are considered occupational nuisances and recognised as vectors of disease. A study was undertaken to evaluate the biocontrol potential of *Bacillus* sp. PHP1601 against *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) larvae. Bioassays were established to determine the biocontrol efficacy of various treatment formulations, viz. vegetative cell (10^2 – 10^{10} cells g^{-1}), endospore (10^2 – 10^{10} endospores g^{-1}), cell-free supernatant (1 – 30% v w^{-1}) and crude biosurfactant extract (5 – 1000 µg g^{-1}) against second instar *L. cuprina* fly larvae reared on milk agar medium. All treatments demonstrated a dose-dependent larvicidal response over a 240 h period. Even at the lowest cell and endospore concentrations tested (i.e., 10^2 cells/endospores g^{-1}) the pupation stage was inhibited, effectively interrupting the fly reproduction cycle. Larvae antagonism elicited by partially purified biosurfactant extracts indicated the likely contribution of bioactive lipopeptide compounds to the mechanism of inhibition. Larvae cadavers recovered from these treatments displayed substantial physiological discolouration and lacked structural integrity. PHP1601 was confirmed to be a strain of *B. velezensis* based on multiple-locus sequence analysis of 16S rRNA, *gyr*A, *rpo*B and *dna*J housekeeping genes. This study constitutes the first report of a strain of *B. velezensis* demonstrating a larvicidal response towards a member of the suborder Brachycera.

Keywords Bacillales, Bacillaceae, *Bacillus velezensis*, lipopeptide biosurfactants, Diptera, *Lucilia cuprina*.

2.2 Introduction

Flies are one of the most prevalent insect groups in terms of population abundance and diversity and are associated with various habitats and ecosystems. Several fly species within the suborder Brachycera are detrimental to humankind and are considered to be a threat in agricultural or livestock farming settings (Kremer and King 2019). Among these, blowflies (Diptera, Calliphoridae) are regarded as pests of biocontrol significance (Prawer et al. 2020).

The blowfly, *Lucilia cuprina* (Wiedemann), is a pest of veterinary and medical importance since it is the primary cause of flystrike or myiasis (Caleffe et al. 2019). This is a condition where blowflies lay their eggs on warm-blooded animals, and the emerging larvae consume the hosts flesh as they develop. This exposes wounds to secondary infections, which could become fatal if left unattended. Although flystrike is a global threat more severe cases are experienced in countries with warmer climates (Leathwick et al. 2019). Furthermore, the occurrence and severity of flystrike is expected to increase as climate changes associated with global warming takes hold. Environmentally friendly control measures incorporating biological control or biopesticide approaches have been advocated in recent years to address this growing challenge (Leathwick et al. 2019).

The *Bacillus subtilis sensu lato* group (Bacillales: Bacillaceae) includes members that have achieved GRAS (generally regarded as safe) status, which is attractive from a biocontrol perspective (Chen et al. 2020). Members of this species complex produce a range of bioactive compounds, including several classes of lipopeptide, which show biocontrol potential towards many phytopathogens (Chowdhury et al. 2015). Several reports in the literature suggest that these lipopeptides may also be active against certain insect groups; however, their insecticidal biocontrol applications appear to be relatively scarce and mainly focus on mosquito (Diptera: Nematocera) larvae control (Mnif et al. 2013; Falqueto et al. 2021). Therefore, members of the *B. subtilis* species complex warrant evaluation as potential antagonists of Brachycera flies of medical and veterinary significance, such as *L. cuprina*.

In the current study, *Bacillus* sp. PHP1601, a strain isolated from a fly larvae cadaver and anecdotally has shown to antagonise fly larvae, was evaluated to assess its biocontrol potential. Based on preliminary 16S rRNA sequence analysis, this strain was putatively classified as a

member of the *B. subtilis* species complex. However, due to the limited resolution of the 16S rRNA gene within this taxonomic grouping, more robust identification methods, such as multiple locus sequence analysis (MLSA), is required (Mohkam et al. 2016). The efficacy of *Bacillus* sp. PHP1601 antagonism towards *L. cuprina* larvae was evaluated through bioassays designed to simulate the application of a biocontrol agent to a substrate infested with fly larvae. The bioassays assessed the effect of a bioassay medium amended with varied cell, endospore, culture-free supernatant and lipopeptide biosurfactant concentrations on larvae mortality achieved. The identification of the strain was determined using MLSA.

2.3 Methods and materials

2.3.1 Bacterial culture

Bacillus sp. PHP1601, hereafter referred to as PHP1601, was obtained from Andermatt Plant Health Products (Strathdean Farm Gowrie Avenue, Nottingham Road, 3280, KwaZulu-Natal, South Africa). PHP1601 was routinely cultured on tryptone soy agar (TSA) or broth (TSB) (Biolab, Merck, Germany) for 24 h at 30ºC. The strain was preserved as 20% (v v^{-1}) glycerol-endospore master culture solutions and stored at -20ºC and -80ºC.

2.3.2 DNA extraction

A vegetative culture of PHP1601 was prepared for DNA extraction by culturing in Luria-Bertani broth (10 g l^{-1} of tryptone, 10 g l^{-1} NaCl, 5 g l^{-1} of yeast extract, pH 7.00) at 150 rpm for 16 h at 30ºC. Approximately, 2×10^9 cells were pelleted (8000 × g for 5 min) and washed with an equal volume of phosphate-buffered before being used for DNA extraction according to the GeneJET protocol for Gram-positive bacteria (Thermo Fisher Scientific, Inc., USA).

2.3.3 MLSA and species-specific PCR identification of PHP1601

PHP1601 was identified by PCR amplification and MLSA targeting the partial 16S rRNA, *gyr*A, *rpo*B and *dna*J genes. The 16S rRNA and *dna*J genes were amplified according to the method described by Ström et al. (2002) and Connor et al. (2010) respectively, whereas the *gyr*A and

*rpo*B genes were amplified as described by Roberts et al. (1994). All PCR reactions contained: ×1 DreamTaq Green Master Mix (Thermo Fisher Scientific, Inc., USA), 0.5 mM of forward and reverse primers (Appendix A: Table A1), 2 ng μl^{-1} of template DNA and was made to 25 µl with nuclease-free water. A G-Storm GS1 thermal cycler (G-Storm, Somerset, UK) was used to run the PCR reaction sequence. The identification of PHP1601 from the MLSA was verified using *B. velezensis* specific endpoint PCR according to the method described by Dunlap (2019). PCR amplification of the targeted gene fragments were confirmed using agarose (1.5% w v^{-1}) gel electrophoresis at 80V for 100 min using a TAE buffer (40 mM Tris, 20 mM Acetic acid, 1 mM EDTA, pH 8.3).

Amplified gene products were sequenced at Inqaba Biotec Laboratories (Pretoria, South Africa) using the ABI 3500XL Genetic Analyzer (Applied Biosystems, USA). The quality of the sequence chromatograms was assessed using Chromas software (v 2.6.6). Consensus sequences from the forward and reverse sequence reads were generated using BioEdit software (v 7.2.6.1). These sequences were then matched to similar gene sequences within the GenBank database using the nucleotide blast algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignment, concatenation of genes and phylogenetic analysis were all performed using MEGA X software (v 10.2.1). The Maximum Likelihood method and Tamura-Nei models were used to construct a phylogenetic tree deduced from the bootstrap consensus of 1000 replicates (Felsenstein 1985; Tamura and Nei 1993; Kumar et al. 2018).

2.3.4 Capture and rearing of blowflies

A wild-caught population of blowflies were reared and maintained in insectaries kept at a constant temperature of 28°C with a 12 h light-dark cycle and relative humidity of 66 ± 5% at the University of KwaZulu-Natal Controlled Environment Research Unit (UKZN-CERU). The fly population was routinely fed 200 g of maggot growth (MG) agar (500 ml l^{-1} UHT milk, 2 g l^{-1} bacteriological agar, pH 6.50) adapted from Johnson et al. (1998). The MG agar was supplemented with 10 g of minced chicken livers weekly to provide a stimulus for oviposition. Fly populations were kept hydrated by provisions of ~20 ml of sterile tap water daily.

2.3.5 Identification of captured flies and assessment of population purity

Flies were characterised phenotypically and their identification was confirmed by barcoding of their cytochrome oxidase subunit 1 (CO1) gene sequence according to the method of Radzevičiūtė et al. (2017). Specimen samples (100 mg) sourced from maggots, pupae and adult flies were used for DNA extraction. Specimens were washed in 5% (v v^{-1}) bleach and rinsed with 40 ml of sterile phosphate-buffered saline (PBS) (136.9 mM NaCl, 5.8 mM Na_2HPO_4 $7H_2O$, 4.2 mM Na_2HPO_4 $7H_2O$, pH 7.00) before being frozen in liquid nitrogen and macerated by pestle and mortar grinding. Forty milligrams of the respective insect paste were processed as per the GeneJET Genomic DNA purification kit protocol for rodent tail. Confirmation of the PCR amplification was performed using agarose gel electrophoresis.

2.3.6 Growth of PHP1601 in MG agar

The growth of *Bacillus* sp. PHP1601 in MG agar was assessed to determine viable concentration ranges of the organism over the course of a biocontrol trial. A 100 ml 16 h TSB culture of PHP1601 was pelleted via centrifugation (10000 × g for 5 min) and resuspended in 10 ml of PBS to create an inoculum stock which was then quantified using a counting chamber (0.01 mm × 0.0025 mm^2). Thereafter, 54 g of ×1.11 MG agar was amended with 6 ml of cell suspension to create 10^2, 10^5, 10^8 and 10^{10} cells g^{-1} tests solutions respectively in 250 ml Schott bottles. The bottles were incubated statically at 28ºC for 10 days. Similarly, autoclave killed cells were prepared equivalent to the cell standards for use as dead cell controls. Cell-free controls were prepared similarly by inoculating MG agar with 6 ml of sterile PBS. An average of triplicate readings was collected for statistical inferences.

The presumptive bacterial load was determined at 48 h intervals by an extinction dilution method adapted from Hidore et al. (1991). The adaptions used were; bead-beating 1 g of MG agar in a McCartney bottle containing 9 ml of TSB and 10 × 2 mm glass beads on a bench top vortex (2 min at 2000 rpm) to free bound cells, which were then serially diluted in TSB.

2.3.7 Larvicidal trials evaluating PHP 1601 vegetative cell and endospore treatments

Plastic containers (300 ml) containing 60 g of MG agar served as the vessels and environment for each trial. As described previously, the MG agar was amended to contain 0, 10^2, 10^5, 10^8 and 10^{10} cells g^{-1} of PHP1601 respectively. Thirty second instar larvae were added to each container to assess their susceptibility to vegetative cells. Bioassays were run for 240 h and maintained under the same conditions used for rearing the flies. Observations of larval mortality were made at 24 h intervals.

Similarly, larvicidal trials using endospore treatments were prepared and assessed as described above. Endospores were obtained by culturing PHP1601 in 100 ml of glucose omitted Schaeffer's broth at 35ºC for 96 h with agitation (225 rpm) and harvested by centrifugation (15000 × g for 10 min) (Leighton and Doi 1971). Endospores were visualised and quantified using a bacterial counting chamber (0.01 mm × 0.0025 mm^2) in conjunction with phase-contrast microscopy. To eliminate vegetative cells from endospore stock solutions a heat shock step (80ºC for 20 min) was used. Dead cell or endospore controls were prepared by autoclaving the inoculum prior to inoculation.

2.3.8 Larvicidal effect of cell-free supernatant (CFS) of PHP1601

Aliquots (1 ml) from an overnight TSB culture of PHP1601 was used to inoculate 100 ml of TSB to achieve a starting OD_{600nm} of 0.050. The culture was shaker incubated at 200 rpm for 18 h at 30ºC. The CFS was harvested by centrifugation at 15000 × g for 10 min and then filter sterilised (GxF 0.2 µm GHP Acrodisc, USA). Larvicidal bioassays were prepared by amending the water fraction of the MG agar with CFS. The final test concentrations evaluated were 0%, 1%, 5%, 10%, 20% and 30% (v w^{-1}) CFS in 60 g MG agar. Similarly, TSB controls were prepared by amending the MG agar with sterile TSB of similar concentrations. To each container, 30 second instar larvae were added to assess their susceptibility to CFS. Observations were made at 24 h intervals for 480 h to determine larvae mortality.

2.3.9 Lipopeptide biosurfactant production and assessment

Lipopeptide production was promoted by culturing PHP1601 in defined Landy medium as per Landy et al. (1948). The biomass was removed from the culture by centrifugation and filter sterilisation to acquire a cell-free solution. Lipopeptide biosurfactants were then harvested by acid precipitation and methanol extraction as described by Yang et al. (2015) to acquire a crude lipopeptide (CLP) extract. The CLP extract was qualitatively assessed for biosurfactant activity using the oil displacement assay described by Khondee et al. (2015).

2.3.10 Larvicidal effect of crude lipopeptides

The larvicidal effect of CLP extract was investigated by amending MG agar to standardised concentrations of CLP (viz., 1000, 100, 20, 10, 5, 0 $\mu g\ g^{-1}$). The CLP extracts were introduced as 1 ml phosphate-buffered lipopeptide stocks to 50 g of molten MG agar (~ 40ºC). Unamended and phosphate buffer supplemented MG agar treatments were established as controls. Thirty second instar larvae were added to each container, and observations were made at 24 h intervals for 480 h to determine larvae mortality.

2.3.11 Statistical analysis

All larvicidal trials were performed for 5 independent replicates. Statistical analysis was performed using IBM SPSS (v. 27.0.0.0) at a 99% confidence level whereby significant differences were determined at $P < 0.01$. Data was initially assessed for normality using the Shapiro-Wilks test. Larvicidal data from CLP treatments required a cube root transformation to achieve normality. Significant differences were detected by one or two-way analysis of variance (ANOVA); thereafter, statistical comparisons were determined using the Tukey's HSD test. Lethal concentrations for 50% larvae mortality (LC_{50}) were determined by Probit analysis. All larvae mortality data, inclusive of those presented in figures, were reported as mean ± standard error (SE) values.

2.4 Results

2.4.1 Identification of PHP1601

Evolutionary relationships derived from the MLSA of the partial 16S rRNA, *rpo*B, *dna*J and *gyr*A gene sequences are shown in Fig. 2.1. Phylogenetic analysis of these housekeeping genes achieved species-level grouping of the reference strains and confirmed that PHP1601 is a member of the *B. velezensis* species. This was verified by the amplification of the 170 bp *B. velezensis* specific sugar kinase amplicon. The partial 16S rRNA, *rpo*B, *dna*J, *gyr*A and *B. velezensis* sugar kinase gene sequences were deposited in GenBank and were assigned with the following accession numbers: OM714837.1, OM776918.1, OM714836.1, OM714835.1 and OM776919.1 respectively.

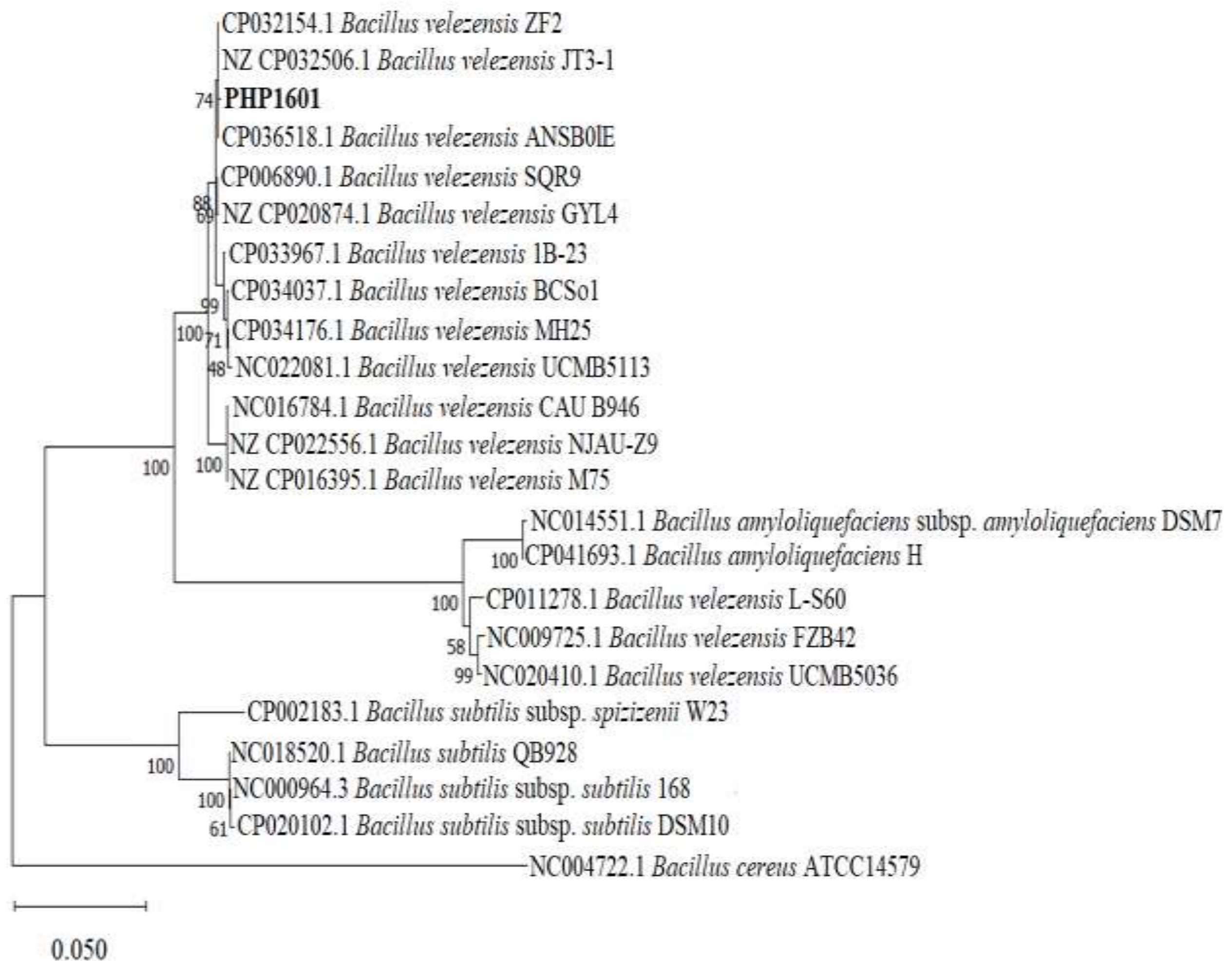

Fig. 2.1 Maximum likelihood phylogenetic tree inferring the evolutionary relationships of PHP1601 and selected reference sequences from NCBI GenBank based on MLSA of partial 16S rRNA, *dna*J, *gyr*A and *rpo*B gene sequences. The final data set comprised concatenated sequences 3035 bp in length. Of the 1000 bootstrap replicates, only values ≥ 50% were included. The scale bar represents 0.050 substitutions per sequence position.

2.4.2 Characterisation and identification of captured flies

Caught flies were indistinguishable from one another and were characterised by having a shiny green abdomen and thorax, three pairs of postsutural acrostichal bristles, hyaline wings and black legs. Phylogenetic analysis of the partial CO1 gene of the adult fly, pupa and larvae samples

confirmed their relatedness (100%) to members of the Diptera, *Lucilia cuprina* (Appendix A: Fig. A1). This also indicated that the population of flies was of the same species. The CO1 gene sequences of the *L. cuprina* larvae, pupa and flies were deposited in GenBank and were assigned the following accession numbers: MW222989.1, MW222990.1 and MW222991.1 respectively.

2.4.3 Larvicidal effect of viable culture of PHP1601

The influence of initial cell concentrations on the titre of PHP1601 inoculated into MG medium was considered an influential variable in assessing its larvicidal activity. Cell concentrations increased by several orders of magnitude over a 240 h time period for each of the concentration ranges tested (Appendix A: Fig. A2). These findings indicated that the degree of larvicidal activity observed for each treatment could not be assigned directly to the initial starting cell concentrations since cell titres increased over time.

The larvicidal effect of cell and endospore treatments are shown in Fig. 2.2. Larvae mortality was observed for all cell and endospore treatments; and interestingly, corresponding cell and endospore treatments exhibited similar larvicidal trends. None of the cell-free or autoclaved culture controls exhibited any instance of larval mortality, which substantiated the mortality observed in the treatments evaluated.

In both sets of experiments, the 10^5 cells/endospores g^{-1} treatments were the only ones to achieve 100% larvae mortality within the study period, even outperforming treatments with higher starting cell/endospore titres. The above-mentioned treatments (10^5 cells/endospores g^{-1}) were, therefore, selected to extrapolate lethal times (LT), which were used to make statistical comparisons.

Lethal time values were determined from linear mortality equations generated from larvicidal observations taken between 48 – 216 h of the bioassay. The mortality equations established for both cell ($y = 0.52x - 13.87$) and endospore ($y = 0.52x - 9.42$) treatments yielded regression coefficients (R^2) of 0.99, which indicated a good correlation to the observed mortality data. The 10^5 cells g^{-1} treatment had LT_{50}, LT_{90}, and LT_{100} times of 121.98 h, 198.38 h and 217.48 h respectively, and correspondingly, 114.30 h, 191.24 h and 210.47 h for the respective endospore treatment (Fig. 2 a and b). Both treatments shared an overall mortality rate of 0.52% per hour.

No significant differences (Two-way ANOVA, $F_{2, 24} = 0.33$, $P > 0.01$) were evident between the 10^5 cell and endospore treatments at each of the respective LT values determined for each treatment. This supports the notion that the endospore treatments underwent rapid germination and performed similarly to their corresponding cell concentration treatments.

One-way ANOVA detected significant differences in larvae mortality between the tested cell concentrations at the LT_{50} ($F_{3, 16} = 12.33$, $P < 0.01$), LT_{90} ($F_{3, 16} = 22.75$, $P < 0.01$) and LT_{100} ($F_{3, 16} = 24.80$, $P < 0.01$) intervals for the 10^5 cells g^{-1} treatment. Significant differences between the different cell concentration treatments were determined by the Tukey's test (Appendix A: Table A2). At the LT_{50} interval, the 10^5 cells g^{-1} treatment exhibited larvae mortality that was not significantly different ($P > 0.01$) to the 10^8 and 10^{10} cells g^{-1} treatments; whereas, at the LT_{90} and LT_{100} intervals, it was significantly ($P < 0.01$) the most potent cell treatment. Interestingly, the 10^2 cells g^{-1} treatment was at least as effective as the 10^8 cells g^{-1} treatment at the tested LT intervals. The endospore treatments followed similar statistical larvicidal trends at the corresponding LT intervals (Appendix A: Table A3).

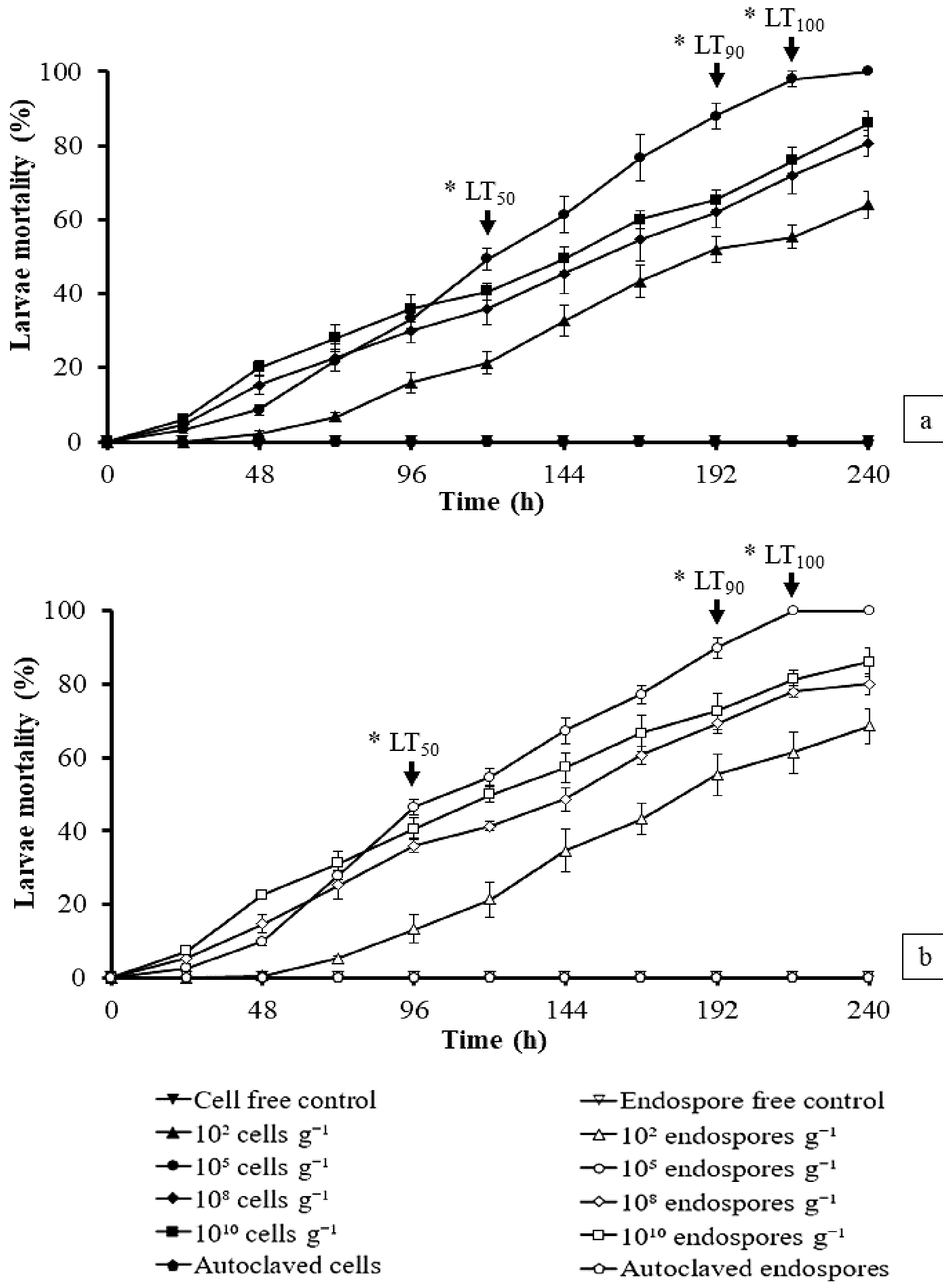

Fig. 2.2 The larvicidal effect of cell (a) and endospore (b) treatments of PHP1601 on *L. cuprina* larvae over a 240-h assay. An asterisk (*) indicates significant differences ($P < 0.01$) detected by one-way ANOVA between the cell or endospore treatments, using intervals that corresponded to the LT_{50}, LT_{90} and LT_{100} of the 10^5 cell/endospore g^{-1} treatment respectively. Error bars represent the SE of the mean.

2.4.4 Larvicidal effect of PHP1601 CFS

For each of the CFS concentrations tested larvae were found to be susceptible to the metabolites contained therein (Fig. 2.3). A dose-dependent larvicidal response was evident over the first 120 h of the experiment. Thereafter, the larvae mortality rates for the 20 and 30% (v w^{-1}) CFS treatments tapered off, plateauing at 81.33 and 78% respectively; whereas, the mortality rates for the 1, 5 and 10% (v w^{-1}) CFS treatments increased over time reaching 76, 86.67 and 100%, respectively by the end of the experiment. The larvae mortality observed was attributed to the action of metabolites present in the CFS, which were absent in the medium and TSB controls.

The 10% (v w^{-1}) CFS treatment exhibited the most effective larvicidal response, and was used to determine LT intervals for statistical comparisons. A logarithmic larvicidal equation, $y = 56.49Ln(x) - 240.87$, was generated based on the observed larvae mortality from 96 – 432 h, which accounted (R^2 = 0.99) for the successive changes in larvicidal rates. The extrapolated LT_{50}, LT_{90} and LT_{100} times were 172.27 h, 349.72 h and 417.45 h respectively.

One-way ANOVA detected significant differences in larvae mortalities observed between the different CFS concentrations at the LT_{50} ($F_{4, 20}$ =17.48, $P < 0.01$), LT_{90} ($F_{4, 20}$ = 9.62, $P < 0.01$) and LT_{100} ($F_{4, 20}$ =16.64, $P < 0.01$) of the 10% (v w^{-1}) CFS treatment. At the LT_{50} and LT_{90} intervals no significant differences ($P > 0.01$) were determined between the 10-30% (v w^{-1}) CFS treatments when compared using the Tukey's test (Appendix A: Table A4). Whereas, at the LT_{100} interval the 10% (v w^{-1}) CFS treatment was significantly better ($P < 0.01$) than the higher CFS concentrations tested. This finding indicated that an increase in the CFS concentration beyond a threshold level does not result in improved larvicidal rates.

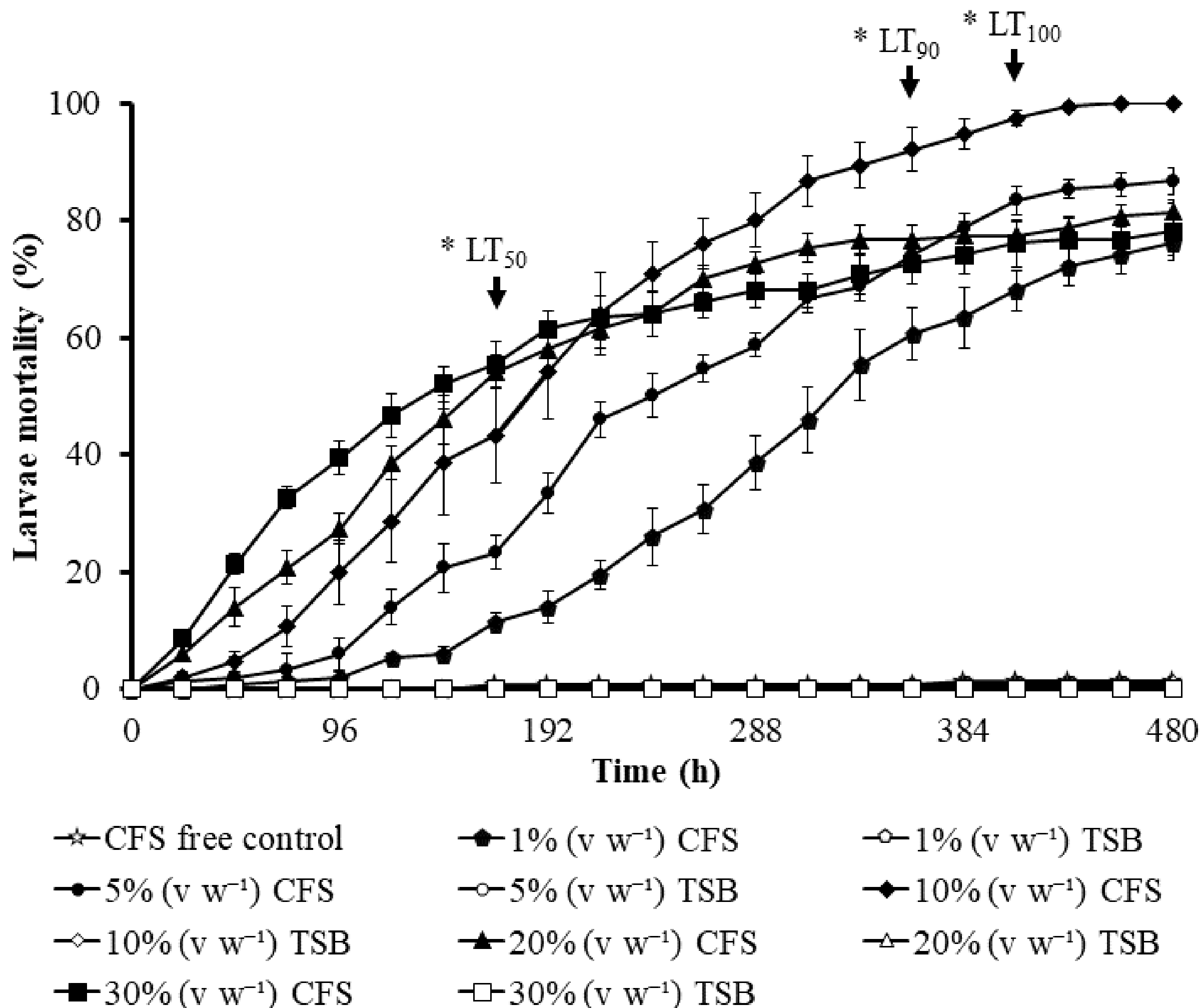

Fig. 2.3 Larvicidal effect of cell-free supernatant of PHP1601 on *L. cuprina* larvae over a 480-h assay. Bioassays investigated a range of cell-free supernatant (CFS) concentrations from 0 – 30% (v w^{-1}). Controls used to verify larvicidal outcomes included a CFS free control and several tryptone soy broth (TSB) controls. An asterisk (*) indicates significant differences ($P < 0.01$) detected by one-way ANOVA between the CFS treatments, using intervals that corresponded to the LT_{50}, LT_{90} and LT_{100} of the 10% v w^{-1} CFS treatment. Error bars represent the SE of the mean.

2.4.5 Larvicidal effect of CLP extracts on *L. cuprina* larvae

PHP1601 yielded 0.87 ± 0.04 mg ml^{-1} of CLP extract after being cultured in Landy medium for 24 h. Aliquots (5 µl) of this extract produced positive results for the oil displacement assay with

zones of 43.40 ± 1.34 cm^2 being achieved. This confirmed the presence of putative lipopeptide biosurfactants in the CLP extract, which could be evaluated in larvicidal bioassays.

Each CLP concentration (5 - 1000 µg g^{-1}) tested demonstrated larvicidal activity with overall mortality rates appearing to be dose-dependent (Fig. 2.4). The 100 and 1000 µg g^{-1} CLP treatments both achieved 100% larvae mortality within 288 h and 192 h respectively. At the end of the trial, the larvae mortality levels for the 5, 10 and 20 µg g^{-1} CLP treatments were 45.33%, 64.66% and 96% respectively. By the end of the experiment, the larvae mortality in the phosphate buffer and medium controls reached an average of 2% (≈ 0.6 out of 30 larvae). This was attributed to natural variance within the larvae population rather than any constituents of the controls. The mortality observed in the CLP treatments was, therefore, attributed to the presence of bioactive biosurfactant compounds.

Since the 1000 µg g^{-1} CLP treatment displayed, on average, the best overall larvicidal activity, it was used to determine LT_{50}, LT_{90} and LT_{100} intervals as reference points for statistical comparisons. The lethal times were determined by extrapolation from a straight-line projection, $y = 0.41x + 20.24$, ($R^2 = 0.99$) of its larvicidal activity over the period 48 – 216 h. The LT_{50}, LT_{90} and LT_{100} times extrapolated were 72.47 h, 169.86 h and 194.21 h respectively. One-way ANOVA detected significant differences in larvae mortality between the tested CLP concentrations at the LT_{50} ($F_{4, 20} = 57.79$, $P < 0.01$), LT_{90} ($F_{4, 20} = 38.84$, $P < 0.01$) and LT_{100} ($F_{4, 20} = 34.41$, $P < 0.01$). Statistical comparisons were further investigated using the Tukey HSD test (Appendix A: Table A5).

At all LT intervals, the 1000 µg g^{-1} CLP treatment exhibited the most potent ($P < 0.01$) larvicidal response; however, at the LT_{90} and LT_{100} intervals the 100 µg g^{-1} CLP treatment had sufficient variance that no significant difference to the 1000 µg g^{-1} CLP treatment could be detected ($P > 0.01$). This indicated that CLP concentrations higher than 100 µg g^{-1} CLP treatment might not be required for an effective larvicidal response to be achieved. The 10 µg g^{-1} CLP treatment had similar ($P < 0.01$) larvicidal responses to the 5 and 20 µg g^{-1} CLP treatment at all LT intervals tested. This indicates that CLP treatments < 20 µg g^{-1} yield less efficient larvicidal responses, as evident by a plateau in larvicidal activity by the 5 and 10 µg g^{-1} CLP treatments.

The lethal concentration for 50% larvae mortality (LC_{50}) at 120 and 240 h was determined to be 138.26 and 17.29 µg g^{-1} respectively. At these intervals, larvae mortality covered the 50%

mortality range which allowed for a more reliable Probit analysis. Interestingly, doubling the incubation time resulted in an 8-fold reduction in the LC_{50} concentration.

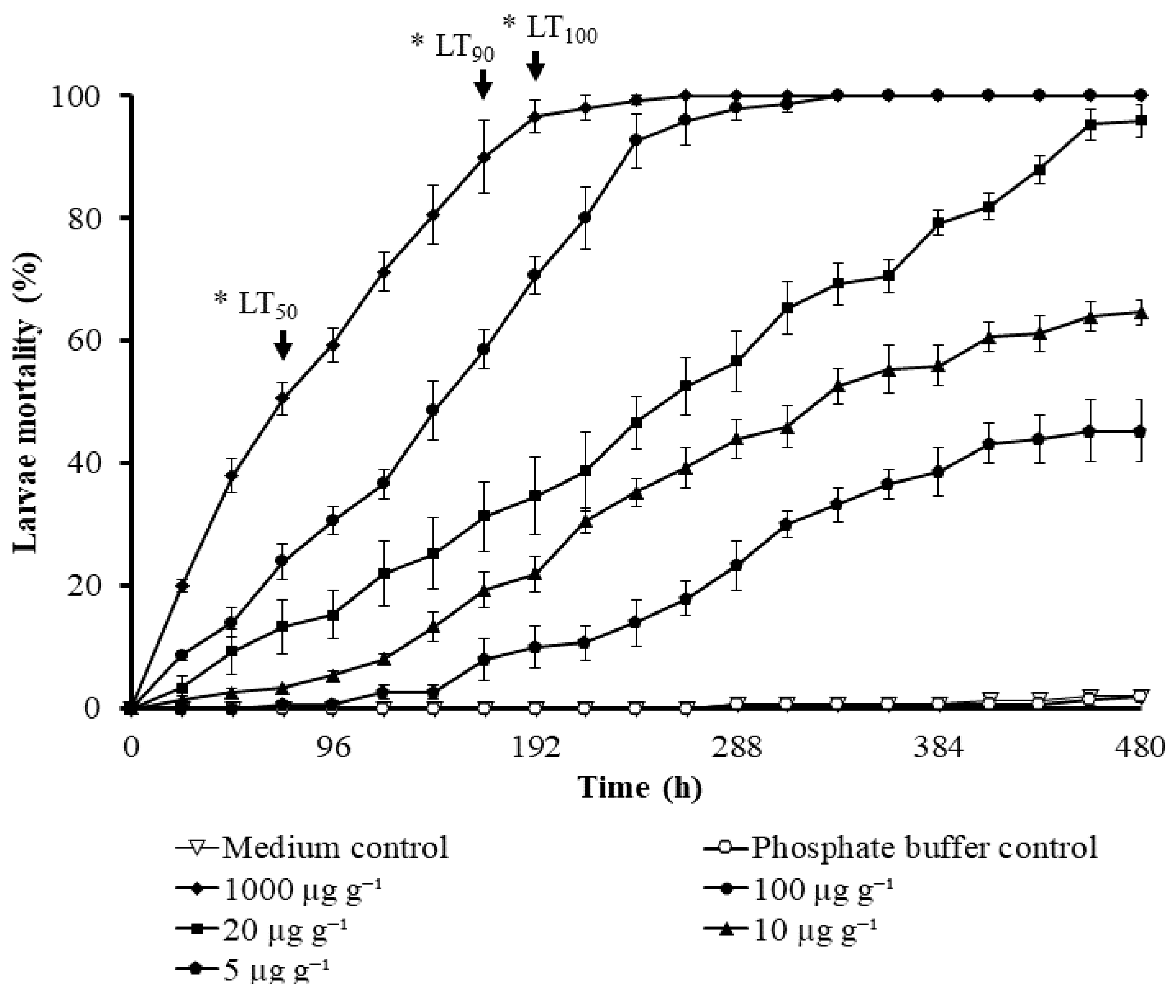

Fig. 2.4 Larvicidal effect of crude lipopeptide extracts from PHP1601 on *L. cuprina* larvae over a 480-h assay. CLP extracts were resuspended in phosphate buffer (pH 7.00) prior to being added to the MG agar. An asterisk (*) indicates significant differences ($P < 0.01$) detected by one-way ANOVA between the CLP treatments, using intervals that corresponded to the LT_{50}, LT_{90} and LT_{100} of the 1000 µg g^{-1} CLP treatment. Error bars represent the SE of the mean.

2.4.6 Behaviour and physiological effect of PHP1601 treatments on *L. cuprina* larvae

Larvae exposed to each of the different treatments displayed a concentration-dependent

behavioural change whereby they progressively avoided the bioassay medium and occupied positions on the walls of the bioassay containers. This occurred at concentrations ≥ the best performing concentration for each treatment. Even though these larvae were no longer in contact with, nor feeding on, the bioassay medium they remained susceptible to their respective treatments. Furthermore, all larvae died after extended periods after the bioassays were completed, and no incidence of pupation was observed. In contrast, larvae in the controls showed no unusual feeding behaviour and were randomly distributed throughout the bioassay medium. These larvae typically entered pupation after approximately 9 days and fly emergence usually occurred 6 days later.

Larvae cadavers recovered from each of the PHP1601 treatments lacked structural integrity, exhibited stunting and displayed dark red-orange to dark brown discolouration. The most severe examples of effected larvae were obtained from the CLP treatments. In contrast, the control larvae were pale cream and firm. The physiological distortions associated with effected larvae was interpreted as a toxic response that could be attributed to bioactive biosurfactant compounds produced by PHP1601.

2.5 Discussion

Bacillus velezensis is a species whose application, or candidacy, as an insect antagonist has not been reported in the literature previously. As such, its role as a potential biological control agent against flies was evaluated. *Lucilia cuprina,* a pest of agricultural and medical significance was selected as an appropriate test organism to assess the fly biocontrol potential of *Bacillus* sp. PHP1601. Larvicidal bioassays were conducted using a milk-based MG medium suitable for PHP1601 growth and the fly larvae development cycle. The influence of the medium on the growth of PHP1601 was assessed to determine if lethal concentrations of cells or endospores could be ascribed. Larvicidal bioassays tested cells, endospores, CFS and CLP extracts to determine the mechanism of larvae antagonism of PHP1601.

PHP1601 was identified as a member of the *B. velezensis* species based on phylogenetic relationships assessed via MLSA of the housekeeping genes tested. MLSA has been used as reliable means for identifying and differentiating closely related *Bacillus* spp. (Glaeser and Kämpfer, 2015; Shrivastava et al. 2018). Generally, a minimum of 4 – 5 genes have been used to

reliably identify members of the *B. subtilis* species complex (Glaeser and Kämpfer, 2015). Tredgold (2021) demonstrated that MLSA targeting the 16S rRNA, *rpo*B, *dna*J and *gyr*A genes were sufficient for reliable identification and species delineation of representatives of the *B. subtilis* species complex. Using the same approach, the taxonomic classification of PHP1601 was confirmed. Additionally, the taxonomic affiliation of PHP1601 was verified using a species-specific endpoint PCR that targets a conserved region of 2-keto-3-deoxygluconate kinase gene, which is unique to the core genome of *B. velezensis* species and absent among other members of the *Bacillus subtilis sensu lato* group (Dunlap 2019).

PHP1601 exhibited a significant larvicidal effect on *L. cuprina* larvae when cell or endospore treatments were evaluated. This was considered as the first demonstration of antagonism of a member of the *B. subtilis* species complex on larvae of Brachycera flies. No significant differences between equivalent vegetative cell and endospore treatments were noted. Since PHP1601 was capable of growing in the bioassay medium, it is likely that the endospores would have germinated and performed similarly to vegetative cell treatments of similar concentrations. The significance of this is that *B. velezensis* can potentially be applied as an endospore formulation, which is advantageous for developing commercial biocontrol products in terms of its shelf-life, ease of application and formulation processing (Zeriouh et al. 2011; Vimala et al. 2021).

Bioassays assessing the effect of CFS treatments indicated that the strain produced larvicidal bioactive metabolites. Strains of *B. velezensis* are commonly associated with the production of a range of lipopeptide compounds, including surfactin, iturin and fengycin (Liu et al. 2020; Chen et al. 2020). Considering that CFS from PHP1601 elicited a significant larvicidal effect on *L. cuprina*, it is likely that bioactive lipopeptide components of the CFS were responsible for, or contributed to this effect. This notion was further supported by evidence that CLP extracts of PHP1601 reduced the surface tension of vegetable oil, which is consistent with the properties of lipopeptide biosurfactant compounds associated with *B. velezensis* (Adu and Hunter 2021). Overall, the 1000 $\mu g\ g^{-1}$ CLP extract demonstrated the most potent larvicidal response of all the treatments tested.

In the current study, LC_{50} values of 138.26 $\mu g\ g^{-1}$ at 120 h and 17.29 $\mu g\ g^{-1}$ at 240 h were calculated for the PHP1601 CLP extract. The impact of bioassay duration on lethal concentration

values is clearly illustrated and indicates the chronic nature of the active compounds involved. Concentrations of CLP as low as 5 µg g^{-1} were sufficient to inhibit pupation and break the fly life cycle even though lower levels of larvae mortality were observed. This finding suggests that larvae mortality measurements probably underestimate the effectiveness of CLP active compounds in controlling fly populations at concentrations below 5 µg g^{-1}. Further studies are required to evaluate the efficacy of using CLP extracts at sub-lethal concentrations to determine its ability to interrupt the fly reproductive cycle.

Lipopeptide biosurfactants produced by members of the *B. subtilis* species complex have been linked previously to biocontrol applications against mosquito larvae (Manonmani et al. 2011; Mnif et al. 2013; Ghribi et al. 2012; Kahia et al. 2021). Reports of this group of compounds showing activity against other insect groups appear to be relatively scarce in the literature. The findings of this study, therefore, contributes to a growing body of work that connects lipopeptide biosurfactant compounds to insect biocontrol applications.

Lipopeptide families such as surfactin, iturin and fengycin, are known to disrupt biological membranes through lysis and/or pore formation (Labiadh et al. 2021). The different lipopeptide families are structurally different and, therefore, have different affinities for the organism or cell lines that they interact with (Mnif and Ghribi 2015). Characterisation of the lipopeptide composition of the CLP extracts of PHP1601 is, therefore, required in order to determine which compound(s) contribute towards the larvicidal response observed. To confirm the central role that lipopeptide compounds play in fly larvae antagonism, mutant strains of PHP1601 deficient in lipopeptide production capabilities could be evaluated for other larvicidal traits.

All larvicidal trials gave rise to larval populations that displayed prominent signs of infection, including structural distortion and discolouration. These morphological distortions were attributed to the bioactive compounds produced by PHP1601. This assumption was substantiated by the CFS and CLP bioassays, which produced similar levels of stunting and discolouration as the vegetative cell and endospore treatments. These observations further supported the hypothesis that the larvicidal activity of PHP1601 could be attributed to the production of extracellular bioactive compounds. Lipopeptide biosurfactants are also known to disrupt cell membranes in a concentration-dependent manner (Dimkic et al. 2017; Rofeal and El-Malek, 2020). Surfactin is regarded to be one of the most potent biosurfactant classes known and is

recognised for its ability to haemolyse red blood cells (Yuan et al. 2018; Wan et al. 2021). If ingested in sufficient quantities, it is possible that such a compound could cause internal damage to the digestive tract of fly larvae.

A significant observation in all biocontrol assays was that the treatments influenced a concentration, or dose-related, change in larvae feeding behaviour over time. Initially, it was proposed that elevated titers of the PHP1601 could have made the medium unpalatable for the larvae; however, subsequent bioassays indicated that the release of extracellular metabolites could have contributed to this effect. This phenomenon has not been reported in the literature previously and could be attributed to an accumulative chronic response. Generally, there is a direct relationship between the concentration of an active biocontrol agent and the level of pest antagonism experienced (Pessanha et al. 2015). This toxic response is an important determinant of the efficacy of PHP1601, which should be considered when developing biological control applications. The toxicological implications of exposing non-target organisms such as livestock animals to lipopeptide extracts also needs to be considered. Toxicity screening using animal model systems such as zebrafish (*Danio rerio*) embryotoxicity studies can be used as a starting point from which to evaluate the toxicity of bioactive compounds produced by PHP1601 (Chahardehi et al. 2020).

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CHAPTER THREE: ELUCIDATION OF LIPOPEPTIDE BIOSURFACTANTS RESPONSIBLE FOR THE LARVICIDAL ACTIVITY OF *BACILLUS VELEZENSIS* PHP1601 TOWARDS *LUCILIA CUPRINA* LARVAE

3.1 Abstract

Lipopeptide biosurfactant compounds derived from cultures of *Bacillus velezensis* PHP1601 (Bacillales: Bacillaceae) show antagonism towards the larval stage of *Lucilia cuprina* (Diptera: Calliphoridae), a blowfly pest of agricultural significance. A study was undertaken to characterise and elucidate the lipopeptide biosurfactant compounds contributing to this effect. Lipopeptide extracts were obtained from cultures grown in Landy medium by acid precipitation and methanol extraction. Thin layer chromatography and UPLC ESI-TOF MS were used to partially purify and characterise the lipopeptides present in the extract. Lipopeptide fractions contained homologues of surfactin (C13 – C17), fengycin (C14 – C17) and iturin (C14 – C17). Each lipopeptide fraction (20 µg g^{-1}) displayed larvicidal activity against second-instar *L. cuprina* larvae, with a highly polar surfactin fraction (Rf: 0.90) being the most effective. The potency of surfactin was confirmed with bioassays incorporating a surfactin standard whereby a LT_{50} of 179.97 h and LC_{50} of 9.87 µg g^{-1} was determined. Interestingly, larvae cadavers recovered from the bioassays displayed significant physiological discolouration and stunting; this was attributed to the biosurfactant nature of the lipopeptide compounds. These findings corroborate the role of lipopeptide compounds, specifically surfactin, in the fly biocontrol mechanism of PHP1601 and constitute the first report of these compounds being insecticidal towards blowfly larvae.

Keywords *Bacillus velezensis*, lipopeptide biosurfactants, microbial pesticides, surfactin, biological control, *Lucilia cuprina*

3.2 Introduction

Members of the *Bacillus sensu lato* group are recognised for their biological control potential due to their ability to produce an array of bioactive lipopeptide biosurfactants (Liu et al. 2020; Cossus et al. 2021; Saggese et al. 2022). Many of these compounds are antagonistic toward a range of organisms including enveloped viruses, bacterial pathogens, fungi and, to a lesser extent, insects (Ghribi et al. 2012; Johnson et al. 2019; Liu et al. 2020; Saggese et al. 2022). Hence, these compounds are promising from a biocontrol perspective.

Reports in the literature detailing the application of lipopeptide biosurfactants as biopesticides to control insect pests are relatively scarce and have mainly been tested as crude extracts. Examples include the biocontrol of mosquito larvae (*Anopheles* spp., *Aedes* spp.; Diptera, Nematocera, Culicidae), African cotton leafworm (*Spodoptera littoralis*; Lepidoptera: Noctuidae) and Mediterranean flour moth larvae (*Ephestia kuehniella*; Lepidoptera: Pyralidae) larvae (Ghribi et al. 2012; Revathi et al. 2013; Parthipan et al. 2018). The paucity of research exploring biocontrol applications of lipopeptides produced by *Bacillus* spp. against members of other insect groups presents an alluring opportunity for further investigation in this field. In a recent study, biosurfactant compounds produced by *B. velezensis* PHP1601 were shown to be larvicidal against blowfly larvae (*Lucilia cuprina*), indicating the potential of these compounds as bio-insecticidal agents (Ramesar and Hunter 2023).

Bacillus-associated lipopeptides are bioactive compounds that exhibit surface-active and amphiphilic properties (Kim et al. 2004; Cossus et al. 2021). Three main lipopeptide families associated with *B. subtilis* sensu lato group are surfactin, fengycin and iturin (Zhao et al. 2017; Kalamara et al. 2018). These compounds are composed of a hydrophobic tail region comprised of fatty acids or sterol rings and a hydrophilic head region made up of covalently cyclised amino acids (Kim et al. 2004; Cossus et al. 2021). This molecular arrangement allows them to interact with, and destabilise, certain membranes by pore formation, which can lead to cellular leakage, disruption of cellular signalling or a cytotoxic response (Cossus et al. 2021).

Bacillus velezensis PHP1601 (Bacillales: Bacillaceae) is a Gram-positive, aerobic endospore-forming bacterium that shows potential as a biocontrol agent against *Lucilia cuprina* fly larvae when cell, endospore, cell-free supernatant and biosurfactant extract treatments were evaluated

(Ramesar and Hunter 2023). Its mechanism of larvae antagonism was putatively attributed to the production of lipopeptide biosurfactants. However, the composition and contribution of biosurfactants to this effect needs to be elucidated further. Accordingly, a study was undertaken to extract, purify and characterise the lipopeptide biosurfactants produced by PHP1601 and evaluate their contribution to its larvicidal effect.

3.3 Methods and materials

3.3.1 Bacterial culture

Bacillus velezensis PHP1601, hereafter referred to as PHP1601, was obtained from Andermatt Plant Health Products (Strathdean Farm, Gowrie Avenue, Nottingham Road, KwaZulu-Natal, 3280, South Africa). PHP1601 was routinely cultured on tryptone soy agar (TSA) (Biolab, Merck, Germany) for 24 h at 30°C or tryptone soy broth (TSB) at 28°C for 18 h at 200 rpm. The strain was cryopreserved in 20% (w v^{-1}) glycerol and stored at -80°C.

3.3.2 Production and extraction of lipopeptide biosurfactants

Lipopeptide biosurfactants were produced by culturing PHP1601 in 10 × 100 ml of defined Landy medium (20 g l^{-1} glucose, 5 g l^{-1} L-glutamic acid, 0.5 g l^{-1} $MgSO_4$, 0.5 g l^{-1} KCl, 1 g l^{-1} KH_2PO_4, 0.15 mg l^{-1} $Fe_2(SO_4)_3$ $6H_2O$, 5.0 mg l^{-1} $MnSO_4$ H_2O, 0.16 mg l^{-1} $CuSO_4$ $5H_2O$, pH 7.00) for 24 h at 30°C (Landy et al. 1948; Yang et al. 2015). Sterile cell-free supernatant was obtained using centrifugation (15000 × g for 10 min) to remove biomass, followed by filter sterilisation (GxF 0.2 µm GHP Acrodisc, USA). The lipopeptide biosurfactants were extracted by acid precipitation and methanol extraction, as described by Yang et al. (2015), to yield a crude lipopeptide (CLP) extract. As a confirmatory step, the CLP extract was assessed for biosurfactant activity using the oil displacement assay described by Khondee et al. (2015).

3.3.3 Detection and characterisation of lipopeptide biosurfactants

Lipopeptide characterisation was performed using ultra-performance liquid chromatography (UPLC) electron spray ionisation time of flight (ESI-TOF) mass spectroscopy (MS) (Waters

Acquity, Milford, USA) (Hunter 2016; Adu and Hunter 2021). Methanol solubilised lipopeptide extracts (0.1 µg μl^{-1}) were loaded onto a Waters Acquity BEH C18 column (2.1 × 100 mm, particle size of 1.7 µm, 35ºC) and fractionated under UPLC conditions. Additionally, surfactin and iturin lipopeptide standards (Sigma-Aldrich, Germany) were processed for identification purposes. The solvent system was comprised of a 0.2 % (v v^{-1}) acetic acid and methanol mobile phase maintained at a ratio of 9:1 and run isocratically at a flow rate of 350 µl min^{-1} for 30 min after sample injection. Thereafter, the methanol concentration in the mobile phase was increased from 10% to 100% (v v^{-1}) from 30 - 38 min before returning to the previous isocratic conditions at 40 min. Compounds in the samples were ionised (positive mode, ESI+) at a constant capillary voltage of 5 MV and a cone voltage of 35 V. A desolvation temperature of 350ºC was maintained with a constant desolvation gas injection rate of 400 l h^{-1} was used. Eluted compounds were detected, and their molecular weights were determined by ESI-TOF MS. The mass-to-charge ratio (m/z) of compounds detected in the 900 – 2000 m/z range were identified based on the corresponding mass data from the lipopeptide standards and those reported in the literature (Koumoutsi et al. 2004; Hunter 2016). Compounds exhibiting a mass spectra relative absorbance of ≥ 50% were considered a major portion of the eluted peak.

3.3.4 Purification of lipopeptide biosurfactants

The composition of CLP extract of PHP1601 was assessed by thin layer chromatography (TLC) according to a method adapted from Hunter (2016). Briefly, 150 µg of CLP extract were spotted on a 25 × 25 cm silica plate (HPTLC 60-F254, Merck, Germany) by adding successive 0.5 µl additions from a 10 mg ml^{-1} methanol solubilised stock solution. For improved resolution, the TLC plate was baked at 100ºC for 30 min and cooled to room temperature before spotting. The plate was placed into a glass TLC tank pre-saturated with a 70:30 propan-1-ol: deionised water solvent system and run for approximately 8 h to allow for band separation. UV active bands were visualised under UV light illumination (252 nm), whilst hydrophobic regions were detected by spraying the plate with atomised deionised water.

To prepare partially purified fractions for further characterisation, 150 mg of CLP extract was spotted along the entire width of the TLC plate in 1 µl aliquots before being resolved using TLC method described previously. Thereafter, the silica region corresponding to each UV active band

was scraped off into 10 ml glass McCartney bottles using a glass microscope slide. The harvested silica regions were mixed with an equal volume of methanol to solubilise the silica-bound lipopeptides. The suspensions were vortexed for 1 min and then centrifuged (10000 × g for 5 min) to pellet out the silica particles. The clear supernatant was transferred into a clean glass ampule and dried to a stable mass at 45ºC. TLC analysis of the semi-purified lipopeptide fractions was performed to confirm lipopeptide extraction; thereafter, UPLC ESI-TOF MS was performed to determine the lipopeptide composition of the TLC fractions.

3.3.5 Fly population

A population of *L. cuprina* flies were reared and sustained at the University of KwaZulu-Natal Controlled Environment Research Unit (UKZN-CERU). The insectaries were housed in a temperature-controlled room set to 28ºC with a 12 h light-dark cycle and relative humidity of 66 ± 5%. The population of flies were fed every 4 days with provisions (~ 200 g) of maggot growth (MG) agar (500 ml l^{-1} UHT milk, 2 g l^{-1} bacteriological agar, pH 6.50) supplemented with 10 g of minced chicken livers to promote oviposition and larvae development (Johnson et al.1998; Ramesar and Hunter 2023).

3.3.6 The larvicidal effect of lipopeptide fractions

Larvicide bioassays were performed in plastic containers (300 ml) containing 50 g of MG agar containing 20 µg g^{-1} of each TLC lipopeptide fraction. Lipopeptide extracts were prepared as phosphate-buffered (5.8 mM Na_2HPO_4, 4.2 mM Na_2HPO4, pH 7.00) lipopeptide stock solutions that were added to molten MG agar medium in 1 ml aliquots. Once the agar solidified, 30 second instar larvae were introduced into each container to assess their susceptibility to the biosurfactants over a 480 h study period under the same conditions the fly population was reared. Observations for larval mortality were taken at 0, 120, 240 and 480 h.

3.3.7 Larvicidal effect of surfactin standard

Larvicidal bioassays were conducted using a surfactin standard (purity > 99%) to confirm the role of surfactin as a larvicidal biosurfactant. Bioassays were prepared as described previously,

and the larvicidal effect of surfactin at concentrations of 0, 5, 10 and 20 µg g^{-1} were evaluated. Trials ran for 480 h with observations of larval mortality being made at 24 h intervals.

3.3.8 Statistical analysis

All larvicidal trials were repeated for 5 independent replicates. Data analysis was performed using IBM SPSS (v. 27.0.0.0), whereby statistical analysis was performed using a 99% confidence level to determine significant differences ($P < 0.01$) in larvicidal responses. Data were assessed for normality using the Shapiro-Wilks test. One-way analysis of variance was performed to detect significant differences in larvae mortality between the different lipopeptide treatments, after which, the Tukey HSD test was performed to ascribe statistical comparisons between the different treatments. Lethal concentrations for 50% larvae mortality (LC_{50}) of the surfactin standard were determined using Probit analysis. All data, including those in figures, were represented as the average ± standard error (SE).

3.4 Results

3.4.1 Characterisation of lipopeptide biosurfactants

PHP1601 produced 0.635 ± 0.016 g l^{-1} of CLP after a 24 h culture period in Landy medium. The CLP had a yellow colouration and dissolved readily in methanol or phosphate buffer. Aliquots (5 µl) of phosphate-buffered CLP stock (100 µg ml^{-1}) solution produced a zone of oil displacement that covered the entire area of the petri dish (≥ 56.48 cm^2). This confirmed that biosurfactant compound(s) were extracted and that the CLP extract could be used for further analysis.

UPLC of the CLP extract of PHP1601 produced several elution peaks (Fig. 3.1) that were identified to contain homologues iturin, fengycin (Appendix B: Fig. B1) and surfactins (Appendix B: Figure B2) when analysed by ESI-TOF MS. The CLP was considered to be predominantly composed of lipopeptide biosurfactants as they accounted for 97.36% of the total peak area, whereas, 2.64% was from an unidentified compound (m/z: 1326.0) that eluted at 31.34 min. Lipopeptides were eluted from 19.50 – 30.00 min whereby, iturins (19.50 – 22.50 min), fengycins (24.50 – 27.00 min) and surfactins (27.50 – 30.00 min) were sequentially eluted.

Surfactins were judged to be the dominant lipopeptide family based on their peak area comprising 90.06% of the total. However, the UV response of lipopeptides differs between families; consequently, absolute comparisons of the proportions of the lipopeptides were not possible and would require further analysis (Geissler et al. 2017).

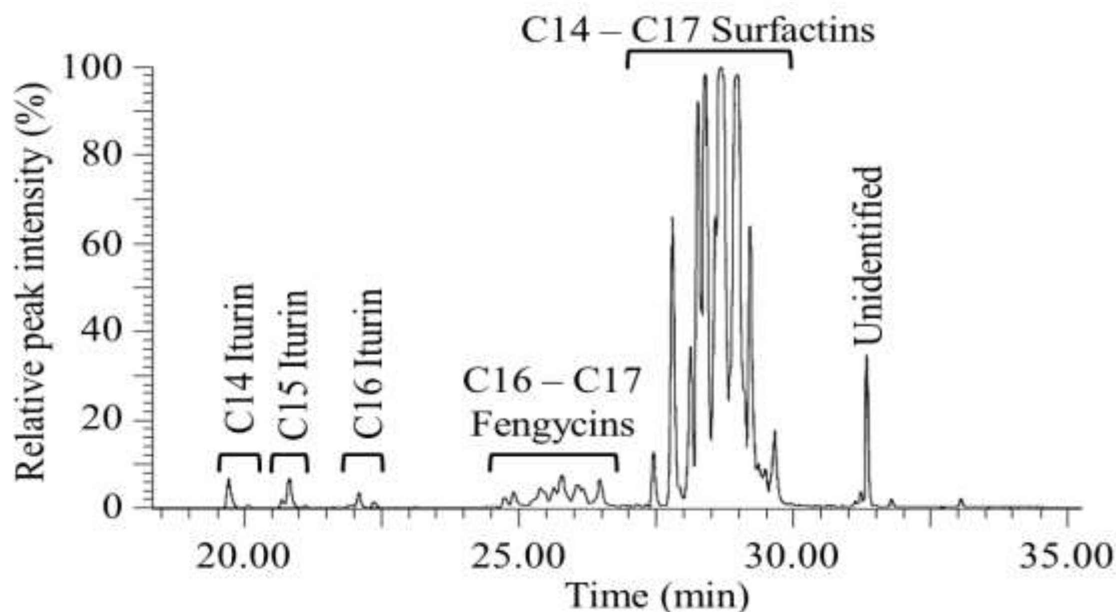

Fig. 3.1 UPLC chromatogram of eluted compounds in the CLP of PHP1601. The compounds present in the elution peaks were characterised by ESI-TOF MS. The detection range was 900-2000 m/z, which caters for lipopeptide biosurfactant compounds.

3.4.2 TLC fractionation and purification of lipopeptide biosurfactants from CLP extracts

The TLC of the CLP extract produced 7 distinct UV active bands (Fig. 3.2), which also tested positive for hydrophobic interactions when sprayed with deionised water. This indicated that the eluted bands possessed similar characteristics to lipopeptide biosurfactants. Bands with retention factors (Rf) of 0.71, 0.81 and 0.90 displayed a more intense UV active colouration, and were presumed to contain higher relative amounts of compound than those with less intense UV responses. No bands were detected in the methanol control, indicating that all UV active and hydrophobic bands could be attributed to lipopeptide compounds in the CLP extract of PHP1601.

TLC purification of the amphiphilic bands recovered 71.26% (106.9 mg) of the compounds present in the original CLP extract. Purification of the TLC fraction demonstrated that compounds from Rf of 0.90, 0.81 and 0.71 existed as the major portions of the extract based on their relative abundance (Table 3.1). Approximately 28.74% (w w^{-1}) was not recovered, which was presumed to be attributed to the removal of unwanted compounds and/or due to the limitation of extracting the fractions from the silica of the TLC plate.

UPLC ESI-TOF MS analysis of TLC purified fractions of PHP1601 indicated that most fractionated bands were composed of a single lipopeptide family (Table 3.1). In all TLC fractions, most of the lipopeptides were detected in a protonated state [M+H]+ and, to a lesser extent, some were detected as sodium adducts [M+Na]+. Interestingly, several lipopeptide homologues were detected (e.g. m/z 1036.7 C15 surfactin [M+H]+, m/z 1464.7 Ala-6-C16 fengycin [M+H]+) where found at different retention factors.

Surfactins were detected as the predominant lipopeptide family in TLC fraction from Rf of 0.37 and 0.90 and were ascribed as S-R0.37 and S-R0.90 respectively (Table 3.1). These fractions mostly contained similar surfactin isoforms (m/z: 1008.7,1022.8, 1036.8, 1050.8, 1061.9, 1064.8). Fengycin isoforms were the predominant constituents associated with TLC fractions extracted from Rf 0.50, 0.60 and 0.65, which were correspondingly ascribed as F-R0.50, F-R0.60 and F-R0.65 (Table 1). TLC fractions from Rf 0.71 and 0.81 contained iturin isoforms and a fengycin Val-6-C17 isoform; accordingly, these fractions were designated IF-R0.71 and IF-R0.80 respectively. ESI-TOF MS characterisation, in conjunction with the relative abundance of the TLC fraction extracts, indicated that the dominant lipopeptide family in the CLP extract was surfactin (45.55%) followed by the iturin-fengcyin mixtures (34.30%) and fengycin fractions (19.36%).

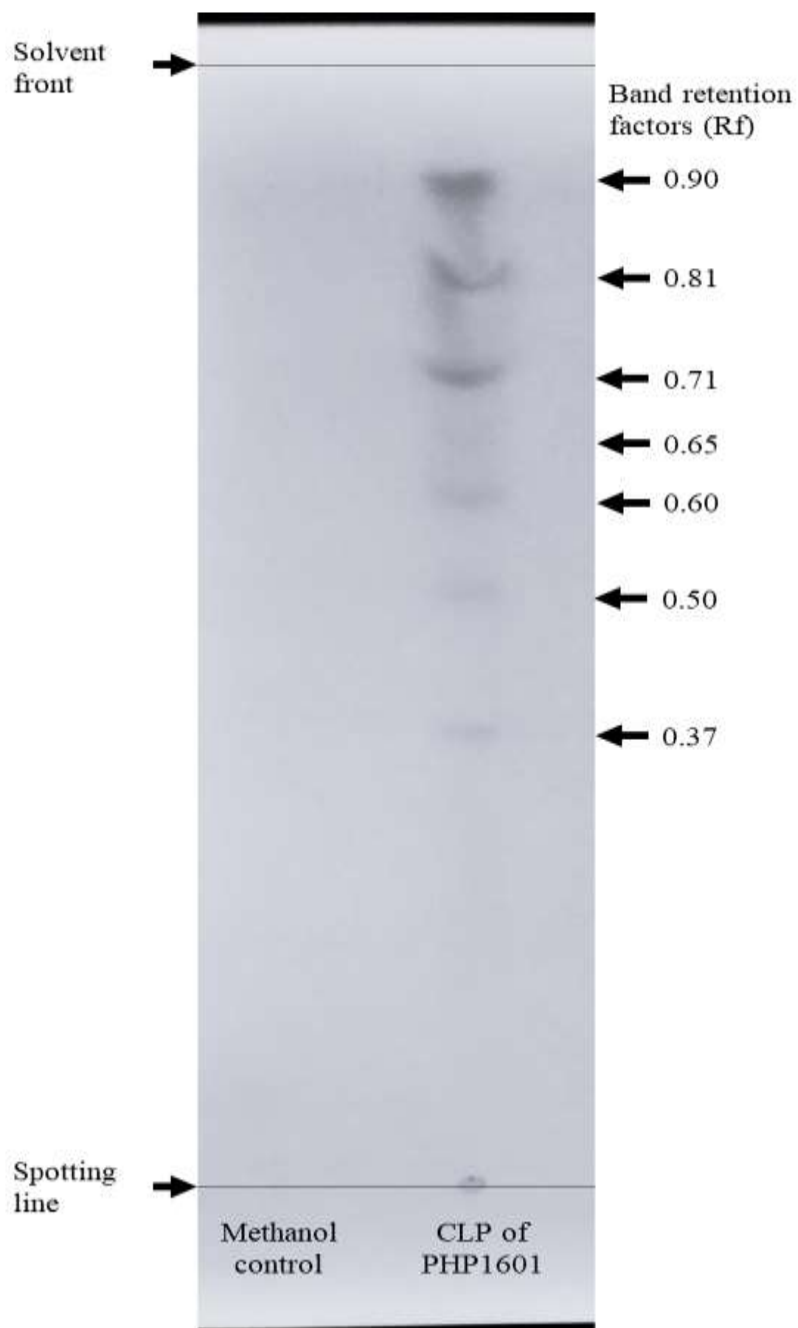

Fig. 3.2 Thin layer chromatography of CLP extract of *Bacillus velezensis* PHP1601. Methanol was used as the solvent to dissolve the CLP extract and subsequently included as a purity control.

Table 3.1 Lipopeptide biosurfactant species detected in the TLC-purified lipopeptide fractions of PHP1601.

TLC retention factor (Rf)	Relative abundance (%)	Band designation	Mass detected (m/z)	Mass assignment
0.37	7.76	S-R0.37	1022.7, 1044.7	C14 surfactin $[M+H, Na]^+$
			1036.7, 1058.7	C15 surfactin $[M+H, Na]^+$
			1050.7	C16 surfactin $[M+H]^+$
			1064.8, 1086.8	C17 surfactin $[M+H, Na]^+$
0.50	7.2	F-R0.50	1436.7	Ala-6-C14 fengycin $[M+H]^+$
			1450.8	Ala-6-C15 fengycin $[M+H]^+$
			1464.7	Ala-6-C16 fengycin $[M+H]^+$
			1478.8	Ala-6-C17 fengycin $[M+H]^+$
0.60	6.64	F-R0.60	1464.7	Ala-6-C16 fengycin $[M+H]^+$
			1478.8	Ala-6-C17 fengycin $[M+H]^+$
			1492.7	Val-6-C16 fengycin $[M+H]^+$
			1506.0	Val-6-C17 fengycin $[M+H]^+$
			1520.9	Val-6-C18 fengycin $[M+H]^+$
0.65	5.52	F-R0.65	1463.9	Ala-6-C16 fengycin $[M+H]^+$
			1493.9	Val-6-C16 fengycin $[M+H]^+$
			1506.0	Val-6-C17 fengycin $[M+H]^+$
0.71	16.28	IF-R0.71	1043.6	C14-iturin $[M+H]^+$
			1085.8	C17-iturin $[M+H]^+$
			1506.0	Val-6-C17 fengycin $[M+H]^+$
0.81	18.02	IF-R0.81	1043.6	C14 iturin $[M+H]^+$
			1071.6	C16 iturin $[M+H]^+$
			1085.8	C17 iturin $[M+H]^+$
			1506.0	Val-6-C17 fengycin $[M+H]^+$
0.90	37.79	S-R0.90	1008.7	C13 surfactin $[M+H]^+$
			1022.7	C14 surfactin $[M+H]^+$
			1036.7	C15 surfactin $[M+H]^+$
			1050.7	C16 surfactin $[M+H,]^+$

Relative abundance values (%) were determined based on the dry weight (mg) of the bands extracted in relation to the total mass of purified fractions (106.9 mg). Mass data of detected lipopeptide biosurfactants were assigned to lipopeptide species based on matching data in the literature (Koumoutsi et al. 2004; Hunter 2016; Adu and Hunter 2021). Fengycin species were detected that differed in their amino acid composition by either the incorporation of alanine (Ala) or valine (Val) moieties.

3.4.3 Larvicidal assays with TLC purified lipopeptide biosurfactants

Larvicidal bioassays indicated that extracts from each TLC fraction induced a significant larvicidal effect at 120 h ($F_{6, 28} = 5.24$, $P = 0.001$), 240 h ($F_{6, 28} = 15.98$, $P < 0.001$) and 480 h ($F_{6, 28} = 20.54$, $P < 0.001$) when compared to the phosphate buffer control (PBC) (Fig. 3.3). All fractions showed larvicidal activity and their levels of larvae mortality increased over time. The IF-R0.81 and S-R0.90 fraction performed the best, albeit with varying levels of significance that improved over time. Differences in larvae mortality were most prominent at last time interval and indicated that surfactin fractions and S-R0.90 performed the best. The IF-R0.81 and S-R0.37 fractions also showed elevated levels of larvae mortality as compared to the remaining fractions; however, their larvicidal performance was statistically similar (all $P > 0.01$). Fengycin fractions showed the lowest levels of larvae mortality and were the least effective treatment when assessed at the 480 h interval. Since no incidence of larvae mortality was observed in the control group, all incidence of larvae mortality was attributed to the constituents of the TLC purified fractions and indicated that surfactins warranted further larvicidal investigation.

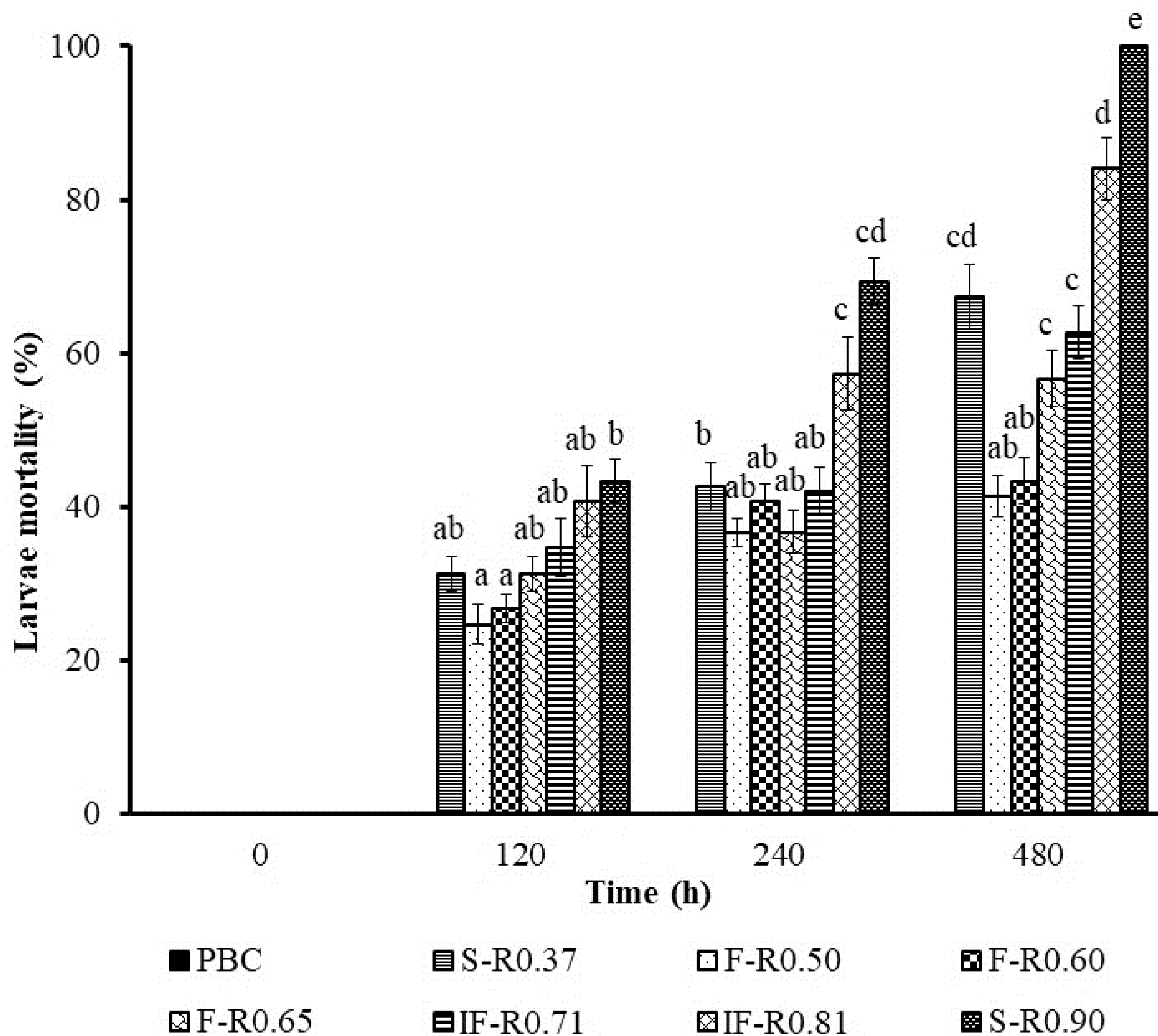

Fig. 3.3 Larvicidal effect of TLC purified lipopeptide fractions from PHP1601 on larvae of *L. cuprina*. All lipopeptide fractions were evaluated at a standardised concentration of 20 µg g^{-1}. A phosphate buffer control (PBC) was included to account for the potential impact the buffer could induce in the bioassay. Significant differences ($P < 0.01$) in larvae mortality were indicated using different letters. Error bars represent the SE of the mean.

3.4.4 Larvicidal effect of surfactin standard

Larvicidal bioassays using a surfactin standard demonstrated that it was able to exhibit a larvicidal effect on *L. cuprina* larvae at all concentrations (5, 10, 20 µg g^{-1}) tested (Fig. 3.4). No incidences of larvae mortality were observed in the medium or phosphate buffer control. This

substantiates that the larvae mortality observed is attributable to the antagonistic effect of surfactin.

The first instance of larvae mortality was observed at 24 h in the 20 µg g^{-1} surfactin treatment (8 ± 1.70%) only. This indicated that a minimum concentration of 20 µg g^{-1} surfactin was required for larvae mortality to be observed in 24 h. Larvae mortalities in the 5 and 10 µg g^{-1} surfactin treatments were only observed from 48 h onwards. Over the ensuing intervals, the 20 µg g^{-1} surfactin treatment exhibited higher average larvae mortalities than the 5 and 10 g^{-1} surfactin treatment. At 120 h significant differences in the larvae mortality between the treatments were detected ($F_{2, 12} = 8.39$, $P = 0.005$), whereby 20 µg g^{-1} surfactin treatment was significantly more effective than the 5 µg g^{-1} surfactin treatment ($P = 0.004$) but not significantly different to the 10 µg g^{-1} surfactin treatment ($P = 0.074$). At 240 h, the 20 µg g^{-1} surfactin treatment was the only treatment that exhibited larvae mortalities > 50%. At this interval, the treatments had significantly different larvicidal responses ($F_{2, 12} = 10.92$, $P = 0.002$), in which the 20 µg g^{-1} surfactin treatment was more effective than the 5 µg g^{-1} surfactin treatment ($P = 0.002$) however, performed similarly to the 10 µg g^{-1} surfactin treatment ($P = 0.032$). By the 456 h interval, the 20 µg g^{-1} surfactin treatment was the only treatment to achieve 100% larvae mortality, whereas the 5 and 10 µg g^{-1} surfactin treatment achieved larvae mortalities of 71.33 ± 4.67% and 90 ± 1.82% respectively. At the final observation interval (480 h) the 10 µg g^{-1} surfactin treatment had larvae mortalities greater than the 5 µg g^{-1} surfactin treatment ($t_8 = 3.60$, 2-tailed $P = 0.007$). Therefore, the order of larvicidal effectiveness at 480 h was 20 > 10 > 5 µg g^{-1} surfactin treatment.

Due to the 20 µg g^{-1} surfactin treatment achieving 100% larvae mortality during the bioassay period, it was considered a suitable concentration to determine lethal time (LT) intervals for 50, 90 and 100% larvae mortality. A predictive line of best fit of its larvicidal activity from 24 – 456 h resembled a second-order polynomial, $y = -2.0\times10\text{-}4x^2 + 0.32x - 1.62$, which accounted ($R^2 = 0.99$) for the change in larvicidal rates at each interval. The extrapolated LT_{50}, LT_{90} and LT_{100} intervals were 179.97 h, 367.49 h and 428.62 h respectively. The LC_{50} values of purified surfactin were determined at 240 h according to the Probit method. This interval contained larvae mortalities ≥ 50%, allowing for reliable extrapolation of the LC_{50} value. The LC_{50} value of purified surfactin at 240 h was 9.87 µg g^{-1}.

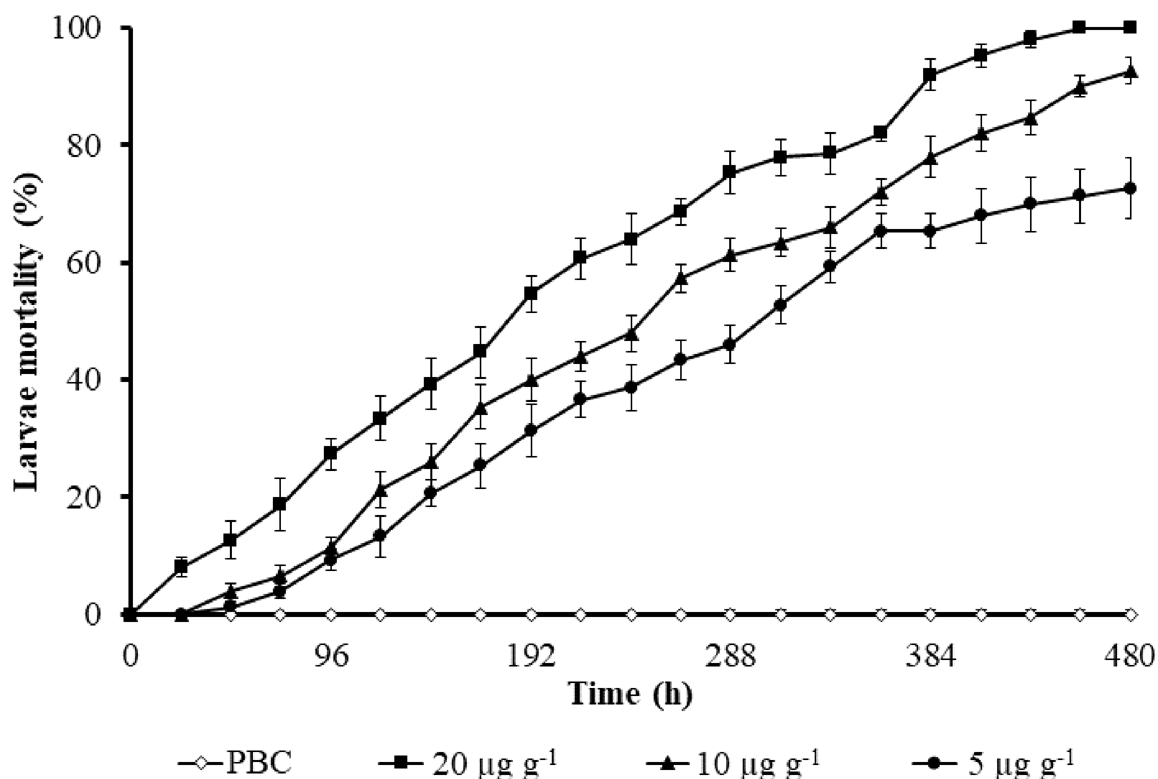

Fig. 3.4 Larvicidal effect of commercial surfactin on larvae of *L. cuprina*. The potential antagonistic effect of the phosphate buffer surfactin was dissolved was accounted for by including a phosphate buffer control (PBC) in the bioassay. Error bars represent the SE of the mean.

3.4.5 Influence of lipopeptide biosurfactants on larvae behaviour and physiology

Larvae exposed to the different TLC purified lipopeptide biosurfactant fractions displayed changes in behaviour and physiology that were not observed in the control group. Larvae in the control groups were pale cream and moved randomly between the bioassay medium and container walls. Larvae were found to have entered pupation after 240 h and by the 480 h interval, flies were present with some even having laid eggs. This was contrasted by larvae in the TLC purified lipopeptide treatments which could not initiate pupation and tended to avoid the bioassay medium from 120 h onwards. Despite the larvae not being in contact with, or consuming the medium as frequently as larvae in the controls, larvae mortality was still

observed. The cadavers of larvae recovered from the treatments were dark red-orange and lacked structural integrity (Appendix B: Fig. B3).

During the course of the surfactin larvicide bioassays, pupation was common in the control groups and usually initiated from 288 h onwards. In contrast, pupation was rarely observed in the surfactin trials, with only ~ 1.33% of larvae being able to do so after 408 h and 504 h for the 5 µg g^{-1} and 10 µg g^{-1} surfactin treatments respectively. All pupa in the control groups emerged into flies from the 600 h interval onwards; however, no fly emergence was observed in any surfactin treatments, even after 110 days of incubation without surfactin. This indicated that concentrations of surfactin as low as 5 µg g^{-1} could inhibit pupation and subsequent fly emergence, thereby breaking the fly life cycle. Cadavers of larvae recovered from the surfactin treatments exhibited similar symptoms of physiological distortions as observed in the TLC purified lipopeptide fractions; these deformities were attributed to the activity of the lipopeptide biosurfactants.

3.5 Discussion

Biosurfactant containing extracts from *B. velezensis* PHP1601 demonstrate a larvicidal effect against *L. cuprina* larvae (Ramesar and Hunter 2023). To better understand the biocontrol mechanism of this larvicidal effect, CLP extract was analysed and partially purified TLC extracts containing discrete lipopeptide families were characterised and assessed for their role in antagonising fly larvae.

UPLC ESI-TOF MS characterisation of the CLP of PHP1601 detected isoforms from all 3 major lipopeptide families associated with *B. velezensis* strains. This alluded to the possibility that the different lipopeptide families or isoforms could work independently but simultaneously to achieve, or facilitate, a larvicidal effect when present in sufficient concentrations. Surfactin was inferred to be the dominant lipopeptide family based on its relative peak area being greater than those corresponding to iturins and fengycins, which are generally more UV active compounds (Geissler et al. 2017).

TLC was used as a simple, cost-effective method for partially purifying lipopeptide compounds in the CLP extract in sufficient quantities to screen for larvicidal activity (Jemil et al. 2017;

Hentati et al. 2019). This method indicated that the strain produced multiple UV active bands that displayed hydrophobic properties and were inferred to be amphiphilic lipopeptide compounds. UPLC ESI-TOF MS analysis of TLC fractions revealed that several isoforms of surfactin, iturin and fengycin could be distinguished. However, similar lipopeptide homologues were detected to have eluted at different retention factors in some instances. This was unexpected but considered to be a consequence of the complexity of biosurfactants in TLC. It was presumed that if the concentration of biosurfactants in the mobile phase was greater than their critical micelle concentration, it is possible that normal and reverse micelles could form between the same or different lipopeptide biosurfactants (Melo et al. 2001; Sumina et al. 2003; Rodrigues 2015). These micelles would have different affinities to the mobile and stationary phases and result in different elution patterns being achieved (Sumina et al. 2003).

Larvicidal bioassays demonstrated that each of the lipopeptide fractions evaluated exhibited a significant larvicidal effect on larvae of *L. cuprina* when tested at a standardised concentration of 20 $\mu g\ g^{-1}$. This outcome was not unexpected as these lipopeptide families are known to disrupt biological membranes through pore formation or exhibit haemolytic activities (Labiadh et al. 2021). The different lipopeptide families differ in affinities for the biological membranes or cell walls they can interact with (Mnif and Ghribi 2015a). Surfactins are effective in disrupting various prokaryotic membranes, while iturin and fengycin are more effective in disrupting the cell wall and membranes of fungi (Ongena and Jacques 2008; Mnif and Ghribi 2015a). Overall, surfactin fractions exhibited higher larvae mortalities than the fengycin fractions; however, the predominantly iturin fractions (IF-R0.71, IF-R0.81) had elevated larvae mortalities that were more effective than the fengycin fractions. The surfactin fraction, S-R0.90, was the most potent lipopeptide fraction and constituted a major part of lipopeptides produced by PHP1601. This indicated that it was most likely a key contributor to the larvicidal effect of PHP1601. Considerations for future investigations would be to assess the levels of impurities in the lipopeptide fractions and account for them larvicide bioassays to provide a more equitable judgement of their larvicidal potential.

Generally, lipopeptide fractions extracted from TLC bands with higher Rf values showed elevated larvicidal activity. Hence, it was inferred that more polar lipopeptides could exhibit a more potent larvicidal effect. A lipopeptide's polarity is determined by the amino acids that

constitute its peptide ring (Seydlová and Svobodová 2008; Mnif and Ghribi 2015a). Lipopeptide biosurfactants with more polar or ionic peptide rings have been associated with increased levels of membrane disruption (Seydlová and Svobodová 2008). Additionally, all lipopeptide fractions had different isoforms present with varied acyl (CH_2) chain lengths. All TLC lipopeptide fractions contained biosurfactants with long 14 – 16 carbons chains lipopeptide isoforms as major components of their lipopeptide fraction. Increases in the length of the lipid tail of lipopeptide biosurfactants have been shown to increase their membrane penetration power and, consequently, exhibits higher levels of membrane disruption by pore formation or emulsification (Seydlová and Svobodová 2008). This is believed to contribute to the insecticidal effect of lipopeptide biosurfactants.

Surfactins are considered as one of the most bioactively potent lipopeptide families due to their membrane disruptive activity and ability to decrease surface tension (Seydlová and Svobodová 2008). This family of compounds is proposed to be responsible for the insecticidal effect of *B. subtilis* VCRC B471 on mosquito larvae and pupa (Geetha and Manonmani 2010). Currently, other lipopeptide families have not been attributed to known insecticidal effects. In this study, the effectiveness of surfactin was confirmed with larvicidal bioassays conducted with purified surfactin and LC_{50} values were determined. These findings, coupled with those of the TLC fraction bioassays, corroborate our hypothesis that lipopeptides are responsible for the larvicidal activity of PHP1601. In addition, surfactin was the major component of the CLP extract, and the results indicate that it is one of the potent lipopeptide families that contributes to the larvicidal effect.

The potency of surfactin as a larvicidal biosurfactant indicates the viability of screening for high surfactin-producing strains of the *B. subtilis* species complex for insect biocontrol options. Lipopeptide production and the production of specific lipopeptides such as surfactin, fengycin or iturin have been used as screening criteria for *Bacillus* spp. being evaluated for biocontrol of phytopathogens or plant growth promotion (Al-Ali et al. 2018; Fan et al. 2018; Trotel-Aziz et al. 2019). Additionally, lipopeptide biosurfactants have a high degree of physicochemical stability and are resistant to abiotic factors such as temperature and UV exposure (Gutiérrez-Chávez et al. 2021). Therefore, evaluating and screening for high surfactin-producing *Bacillus* strains appears

to be a viable avenue for selecting candidate biocontrol agents of current and upcoming insect pests.

Larval cadavers recovered from the treatments of the semi-purified or surfactin bioassays exhibited severe signs of intoxication. Symptoms included severe discolouration, loss of structural integrity, changes in feeding behaviour and termination of successful pupation and fly emergence. Cadavers were extremely fragile and disintegrated upon removal from the bioassay medium, which made dissections and tissue microscopy impossible. It is hypothesised that the larval mortality could be attributed to the consumption of lipopeptide compounds in sufficient quantities that cause damage to the tissue lining of the larvae digestive tract. This is based on the evidence that lipopeptides can disrupt the integrity of cell membranes in both prokaryotic and eukaryotic organisms, leading to cell leakage and haemolysis of blood cells (Seydlová and Svobodová 2008; Mnif and Ghribi 2015b; Chen et al. 2015).

In a biocontrol study targeting carob moth (*E. ceratoniae*) larvae, biosurfactants produced by *B. subtilis* SPB1 were found to target the host's intestinal tract (Mnif et al. 2013). The histopathological effects reported included vacuolisation of the cytoplasm, lysis of columnar cells, destruction of the brush border membrane, increased vesicle formation in the cells located in the midgut lumen region, which was accompanied by intensive solubilisation and destruction of cells. It is feasible that lipopeptides from PHP1601 could have a similar impact on the digestive tract of fly larvae if consumed in sufficient quantities. Histopathological studies would need to be conducted to confirm this assumption.

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CHAPTER FOUR: OLFACTORY RESPONSE OF *LUCILIA CUPRINA* LARVAE TO VOLATILE ORGANIC COMPOUNDS PRODUCED BY *BACILLUS VELEZENSIS* PHP1601

4.1 Abstract

Biological control is a dynamic and intimate process between a biocontrol agent and the pest of interest, which can be influenced by the scent or odours produced by the biocontrol agent. *Bacillus velezensis* PHP1601 (Bacillales: Bacillaceae) is a strain of biocontrol significance that demonstrates larvicidal activity against *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) blowfly larvae. This investigation aimed to determine the impacts of odours produced by PHP1601 when cultured on selected media on the preference of second-instar *L. cuprina*. Scent cues were prepared by growing PHP1601 on tryptone soy agar, MG agar bioassay medium and inoculating it into pig manure. Olfactory choice tests were run at 0, 1 and 5 d time intervals to evaluate the odour-mediated response of larvae. A significant level of larvae attraction was observed for TSA and MG agar after being fermented for 1 and 5 d respectively. Additionally, no decrease in larvae preference towards fermented manure was observed at all culture intervals tested. This indicated that odours produced by PHP1601 have the potential to attract *L. cuprina* fly larvae and do not threaten its biocontrol effect. The scent profile of PHP1601 was determined using GC-MS and was found to comprise at least 25 compounds, of which ketones were the dominant fraction (82.20%). The study demonstrated that odours produced cultivation of PHP1601 in various substrates do not negatively impact the larvae preference to the bioassay medium and were unlikely to hinder its larvicidal effect. The effect and implications of volatile organic compound odour profiles produced by members of the *B. subtilis* species complex on fly biocontrol applications have not been documented previously. Hence, this study constitutes the first report of a *B. velezensis* exhibiting an odour-mediated-larvae attraction.

Keywords Fly biocontrol, larvicide, olfactory choice tests, *Bacillus* volatile organic compounds, gas chromatography mass spectroscopy

4.2 Introduction

Members of the *Bacillus subtilis sensu lato* group have been pursued as promising biocontrol candidates of a variety of phytopathogens due to the production of various bioactive compounds such as lipopeptide biosurfactants, polyketides and other antimicrobial peptides (Fan et al. 2018). Additionally, volatile organic compounds (VOC) produced by members of this species complex are associated with phytopathogen antagonism, plant growth promotion, and the induction of plant immune responses to protect against infection (Fan et al. 2018; Calvo et al. 2020). To a much lesser extent members of this species complex have been reported to demonstrate antagonism towards certain insect groups that is attributed to the production of lipopeptide biosurfactants (Mnif et al. 2013; Ramesar and Hunter 2023a). The role of VOCs produced by this group of organisms in insect biocontrol and their impact on pest behaviour has not been explored previously.

The application of members of the *B. subtilis sensu lato* group as fly biocontrol agents is an emerging field in biocontrol. The influence of how these species alter the local environment that the larvae or pest co-inhabit and the resulting pest-response is currently unexplored; but, are factors that can impact their biocontrol effectiveness against fly species. VOCs produced by *Bacillus* spp. were shown to exhibit antifungal effects towards agricultural pathogens such as *Botrytis cinerea* (Helotiales: Sclerotiniaceae), *Monilinia* spp. (Helotiales: Sclerotiniaceae), *Penicillium* spp. (Eurotiomycetes: Aspergillaceae) and towards the root-knot-nematocidal *Meloidogyne hapla* (Chitwood) (Tylenchida: Heteroderidae) (Calvo et al. 2020; Wu et al. 2023). Therefore, it's possible that VOCs produced by PHP1601 can make the co-inhabited environment of the larvae toxic, which could improve its biocontrol potential or result in a loss of larvae preferences towards the environment, causing the larvae to migrate to a more suitable environment. The latter is more concerning as it can diminish a biocontrol agent's efficacy and render it ineffective.

Recently, *Bacillus velezensis* PHP1601 was shown to exhibit a larvicidal effect on *Lucilia cuprina* blowfly larvae by producing lipopeptide biosurfactants (Ramesar and Hunter 2023b). During the course of the larvicide bioassays, a concentration-dependent larvae repulsion effect was observed, whereby the larvae avoided the bioassay medium with PHP1601. It was hypothesised that this feeding behaviour was either due to a toxic response of lipopeptide

biosurfactants accumulating in the medium or due to an inhibitory effect caused by VOCs released by the strain as it developed in the bioassay medium (Ramesar and Hunter 2023a). Hence, the impact of VOCs released by PHP1601 was considered an aspect of consideration in its evaluation as a biocontrol candidate.

This research aims to assess the influence of odours produced by *Bacillus velezensis* PHP1601 on *Lucilia cuprina* fly larvae to determine their biocontrol implications. This was assessed by establishing odour-mediated larvae choice tests that compared the larvae preference to tryptone soy agar, a milk-based larvae bioassay medium and pig manure fermented with PHP1601. These substrates were chosen to represent media commonly used to culture *Bacillus* spp., perform larvicide bioassays, and as an environmental source associated with biocontrol application to control fly infestations, respectively. Gas chromatography mass spectrometry (GC-MS) was performed to elucidate the VOC odour profile of *B. velezensis* PHP1601.

4.3 Methods and Materials

4.3.1 Bacterial culture

Bacillus velezensis PHP1601, hereafter referred to as PHP1601, was obtained from Andermatt Plant Health Products (Strathdean Farm, Gowrie Avenue, Nottingham Road, KwaZulu-Natal, 3280, South Africa). PHP1601 was routinely cultured on tryptone soy agar (TSA) (Biolab, Merck, Germany) for 24 h at 30ºC or in tryptone soy broth (TSB) at 28ºC for 18 h at 200 rpm. PHP1601 was preserved as endospore master culture solutions in 20% (v v^{-1}) glycerol that was stored at -80ºC or as standardised stock solutions (1×10^7 endospore ml^{-1}) prepared in deionised water and stored at 4ºC. Endospores were produced by inoculating 20 ml of TSB with a colony of PHP1601 and incubating the broth with agitation (250 rpm) for 24 h at 35ºC. Thereafter, the culture (20 ml) was transferred into a 1 l Erlenmeyer flask containing 80 ml of TSB and incubated similarly for 4 d. The culture was heat treated at 60ºC for 20 min to eradicate non-sporulated cells, and the endospores were harvested by centrifugation (15000 × g for 10 min) and suspended in 20 ml of deionised water. Endospores were quantified using a counting chamber (0.01 mm × 0.0025 mm^2) and their viability was assessed by spread plating (100 µl) various

endospore dilutions on a TSA plate and assessing the percentage of colonies that emerged after an 18 h incubation at 30ºC.

4.3.2 Fly population

Lucilia cuprina blowfly larvae were sourced from a population reared and sustained at the University of KwaZulu-Natal's Controlled Environment Research Unit (Ramesar and Hunter 2023a). Harvested eggs were transferred into cylindrical transparent plastic containers (height: 95 mm × diameter: 70 mm) containing 50 g of malt extract agar (100 g l^{-1} brewing malt extract, 15 g l^{-1} agar, pH 6.5) and incubated for 48 h at 30ºC to promote larvae emergence and development.

4.3.3 Production of PHP1601 scent cues

Scent cures were prepared by fermenting different media with PHP1601 and assessing the preference of larvae to them from olfactory choice bioassays. In the context of this research, fermentation is defined as the microbial breakdown or decomposition of a substrate releasing products of metabolism, such as VOCs. The first medium tested was TSA as a standard bacteriological culture agar. In a 20 ml McCartney vial, 20 g of molten TSA (~ 45ºC) was inoculated to a concentration of 10^5 endospores g^{-1} using a 200 µl endospore-deionised water suspension. Afterwards, the medium was poured into a sterile petri dish (inner diameter: 8.9 cm) and allowed to solidify at room temperature (25 – 27ºC). Uninoculated TSA controls were prepared by substituting the inoculum with sterile deionised water. The plates were sealed with parafilm tape and incubated at 30ºC for 24 h. After incubation, the agar was cut into 5 × 5 mm squares with a sterile razor blade for use in larvae olfactory choice bioassays. Five plates of uninoculated and fermented TSA were prepared for use in the bioassays.

The impact of odours from cell and endospore biomass were assessed using amended water agar (15 g agarose l^{-1} deionised water) as the bioassay medium. Cell and endospore biomass scent cues were prepared by amending water agar to ~1×10^9 cells/endospores g^{-1} using 200 µl of culture. Water agar inoculated with 200 µl of sterile water was used as the uninoculated control.

These controls were tested immediately after the agar was solidified and cooled to room temperature (~ 30 min after inoculation).

The scent produced by fermenting a larvicidal bioassay medium, MG agar (500 ml l^{-1} UHT milk, 2 g l^{-1} bacteriological agar, pH 6.50), and pig manure (sourced from University of KwaZulu-Natal Ukulinga Research Farm) were assessed. The MG agar medium was prepared, inoculated and incubated as described previously for TSA. Pig manure samples (25 g; moisture content: 73.62%) were mixed into a paste using a metal spatula after inoculation (250 µl; starting concentration of 10^5 endospores g^{-1}) to ensure the inoculum was homogenously incorporated into the substrate. Thereafter, 20 g of inoculated manure, or manure controls inoculated with sterile distilled water, were transferred into a petri dish, sealed with parafilm and incubated for 0, 1 and 5 d at 30°C. Fifteen plates of each substrate were individually prepared to determine larvae olfactory preference after each time interval for 5 replicates. After a plate was tested, it was discarded from further use.

The concentration of PHP1601 reached during the fermentation was determined using a culture-based method. Briefly, 1 g of substrate (TSA, MG agar or pig manure) was transferred into a 15 ml centrifuge tube containing 9 mL of 0.8% (w v^{-1}) NaCl, 10 × 5 mm glass beads and 1 g of molecular grade sand and bead-beated for 2 min at max speed on a bench top vortex to free the cells bound in the substrate. The suspension was serially diluted 10-fold to a final dilution of 10^{-8}. From each dilution, 100 µl was transferred onto TSA and using a standard spread-plating method. Colonies that emerged after 18 h at 30°C, within a valid countable range of 30 – 300, were used to determine the concentration of PHP1601 as colony-forming units per gram (CFU g^{-1}) of agar or manure. Additionally, colonies that emerged from the pig manure samples were tested to determine whether they were *B. velezensis* using the *B. velezensis*-specific qPCR described by Ramesar and Hunter (2023c).

4.3.4 Scent choice tests

Larvae olfactory choice tests were performed in glass petri dishes (inner diameter of 18 cm) using the method adapted from Saumweber et al. (2011). The principle of the bioassays involves assessing the preference of larvae placed in the centre of the petri dish to scent cues or controls, placed opposite each other at the petri dish inner circumference, by monitoring the proportion of

larvae that move to the scent cues, control, or showed no attractant/repellent behaviour over a period of time. When more larvae migrated to the scent cue than its control, the scent cue was considered to attract larvae. Whereas, if the opposite was observed, the scent cue was considered to repel larvae. Larvae that showed no response to either scent cues or control and remained in the middle of the petri dish were categorised as residual larvae.

The petri dishes used in the choice bioassays were lined with 20 ml of 1% (w v^{-1}) agarose to provide soft surface that larvae can move unhindered on. After that, 1 g of the prepared scent cue(s) medium or their controls were placed on one or opposite ends of the petri dish, when tested in combination, followed by adding 20 second instar larvae to the centre of the petri dish. Second instar larvae were preferred to first instars as their developmental size was more consistent and bodies were stronger, offering greater ease of handling without mortality or injury which would impact the results of the assay.

Choice assays were conducted in a dimly lit, well-ventilated room maintained at 25°C. Petri dish lids were removed to prevent an odour saturation effect. Observations of larvae preferences were taken every minute for 5 min for odours produced by fermenting TSA. The larvae preference for odours produced by fermenting MG agar and pig manure was evaluated with observations of larvae preference taken after an assay duration of 5 min. All assays were replicated 5 times.

4.3.5 Scent profiling of PHP1601

VOCs produced by PHP1601 were collected using a dynamic headspace extraction method adapted from Schmitt et al. (2020) and analysed with an adapted method from Johnson et al. (2020) using coupled gas chromatography mass spectroscopy (GC-MS). For VOC sampling, PHP1601 was cultured in 1 l Erlenmeyer flasks, whereby 200 g of molten TSA (~ 45°C) was inoculated with 2 ml of endospore-deionised water suspension to achieve a starting concentration of 10^5 endospores g^{-1}. Similarly, sterile TSA controls were prepared using sterile deionised water. The neck of each flask was covered with aluminium foil and secured with an elastic rubber band. The flasks were incubated for 24 h at 30°C before headspace sampling was performed. All samples and controls were prepared in triplicate.

After incubation, the foil caps were removed from the flasks and a 10 × 20 cm polyacetate bag (NaloPhan, Kalle, Germany) was used to connect the neck of the flask to a PAS500 Personal Air Sampler (Spectrex, Redwood City, CA, USA) such that air from the bag was sucked through a small glass cartridge (~ 30 mm) filled with a 1:1 adsorbent matrix of 1.5 mg of Tenax® TA (60/80; SupelcoTM; Bellefonte, PA, USA) and 1.5 mg of Carbotrap® B (20-40 mesh; Sigma-Aldrich Co., St Louis, MO, USA) at a flow rate of 50 ml min^{-1} for 30 min.

GC-MS analysis of the samples was carried out using a Scion 436 GC coupled to a Scion SQ single quadrupole MS operated in electron-impact ionisation mode at 70 eV (Scion Instruments, Goes, Netherlands). VOCs were separated using a SGE SolGel wax standard polar capillary column (30 m × 0.25 mm ID, film thickness: 0.25 µm). VOCs were thermally desorbed directly from cartridges placed in a Scion 1079 PTV injector port modified with a 'ChromatoProbe' thermal desorption device (Amirav and Dagan 1997). Helium was used as the carrier gas for each sample at a flow rate of 1 ml min^{-1}. The injector was held at 40ºC for 2 min with a 20:1 split and then increased to 200ºC at a rate of 200ºC min^{-1} in the split-less mode for thermal desorption. Meanwhile, the GC oven was held at 40ºC for 3 min, then ramped up to 240ºC at 10ºC min^{-1} and held at 240ºC for 12 min. Compounds were identified using MS Workstation software (Scion Instruments, v. 8.2.1) in conjunction with the NIST2020 mass spectral library (v 2.4). Compound identifications were verified, where possible, using retention times of authentic standards and published n-alkane linear retention indices (Dool and Kratz 1963; Babushok et al. 2011; Babushok 2015). Compounds present at similar abundance in samples from the control flasks were considered to emanate from the TSA medium or represent background contaminants and hence, were excluded from the analysis. The proportions of the detected compounds were described based on their frequency of occurrence, describing how often a compound was detected in all samples (n = 3), and their average relative abundance (RA) as a percentage (%) of the total peak area.

All glassware used for scent production, headspace sampling and in bioassays were pre-treated before use to remove contaminating VOCs. Briefly, glassware was washed using commercial detergent and rinsed liberally with deionised water. Thereafter, they were sequentially rinsed with MS-grade methanol and acetone before being baked at 150ºC for 6 h.

4.3.6 Statistical analysis

Statistical analysis of the larvae olfactory data was performed using R (v 4.2.1) (R Core Team 2023) through the RStudio integrated development environment (v 2022.07.1, build 554). All data were analysed at the 95% confidence level. Larvae preference data were assessed for normality based on the Shapiro-Wilks test. Significant differences in the larvae responses were determined using the t-test or one-way analysis of variance (ANOVA) followed by the Tukey HSD test. The Kruskal-Wallis (KW) test, followed by the Conover-Iman test (R package: conover.test; Dinno 2017) for pairwise comparisons, were conducted as non-parametric alternatives when the assumptions of normality were not met. All data, including those shown in the figures, were of the mean ± standard error (SE).

4.4 Results

4.4.1 Olfactory choice bioassays of VOCs from fermented TSA

The bioassays assessing the olfactory preference of larvae towards VOCs produced by PHP1601 are shown in Fig. 4.1. Initially, choice bioassays were conducted with uninoculated TSA and TSA fermented by PHP1601 only (Fig. 4.1a, b) to determine their impact on larvae without other stimuli. Larvae were attracted to, and moved towards, the TSA sample linearly (time interval: 0 – 5 min) as defined by $y = 12.57x - 3.43$ ($R^2 = 0.99$). After 5 min, 60 ± 5.70% of larvae were attracted towards the TSA sample, which was significantly higher (t-test: $t_8 = 2.98$, $P = 0.018$) than larvae that showed no response to the scent of TSA. This indicated that the scent emanating from TSA was attractive to fly larvae. Scent preference assays using TSA fermented with PHP1601 had a larvae attraction rate that was described by the equation $y = -1.57x^2 + 21.97x + 1.143$ ($R^2 = 0.99$) (time interval: 0 – 5 min) and resulted in visibly higher levels of larvae attraction towards the fermented TSA from 3 to 5 min. At the 5 min interval, 72 ± 6.04% of larvae were attracted to the agar, which was significant (t-test: $t_8 = 5.15$, $P < 0.001$) compared to the residual larvae that showed no response. These investigations indicated that the scent of both substrates attracted larvae and required subsequent choice assays to compare the levels of attraction between TSA and TSA fermented by PHP1601 and that conducting the assays for 5 min was sufficient to assess the larvae olfactory response.

Larvae odour choice assays evaluating the odour preference of cell and endospore biomass were performed to determine whether their associated scents could have been detected and influential in the larvae attraction (Fig. 4.1c, d). Larvae choice assays were conducted testing 10^9 cells or endospores g^{-1}, which is similar to the concentration of PHP1601 achieved in TSA prepared for choice assays ($9.84 \pm 0.88 \times 10^8$ CFU g^{-1}). Significant differences in larvae preference were detected in the cell (KW: $\chi^2_2 = 9.97$, P = 0.007) and endospore (KW: $\chi^2_2 = 10.20$, P = 0.006) biomass choice assays at the 5 min interval. In both cases, no significant difference in larvae preference was observed between the cell/endospore biomass and the water agar control (P > 0.05), while a significant proportion of larvae (P < 0.001) (≥ 89%) showed no response to or could not detect the biomass-amended agars or their controls and were randomly distributed in the petri dish. This indicated the scent of cell or endospore biomass was not influential in attracting fly larvae.

Choice tests comparing the preference of larvae to the scent of TSA and PHP1601 fermented TSA showed significant differences (KW: $\chi^2_2 = 17.163$, P < 0.001) (Fig. 4.1 e). Significantly more larvae (P = 0.040) were attracted to the fermented TSA compared to the TSA control (27±2.0%) and resulted in significantly fewer larvae showing no response to either of the tested substrates (12±3.39%) (P < 0.05). This indicated that odours released by PHP1601 contained compounds that are attractive to *L. cuprina* larvae.

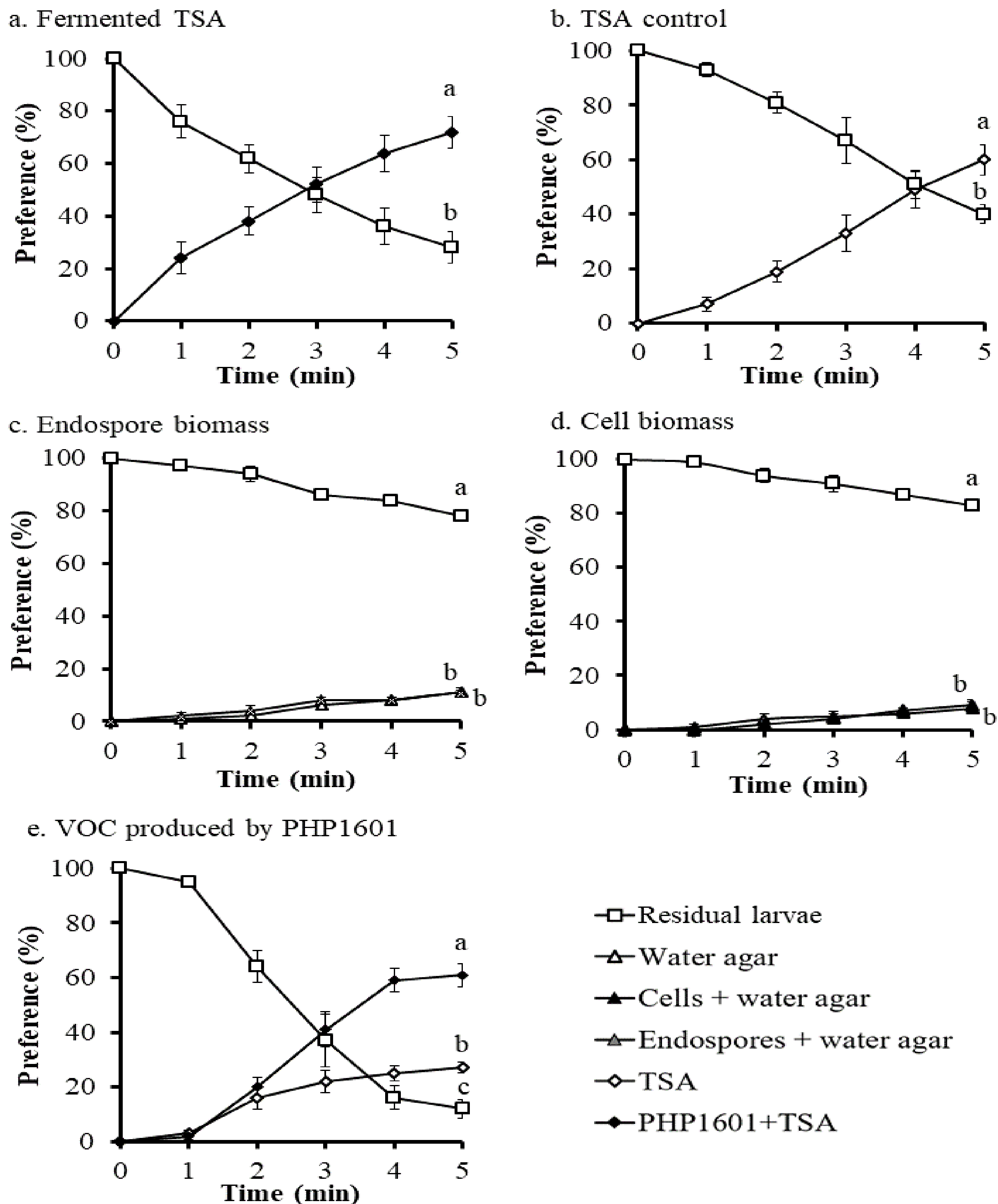

Fig. 4.1 Larvae preference (mean ± SE) towards VOCs produced by PHP1601 fermented TSA only (a), uninoculated TSA only (b), endospore biomass (c), cell biomass (d) and comparatively between fermented TSA and the TSA control (e). Controls used in the study to substantiate the response of the larvae to the scent of PHP1601 fermented TSA (PHP1601+TSA)

included an uninoculated TSA control (TSA), water agar and water agar with cell (cells + water agar) or endospore (endospores + water agar) as biomass controls. Larvae that remained on the petri dish, showing no preference to the controls or treatments, were categorised as the residual larvae group. Letters represent significant differences detected by $P < 0.05$ at the final observation interval (5 min).

4.4.2 Olfactory choice bioassays of VOCs from fermented MG bioassay medium

Larvae scent choice assays using MG agar as the bioassay medium are shown in Fig. 4.2. At the start of the choice assay (0 d), no significant difference in larvae preference (ANOVA: $F_{2, 12} = 3.15$, $P = 0.080$) was observed between the MG agar control, the inoculated agar and the residual larvae displayed no attractant behaviour. After a 1 d fermentation period, no significant differences in the preference of larvae between the fermented MG agar (35±1.58%), the MG agar control (37±255%) and the residual larvae (28±3.74%) in the petri dish (ANOVA: $F_{2, 12} = 2.91$, $P = 0.093$) were observed. The titer of PHP1601 increased from $1.07 \pm 0.05\times10^5$ at 0 d to $7.44 \pm 0.40\times10^7$ CFU g^{-1} after 24 h, which is ~ 13.23 times lower than that reached in TSA.

After a 5 d fermentation period, the titer of PHP1601 in the MG agar reached $6.52 \pm 0.22\times10^8$ CFU g^{-1}, and significant differences (ANOVA: $F_{2, 12} = 43.82$, $P < 0.001$) in the preference of the larvae were detected (Fig 4.2). The fermented MG agar had significantly higher levels of larvae preference (48±2.55%) than the MG agar control (34±1.87%) ($P = 0.003$) and both samples were significantly distinct ($P < 0.001$) from the larvae that showed no preferential response to both stimuli (17±2.55%). This indicated that with prolonged fermentation in the bioassay medium, VOCs produced by PHP1601 could reach levels that could attract *L. cuprina* larvae.

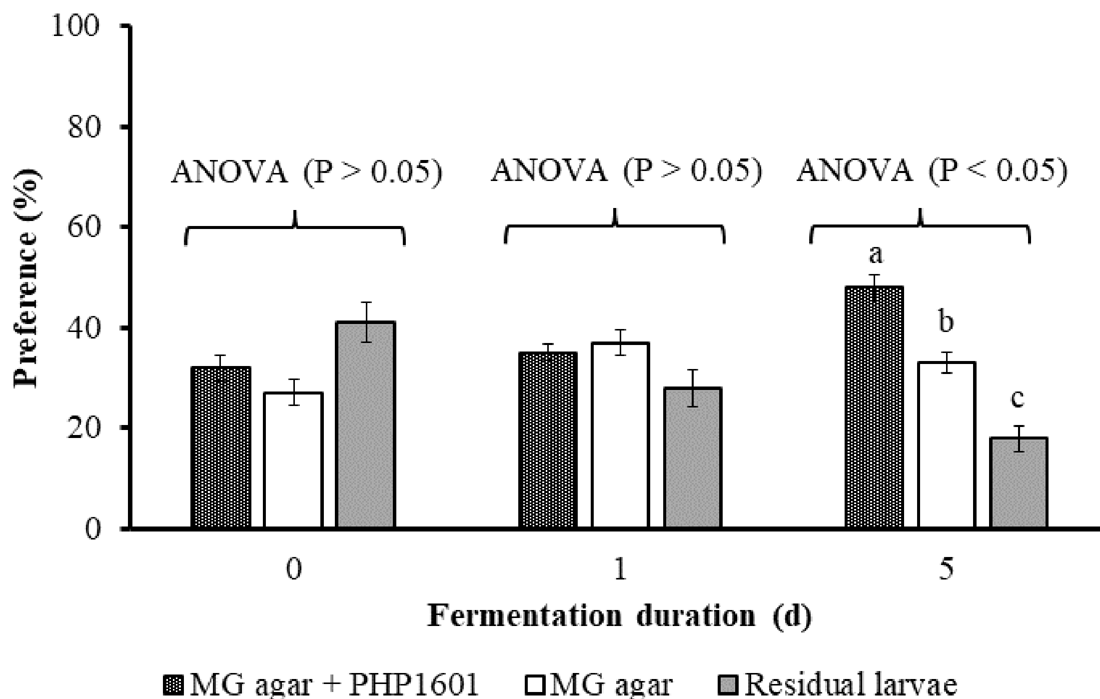

Fig. 4.2 Larvae preference (mean ± SE) towards VOC produced by PHP1601 from the fermentation of the MG agar bioassay medium. The preference (%) of the larvae towards VOC produced by PHP1601 fermentation of MG agar (MG agar + PHP1601) was substantiated by the levels of larvae attraction to the uninoculated MG agar control (MG agar) and compared against the proportion of larvae that remained uninfluenced on petri dish (residual larvae). Letters denote significant differences ($P < 0.05$) detected by the Tukey HSD test.

4.4.3 Preference of larvae towards the scent of PHP1601 cultured on pig manure

Larvae scent choice tests using pig manure as the assessment medium are shown in Fig. 4.3. Bioassays using pig manure as the test substrate showed elevated levels of larvae attraction, whereby the bioassays were completed in 1.54 ± 0.08 min with all larvae responding to either the control or fermented manure samples. However, no significant differences in the preference of the larvae for fermented manure or the control were detected when fermented for 0 d (t-test: $t_8 = 0.76$, $P = 0.471$), 1 d (t-test: $t_8 = 1.66$, $P = 0.135$) or 5 d (t-test: $t_8 = 1.11$, $P = 0.300$). Over these intervals, titers of *B. velezensis* increased from $9.82 \pm 0.39 \times 10^5$ CFU g^{-1} (0 d) to $1.55 \pm 0.05 \times 10^9$ CFU g^{-1} and $3.82 \pm 0.12 \times 10^9$ CFU g^{-1} at 1 and 5 d respectively. Since no *B. velezensis* colonies were detected in the uninoculated manure controls, it was presumed that the colonies

that emerged in the treatment were of strain PHP1601. This indicated that although PHP1601 was able to grow and ferment the pig manure, the natural scent of the manure and/or fermentation by its inhabiting microbes masked the scent of PHP1601.

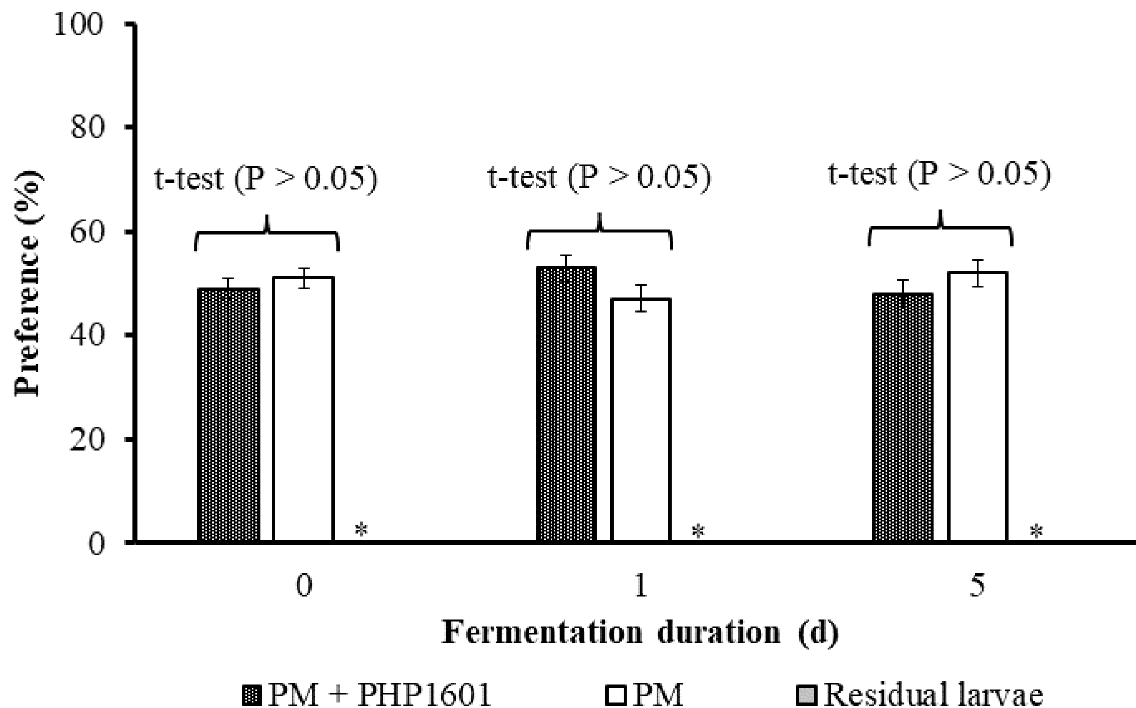

Fig. 4.3 Larvae preference (mean ± SE) towards VOC produced by PHP1601 from the fermentation of pig manure. The preference (%) of the larvae towards VOC produced by PHP1601 fermentation of pig manure (PM + PHP1601) was substantiated by the levels of larvae attraction to the uninoculated pig manure control (PM) and compared against the proportion of larvae that remained uninfluenced on petri dish (residual larvae). Significant differences ($P < 0.05$) in larvae preference were assessed using the t-test. Asterisks indicate no (0%) larvae preference.

4.4.4 Identification of VOC produced by PHP1601

The profile of VOCs produced by PHP1601 from the fermentation of TSA is presented in Table 4.1. The identified VOC included secondary alcohols (n = 2), ketones (n = 16), carboxylic acids (n = 2) aromatics (n = 1) and sulphides (n = 1). The headspace was dominated by aliphatic ketones, with heptan-2-one (RA: 34.17 ± 12.07 %), nonan-2-one (RA: 9.96 ± 2.58%), undecane-

2-one (RA: 7.48 ± 2.34 %) and an unidentified ketone (RA: 8.33 ± 3.15%) exhibiting mass fragments (m/z: 58,43,71,105,81,95,70,41,85,55) consistent with a similar ketone structure all occurring in relatively high abundance (> 5%). Ketones were the dominant class of compound in terms of both diversity (n = 16) and relative abundance (82.20%). The majority of compounds were consistent across samples, although the carboxylic acid, 2-methylpropanoic acid, was only detected in 1 of the 3 samples.

Table 4.1 Qualitative analysis of volatile organic compounds produced by *B. velezensis* PHP1601

Compounds	LRI	RA (%)
Aliphatic alcohols		
Nonan-2-ol	1531	3.48 ± 0.41 (3/3)
Undecan-2-ol	1738	1.84 ± 0.35 (3/3)
Aliphatic ketones		
Heptan-2-one [S]	1172	34.17 ± 12.07 (3/3)
Acetoin [S]	1263	2.17 ± 1.32 (3/3)
Nonan-2-one	1372	9.96 ± 2.58 (3/3)
Decan-2-one	1440	2.76 ± 0.61 (3/3)
Undecan-2-one	1579	7.48 ± 2.34 (3/3)
Dodecan-2-one	1649	2.96 ± 2.29 (3/3)
Tridecan-2-one	1787	1.49 ± 0.62 (3/3)
Tetradecan-2-one	1857	0.24 ± 0.11 (3/3)
Hexadecan-2-one	2066	0.03 ± 0.02 (3/3)
m/z: 58,43,71,105,81,95,70,41,85,55	1224	8.33 ± 3.15 (3/3)
m/z: 43,71,58,104,70,103,78,57,105,55	1243	3.2 ± 1.28 (3/3)
m/z: 142*,58,43,82,71,105,59,57,84,45,41	1323	2.16 ± 0.62 (3/3)
m/z: 156*, 58, 43, 71, 59, 82, 57, 55, 146, 81	1428	2.5 ± 0.55 (3/3)
m/z: 184*,58,43,71,59,82,57,55,156,146,81	1635	4.39 ± 1.62 (3/3)
m/z: 184*,58,59,43,71,57,85,184,55,61,56	1683	0.09 ± 0.02 (3/3)
m/z: 212*,58,59,43,71,57,96,55,85,69	1842	0.27 ± 0.13 (3/3)
Carboxylic acids		
2-Methylpropanoic acid	1541	1.11 ± 1.11 (3/3)
2-Methylhexanoic acid	1640	3.9 ± 3.73 (1/3)
Aromatic compounds		
2-Phenylethyl alcohol [S]	1941	1.88 ± 0.75 (3/3)
Sulphides		
Dimethyl disulphide [S]	1065	4.87 ± 1.78 (3/3)
Unidentified sesquiterpenes		
m/z: 204*, 79, 109, 107, 122, 91, 81, 133, 95, 67	1976	0.58 ± 0.28 (3/3)
m/z: 204*,79, 109, 95, 122, 77, 81, 91, 105, 107	2053	0.07 ± 0.03 (3/3)
Unidentified compounds		
m/z: 136,121,77,91,93,58,43,135,41,83	1834	0.08 ± 0.03 (3/3)

LRI - The linear *n*-alkane retention index (Van den Dool and Kratz 1963).
The relative abundance (RA) of compounds were reported as the mean ± SE, accompanied with the compound’s frequency of occurrence in parenthesis, describing in how many samples (n = 3) it was detected.

Mass fragments for unidentified compounds are listed with the molecular ion first (where possible, tentatively identified from the mass spectrum marked with asterisk), followed by the base peak and other fragments in decreasing order of abundance.

[S] Library match confirmed with injection of synthetic standard.

The contribution of alcohols, ketones, aliphatic acids, aromatics, sulphides, unidentified sesquiterpenes and unidentified compounds to the VOC composition was 5.32, 82.20, 5.01, 1.88, 4.87, 0.65 and 0.08% respectively.

4.5 Discussion

The impacts of VOCs produced by microbial biocontrol agents are significant for understanding their interactions with their target pest(s). In particular, the odours of *B. velezensis* PHP1601 was studied to determine whether it was linked to the larvae repulsion effect documented in previous studies and whether it could negatively impact its biocontrol potential (Ramesar and Hunter 2023a). The VOCs that constitute these odours were considered to be influenced by the substrate that PHP1601 fermented; hence, larvae olfactory choice assays and GC-MS were conducted to determine their preference towards these scents and to identify the constituting VOCs respectively.

Initial larvae choice assays using TSA as the base substrate showed an attraction effect of the larvae towards TSA fermented for 24 h. It was further determined that larvae were attracted to the scent profile produced by PHP1601 since they demonstrated no significant attraction towards the biomass controls or uninoculated TSA. When PHP1601 was cultured in MG agar, significant levels of larvae attraction were only observed after 5 d of fermentation. This was attributed to variations in nutrient composition of the two media and the likelihood that the strain grew at a slower growth rate in the bioassay medium as compared to TSA, resulting in a less pronounced odour profile to attract larvae during the early phases of the bioassay. In previous studies, a repulsion effect was observed whereby *L. cuprina* larvae increasingly avoided MG agar used in larvicide bioassays over time (starting concentration $\geq 10^5$ PHP1601 cells/endospores g^{-1}) (Ramesar and Hunter 2023a). Considering the significant levels of larvae preference for fermented agar, it is unlikely that the odours produced by fermentation were contributors to this effect.

The impact of the fermentation stage (viz. lag, exponential, death phase) on the pungency and composition of VOCs in the odours produced are factors considered for future investigation of the odour-mediated larvae attraction by PHP1601. A study conducted by Ng (2015) reported that flies were attracted to the odours released from broth fermented by *B. subtilis* NRS-762 and described that an increase in fly attraction was associated with the strain entering the stationary growth phase. It is likely that changes in the growth stage and growth parameters can influence the type and pungency of odours released.

Larvae choice tests using pig manure samples indicated no significant differences in larvae attraction were observed between the inoculated manure and the control, even with extended fermentation duration and titers of PHP1601 reaching > 3 times that achieved in TSA. Pig manure is a natural fly attractant and has been associated with higher levels of oviposition with certain fly species as well as promoting fly larvae development (Nyakeri et al. 2017; Aliu and Esume 2022). Therefore, it is likely that the larvae had a higher affinity to the manure, regardless of the presence of PHP1601, or that the odour of the manure overwhelmed those produced by PHP1601, which could account for no significant larvae attraction being observed. Additionally, the results motivate that larvae repulsion is unlikely to occur and therefore does not pose a detriment to the strains biocontrol efficacy.

The fly/larvae olfactory system is complex and, therefore, the attraction or response of larvae to a scent cue might not necessarily suggest a similar response with adult flies. The larvae olfactory system, modelled on *Drosophila* spp. (Diptera: Drosophilidae), is typically composed of 21 olfactory sensory neurons housed in the dorsal organ of the larvae head and express 25 components of the odorant receptor family (Ramaekers et al. 2005; Dweck et al. 2018). Of these, 13 are also expressed in adult flies (Ramaekers et al. 2005). Therefore, it is possible for larvae and flies to have different preferences towards the same scent cue. Therefore, to better evaluate the biocontrol implications of odours of PHP1601 on manure applications, larvae and adult fly scent preference tests need to be conducted. Other substrates associated with promoting fly infestations include different types of manure, hay padding of cattle pens or compost, which requires assessment to determine whether fly or larvae-luring odours can be produced (Cruz-Vázquez et al. 2007; Tangtrakulwanich et al. 2015). Additionally, evaluating whether these

odours can offer enhanced biocontrol effects, such as increasing the retention time of larvae on treated substrates, are warranted.

GC-MS analysis of VOCs produced by PHP1601 indicated the presence of 25 compounds which constituted the odour profile of PHP1601 when cultured in TSA. Ketones were detected as the major component of the VOCs produced by PHP1601 and were presumed to be the most influential in attracting larvae of *L. cuprina* based on their relative abundance. Alcohols are readily oxidised to ketones, catalysed by alcohol dehydrogenases, which could explain the relatively low abundance of alcohols compared to ketones (Patel et al. 1979). Ketones are biochemically important compounds for energy production by being converted to acetyl-CoA through β-oxidation, thereafter, thereafter oxidised through the Krebbs cycle to produce ATP (Patel et al. 1979; Martínez-Herrera et al. 2023). The role of these compounds on *L. cuprina* larvae and flies is either scarcely documented or has not been reported, which hinders ascribing key VOCs of interest. The scent of decaying carrion is composed of a mixture of alcohols, ketones, aldehydes, fatty acids, esters, sulphides, aromatic and cyclic compounds, which constitute an odour that saprophagous flies, like *Lucilia* spp., are attracted to (Kasper et al. 2015). However, the direct impact of many of these VOCs on fly behaviour are not fully understood (Kasper et al. 2015). Sulphides such as dimethyl disulphide and dimethyl trisulphide are VOCs associated with decaying flesh and have been shown to attract fly larvae of *L. sericata, L. cuprina* and other saprophagous flies (Liu et al. 2016; Verheggen et al. 2017; Sajan et al. 2022). Similar compounds to the VOCs produced by PHP1601 have been studied on relatively few fly spp., mainly on *Drosophila* spp. and indicate that the olfactory preference of larvae and adult flies varies considerably between genera, species, strain and even the sex of the fly (Cobb 1999; Khurana and Siddiqi 2013; Li et al. 2020). Hence, scent choice tests using standards of these compounds are required to reliably determine their contribution to the preference of *L. cuprina* larvae or flies.

VOCs produced by PHP1601 allude to a wider biocontrol potential. Ketones within the VOC mixture such as nonane-2-one, decan-2-one, heptan-2-one, nonan-2-one and undecane-2-one have shown broad-spectrum antifungal properties, namely against members of the Sclerotiniaceae (order: Helotiales) which are associated with postharvest rot (Calvo at al. 2023). Therefore, research in to the extraction, quantification and assessment of these antifungal

compounds would be interesting and valuable in expanding the biocontrol value or range of PHP1601. Additionally, microbially produced VOCs play an important role in intra- and inter-kingdom interactions, such as attracting parasitoid wasps, which can offer additional biocontrol benefits (Đurović et al. 2021). Due to the lack of studies evaluating the odours or VOCs produced by members of the *B. subtilis* species complex in insect biocontrol applications, the synergistic biocontrol potential of these VOCs are unknown. Future investigations assessing the potential effects of VOCs produced by PHP1601 on attracting parasitoid wasps and fly spp. for an enhanced biocontrol effect would be valuable in addressing the gap in the literature.

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CHAPTER FIVE: MANURE TRIAL EVALUATION OF *BACILLUS VELEZENSIS* PHP1601 AS A VIABLE BIOCONTROL CANDIDATE AGAINST NUISANCE FLY SPECIES AT AN OPERATIONAL PIGGERY

5.1 Abstract

Fly infestations threaten farming and livestock operations, contributing to animal irritation and the spread of serious diseases. In this study, the application of *Bacillus velezensis* PHP1601 (Bacillales: Bacillaceae) treatments (10^5 and 10^{10} endospore g^{-1}) to pig manure slurries was evaluated as a means of controlling fly reproductive cycles. Two cycles of fly emergence were evaluated over a 33-d period for each replicated trial. For both treatments, the first emergence event resulted in a significant reduction in the percentage of flies emerging compared to an un-treated control. Extended monitoring of the biocontrol containers revealed that fly emergence was completely eliminated by the time that the second round of fly emergence was observed for the control. A *B. velezensis*-specific real-time PCR method was developed and used to assess the population dynamics of the applied biocontrol agent over the course of the experiment. Strain PHP1601 remained viable in the manure and cell numbers increased by several orders of magnitude. REP-PCR fingerprinting was used to confirm the clonality of endospores recovered from the manure. Fly species recovered from the trials were identified by cytochrome oxidase gene barcode sequencing. Several species of veterinary and medically significant flies were identified; these were all deemed to be susceptible to treatments with PHP1601 and constituted part of the strain's host range. The study demonstrated the effectiveness of *B. velezensis* PHP1601 as a promising biocontrol agent for controlling fly infestations under conditions similar to its intended use.

Keywords Bacillales, Bacillaceae, *Bacillus velezensis*, Diptera, quantitative real-time PCR, fly infestation, biological control

5.2 Introduction

Livestock and poultry farming generate considerable quantities of manure, which promotes the development of fly infestations (Tangtrakulwanich et al. 2015). Fly infestations negatively impact farming operations through pathogen transmission and animal irritation, leading to significant economic losses (Meerburg et al. 2007; Taylor et al. 2012). The control of fly populations within animal production facilities is an important goal for promoting animal health and ensuring food safety. Increasingly, eco-friendly alternatives to traditional chemical-based control methods that pose risks to human health and the ecosystem have been advocated (Brown 2006; Manrakhan and Addison 2014; Hinckle and Hogsette 2021). Biological control strategies provide opportunities for establishing sustainable control measures that safeguard against and reduce losses related to fly infestations in a non-toxic manner that does not compromise the safety of livestock, farm workers or the environment.

Fly species commonly associated with fly infestation include house flies, *Musca domestica* (Linnaeus) (Diptera: Muscidae), stable flies, *Stomoxys calcitrans* (Linnaeus) (Diptera: Muscidae), and blowflies (Diptera: Calliphoridae). House flies, for instance, are notorious carriers of numerous pathogens, including bacteria, viruses, and parasites, which they can transmit to animals and humans (Hinckle and Hogsette 2021). Stable flies can inflict painful bites, which can cause stress and discomfort to livestock, leading to reduced feed intake, weight loss, and decreased milk production (Taylor et al. 2012). Blowflies can cause flystrike or myiasis, whereby they lay their eggs on wounds, leading to maggot infestations and complications in livestock (Khater and Geden 2018). This can result in economic losses attributed to poor hide or wool quality, reduced weight gain, and decreased livestock fertility (Soyelu and Masika 2009). Since nuisance flies in animal production facilities can negatively impact animal welfare and disrupt operations, establishing effective control measures is paramount.

Bacterial species of the *Bacillus subtilis sensu lato* group (Bacillales: Bacillaceae) are gaining attention as candidate fly biocontrol agents (Torres et al. 2022; Ramesar and Hunter 2023b). Many members of the *B. subtilis* species complex have achieved "generally regarded as safe" status and are non-pathogenic to humans or animals (Abdallah et al. 2019; Lin et al. 2020). In a recent study, *B. velezensis* PHP1601 (Bacillales: Bacillaceae) was shown to be effective in

controlling *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) fly larvae through the activity of its lipopeptide biosurfactants (Ramesar and Hunter 2023a). Lipopeptide fractions containing surfactin homologues demonstrated the highest levels of larvicidal activity (Ramesar and Hunter 2023b). Larvicide bioassays (240 h) using purified surfactin resulted in an LC_{50} of 9.87 µg g^{-1}. *Bacillus velezensis* PHP1601 was, therefore, considered a promising candidate for controlling problematic flies associated with farming operations and was selected for further evaluation.

Piggery operations provide an ideal environment for fly proliferation (Meerburg et al. 2007); hence, a study was initiated with the aim of assessing the biocontrol potential of *B. velezensis* PHP1601 treatments in controlling fly infestation associated with pig manure. Trials were established at an operational piggery at the University of KwaZulul-Natal's Ukulinga Research Farm, where endospore treatments were applied to pig manure slurry samples and evaluated for their efficacy in disrupting fly reproductive cycles. Additional objectives included, determining the potential host range of flies susceptible to *B. velezensis* PHP1601; and, assessing the population dynamics of the applied biocontrol agent over the course of the experiments.

5.3 Methods and materials

5.3.1 Bacterial strains

Bacillus velezensis PHP1601 was obtained from Andermatt PHP (Pty) Ltd (Strathdean Farm, Nottingham Road, 3280, KwaZulu-Natal, South Africa). Reference *Bacillus* strains used in the study were sourced from an in-house culture collection within the Discipline of Microbiology, School of Life Sciences, University of KwaZulu-Natal (Pietermaritzburg, 3209, KwaZulu-Natal, South Africa) and included *B. velezensis* FZB42^{T}, *B. subtilis* DSM10^{T} and *B. cereus* DFbc. Strains were routinely cultured on tryptone soy agar (TSA) (30°C for 18 h) or in tryptone soy broth (TSB) (Biolab, Merck, Germany) at 28°C for 18 h at 200 rpm. Master cultures of each strain preserved in 20% (w v^{-1}) glycerol solution were stored at -80°C. Sporulation was promoted by culturing strains in 100 m1 of glucose omitted Schaeffer's broth as described by Leighton and Doi (1971).

5.3.2 Trial design

Manure trials were conducted at a piggery facility located at Ukulinga Research Farm, University of KwaZulu-Natal, South Africa (29º39'45"S, 30º24'10"E) during the summer months of December 2021 to March 2022 when fly activity was at its height. The trial design was adapted from Nurita and Hassan (2013) and utilised standardised 1 l black plastic bucket containers (12 cm tall × 10.5 cm wide) (Appendix C: Fig. C1). Each container lid had a 3 cm hole at it centre, to which a clear plastic bottle (14 cm tall × 4.5 cm wide) was glued. The base of each bottle was removed and covered with fine gauze net material, which allowed air to diffuse into the container while preventing flies and larvae from escaping. This allowed the emergence of flies to be observed and recorded over time.

5.3.3 Manure trial

Approximately 12 - 14 kg of pig manure was collected from the facility and mixed with a metal hand spade in a 15 l plastic bucket for 10 min to create a homogenous mixture. This mixture was kept in a closed container for 3 d at ambient temperature (~26ºC) to assess for the emergence of fly larvae, which could interfere with the investigation. The dry mass of the manure was determined by drying 5 × 20 g samples at 75ºC (Labotec EcoTherm Industrial Oven, Labotec, RSA) for 2 d; to facilitate accurate endospore dosages to be calculated and applied (Wong et al. 1999).

Prior to setting up each treatment, the manure sample was homogenised as described previously. Four hundred grams of unamended manure was added to each container and five replicates established for each treatment. Two treatments of *B. velezensis* PHP1601 were evaluated, viz., 10^5 and 10^{10} endospores g^{-1}; these treatments were prepared by amending the manure with 10 ml of an endospore suspension, in deionised water. Initial endospore titres of PHP1601 cultured in Schaeffer's broth were determined using a bacterial counting chamber (0.01 × 0.0025 mm^2; Neubauer, Marienfeld, Germany) before being adjusted to the desired concentration via centrifugation and dilution. Controls used included an autoclaved 10^{10} endospore g^{-1} treatment, and an endospore-free control amended with 10 ml of deionised water; these were included to substantiate any differences in fly emergence observed between treatments. Strips of shredded

black plastic refuse bag (~5.0 g) were introduced into each container to provide a surface for fly visitation and oviposition. An additional set of controls and treatments were prepared to assess changes in moisture content at various intervals (0, 16 and 33 d) to supplement qPCR and endospore quantification assays without disturbing the trial itself.

Prior to starting the trial, buckets were grouped in 5 replicate sets containing a representative of each treatment and control. These were placed at several sites within the piggery environs and opened to attract flies and promote oviposition. Over a 5 d period, bucket sets were switched between locations daily to increase the likelihood of an even distribution of fly visitation. Subsequently, buckets were covered and relocated to an unused pen within the piggery for the duration of the trial (33 d). Buckets were monitored for fly emergence every 3 d, as evidenced by the appearance of flies within the clear plastic bottle attached to the container lid. The bottles could be unscrewed to take samples for counting or identification purposes.

5.3.4 Identification of captured flies

Emerging flies were collected and sorted into morphologically similar groups. Representative specimen samples (~ 100 mg) were homogenised by liquid nitrogen maceration; thereafter, DNA was extracted following the Thermo Scientific GeneJet DNA extraction kit (Thermo Fisher Scientific, USA) protocol for rodent tails. Flies were identified by PCR amplification and sequence analysis of cytochrome oxidase genes according to the method of Radzevičiūtė et al. (2017). All PCR reactions were prepared using DreamTaq Green PCR Master Mix reagents (Thermo Fisher Scientific, USA).

5.3.5 Detection and quantification of *B. velezensis* in pig manure slurries by qPCR

A qPCR method based on *B. velezensis* specific primers designed by Dunlap (2019) was used to detect and monitor *B. velezensis* populations in pig manure, as a proxy for monitoring changes in *B. velezensis* PHP1601 concentration. At 0, 16 and 33 d intervals, 2 g of manure slurry was sampled from three replicates of each treatment for DNA extraction and qPCR analysis. Additionally, DNA from duplicate sets of pig manure spiked with 1 - 10^9 cells g^{-1} of *B. velezensis* PHP1601 and 10^9 cells g^{-1} of *B. velezensis* FZB42^T were extracted to determine DNA

extraction efficiency.

Prior to DNA extraction, manure samples were treated to remove exogenous DNA and water soluble contaminants. Briefly, 100 mg of manure was suspended in saline solution (1.5 ml of 0.8% (w v^{-1}) NaCl) in a 2 ml screw cap microfuge tube and vortexed for 1 min to dissolve exogenous DNA; thereafter, particulate matter was collected by centrifugation (6000 × g for 5 min). This washing step was repeated three times. Microbial DNA was extracted from pelleted cells and particulate matter using a DNeasy PowerSoil kit (Qiagen, Germany) as per the manufacturer's instructions and recommendation for hard to lyse cells. An amendment was made to the sample homogenisation step, which included bead-beating samples at 7 m s^{-1} for 1 min (Bead Blaster 24, Benchmark Scientific, USA). Eluted DNA was quantitatively and qualitatively assessed using UV spectral analysis (Nanodrop, Thermo Fisher Scientific, USA), and agarose (1.5% w v^{-1}) gel electrophoresis at 80V for 100 min in a TAE buffer (40 mM Tris, 20 mM Acetic acid, 1 mM EDTA, pH 8.3).

A qPCR hydrolysis probe was designed using Primer3 (https://bioinfo.ut.ee/primer3-0.4.0/primer3/) to align within the *B. velezensis* specific 2-keto-3-deoxygluconate kinase (*kdg*K) gene region. Potential probe sequences were queried in silico against a sample of 75 *B. velezensis* genomes (NCBI GenBank, https://www.ncbi.nlm.nih.gov/) using the Blastn algorithm (https://blast.ncbi.nlm.nih.gov/) to confirm specificity. The resulting probe, Bvel-P, was synthesised incorporating a 5' FAM fluorophore and a 3' Blackhole quencher 1 (BHQ1) to give a final sequence of (5'-3') FAM-TCCCCAGTCTGCTGACCCACT-BHQ1 (Inqaba BioTech Laboratories, Pretoria, South Africa).

All qPCR reactions contained per 20 µl: 1× HOT FIREPol Probe qPCR Mix (ROX) Plus (Solis BioDyne, Estonia); 0.4 mM Bvel-F (5'CCTTTGCGTTTTGTTACCC-3') and Bvel-R (5'-CACATCAATTCCTTCTCC-3') primers; 0.2 mM of Bvel-P hydrolysis probe; and, 1 ng µl^{-1} of template DNA. Reactions were performed using a QuantStudio 5 Real-time PCR system, which included an initial denaturation step at 95°C for 12 min followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. QuantStudio Scientific Design and Analysis software (v. 1.5.1) (Thermo Fisher Scientific, USA) was used to normalise amplification curves against the ROX dye and to determine Ct values, qPCR efficiencies and standard error values. Controls included a no template control to assess PCR contamination, and non-specific amplification controls using

genomic DNA from *B. subtilis* DSM10^T and *B. cereus* DFbc to confirm primer specificity. *Bacillus velezensis* qPCR standards (1 - 10^9 cells reaction^{-1}) were included in triplicate, in each set of qPCR reactions for quantification purposes. Standards were prepared from an overnight culture of *B. velezensis* PHP1601 (TSB, 30ºC, 150 rpm) that was quantified using a bacterial counting chamber (Neubauer, Marienfeld, Germany) prior to DNA extraction according to a CTAB method described by Minarovičová et al. (2018).

Since endospores of *B. velezensis* were not lysed efficiently using the DNeasy PowerSoil kit, a plate count method was used to quantify viable endospores present in manure samples collected in triplicate. Briefly, a serial dilution (10^{-1} – 10^{-8}) of 1 g of manure in saline solution was prepared in McCartney bottles and heat treated at 80ºC for 20 min in a water bath to eliminate vegetative cells and select for endospores. Aliquots (100 µl) from each dilution were spread-plated onto TSA and incubated for 18 h at 30ºC. The resulting colonies, within a range of 30 – 300, were picked off from the highest dilutions plated and suspended in 100 µl of nuclease-free water and heated at 94ºC for 10 min to lyse the cells and release their DNA. Cellular debris was removed by centrifugation (15000 × g for 10 min) and 1 µl of the supernatant was used to identify colonies of *B. velezensis* as per the method of Dunlap (2019). Isolates that tested positive as *B. velezensis* were fingerprinted according to the REP-PCR method described by Versalovic et al. (1994), and compared to *B. velezensis* PHP1601. REP-PCR fingerprints were resolved by agarose gel (1.5% w v^{-1}) electrophoresis at 80V for 100 min using a TBE buffer (89 mM tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) system.

5.3.6 Statistical analysis

The manure trials were performed comprising of 5 replicates of each treatment and control. Fly antagonism was assessed by comparing the averaged total number of flies emerged in each treatment to fly emergence events recorded for the controls. The trial was repeated 2 weeks after the first trial to substantiate the results acquired. Statistical analysis was performed using R (v 4.2.1) (R Core Team 2022) through the RStudio integrated development environment (v 2022.07.1, build 554). Fly emergence data was initially assessed for normality using the Shapiro-Wilks test; thereafter, one-way analysis of variance (ANOVA) followed by Tukey's HSD test was performed to determine statistical differences ($P < 0.01$) between treatments and the controls

tested. The data presented for cell and endospore concentrations enumerated from manure treatments were determined from 3 replicates sampled at each sampling interval and reported as the mean ± standard error (SE) per gram (dry weight) of manure. The Kruskal-Wallis test (KW) followed by the Conover-Iman test for pairwise comparisons were performed to determine statistical differences between cell and endospore treatments using the "conover.test" package (v 1.1.5) in R (Dinno 2017).

5.4 Results

5.4.1 Influence of treatments on fly oviposition

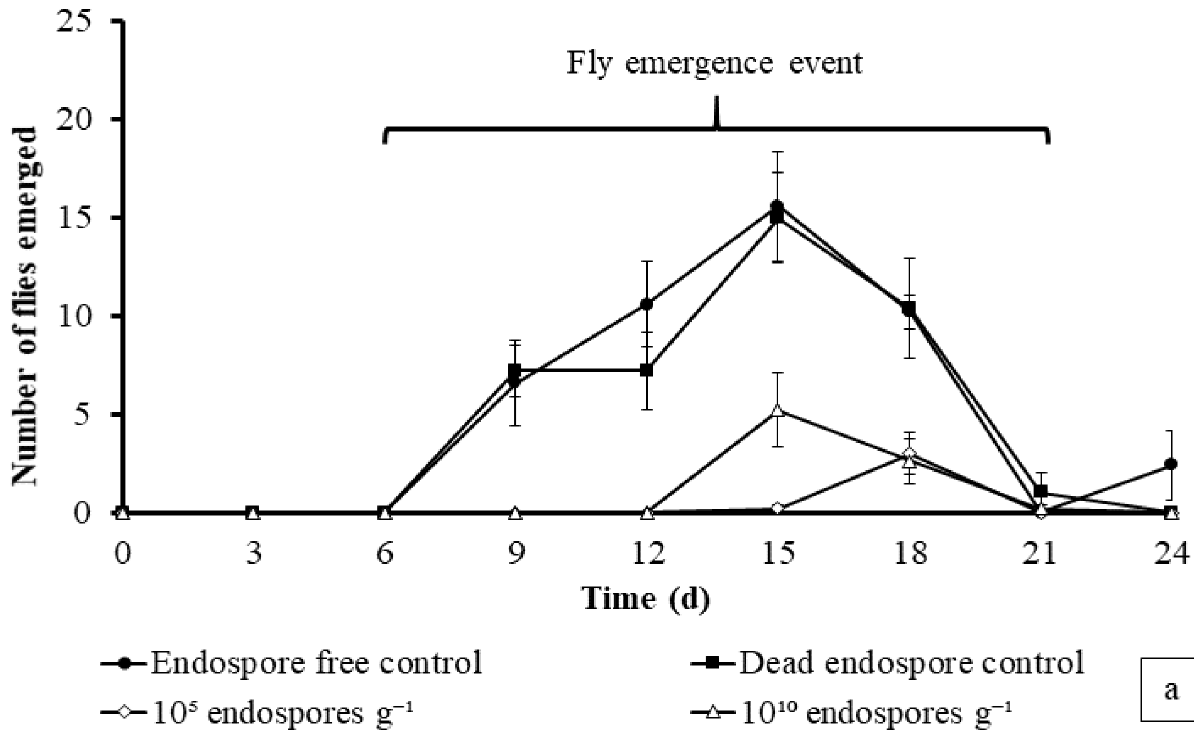
Prior to conducting the manure trial, the influence of the different treatments and controls on fly oviposition levels was assessed. The average number of eggs laid, between the controls and treatments after 24 h of the buckets being left open ranged between 46.80 ± 12.94 – 53.40 ± 4.88; however, no statistical difference in the number of eggs laid were detected ($F_{3,\ 16} = 0.05$, $P > 0.01$). Subsequently, it was concluded that neither the controls nor treatments introduced a bias in fly attraction or oviposition that could interfere with the results acquired from the trials.

5.4.2 Manure biocontrol trial 1

The effect of the endospore treatments on fly emergence is shown in Fig. 5.1a. The first indication of fly antagonism was a delay to the start of fly emergence observed in the endospore treatments compared to the controls. Fly emergence was evident from 6 - 21 d for the controls, whereas fly emergence in the 10^5 and 10^{10} endospore g^{-1} treatments was observed from 15 d – 21 d and 12 d - 21 d respectively. Significant differences ($F_{3,\ 16} = 52.84$, $P < 0.001$) in the total number of emergent flies was evident between the controls and treatments over this period (Fig. 5.1b). On average, the highest number of flies that emerged were from the endospore free (43.00 ± 4.60 flies) and non-viable endospore controls (39.80 ± 2.73 flies). Both controls displayed significantly higher levels of fly emergence (all $P < 0.001$) than each of the endospore treatments. This substantiated that neither the presence of the endospore biomass nor the use of deionised water to prepare the endospore solutions negatively impacted the ability of the eggs or larvae or pupae to develop into flies. Overall, the total number of flies that emerged in endospore

treatments were 5.01 – 12.44 times lower than in the controls. The levels of fly emergence for the two endospore treatments, over the duration of the trial, were not significantly different ($P = 0.808$); however, it was observed that the 10^5 endospore g^{-1} treatment required a longer time (3 d) for fly emergence to be detected.

The trial ended abruptly after 24 days due to unforeseen animal interference of the trial area, resulting in the destruction of many of the trial containers. At this stage in the investigation, evidence substantiated that the treatments of *B. velezensis* PHP1601 significantly inhibited fly emergence.



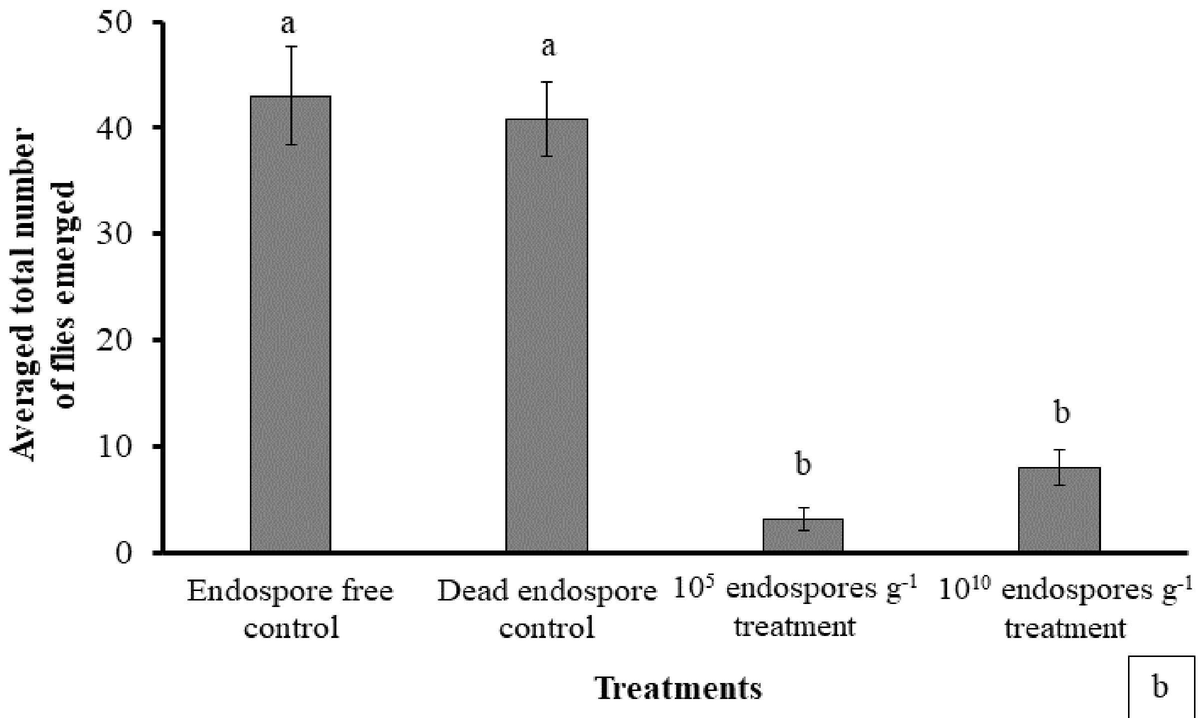


Fig. 5.1 Effect of *B. velezensis* PHP1601 endospore treatments on fly emergence trends over a 24-d period (a), and averaged total number of flies counted during the primary fly

emergence event (b). Error bars represent the SE of the mean. Means denoted by a different letter indicate significant differences between treatments ($P < 0.01$). Based on fly emergence patterns observed for the controls, the primary fly emergence event was judged to occur from 6 - 21 d.

5.4.3 Manure biocontrol trial 2

To verify the findings of the first fly emergence trial, the trial was repeated. Two fly emergence events were observed in the controls, whereas fly emergence associated with the endospore treatments were only evident during the first emergence event (Fig. 5.2a). Over the course of the trial, significant differences in fly emergence was observed ($F_{5, 25} = 33.20$, $P < 0.001$). During the primary fly emergence interval, significant differences in fly emergence were observed whereby the 10^5 endospore g^{-1} treatment had the lowest number of flies when compared to the controls (all $P < 0.01$) (Fig. 5.2b). In contrast, fly numbers associated with the 10^{10} endospore g^{-1} treatment were not statistically different to either of the controls or the 10^5 endospore g^{-1} treatment ($P > 0.01$) during the primary emergence period. This indicated that the 10^5 endospore g^{-1} treatment was the best treatment tested and that the biocontrol effectiveness could be negatively impacted when a threshold dosage was exceeded.

Fly emergence during the second emergence event was limited to the controls and showed a significant increase in the numbers of flies emerged (all $P < 0.001$) compared to their respective primary emergence events (Fig. 5.2b). This indicated that the trial design was suitable for a population of flies to complete their life cycle; however, all larvae in the 10^5 and 10^{10} endospores g^{-1} treatments of *B. velezensis* PHP1601 were confirmed to be dead upon inspection at the 33 d interval. This confirmed that the *B. velezensis* PHP1601 treatments are effective in controlling various fly populations.

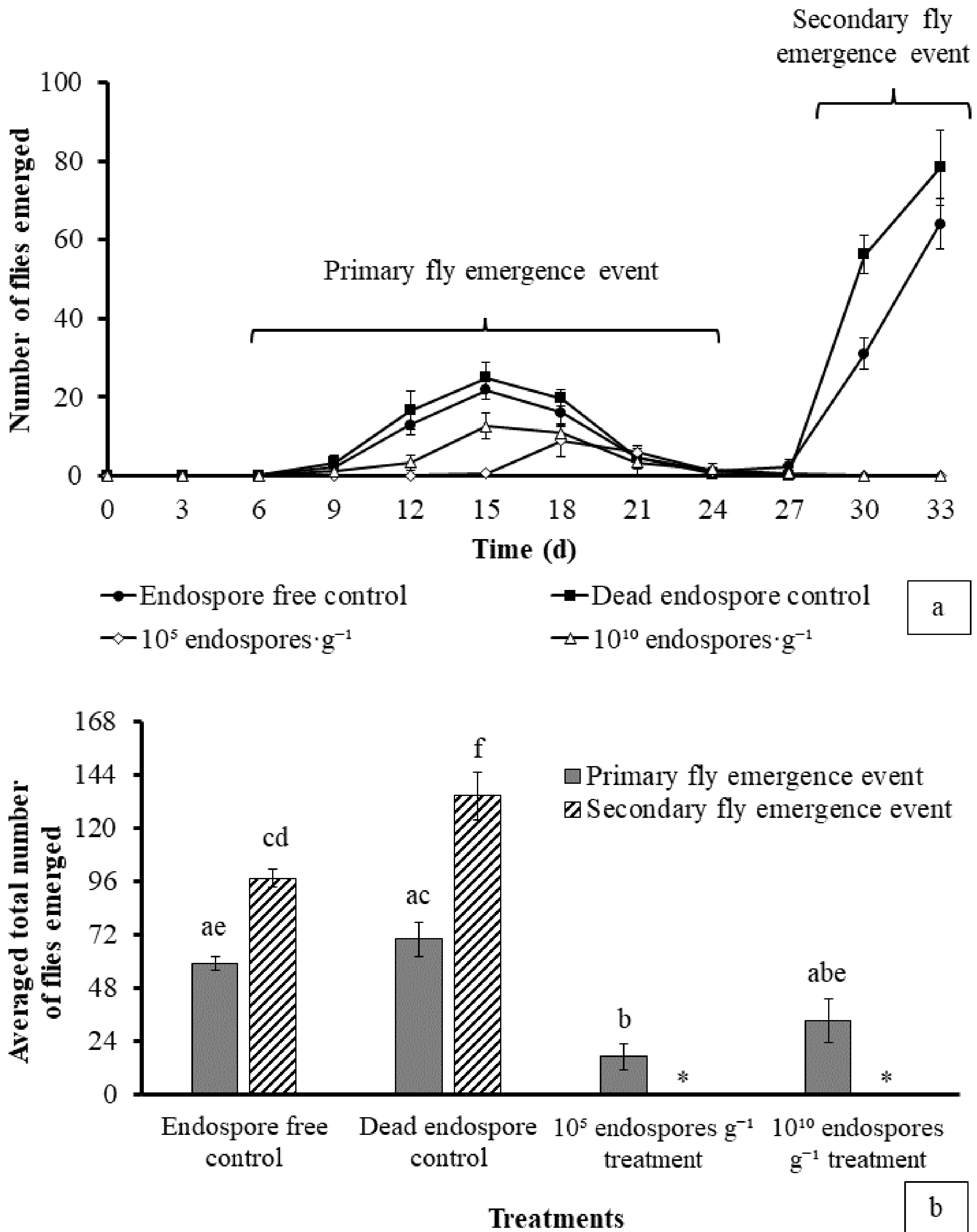


Fig. 5.2 Effect of *B. velezensis* PHP1601 endospore treatments of on fly emergence trends over a 33-d period (a), and averaged total number of flies counted during each fly emergence event (b). Error bars represent the SE of the mean. Means denoted by a different

letter indicate significant differences in fly emergence between treatments ($P < 0.01$) across the primary and secondary fly emergence events. Asterisks indicate treatments that had no fly emergence. Based on fly emergence patterns observed for the controls and treatments, primary fly emergence was judged to occur from 6 - 24 d and a secondary emergence event from 27 - 33 d.

5.4.4 Identification of the host range of flies antagonised by *B. velezensis* PHP1601

Seven distinct fly morphologies were distinguished when fly emergence was recorded over the course of the second field trial. These flies were identified based on analysis of their CO1 gene sequence as *L. cuprina* – the Australian blowfly, *Dacus bivittatus* (Bigot) (Diptera: Tephritidae) – the common fruit fly, *Sarcophaga ruficornis* (Fabricius) (Diptera: Sarcophagidae) – the flesh fly, *Megaselia scalaris* (Loew) (Diptera: Phoridae) – the scuttle fly, *Muscina stabulans* (Fallén) (Diptera: Muscidae) – the false stable fly, *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) - oriental latrine fly, and *Synthesiomyia nudiseta* (Wulp) (Diptera: Muscidae). Sequences were deposited into GenBank with the following accession numbers: OP355338.1, OP356210.1, OP356702.1, OP356703.1, OP356704.1, OP356705.1 and OP356706.1 respectively.

All fly species were found in the control groups; however, only *L. cuprina*, *D. bivittatus*, *S. ruficornis*, *C. megacephala*, *S. nudiseta* and *M. stabulans* were observed in the treatments during the first fly emergence event (Appendix C: Fig. C2). Subsequently, all larvae in the treatments were assessed to have been deceased by the 33-d interval; hence, indicating their susceptibility to the *B. velezensis* PHP1601 treatments.

5.4.5 Growth of *B. velezensis* PHP1601 in pig manure slurries

The *B. velezensis* cell standard prepared using the CTAB method produced DNA of high quality ($A_{260:280}$: 1.82 ± 0.02, $A_{260:230}$: 1.35 ± 0.16) suitable for qPCR analysis. The qPCR standard curve (10 – 10^9 cells reaction^{-1}) yielded a straight line curve ($y = -3.388x + 35.961$, $R^2 = 0.999$) that had good qPCR efficiency (97.307%) (Appendix C: Fig. C3). The limit of detection was 10 cells reaction^{-1} and concentrations of *B. velezensis* within the 10 – 10^9 cells reaction^{-1} range could be

quantified.

DNA extracted from *B. velezensis* spiked manure (1 – 10^9 cells g^{-1}) had detectable levels of co-extracted organic compounds ($A_{260:280}$: 1.68 ± 0.03, $A_{260:230}$: 0.95 ± 0.21), which was considered to be a consequence of the complex nature of the manure constituents. An attempt was made to further purify the DNA as per the kit's recommendations; however, no significant improvements were gained.

qPCR successfully detected *B. velezensis* in the 10^4 to 10^9 cells g^{-1} spiked manure standards and reaffirmed their respective concentrations (Appendix C: Fig. C4). No endogenous *B. velezensis* were detected in unamended manure samples by qPCR assays or culturing, and were considered to not impact qPCR analysis. This indicated that the limits of *B. velezensis* detection and quantification are within the 10^4 to 10^9 cells g^{-1} range. Additionally, the qPCR derived concentration of *B. velezensis* FZB42^T in the spiked manure control (1.27 ± 0.15 × 10^9 cells g^{-1}) was similar to that acquired from the corresponding *B. velezensis* PHP1601 spiked manure standard (1.15 ± 0.31 × 10^9 cells g^{-1}), which indicated that variance in DNA extraction or qPCR sensitivity between different *B. velezensis* strains were unlikely. Subsequently, the *B. velezensis*-specific qPCR protocol was considered suitable to assess the population dynamics of the best-performing treatment (viz., 10^5 endospore g^{-1}) in pig manure.

Throughout the study, no evidence of *B. velezensis* cells, nor endospores, was detected in the dead endospore control; whereas, in the treatment, the population increased significantly (KW: $\chi^2_4 = 13.52$, $p < 0.01$) by several orders of magnitude over a 16 d period (Table 5.1). This indicated that the endospores germinated and were in a bioactive state. By the 16-d interval, ~ 0.62% of the *B. velezensis* population had sporulated, which is interesting as it suggests that nutrient exhaustion had not yet been reached. By the end of the trial (33 d) there was a substantial decrease in the concentration of vegetative cells (99.48%) ($P < 0.001$), accompanied by increased sporulation levels to 2.48 ± 0.77×10^8 endospores g^{-1} (sporulated population: 98.94%) ($P < 0.001$). Furthermore, REP-PCR fingerprinting indicated that the genomic profiles of the *B. velezensis* colonies recovered were identical to that of the *B. velezensis* PHP1601, which confirmed that the population change determined for *B. velezensis* was attributable to the PHP1601 strain (Fig. 5.3). The amplification of the ~169 bp *B. velezensis* specific *Kdg*K region detected in all qPCR reactions was verified by agarose gel electrophoresis (Appendix C: Fig.

C5).

Table 5.1 Population dynamics of *B. velezensis* over the course of the manure trial

	10^5 endospore g^{-1} treatment		Dead endospore control	
Time (d)	Cells (cells g^{-1})	Endospores (endospore g^{-1})	Cells (cells g^{-1})	Endospores (endospore g^{-1})
0	ND	$1.14 \pm 0.19 \times 10^{5}$ [a]	ND	ND
16	$5.04 \pm 0.17 \times 10^{8}$ [b]	$3.17 \pm 0.31 \times 10^{7}$ [c]	ND	ND
33	$2.66 \pm 0.16 \times 10^{6}$ [d]	$2.48 \pm 0.77 \times 10^{8}$ [e]	ND	ND

Cell concentration was determined using qPCR, whereby all Ct values were determined once the respective amplification curves passed the threshold of 0.04 within 40 PCR cycles. The concentration of endospore of *B. velezensis* were determined using a plate count method and verified using *B. velezensis*-specific qPCR. When no amplification occurred or no endospores of *B. velezensis* were isolated, it was interpreted that *B. velezensis* was not detected (ND). The data represents the mean ± SE of concentrations of cells or endospore per gram (dry weight) of manure determined from 3 replicates. Letters indicate significant differences ($P < 0.01$) between the cell and endospore treatments achieved over the duration of the trial (33 d)

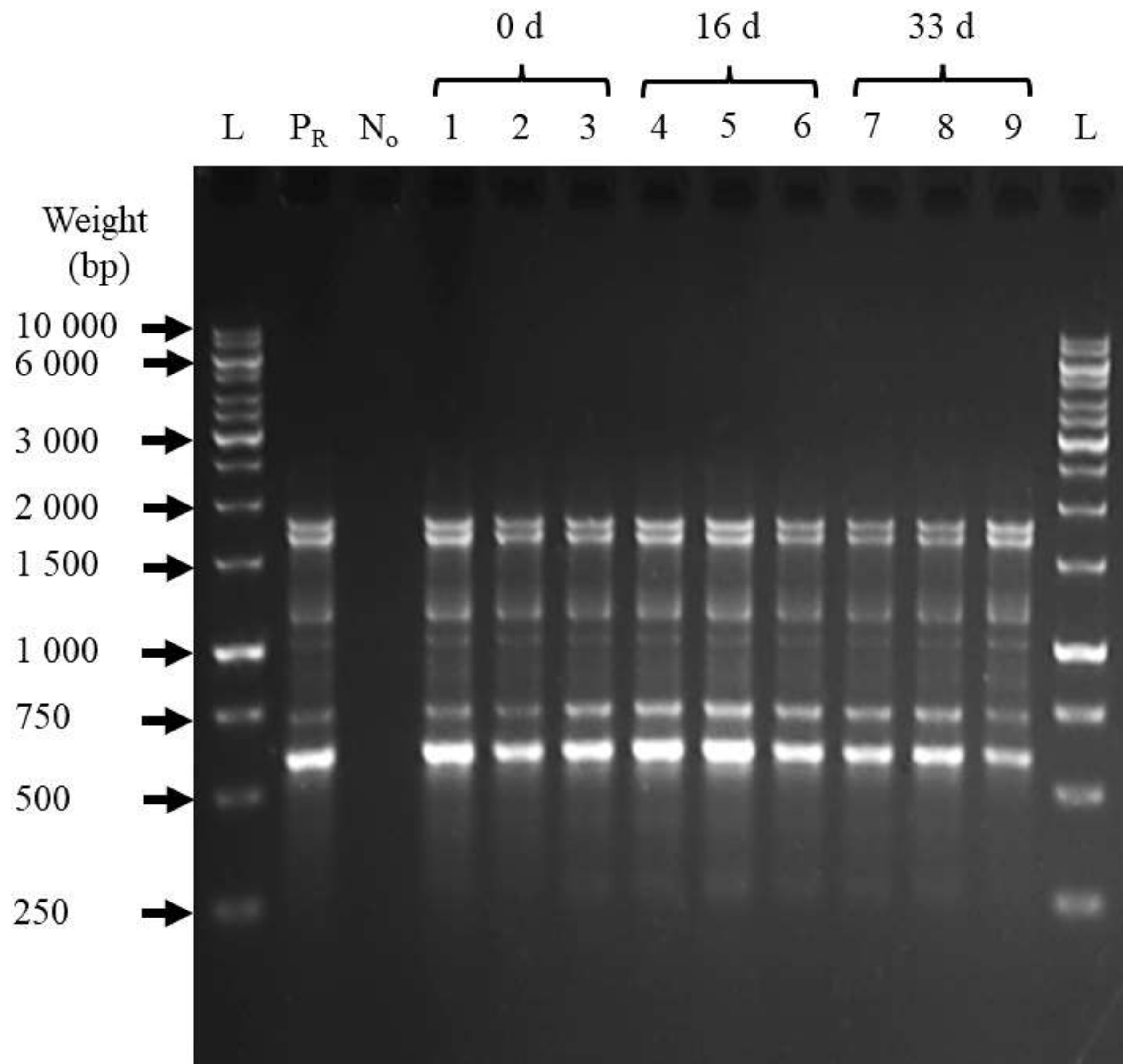


Fig. 5.3 REP-PCR genomic fingerprints of representative *B. velezensis* colonies isolated from a 10^5 endospore g^{-1} manure treatment at 0, 16 and 33 d. Bacterial colonies were confirmed to be strains of *B. velezensis* using species-specific qPCR targeting the *kdg*K gene, prior to selection for REP-PCR. *Bacillus velezensis* PHP1601 was included as a reference control (P_R), along with a no template control (N_o) and 10 kb molecular weight ladders (L). Representative fingerprints of isolates from the treatment at 0, 16 and 33 d are shown in lanes marked 1 - 3, 4 - 6 and 7 - 9 respectively.

5.5 Discussion

Manure trials were conducted to assess the effectiveness of *B. velezensis* PHP1601 as a viable biocontrol agent against fly species commonly associated with a piggery setting. Many biocontrol candidates have been reported to exhibit poor insect antagonism and/or inconsistent results when assessed under farm, semi-field or field trial conditions due to factors such as changes in temperature, humidity, desiccation, and exposure to UV radiation (Besset-Manzoni et al. 2019) Therefore, the manure trials undertaken were considered an important milestone in assessing the biocontrol candidacy of *B. velezensis* PHP1601 under field conditions that were representative of their intended use.

The trials were conducted using closed containers to house the various treatments and contain the flies that emerged, whilst still being subjected to the prevailing temperatures found within the piggery. This approach was adopted to ensure that a reasonable level of consistency was retained within, and between, replicated treatments whilst limiting the influence of independent variables (e.g. moisture content, repeated fly visitation and oviposition) that could have interfered with the findings of the trial. This allowed the trial to be operated at a smaller scale and yet incorporate essential elements of a field trial.

Pig manure slurry was considered a suitable medium for the trials because it is known to attract flies and contributes to the development of fly infestations (Tangtrakulwanich et al. 2015). Chicken and cow manure slurries are also important nutritional sources for flies, which contribute to the development of fly infestations if left unattended (Cruz-Vázquez et al. 2007; Tangtrakulwanich et al. 2015). Therefore, these types of manure could be used as suitable media for future field trial assessments.

The first trial showed a significant reduction of fly emergence by both endospore treatments tested. This was considered to be a positive indication that PHP1601 could elicit a fly antagonistic effect under the prevailing environmental conditions. Similar results were obtained when the trial was repeated. When the second trial was extended beyond 24 d, it was found that none of the survivors of the first fly emergence event associated with the endospore treatments were able to produce a second generation of flies and all remaining larvae were deceased by the end of the trial. Hence, the larvicidal effect of *B. velezensis* PHP1601 was described as a chronic

antagonistic effect rather than an acute one. As far as the authors are aware, no similar studies have been reported in the literature; therefore, this constitutes the first report of a *B. velezensis* exhibiting real-world potential as a fly biocontrol agent.

In both sets of trials, the endospore treatments displayed statistically similar levels of fly inhibition when compared to each other. The 10^5 endospores g^{-1} treatment consistently displayed the most prolonged period for fly emergence, and the lowest numbers for fly emergence. As such, it was considered the more effective of the two treatments tested. Generally, higher doses of biocontrol agents are associated with higher levels of pest antagonism (Fite et al. 2019). However, in previous studies with *B. velezensis* PHP1601 it was noted that the feeding behaviour of *Lucilia cuprina* larvae was negatively impacted at elevated cell concentrations resulting in larvae progressively moving away from the bioassay medium (Ramesar and Hunter 2023b). This was attributed to an accumulation of lipopeptide compounds, which may have reduced the palatability of the medium. Therefore, we hypothesise that when *B. velezensis* PHP1601 was applied to the pig manure slurry at the lower endospore concentration (10^5 endospores g^{-1}), the substrate medium was more palatable, allowing the larvae to feed for more extended periods resulting in a more effective larvicidal response. It was likely that after a specific threshold concentration of *B. velezensis* PHP1601 or lipopeptides was reached in the pig manure slurries, no further biocontrol benefit could be achieved from higher dosages. This finding is promising as it suggests that lower application dosages of *B. velezensis* PHP1601 are preferable for effective fly antagonism.

qPCR assays indicated that *B. velezensis* PHP1601 endospores germinated, grew and multiplied in the pig manure slurry, reaching $>10^8$ cells g^{-1} by the 16-d interval. This is promising because it showed that the strain could grow in a medium that accommodates its target for biocontrol, i.e. fly species (Tangtrakulwanich et al. 2015). It also indicates that strain PHP1601 was metabolically active and likely produced lipopeptide biosurfactants, which brought about the fly antagonism, as reported previously in controlled in vitro lab studies (Ramesar and Hunter 2023b). To confirm this, analytical methods for the detection and quantification of lipopeptide biosurfactants in environmental samples still need to be developed and evaluated in future studies.

REP-PCR profiling of colonies derived from endospores recovered from the pig manure

confirmed that they were clonally identical to PHP1601. This supported the notion that the fly antagonism could be attributed to the application of the *B. velezensis* PHP1601. Furthermore, the ability to recover endospores of *B. velezensis* PHP1601 allows it to be isolated and quantified, which is attractive from a population dynamics and environmental persistence point of view.

qPCR has been used as an efficient means of detecting and quantifying microbes in environmental sources, provided that the microbes are amenable to DNA extraction methods. Using species-specific PCR primers targeting the *Kdg*K gene, a qPCR method was developed that integrated a hydrolysis probe to detect and quantify *B. velezensis* in manure samples. The limits of detection and quantification was similar to those reported in the literature for *B. velezensis* qPCR studies (Zhang et al. 2018). *Bacillus velezensis* is a species commonly studied for its biocontrol potential against many phytopathogens; subsequently, the qPCR protocol was considered a valuable tool that could be used in other studies related to the quantification of *B. velezensis*. Future research would involve exploring means to efficiently extract DNA from *B. velezensis* endospores in environmental samples to allow for qPCR quantification.

Fly species that fell into the potential host range of *B. velezensis* PHP1601 included: *L. cuprina*, *D. bivittatus*, *S. ruficornis*, *C. megacephala*, *S. nudiseta* and *M. stabulans*. Strains of *S. nudiseta*, *C. megacephala*, *L. cuprina*, and *Sarcophaga* sp. are commonly attracted to the stench of faecal material and, as such, their presence in the trial was not unexpected (Nurita and Hassan 2013). These flies are considered nuisance pests and can pose severe threats to farming practices. *Lucilia cuprina* and *S. ruficornis* are both causative agents of myiasis or flystrike, which are significant threats to the livestock industry (Azevedo et al. 2015). These flies contribute to economic losses from reduced milk production from dairy farms, decreased animal and livestock fertility and reduced hide quality (Mukandiwa et al. 2012). *Dacus* spp. are recognised as common pests found in fruit farms in South Africa (Grové and de Beer 2014). Further economic loss is attributed to the cost of purchase and application of chemical pesticides, which are losing effectiveness in controlling these species due to insect resistance (Mukandiwa et al. 2012). Therefore, the ability of *B. velezensis* PHP1601 to control these fly species is promising and warrants that the strain undergoes further field trial evaluation. A comprehensive evaluation of its fly host range is also recommended.

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CHAPTER SIX: TOXICOLOGICAL ASSESSMENT OF *BACILLUS VELEZENSIS* PHP1601 BASED ON ZEBRAFISH EMBRYO TOXICITY ASSAYS AND GENOME MINING

6.1 Abstract

Bacillus velezensis PHP1601 is a promising fly biocontrol candidate that produces lipopeptide biosurfactants exhibiting larvicidal properties. In evaluating its biocontrol candidacy, a toxicological assessment of this strain and its bioactive metabolites was warranted. A zebrafish (*Danio rerio*) embryo toxicity (ZET) evaluation of biosurfactant compounds produced by PHP1601 was undertaken in conjunction with genome mining for pathogenic factors, toxin production and antibiotic resistance genes. Lipopeptide compounds were produced by culturing PHP1601 in Landy medium and harvested by acid precipitation and methanol extraction. These compounds were characterised by UPLC ESI-TOF MS and quantified using RP-HPLC. Surfactins and iturins were produced with yields of 122.68 mg l^{-1} and 168.10 mg l^{-1} respectively; whereas, fengycins were present at concentrations below the threshold for accurate quantification. From the zebrafish embryo toxicity assays, crude lipopeptide biosurfactants were characterised as being slightly toxic (LC_{50}: 22.77 µg ml^{-1}); however, they exhibited no developmental abnormalities in surviving embryos. Additionally, PHP1601 did not exhibit any growth nor biosurfactant production when incubated in river water or artificial freshwater medium, indicating that it was unlikely to cause an aquatic threat. ANI cluster analysis grouped PHP1601 in a clade distinct from recognised pathogenic strains of *Bacillus* spp, which verified its taxonomic grouping and highlighted its close association with recognised GRAS species. VFDB genome screening indicated that genes related to toxin production and pathogenic behaviour were absent. Several antimicrobial resistance genes (*tet*, *van*T, *van*Y, *qac*G, *qac*J, *bc*I, *clb*A) were detected using CARD that could, putatively, confer resistance towards antibiotics and disinfectants. Genes for metabolite production were screened for using AntiSMASH; and it indicated that the strain contained several complete gene clusters associated with various compounds of biocontrol significance such as bacillaene, macrolactin H, bacilysin, difficidin, bacillomycin D, fengycin and surfactin. The results of ZET assays and genome mining indicated that neither PHP1601 nor its metabolites pose a significant ecotoxicological threat in its application as a larvicidal biocontrol agent.

Keywords Zebrafish toxicology, biopesticide, lipopeptide biosurfactants, surfactin, genome mining

6.2 Introduction

Members of the *Bacillus subtilis* species complex have been perused as biocontrol candidates against various organisms, ranging from microbial phytopathogens to insect larvae (Zhu et al. 2020; Falqueto et al. 2021). This is largely attributed to their ability to produce bioactive lipopeptide biosurfactants such as surfactin, iturin and fengycin, and due to the fact that several species have achieved "generally regarded as safe" (GRAS) status (Devi et al. 2019; Quach et al. 2021). However, pathogenic or toxicological assessments are not commonly done despite being important considerations necessary to evaluate the risks associated with a biocontrol agent or biopesticide (Ongena and Jacques 2008; Chen et al. 2020; Falqueto et al. 2021).

The difficulties associated with toxicological studies include complex application procedures for ethical clearance, financial constraints for rearing model organisms (eg. mice or rabbits), operational costs for the bioassays and requiring long study periods (Nishimura et al. 2016; Horzmann and Freeman 2018). Consequently, the routine toxicological assessment of biocontrol candidates are not commonly performed. However, advancements in genome sequencing, bioinformatics and criteria for selecting and assessing model organisms to be used have improved the feasibility and practicality of evaluating the toxicological risk associated with a biocontrol agent or pesticide (Nishimura et al. 2016; Horzmann and Freeman 2018).

Zebrafish, *Danio rerio* (Cypriniformes: Cyprinidae), are considered as excellent model organisms for biomedical investigations, developmental toxicity and ecological toxicity (Nishimura et al. 2016; Zhang et al. 2020). The success of zebrafish as a model organism for toxicological assays can be attributed to several contributing factors. These include their developmental process, which bears significant comparability to mammals owing to the highly conserved nature of vertebrate development (Nishimura et al. 2016; Zhang et al. 2020). Additionally, zebrafish exhibit a high degree of genetic similarity with humans, enabling the evaluation of mutagenic effects (Falcão et al. 2018). Furthermore, zebrafish can be efficiently and economically reared in captivity, further establishing their suitability as a model organism in

this context (Nishimura et al. 2016; Horzmann and Freeman 2018; Zhang et al. 2020). Therefore, zebrafish are viable model organisms for the risk assessment of a biocontrol candidate.

Improvements in whole genome sequencing and genome mining have made it possible to assess the risks associated with a biocontrol candidate without the need for ethical clearance applications and the costs associated with them. Databases such as the Virulence Factor Database (Liu et al. 2022), the Comprehensive Antimicrobial Resistance Database (Jia et al. 2017), and AntiSMASH (Blin et al. 2021) are among the most common bioinformatic tools for the assessment of pathogenic factors, antimicrobial resistance genes and genes related to the production of secondary metabolites respectively. These databases are hosted online, globally available and have a simple user interface that encourages their adoption in genome mining applications. Therefore, the information contained in a biocontrol candidate's genome can be mined for risk factors to improve the design and supplement the results of in vitro toxicological assays (de Nies et al. 2021).

Bacillus velezensis PHP1601 is a member of the *B. subtilis* species complex that shows fly larvicidal activity due to the production of lipopeptide biosurfactants (Ramesar and Hunter 2023a, b). In order to determine the potential risk factors associated with PHP1601, zebrafish embryo toxicity assays of its larvicidal lipopeptide biosurfactants. Additional objectives included genome sequencing and mining for potential pathogenic factors, antimicrobial resistance genes and secondary metabolite production.

6.3 Methods and materials

6.3.1 Bacterial culture

Bacillus velezensis PHP1601, hereafter referred to as PHP1601, was obtained from Andermatt Plant Health Products (Strathdean Farm, Gowrie Avenue, Nottingham Road, KwaZulu-Natal, 3280, South Africa). The strain was routinely cultured on tryptone soy agar (TSA) for 18 h at 30°C or in tryptone soy broth (TSB) (Biolab, Merck, Germany) at 200 rpm for 18 h at 30°C.

6.3.2 Lipopeptide biosurfactant production

Lipopeptide biosurfactants were produced and extracted according to the method described by Ramesar and Hunter (2023b). Crude lipopeptide extract (CLP) was qualitatively assessed for biosurfactant activity using an oil displacement assay (Khondee et al. 2015).

6.3.3 Identification and quantification of lipopeptide biosurfactants produced by PHP1601

Lipopeptide species in the CLP extract of PHP 1601 were identified using ultra-performance liquid chromatography (UPLC) in conjunction with electron spray ionisation time of flight mass spectroscopy (ESI TOF MS). Methanol solubilised CLP extracts or lipopeptide standards (100 µg ml-1) were loaded onto a Waters Acquity BEH C18 column (2.1 × 100 mm, particle size of 1.7 µm, 35°C) and fractionated under UPLC conditions (Waters Acquity, Milford, USA). Chromatography was performed using an 80% acetonitrile and 20% 0.1% (v v^{-1}) acetic acid solvent system for 40 min with a flow rate of 0.8 ml min^{-1}. Compounds in the samples were ionised (positive mode, ESI+) at a constant capillary voltage of 5 MV and a cone voltage of 35 V. A desolvation temperature of 350°C was maintained with a constant desolvation gas injection rate of 400 l h^{-1}. Eluted compounds were detected, and their molecular weights were determined by ESI-TOF MS. Compounds detected in the 900 – 2000 m/z range were identified based on corresponding mass data from iturin and surfactin standards (Sigma-Merck, Germany) and from mass data presented in the literature (Koumoutsi et al. 2004; Hunter 2016; Adu and Hunter 2021).

Lipopeptide biosurfactants in CLP extracts were quantified using a reverse-phase high-performance liquid chromatography (RP-HPLC) UV-vis quantification method (Geissler et al. 2017). The RP-HPLC apparatus (Shimadzu HPLC, Shimadzu Corporation, Japan) was equipped with a UV-vis diode array detector (SPD-M20A) and operated with LCsolution software (v. 1.25) (LabSolutions, Shimadzu Corporation, Japan). Chromatography was performed at 25°C on a C18 reverse-phase column (2.6 µm, 100 × 4.6 mm, 100 Å, Kinetex, Phenomex, USA). An isocratic method was applied with 80% acetonitrile and 20% 3.8 mM trifluoroacetic acid for 30 min with a flow rate of 0.8 ml min^{-1}. Surfactin and iturin standards were prepared (5 – 500 µg

ml^{-1}) in HPLC grade methanol to facilitate the quantification of lipopeptide families present in 100 µg ml^{-1} CLP solution. Samples and biosurfactant standards were injected as 30 µl volumes for RP-HPLC analysis, and the eluted biosurfactant peaks were detected at 210 nm. All standards and samples were prepared independently and run as 3 replicates. Peaks eluted between 1.5 – 2.5 min, 4 – 11 min and 11 – 30 min were assigned as iturin, surfactin and fengycin isoforms respectively (Geissler et al. 2017).

6.3.4 Zebrafish embryo toxicity assays

Zebrafish (*Danio rerio*) embryo toxicity (ZET) assays were conducted at the University of KwaZulu Natal Zebrafish Unit (KwaZulu Natal, South Africa), which also catered for the rearing of zebrafish and providing embryos for ZET assays. CLP treatments (0, 0.1, 1, 5, 10, 20, 50 ug ml^{-1}) tested were prepared in E3 medium (5 mM NaCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ $7H_2O$, 0.17 mM KCl) from phosphate-buffered (116 mM Na_2HPO_4 $7H_2O$, 84 mM Na_2HPO_4 $7H_2O$ pH 7.00) CLP (2% w v^{-1}) stock solution. ZET bioassays were conducted in 24-well culture plates in which 10 embryos at the 3 h post fertilisation (hpf) development stage were transferred into wells containing 500 µl of each treatment. The plates were incubated at a temperature of 28 ± 0.5°C, an atmospheric humidity of 45%, and 10:14 light-dark cycle for 96 h. At 24 h intervals, the embryos were transferred into new wells, and the bioassay medium was replaced with fresh E3 medium amended with the corresponding CLP concentration to reduce the influence of waste accumulation or evaporation of the bioassay medium. Observations for embryo mortality or developmental deformities were made at the 3, 24, 48, 72 and 96 hpf interval. ZET trials were performed as 3 independent replicates using embryos acquired from 3 different breeding sets. All aspects of zebrafish rearing and ZET assays were conducted in compliance with the University of KwaZulu Natal Animal Research Ethics Committee (reference number: AREC/029/019).

PHP1601 was cultured in E3 to determine whether it could grow and produce similar titers of lipopeptide biosurfactants associated with zebrafish mortality. Cells from an 18 h TSB culture were washed twice by centrifugation (10000 × g for 10 min) and resuspended in sterile E3 medium. The concentration of cells were determined using a bacterial counting chamber (Modified Thoma, Marienfield, Germany) and 1 ml culture suspension was used to inoculate 99 ml of E3 medium to a starting concentration of 10^5 cells ml^{-1}. The medium was shaker incubated

for 96 h at 28°C (100 rpm). Similarly, E3 medium was inoculated with autoclave-killed cells as a control. At 24 h intervals, 1 ml aliquots were taken, and the total cell concentration was determined by sequentially diluting the sample in PBS to 10^{-1} – 10^{-6} and spread plating 100 µl from each dilution on TSA plates in triplicate. Endospores were enumerated by heating the dilutions at 80°C for 20 min before plating. Both treatments and controls were tested as 3 independent replicates. A similar investigation was performed using autoclaved water (pH: 6.33, suspended solids: 31.26 ± 3.09 mg l^{-1}) from a nearby river (29°57'16" S, 30°35'46" E) as a representative source of natural freshwater.

6.3.5 DNA extraction

Genomic DNA of PHP1601 was extracted from an 18 h TSB culture using the PureLink™ Genomic DNA Mini kit (Invitrogen, Thermo Fisher Scientific, USA) as per the manufacturer's instructions for Gram-positive bacteria. Genomic DNA was eluted with nuclease-free water and analysed by UV spectroscopy (Nanodrop, Thermo Fisher Scientific, USA) and agarose (1% w v^{-1}) gel electrophoresis at 80V for 100 min using a TAE buffer system (40 mM tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3).

6.3.6 Whole genome sequencing and assembly

The genome sequencing of PHP1601 was performed using the Pacific Biosciences (PacBio, USA) Sequel sequencer at Inqaba Biotec Laboratories (Pretoria, South Africa). Briefly, the genomic DNA was quantified using Qubit HS 2.0 fluorometer (Life Technologies, USA) and qualified on TapeStation (Agilent, USA). The average genome fragment size was ~7-10 kbp long as observed on the TapeStation; therefore, shearing was not required. Library preparation was carried out using the SMRTBell Express template Prep kit 3.0, followed by sequencing using the SMRT Cell 1M (PacBio, USA). HiFi sequence data was assembled on the PacBio SMRT Link software (v. 11.0.0) with the Microbial Assembly tool at default parameters. The assembled genome was submitted to GenBank (https://www.ncbi.nlm.nih.gov/genbank/) , followed by automatic annotation using the NCBI Prokaryotic Genome Annotation Pipeline (v. 6.1).

6.3.7 Bioinformatic assessments

The genome of PHP1601 was compared with reference and type *Bacillus* strains using OrthoANIu average nucleotide identity (ANI) software (Yoon et al. 2017) to construct a similarity matrix, which, was used to construct a dendrogram using the R – Shipunov package (v 1.17) (Shipunov et al. 2023). The Manhattan model was used to determine the level of dissimilarity between ANI comparisons, followed by clustering using the Complete agglomeration method. A consensus dendrogram tree was constructed from 1000 bootstrapped replicates.

Toxin production and bacterial virulence factors were screened for using the Virulence Factor Database (VFDB) (http://www.mgc.ac.cn/VFs/). The Comprehensive Antimicrobial Resistance Database (CARD) (v. 3.2.5) (https://card.mcmaster.ca/analyze/rgi) was used to screen the genome for antimicrobial resistance (AMR) genes using the Resistance Gene Identifier (RGI) (v. 6.0.0) algorithm set to strict and high-quality AMR gene detection. AntiSMASH (v. 6.0) (https://antismash.secondarymetabolites.org/) was used to detect secondary metabolite production using the strict and loose query level and to detect well-defined gene clusters, of which, only complete gene clusters identified by MIBiG gene cluster analysis were reported.

6.3.8 Statistical analysis

All statistical analysis was performed using R (v 4.2.1) (R Core Team 2022) through the RStudio integrated development environment (v 2022.07.1, build 554). All data sets were assessed for normality using the Shapiro-Wilks test before proceeding with statistical analysis. A one-way analysis of variance followed by the Tukey HSD test was performed to detect significant differences ($P < 0.05$) between the treatments and controls. All data were reported as the mean ± standard error (SE).

6.4 Results

6.4.1 Quantification and identification of lipopeptide biosurfactants produced by PHP1601

PHP1601 produced 635.54 ± 16.21 mg l^{-1} of CLP when cultured in Landy medium for 24 h. Aliquots (5 µl, 100 µg ml^{-1}) of CLP produced a zone of oil displacement that was ≥ the area of the petri dish (~ 56.48 cm^2) which indicated that biosurfactant compounds were extracted.

UPLC of the CLP produced several peaks that eluted from 0.75 – 13 min, which were identified using ESI-TOF MS to contain homologues of surfactin and iturin (Appendix D, Fig. D1). Most of these compounds were detected in their protonated state, [M+H]+, or to a lesser extent, in association with sodium adducts, [M+Na]+. Isoforms of fengycin were detected, but in relatively lower abundance. Therefore, the presence of lipopeptide biosurfactants was confirmed and indicated that quantification by RP-HPLC could proceed.

RP-HPLC was used to quantify surfactins and iturins present in the CLP of PHP1601 (Fig. 6.1). Peaks corresponding to fengycins were not detected and considered to be below the limits of detection using the Shimadzu HPLC system. Standards were used to correlate the total peak area to the concentration (mg ml^{-1}) of surfactin ($y = 14492x$; $R^2 = 0.998$) or iturin ($y = 31396x + 10^6$; $R^2 = 0.995$) respectively. The RP-HPLC chromatogram of the CLP from *B. velezensis* PHP1601 showed a single iturin-associated peak (1 – 2 min) and 8 surfactin-associated peaks (3.5 – 11 min) (Fig. 6.1). The amount of surfactin and iturin present in the CLP of *B. velezensis* PHP1601 was calculated to be 19.32 ± 1.14% and 26.02 ± 0.35% respectively, constituting ~ 45.34% of the mass of the CLP extract. Therefore, within a 24 h culture period in Landy medium, the strain produced 122.79 mg l^{-1} of surfactin and 165.37 mg l^{-1} of iturin.

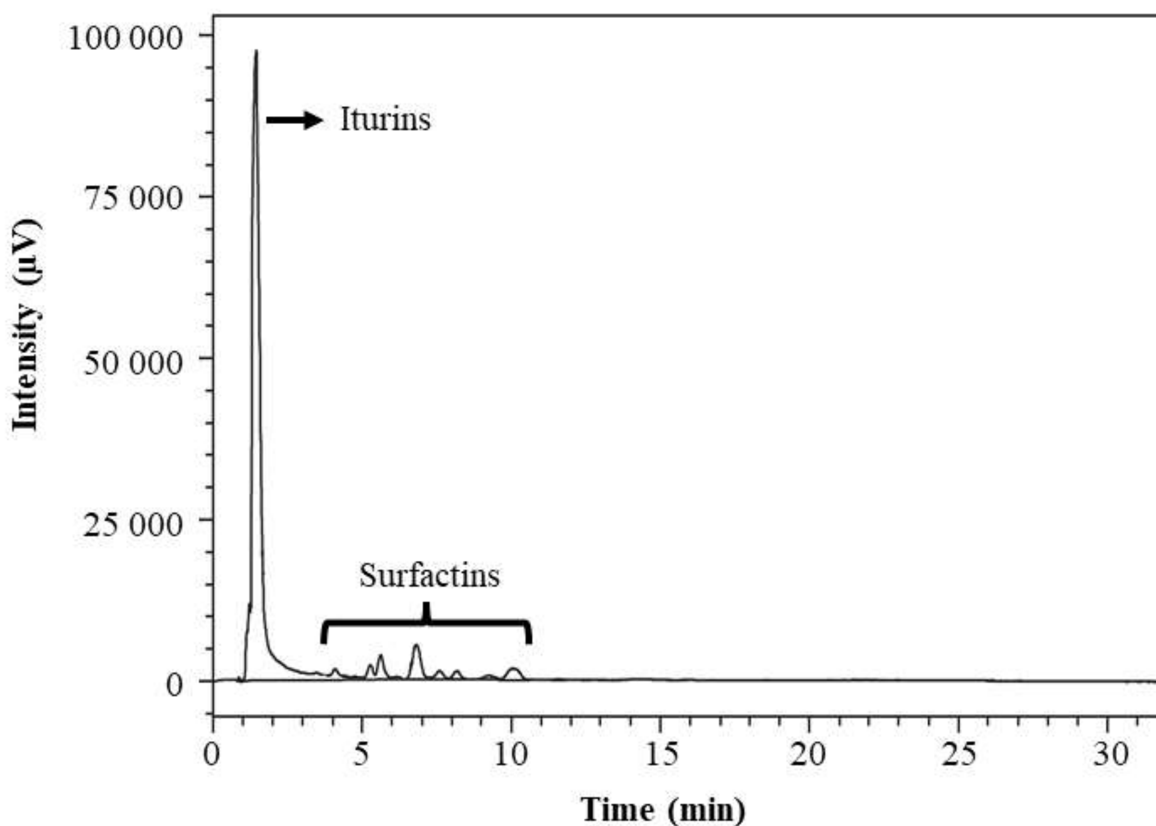

Fig. 6.1 Representative RP HPLC chromatogram of lipopeptide biosurfactant compounds present in the CLP of PHP1601. The CLP of PHP1601 was dissolved in methanol and run through RP-HPLC at 100 µg mL^{-1}.

6.4.2 ZET bioassays

The ZET assays were initially run testing 200, 100, 50, 20, 10, 5, 1, 0.1 and 0 µg ml^{-1} of CLP to determine the presumptive lethal concentration for 50% mortality (LC_{50}), which would be used as the highest concentration tested in ZET assays. This was done following AREC ethical guideline to limit unnecessary use and euthanisation of embryos. Subsequently, the concentration range tested was 0.1 – 50 µg ml^{-1} of CLP (Fig. 6.2).

ZET assays indicated that embryo mortality (53.33 ± 3.33%) was only observed at the 24 hpf interval in the 50 µg ml^{-1} CLP treatment. Accordingly, the 50 µg ml^{-1} CLP treatment was ascribed as the LC_{50} at 24 hpf and an extrapolated biosurfactant LC_{50} of 22.77 µg ml^{-1} was

inferred from its RP-HPLC compositional analysis. Over the ensuing observation intervals, no further mortality were observed up to the final interval at 96 hpf.

The physical development of the embryos was monitored throughout the ZET assays and all surviving embryos showed no developmental hindrances that could be attributed to the CLP treatments. All surviving embryos from the 24 hpf interval were able to develop into zebrafish larvae that were indistinguishable from those in the CLP-free control (Fig. 6.3). This indicated that the developmental stages were not negatively impacted based on visual observations of development (viz., developed head, circular eyes, pigmentation, beating heart, straightened tails, spontaneous movement).

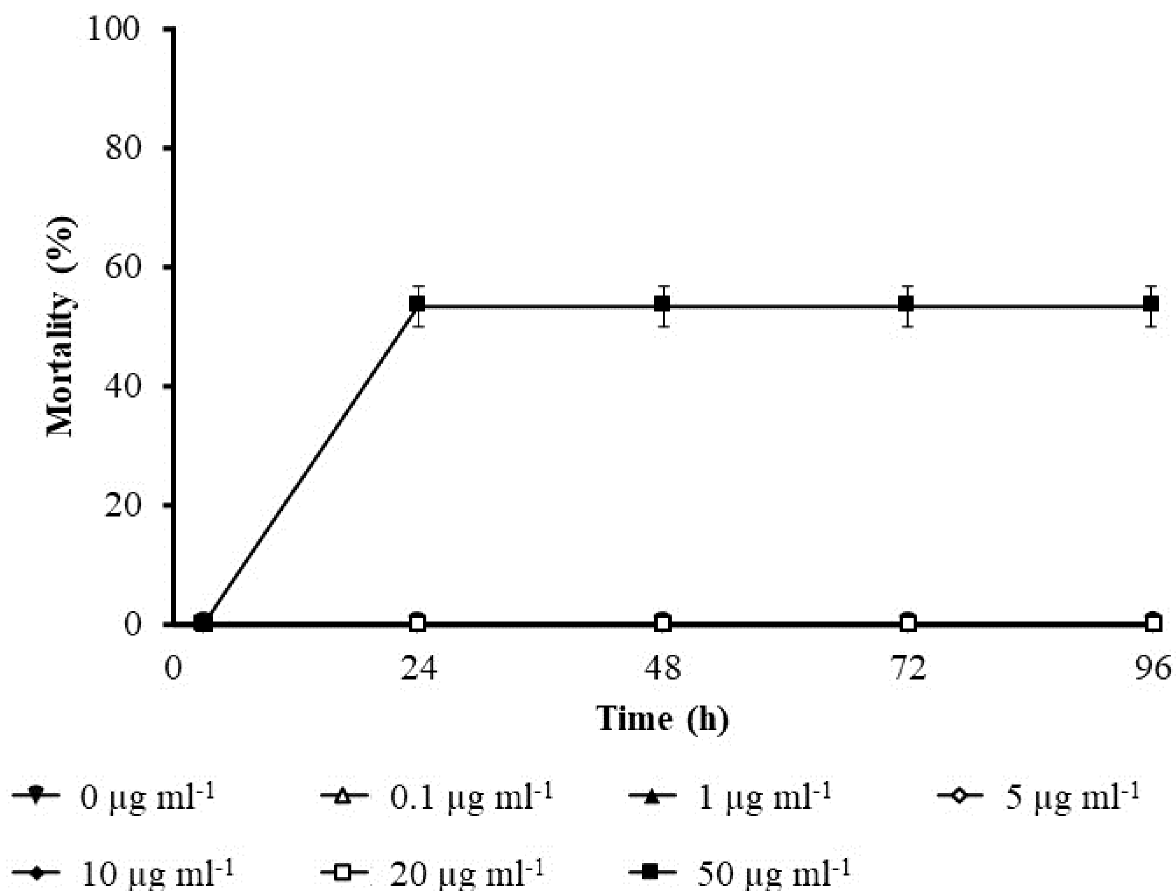

Fig. 6.2 Mortality (%) of zebrafish exposed to CLP extracts of PHP1601 for 96 h. Data from an 3 ZET assays testing 10 embryos per treatment were reported as the average (± SE) percentage of embryo mortality.

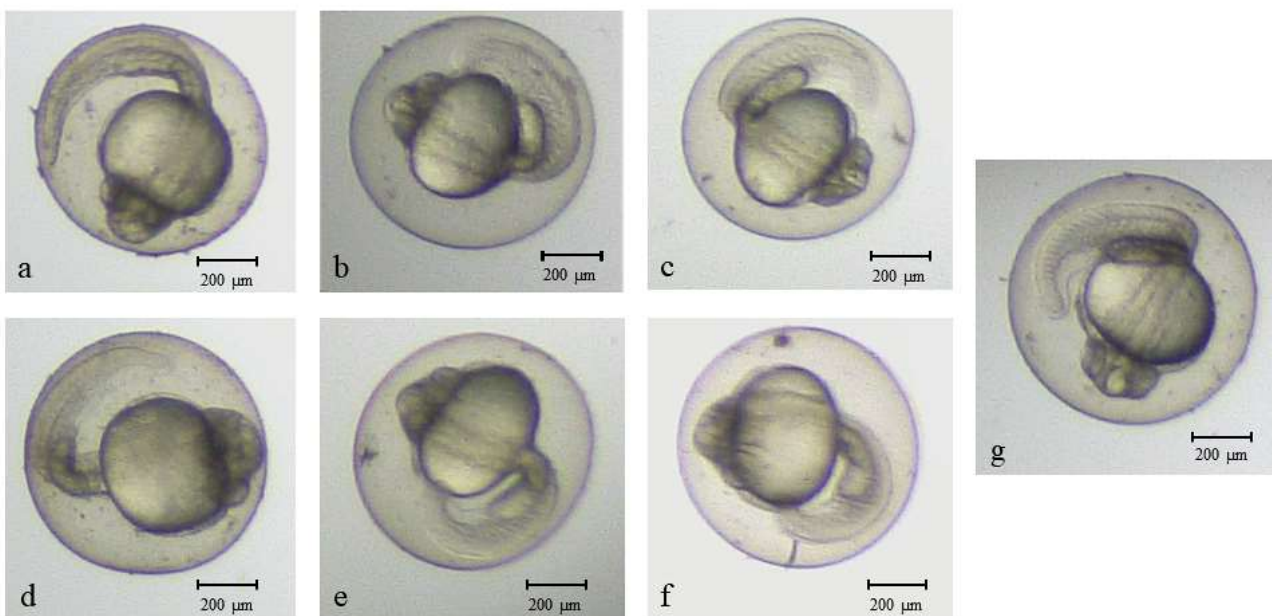

Fig. 6.3 Zebrafish embryos exposed to CLP extracts of PHP601 for 24 h. Images of zebrafish embryos were taken of the survivors a the 24 hpf interval which were exposed to 50, 20, 10, 5, 1, 0.1 and 0 µg mL^{-1} of CLP extract (images: a - g) of *B. velezensis* PHP1601. The scale bar corelated size in 200 µm intervals.

6.4.3 Growth of PHP1601 in freshwater media

The growth of PHP1601 in E3 medium and river water medium was assessed under similar conditions to ZET assays to determine whether toxic levels of lipopeptide could be produced (Fig. 6.4). This showed that PHP1601 was unable to grow in freshwater media, as shown by a significant decrease ($F_{7, 16} = 9.023$, $P = 0.004$) in total cell counts, resulting in total sporulation from 48 h in E3 medium and river water ($F_{7, 16} = 50.60$, $P < 0.001$). The level of sporulation achieved in E3 medium and river water were $3.33 \pm 0.17 \times 10^3$ and $2.35 \pm 0.11 \times 10^3$ endospores ml^{-1} respectively. This indicated that 1.95 – 2.78% of the cells were able to sporulate into viable endospores. Additionally, no indication of lipopeptide biosurfactant activity was detected by oil displacement assays nor harvested by acid precipitation and methanol extraction (data not shown).

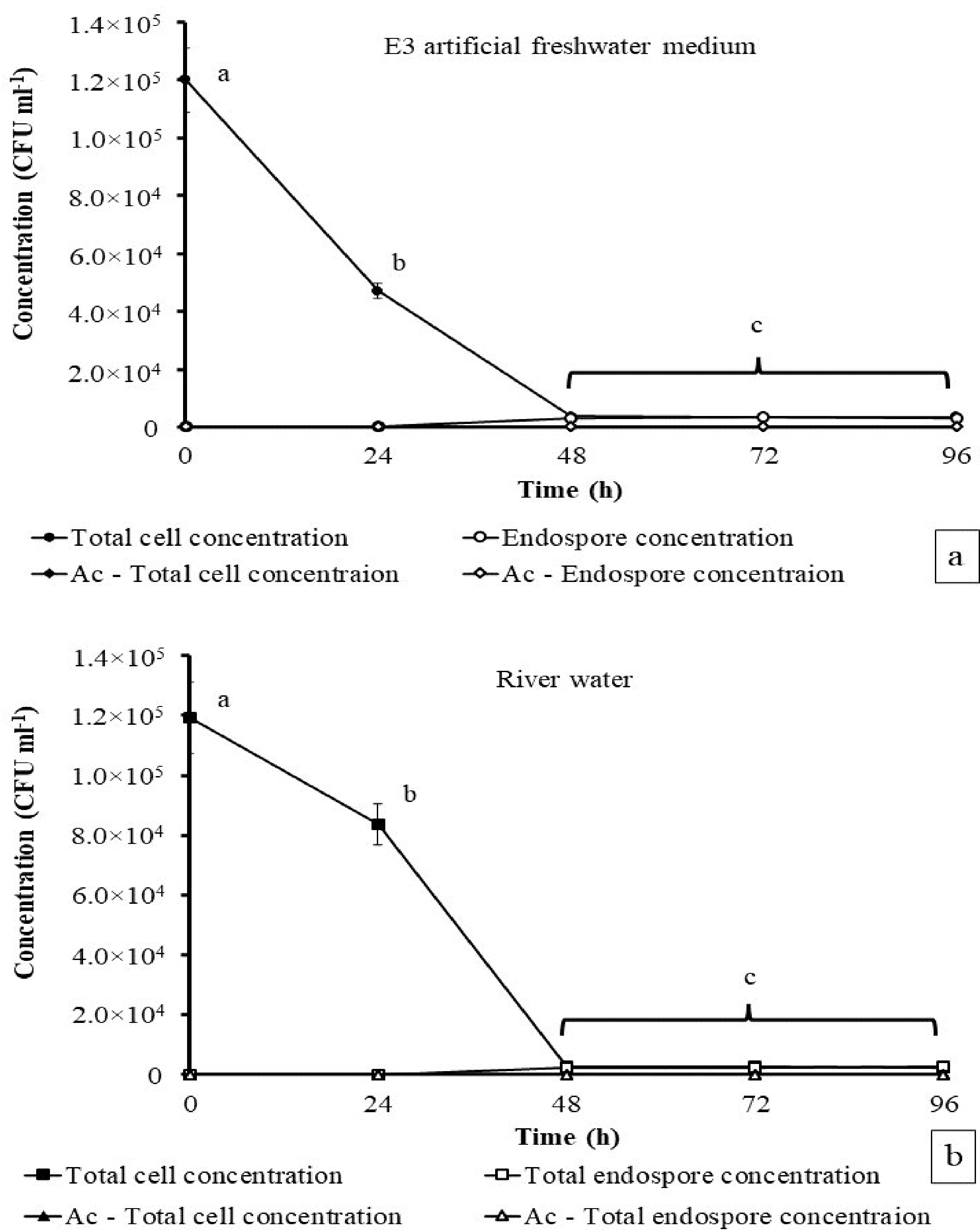

Fig. 6.4 Survival of *B. velezensis* PHP1601 in river water and artificial freshwater. Autoclave killed cells (Ac) were included as dead cell controls. All experiments were independently repeated 3 times. letters represent significant differences in the concentration of PHP1601. Data points represents the average concentration of cells or endospores and colony forming units (CFU) ± SE.

6.4.4 Genome description

Whole genome sequencing of PHP1601 produced 237 037 reads representing × 485 genome coverage. The final genome was 3 929 782 bp in size and had a G+C content of 46.50%. A total of 3 891 genes were identified using PGAP, of which 3 670 accounted for protein-coding regions, 118 for RNA-related genes, and 103 were pseudogenes. The genome of PHP1601 was submitted to GenBank and was assigned the following accession number CP100391.

6.4.5 Average nucleotide identity (ANI) facilitated species clustering of PHP1601

ANI values indicate the similarity of the genome sequence of PHP1601 was similar (98.87± 0.57%) to strains of *B. velezensis*, which substantiated its affiliation to the *B. velezensis* species, as compared to, strains of *B. amyloliquefaciens* (94.02 ± 0.03%), *B. subtilis* (77.04 ± 0.06%) and representative pathogenic strains of *B. anthracis* (66.77 ± 0.06%), *B. cereus* (66.57 ± 0.04%), *B. thuringiensis* (66.47 ± 0.03%). Furthermore, cluster analysis of the ANI similarity matrix grouped PHP1601 within a *B. subtilis sensu lato* clade and was positioned on a *B. velezensis* homogenous branch 100% of the bootstrapped analysis (Fig. 6.5). This confirms that PHP1601 was correctly assigned as a strain of *B. velezensis* and is distinct from commonly associated pathogenic *Bacillus* spp.

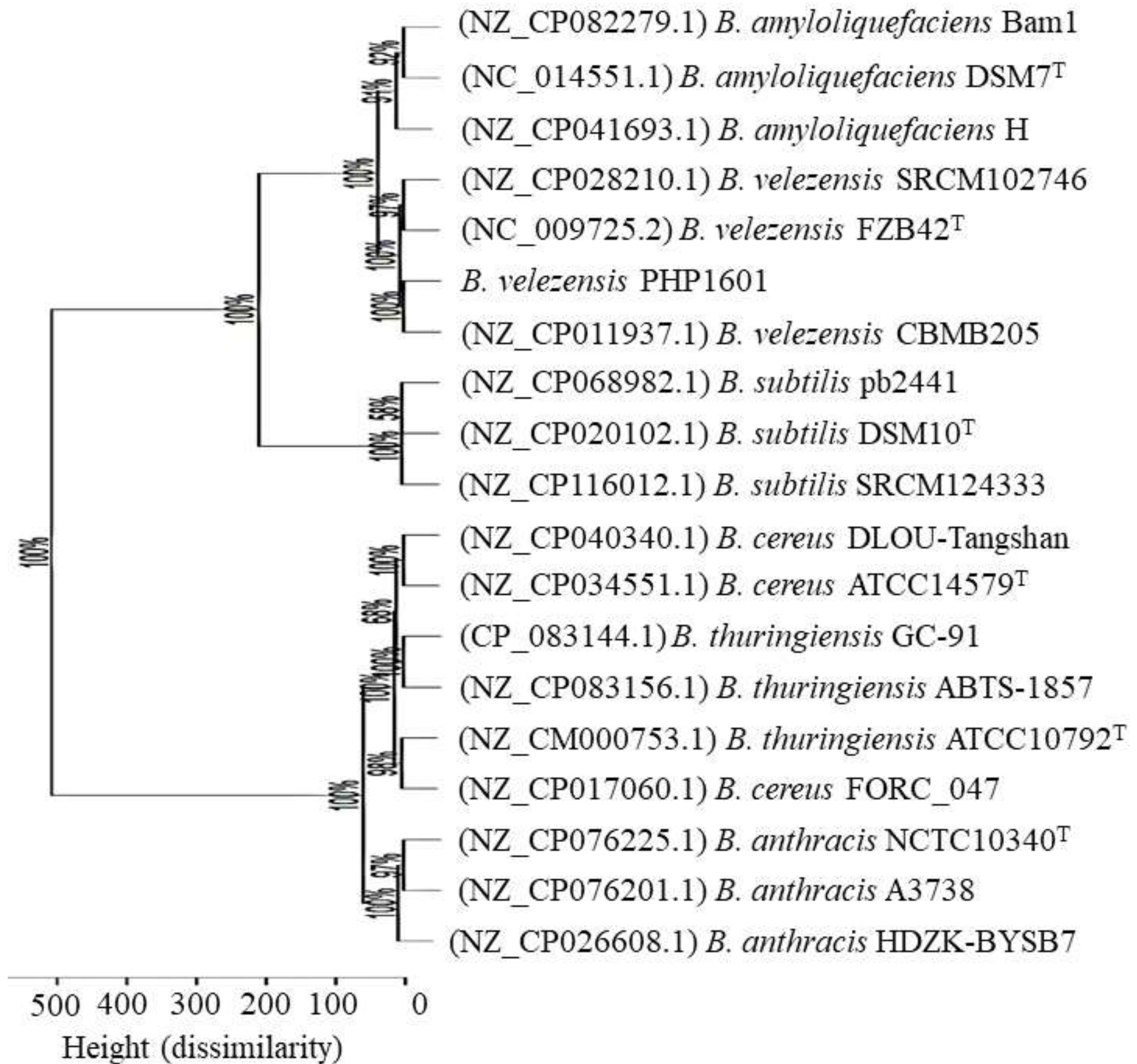

Fig. 6.5 Dendogram of whole genome ANI similarity of *B. velezensis* PHP1601 compared to representative *Bacillus* spp. ANI values (%) were determined using OrthoANIu whereby the core genomes of reference and type (^{T}) strains of *Bacillus* spp. from GenBank and *B. velezensis* PHP1601 were compared. The level of ANI dissimilarity (%) was transformed to distance values using the Manhattan model, and then a dendogram was constructed from a bootstrap consensus of 1000 replicates using the Complete clustering model. Percentages above nodes represent the frequency (%) the branches were clustered together.

6.4.6 Genome mining for toxin production and pathogenicity

The genome of PHP1601 was screened using VFDB to detect potential genes related to toxin production. This indicated that PHP1601 was not heavily invested in toxin production. Specifically, it lacked genes for the production of Anthrax toxin (*cya*, *lef*, *pag*A), classes of hemolysins (*hly*I, *hly*II, *hly*IV), vegetative insecticidal proteins (*vip*), *B. thuringiensis* toxins (Bt toxins: *cry* or *cyt*), cereulide (*ces*A-*ces*D, *ces*H, *ces*P, *ces*T), certhrax (*cer*) and *Bacillus* associated hemolytic (*nbl*) and non-hemolytic (*nhe*) enterotoxins. However, a gene encoding for a class 3 hemolysin (*hly*III) was detected.

VFDB also screened for genes related to virulence factors which are associated with pathogenic *Bacillus* spp. (Table 6.1). This indicated that the strain does not have recognised genes to suppress or regulate immune responses, regulation of virulence factors or toxin-related secretory systems; however, genes related to the production of monomers involved in the production of an extracellular capsule associated with immune evasion, genes involved in surface adhesion, mineral acquisition and oxygenic stress adaptation were detected. The presence of these genes does not substantiate that the strain is capable of attacking the hosts immune system nor initiating pathogenic behaviour; subsequently, the strain does not appear to possess sufficient virulence-related genes for it to be considered pathogenic. The location genes predicted by the VFDB are provided in Appendix D (Table D1).

Table 6.1 Virulence factors detected in the genome of *B. velezensis* PHP1601

Virulence class	Virulence factor	Detected genes
Adherence	Biofilm surface layer protein A (*bsl*A)	-
	Fibronectin-binding protein (*fbp*A)	*fbp*A
	Lipoprotein-specific signal peptidase II (*lsp*A)	*lsp*A
Virulent enzymes	Immune inhibitor A metalloproteinase (*inh*A)	-
	Phosphatidylcholine-preferring phospholipase C (*plc*A)	-
	Phosphatidylinositol-specific phospholipase C (*pip*LC)	-
	Sphingomyelinase (*sph*)	-
Immune system evasion	*B. cereus* exo-polysaccharides (*bsp*A - *bsp*H, *bsp*X)	-
	Hyaluronic acid capsule (*has*A - *has*C)	*has*C
	Polyglutamic acid capsule (*cap*A - *cap*E)	*cap*A - *cap*D
Mineral acquisition	Bacillibactin (*dhb*A - *dhb*F)	*dhb*A - *dhb*F
	heme-acquisition leucine-rich repeat protein (*hal*)	-
	Iron regulated leucine-rich surface protein (*ils*A)	-
	Copper - Cation transport protein (*ctp*V)	*ctp*V
	Petrobactin (*asb*A - *asb*F)	-
Regulation	Acyl carrier protein (*acp*A, *acp*B)	-
	B. anthracis toxin expression regulator (*atx*A)	-
	B. anthracis protective antigen protein (pagR-pXO1, pXO2)	-
	Quorm sensing regulators (*pap*R, *plc*R)	-
Secretory systems	Type VII (*ess*C, *esx*B, *esx*L)	-
Stress adaptation	Catalase (*kat*A)	*kat*A

The genome of PHP1601 was screened for genes associated with virulence and pathogenicity deposited in the VFDB.

6.4.7 Genome mining for AMR genes using CARD

Several potential AMR genes were detected in the genome of PHP1601 using CARD (Table 6.2). Putative AMR genes against tetracycline (*tet*), vancomycin (*van*T, *van*Y), cephalosporin (*bcl*), chloramphenicol-florfenicol (*clb*A) and quaternary ammonium compound-resistance compounds (*qac*G, *qac*J) where detected in the genome of PHP1601. Interestingly, the *clb*A gene

encoding, a 23S rRNA methyltransferase, was the only gene detected with a high level of gene identity (99.43%) and coverage (100%), accordingly, has the highest likelihood of being correctly identified in the genome of PHP1601. Locations of the AMR genes are provided in Appendix D, Table D2.

Table 6.2 AMR gene profile of PHP1601 determined by CARD analysis

AMR gene	AMR gene family	Drug class	Resistance mechanism	Sequence identity (%)	Sequence length (%)
tet	major facilitator superfamily	tetracycline antibiotic	antibiotic efflux	75.49	100
*van*T	glycopeptide resistance gene	vancomycin antibiotic	antibiotic target alteration	33.96	55.2
*van*T	glycopeptide resistance gene	vancomycin antibiotic	antibiotic target alteration	34.79	54.63
*van*Y	glycopeptide resistance gene	vancomycin antibiotic	antibiotic target alteration	34.87	101.49
*qac*G	small multidrug resistance family	disinfecting agents	antibiotic efflux	37.25	97.2
*qac*G	small multidrug resistance family	disinfecting agents	antibiotic efflux	42.45	113.08
*qac*J	small multidrug resistance family	disinfecting agents	antibiotic efflux	37.37	97.2
*qac*J	small multidrug resistance family	disinfecting agents	antibiotic efflux	44.9	109.35
*bc*I	*B. cereus* beta-lactamase	cephalosporin, carbapenem antibiotics	antibiotic inactivation	63.07	99.35
*clb*A	*cfr* 23S ribosomal RNA methyltransferase	lincosamide, streptogramin, oxazolidinone, phenicol, pleuromutilin antibiotic	antibiotic target alteration	99.43	100

Detected AMR genes in the genome of PHP1601 were ascribed sequence identity and length values (%) based on reference ARM genes present in the Comprehensive Antibiotic Database (date accessed: 7 November 2022). Detected AMR gene families included

6.4.8 Genome screening for metabolite production using AntiSMASH

AntiSMASH was used to determine complete gene clusters for metabolite production. This indicated that PHP1601 contained complete gene clusters for the production of 2 antimicrobial compounds (bacilysin and difficidin), 3 lipopeptide biosurfactants families (bacillomycin D, fengycin, surfactin) and polyketides compounds (bacillaene, macrolactin H) (Table 6.3). The location of predicted metabolite gene clusters are provided in Appendix D, Table D3.

Table 6.3 Gene clusters identified for secondary metabolite production in the genome of *B. velezensis* PHP1601 by AntiSMASH genome mining

Gene cluster	Type	Genes
Bacilysin	Antibiotic	*ywf*A, *ywf*G, *bac*A-*bac*E
Difficidin	Antibiotic	*dif*A-*dif*O
Bacillomycin D	Biosurfactant	*ynf*F, *xyn*D, *bmy*A-*bmy*D, *xyj*C, *xyj*F,*sco*A, *aco*B
Fengycin	Biosurfactant	*yng*E-*yng*L, *fen*A-*fen*E, *dac*C
Surfactin	Biosurfactant	*yci*C, *yx*01,*yx*02, *yck*C-*yck*D, *yck*I, *yck*J, *nin*, *nuc*, *hxl*A, *hxl*B, *hxl*R, *srf*AA-*srf*AD,*srf*P, *com*S, *aat*, *ycx*C, *ycx*D, *ycz*E
Bacillaene	Polyketide	*bae*B-*bae*E, *bae*G-*bae*N, *bae*S, *bae*R
Macrolactin H	Polyketide	*pks*2A-*pks*2I, *pdh*A

Genes coding for metabolite clusters were compared to reference genes present in AntiSMASH (date accessed: 15 August 2022).

6.5 Discussion

Bacillus velezensis PHP1601 is a strain that shows effective control of fly species through the fly larvicidal effect of its CLP extracts (Ramesar and Hunter 2023a, b). Subsequently, evaluating potential risk factors associated with this strain and its CLP extracts were necessary steps in pursuing its candidacy as a biocontrol agent. Toxicity associated with its larvicidal lipopeptide biosurfactants was assessed through ZET bioassays, and genome mining was used to screen for potential virulence factors that were not innately apparent.

CLP extracts of PHP1601 contained lipopeptide biosurfactant species predominantly of surfactin and iturin. Fengycins were detected by UPLC ESI-TOF MS; however, in relatively lower

amounts that were below limits of detection and quantification by RP-HPLC. PHP1601 produced 635.54 mg l^{-1} CLP of which 122.79 mg l^{-1} was composed of surfactins and 165.37 mg l^{-1} of iturins. Compared to lipopeptide production levels reported in the literature for members of the *B. subtilis* species complex, PHP1601 was regarded as a high-yielding lipopeptide-producing strain (Sarwar et al. 2018; Ayed et al. 2019; Li et al. 2019). Strains that produce elevated amounts of lipopeptide biosurfactant have been shown to be more effective biocontrol agents (Sarwar et al. 2018). The production of elevated levels of lipopeptide biosurfactants substantiated the need for ecotoxicological assessments.

ZET assays indicated that 50 µg ml^{-1} CLP treatment, corresponding to 22.77 µg ml^{-1} of lipopeptide biosurfactant, resulted in ~ 50% embryo mortality at the 24 hpf interval and exhibited no subsequent mortality. Additionally, no developmental abnormalities were observed till the final observation interval at 96 hpf. The results of the ZET assays were accepted as the survivability of embryos in the control group was 100% at all intervals tested and greater than the minimum required threshold (90%) as per the OECD guidelines (Jin et al. 2018; Xu et al. 2022). In accordance with the United States Environmental Protection Agency classification, the toxicity of a pesticide is categorised as highly toxic, moderately toxic or slightly toxic if its LC_{50} value ranges between 0.1 - 1 µg ml^{-1}, 1 - 10 µg ml^{-1} and 10 – 100 µg ml^{-1} respectively (Jin et al. 2018). Considering that the strain could not grow and produce lipopeptide biosurfactants in the artificial freshwater E3 medium, it is unlikely that PHP1601 poses a significant threat to the aquatic system. The LC_{50} value of bacillomycin D, an iturin class biosurfactant, purified from CLP extracts of *B. amyloliquefaciens* HAB-2 (22.2 µg ml^{-1}) was similar to the corrected LC_{50} of PHP1601 (Jin et al. 2018). This substantiated that mortality observed in the ZET assays was due to the presence of lipopeptide biosurfactants and not contributed by other metabolites in its CLP extract. However, this identifies a need for ZET evaluation of purified surfactin, iturin and fengycin biosurfactants as well as other Bacillus-associated metabolites such as difficidin, bacilysin and polyketides.

Ecotoxicological assays on the non-target microcrustacean, *Daphnia magna* (Straus) (Anomopoda: Daphniidae), and freshwater shrimp, *Dendrocephalus brasiliensis* (Daday) (Anostraca: Thamnocephalidae), indicated that surfactin, fengycin and iturin are > 6.7 times less toxic as compared to chemical surfactants such as sodium dodecyl sulphate and Triton-X 100

based on LC_{50} comparisons (Santos et al. 2019). Surfactins are considered one of the most potent lipopeptide families due to their membrane disruptive activity and ability to decrease surface tension (Ongena and Jacques 2008; Seydlová and Svobodová 2008). Toxicological studies using mice determined that the LC_{50} (at 14 d) for surfactin C was greater than 2500 mg kg^{-1} mouse body weight (Park et al, 2006). According to the Hodge and Sterner toxicity scale, this classifies surfactin C as a slightly toxic substance since its LC_{50} value is within the 500 – 5000 mg kg^{-1} corporal weight range (Hodge and Sterner 1949). Subsequently, the findings of this study complement the notion that lipopeptide biosurfactants are not significant ecotoxicological threats.

Bioinformatic assessments of the genome of PHP1601 were a valuable tool in screening for additional risk factors. ANI cluster analysis confirmed that the strain was correctly assigned to its taxon and was distinct from representative pathogenic *Bacillus* species. Species of *B. subtilis* species complex have achieved "generally regarded as safe" (GRAS) status and are not recognised as being innately pathogenic (Devi et al. 2019; Quach et al. 2021a). Strains of species that have achieved GRAS status are more readily accepted during the regulatory and registration process of a biocontrol agent. This is contrasted by strains of the *B. cereus* species complex, which pose a significant threat to humans and animals, according to the United States Federal Select Agent Program (Baldwin 2020).

Genome screening using VFDB indicated that PHP1601 lacked significant genes for toxin production. Genes coding for anthrax toxins, certhrax, and non/hemolytic enterotoxins are produced by pathogenic strains of *B. anthracis* and *B. cereus* (Baldwin 2020; Amoutzias et al. 2022). Additionally, PHP1601 lacked the genes for the production of the insecticidal crystalline Bt toxins (cry and cyt). These genes are generally not found in the genomes of members of the *B. subtilis sensu lato* group; however, it supports that they were not acquired by horizontal gene transfer. A gene for a class 3 hemolysin was detected; however, this gene is not an independent determinant for hemolysis, nor has it been documented as a contributing virulence factor in *B. velezensis* strains (Jeong et al. 2017; Na et al. 2022). Contrastingly, pathogenic strains of *B. cereus* have been reported to have multiple copies of different types of hemolysins, complementing their hemolytic activity and pathogenic behaviour (Zhao et al. 2021). Hence, it is

unlikely that PHP1601 can exhibit hemolysis attributed to the production of hemolysins or other *Bacillus*-associated toxins.

The VFDB additionally provided insight into potential virulence-related genes present in the genome of PHP1601. This indicated that the strain does not possess genes related to suppressing immune responses, regulation of virulence factors or toxin-related secretory systems, which are present among various pathogens (Yuan et al. 2020; Zhao et al. 2021). This substantiates that PHP1601 lacks significant pathogenic genes and is not an ecotoxicological threat. Genes associated with the production of hyaluronic (*has*C) and poly-glutamic acids associated with capsule production, surface adhesion proteins (*fbp*A, *lsp*A), bacillibactin siderophore (*dhb*A-*dhb*F), a cation transport protein (*ctp*V) and catalase (*kat*) (Table 6.1) where detected. When accompanying virulence factors or toxins are present, these aforementioned genes can facilitate counter actions against the immune system and contribute towards pathogenicity as seen in pathogenic strains of *B. anthracis*, *Mycobacterium tuberculosis* (Mycobacteriales: Mycobacteriaceae) and other pathogens (Ward et al. 2010; Begg 2019). However, individually, these genes do not dictate pathogenicity as they are not independent determinants of pathogenicity because they, and their translated products, may be present or produced by GRAS species or constitute part of microbial environmental adaptation survival strategies (Arimoto and Igarashi 2008; Ward et al. 2010; Begg 2019; Cortese et al. 2021; Quach et al. 2021). For instance, the bioproduction of hyaluronic acid has shifted from using pathogenic strains of *Streptococcus equisimilis* (Lactobacillales: Streptococcaceae) and *B. anthracis* to strains of *B. subtilis* due to concerns about toxin carryover (Amjad et al. 2022).

The AMR profile of a biocontrol candidate is used to determine its risk associated with contributing to the spread and development of resistance genes and, subsequently, is crucial in the regulatory registration process of a biocontrol agent (Joly et al. 2021). CARD screening indicated that PHP1601 has putative AMR genes related to resistance towards several classes of antibiotics and disinfectants. AMR towards these compounds has been reported in various organisms, including strains of the *B. subtilis* species complex (Bragg et al. 2014; György et al. 2021; Joly et al. 2021); therefore, their detection in PHP1601 was not unexpected. However, it is necessary to evaluate the functional AMR potential of PHP1601 through culture-based assays to determine the antimicrobial compounds PHP1601 is susceptible and resistant towards and,

subsequently, the likely resistance it could confer. This was considered an important aspect for future investigations.

AntiSMASH detected complete gene clusters for producing various metabolites of biocontrol significance. These include antimicrobial peptides such as difficidin and bacilysin, polyketides such as bacillaene and macrolactin H and lipopeptide biosurfactants such as surfactin, bacillomycin and fengycin. These compounds have been shown to exhibit effective antagonism of various microbial pathogens and allude to PHP1601 extending biocontrol applications beyond the control of flies (Wu et al. 2015; Zhao et al. 2017; Pandey et al. 2022). Genes for the production of lipopeptide biosurfactants were confirmed to be functional due to the detection of surfactin, fengycin and iturin biosurfactants by UPLC ESI-TOF MS. Future investigations would assess the production of the aforementioned metabolites and determine their biocontrol efficacy against clinical and phytopathogens.

Members of the *Bacillus subtilis* species complex are beneficial to humans and animals forms other than biocontrol agents. These species, especially strains of *B. velezensis*, are used as probiotics which exhibit antagonism towards a range of fungal (e.g. *Aspergillus flavus*) and bacterial (e.g. *Vibrio cholerae*) pathogens, decreasing the severity of symptoms associated with certain fungal toxins (e.g. alpha toxin and zearalenone), improve nutrient uptake without disrupting the hosts intestinal microbiome or intestinal epithelial cell viability (Wang et al. 2018; Khalid et al. 2021; Soni et al. 2021; Zhu et al. 2021; Ji et al. 2023, Cai at al. 2024). This complements their benign nature and motivates their development as biocontrol agents.

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CHAPTER SEVEN: GENERAL OVERVIEW AND CONCLUSION

7.1 Summary of findings

Many species of blowflies are considered as relevant pests due to their medical and economic significance (Gomez-Puerta et al. 2021; Heve et al. 2021). Threats associated with these flies include the risk of spreading of pathogens, affliction of livestock and the agricultural sector. This is exacerbated by reduction in yields and quality of crops, animal hide and animal products as a consequence of fly infestations (Kremer and King 2019; Gomez-Puerta et al. 2021; Heve et al. 2021). Of these species, *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) has been identified as a significant pest and has a need for effective control measures due to its role in flystrike or myiasis (Brett et al. 2021). This species of blowfly was captured and used as a representative Brachycera fly for the conducted biocontrol study.

Advancements in the field of biocontrol has resulted in higher control rates and improved chances of successful pest antagonism beyond lab-scale testing (Singh et al. 2017). Many biocontrol agents have gained acceptance in the pest-control market due to their low environmental impact, low dosage or application requirements, and few to no occurrences of pest resistance (Singh et al. 2019). This occurs at a time when there are growing concerns about the longevity of the effectiveness of chemical control agents and their negative impacts on the environment (Anjum et al. 2019; Singh et al. 2019). Of the bacterial biocontrol agents developed, members of the aerobic endospore-forming bacteria are in the vast majority (Singh et al. 2019). These include *Paenibacillus popilliae* (Bacillales: Paenibacillaceae) (biocontrol of members of Coleoptera), *Bacillus thuringiensis* (Bacillales: Baciliaceae) (biocontrol of members of the Lepidoptera, Coleoptera and Culicidae) and *Lysinibacillus sphaericus* (Bacillales: Bacillaceae) (biocontrol of Culicidae) (Singh et al. 2019).

This study was undertaken to evaluate the biocontrol efficacy *B. velezensis* PHP1601 against *Lucilia cuprina* larvae as a representative of Brachycera flies. Currently there have been no documented strains of the *B. subtilis sensu lato* group that have shown antagonism towards representatives of this order. To the best of the authors knowledge, this is the first study to demonstrate the biocontrol effectiveness of a strain of *B. velezensis* towards fly species.

The major findings from this investigation revealed the following:

- The identification of *Bacillus* spp. strain PHP1601 was confirmed to be *B. velezensis* PHP1601, by multiple locus sequence analysis and a *B. velezensis* specific end point PCR method.
- Culture based bioassays indicated that both cell and endospore treatments exhibited a significant ($P < 0.01$) larvicidal effect on second instar *L. cuprina* larvae (10^2, 10^5, 10^8 and 10^{10} cells/endospores g^{-1}). The optimal starting concentration was 10^5 cells/endospores g^{-1} and was the only concentration that achieved total larval mortality within the bioassay duration. Higher concentrations suffered from lower larvicidal efficiencies as a consequence of a dose-related larvae repulsion effect. Corresponding cell and endospore treatments showed no significant difference ($P > 0.01$) in larvae mortality which was promising as it indicated that endospore could be used for further development as the active agent of a biocontrol formulation without loss in efficacy compared to cells. The cadavers of larvae recovered from the various cell/endospore treatments exhibited an orange – dark red discolouration and lacked structural integrity. The physiological distortions were considered to be an indication of the toxic effect the larvae had succumbed to when treated with PHP1601 cells or endospores.
- Bioassays investigating the larvicidal effect of the cell-free supernatant (CFS) of PHP1601 were conducted to determine the presence of extracellular larvicidal metabolites. The study confirmed that metabolites in the CFS treatments (1%, 5%, 10%, 20% and 30% (w v^{-1}) CFS) were larvicidal and exhibited a dose-dependent larvicidal effect from 1% - 10 % (w v^{-1}) CFS. Further increase in concentration of CFS provoked an altered feeding behaviour in the larvae, which resulted in them being repelled from the bioassay medium. This alluded that the previously observed repulsion effect depended on the metabolites produced by PHP1601. Consequently, the 10% (w v^{-1}) CFS treatment had significantly ($P < 0.01$) higher larvicidal rates and larvae mortalities than the other treatments. Larvae cadavers from the various CFS treatments showed darkening of their corpse and had compromised structural integrity, which became recognised as an indication of PHP1601 larvae toxicity. Studies in the literature indicate that lipopeptide biosurfactants produced by certain members of the *B. subtilis sensu lato* group had insecticidal properties. Therefore, these compounds were considered viable compounds

to investigate as the potential larvicidal metabolites produced by *B. velezensis* PHP1601.

- Lipopeptide biosurfactants of *B. velezensis* PHP1601 were assessed as crude lipopeptide (CLP) extracts (5, 10, 20, 100 and 1000 µg g^{-1}) to determine their role in its larvicidal activity. Bioassays showed that the CLP extract had a significant ($P < 0.01$) larvicidal effect that appeared to be dose-related and cadavers exhibited similar symptoms but at a greater severity of physiological damage as observed in previous bioassays. This confirmed that the extracts contained larvicidal metabolite(s). The composition of the CLP extracts was assessed using thin layer chromatography (TLC) and electron spray ionisation time of flight (ESI-TOF) mass spectroscopy (MS). This indicated that surfactin was the dominant lipopeptide present, followed by fengycin and iturin.
- Lipopeptides from the CLP of *B. velezensis* PHP1601 were extracted via TLC and characterised using ESI-TOF MS before being assessed in larvicide bioassays to determine the potential larvicidal properties of each lipopeptide fraction. The extracted lipopeptide fractions contained either surfactin, fengycin or a fengycin-iturin mixture. Bioassays performed with these extracts indicated that each lipopeptide fraction tested displayed a larvicidal effect; and, of which, the surfactin fraction (S-R0.90), proved to be the most potent. The potency of surfactin was assessed through bioassays conducted with purified surfactin (>99% purity, Sigma-Aldrich). This confirmed that surfactin was a larvicidal lipopeptide and that it could elicit a significant ($P < 0.01$) larvicidal effect independently of other lipopeptide families being present Larvae cadavers recovered from the bioassays with commercial surfactin displayed similar levels of physiological distortion as those collected from the CLP bioassays.
- Larvae choice assays indicated that the odour profile produced by PHP1601 was not responsible for the dose-related larvae repulsion effect observed in larvicidal bioassays. Significant levels ($P < 0.05$) of larvae attraction was observed using TSA fermented for 24 h and MG agar fermented for 5 d. Additionally, fermented pig manure samples showed no significant larvae attraction, nor repulsion effect relative to the controls, which indicated that the volatile organic compounds (VOCs) produced by PHP1601 were not responsible for the larvae repulsion effect or detrimental to its biocontrol efficacy. Furthermore, the hypothesis of the larvae-repulsion effect was revised and presumed to be associated with a toxic response due to the lipopeptide biosurfactants. GC-MS analysis

of the VOCs produced by PHP1601 indicated that ketones were present in the highest relative abundance and presumably a significant contributor to the larvae attraction effect observed.

- The real-world effectiveness of PHP1601 was assessed through field trials conducted in bucket containers using pig manure slurries as the assessment medium. Manure trial assessments demonstrated that endospore treatments (10^5 or 10^{10} endospores g^{-1}) of PHP1601 were able to significantly reduce ($P < 0.01$) the number of flies that emerged and inhibit the emergence of the second generation of flies. The 10^5 endospores g^{-1} treatment was found to be more effective than the 10^{10} endospores g^{-1} treatment. Its reduction in fly emergence was statistically distinct from the controls and displayed consistent levels of fly antagonism when the trials were repeated independently. The manure trials elucidated several fly species that fall into the potential host range of PHP1601, including *L. cuprina*, *Dacus bivittatus* (Bigot) (Diptera: Tephritidae), *Sarcophaga ruficornis* (Fabricius) (Diptera: Sarcophagidae), *Megaselia scalaris* (Loew) (Diptera: Phoridae), *Muscina stabulans* (Fallén) (Diptera: Muscidae), *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae), and *Synthesiomyia nudiseta* (Wulp) (Diptera: Muscidae).
- A *B. velezensis* specific qPCR method was developed from the method described by Dunlap (2019) and was able to monitor the change in *B. velezensis* concentrations over time in the pig manure slurries. This indicated that the strain was able to propagate and grow by several orders of magnitude in the manure. Additionally, culture based methods, in conjunction with REP PCR fingerprinting, confirmed the recovery of PHP1601 from the manure slurries. This indicated that PHP1601 can be monitored in environmental samples it would be applied in.
- Zebrafish embryo toxicological (ZET) studies indicated that PHP1601 were slightly toxic at 24 hpf (LC_{50}: 50 µg ml^{-1} CLP) however, exhibited no developmental abnormalities over the remainder of the assay (96 hpf). Reverse phase HPLC indicated that the strains CLP extracts were composed of 19.32 ± 1.14% surfactin and 26.02 ± 0.35% iturin; however, fengycins were below the limits of quantification. Therefore, the extrapolated LC_{50} of lipopeptide biosurfactant at 24 h was 22.77 µg ml^{-1}. Endpospores of PHP1601 were unable to germinate, grow or produce lipopeptide biosurfactants in river water or

the E3-ZET freshwater medium, which indicated that they were unlikely to contribute directly to the toxic response seen in the ZET assays. Additionally, genome mining indicated that the strain does not comprise any notable virulence or pathogenic factors; consequently, the strain was not considered to pose a biological threat.

7.2 Contributions of the study and recommendations for future assessments of fly biocontrol *B. subtilis sensu lato* candidates

Biological control aims to achieve an ecological and environmentally friendly means of pest management, which has been through the use of pest specific pathogens or predators (Jaiswal et al. 2022). The evaluation of PHP1601 harmonises with this purpose as members of this group are utilised as plant growth promoters, antagonists of phytopathogens and now as viable bioinsecticidal candidates; thereby contributing positively to the field of biological control. Before this study, members of the *B. subtilis* species complex were generally not considered as candidates for insect biocontrol, apart from mosquitos, and overlooked compared to members of the *B. cereus* species complex such as *B. thuringiensis* (Falqueto et al. 2021). This was attributed to gaps in the literature about their ability to antagonise insects, mechanisms of action and effectiveness under real-world conditions. This study addressed these gaps through the evaluation of PHP1601 as a fly biocontrol agent; resultingly, PHP1601 can be considered as one of the few members of the *B. subtilis* species complex recognised as a candidate for fly biocontrol development.

The most successful results of biological control have been achieved through the systematic application of a variety biocontrol products, at varied dosages and intervals as part of an integrated pest management (IPM) program. IPMs are designed to achieve optimal pest control whilst factoring the influence of the environment, climate, pest pressure and pest control rates (Thilagam et al. 2023). This allows for the short falls in time-to-kill and environmental adaptability to be compensated and challenge pest control efficacy achieved with chemical pesticides. (Jaiswal et al. 2022; Araújo et al. 2023; Khulbe and Batra 2024). PHP1601 is similar to many biocontrol products in this regard as its LC_{50} range within 4-5 d and total mortality taking longer to achieve. Therefore, PHP1601 is not viewed as a replacement to chemical pesticides, rather, as a constituent of a fly control IPM program where its lag in pest mortality can be compensated with other biocontrol agents (Araújo et al. 2023; Thilagam et al. 2023; Russi

et al. 2024). It also offers unique mode of larvae mortality, surfactant production as compared to infection or insecticidal-toxins, which lends itself to decreasing bio-insecticide resistance and improving mortality rates.

This study has shown that endospore treatment are viable forms for biocontrol assessment through lab and manure trial evaluation. This is significant because they are tough dormant structures that are resilient to environmental factors and amenable to various application and formulation development processes without incurring significant loss to viability, therefby increasing product shelf life (Zeriouh et al. 2011; Vimala et al. 2021; Russi et al. 2024). Additionally, sporulation is a natural part of the life cycle of *Bacillus* spp. and is readily achieved through nutrient depletion or extended culturing. Hence, major interventions to upscaling or batch production are unlikely, which is a significant consideration in the production process of a biocontrol product (Russi et al. 2024).

Lipopeptide biosurfactants are biologically valuable compounds that are significant in functioning of plant growth promoting *Bacillus* spp., control of phytopathogens and, recently, the control of fly larvae (Rabbee et al. 2023). Therefore, lipopeptide biosurfactant producing members of the *B. subtilis sensu lato* group evaluated for insect biocontrol should not be viewed in isolation of the other applications that they could be used for as well. Hence, it can be stated that members of this sensu-lato group need to be efficient lipopeptide producing strains in order to be effective biocontrol agents. Therefore, lipopeptide biosurfactant production should be used as an indicator of biocontrol potency or effectiveness amongst members of the *B. subtilis sensu lato* group.

Lab scale bioassays revealed the impact of PHP1601 treatments on larvae behaviour, namely the dose-related repulsion effect. This is beneficial because it provided another set of criteria to assess the effective concentration (LC_{50}) or exposure time of a treatment (LT_{50}) and advised on how field trials can be developed and the applied dose of the biocontrol product. Generally, these interactions are not reported in the literature, which, creates a false impression of the pest response to the biological or chemical pesticide. Hence, the larvae-odour choice tests were necessary as it elucidated how PHP1601 treated media could affect the preference of the larvae to it. Arguably, if the knowledge of these interactions were more readily documented it could work positively in reducing the over/under-application of pesticides in general.

Manure trials demonstrated the potential for real-world fly biocontrol success using endospore treatments of PHP1601 and elucidated the fly spp. in its host range. This is an important milestone in the evaluation of a biocontrol candidate because it demonstrated the effectiveness of the strain beyond controlled lab conditions. The succession of studies from the controlled laboratory level to environmental conditions, or conditions typical of its application, are not common in the literature, which hinders the adoption of reported biocontrol candidates into product development and commercialisation (Ali et al. 2023). The conducted manure trials used cheaply sourced resources that were easy to assemble into bioassay containers. This has the potential to increase the accessibility of similar fly biocontrol assays and provide a more realistic view of the performance of a biocontrol candidate.

The qPCR quantification method of *B. velezensis* in pig manure slurries was developed with the intentions of facilitating an accurate and high throughput monitoring of the population dynamics of vegetative *B. velezensis* in environmental samples it was applied in. This has the potential to mostly replace culture based quantification methods which are time-consuming, resource intensive, especially when testing many replicated treatments, and requires further molecular characterisation to identify the species isolated (Veilleux et al. 2021). Also, the extracted DNA from the sample would be stable indefinity when cryopreserved in a stable buffer (eg. Tris-EDTA), offering the potential to rested the DNA at a later stage to verify the result, contrasted by storing environmental samples. Endospores are complex structures which do not lyse easily and make qPCR analysis of endospores difficult. However, the resilient trait of endospores make them easy to isolate and confirm sites where the biocontrol agent was applied using methods like REP-PCR fingerprinting from colony material. Collectively, qPCR and REP-PCR fingerprinting provides a means of assessing the persistence of a biocontrol agent in the environment which can be used to refine treatment parameters such as the dosage, frequency of application and advise on how to adjust the dosage in association with the pest pressure. Therefore, these methods have the potential to accelerate the environmental assessments of a biocontrol candidates.

The toxicological assessment of PHP1601 through ZET assays and genome mining contributed to the consensus that members of the *B. subtilis* species complex are benign organisms to be sought after for biocontrol applications. ZET assays are used as proxies for aquatic risk assessments and mammalian toxicological studies because they use complex eukaryotic

organisms which exhibit reproducible results when tested independently in different labs (Falcão et al. 2018). The framework for these assays is standardised by the OECD and offer direct adoption into records for risk assessment of a biocontrol candidate, making them essential for evaluating the risks associated with a biocontrol candidate. These assays are, however, dependent on ethical clearance which can limit them as routine assessments in evaluating biocontrol candidates. Whole genome sequencing has become considerably more cost effective and mining for pathogenic factors, metabolites of interest, antimicrobial resistance genes and metabolic pathways of interest. This an auxiliary toxicological screening route to ZET assays, or other toxicological assays, that can be done without the need for ethical clearance and can provide valuable information before hand that can advise on what risk criteria can be wavered or needs further investigation. The genome mining pipelines used in this study are free to use, simple to operate and understand, server based and globally available, which removes the barriers from genome analysis studies, especially in the context of a risk assessment (Jia et al. 2017; Blin et al. 2019; Liu et al. 2022).

In order to facilitate the screening and assessment of members of the *B. subtilis sensu lato* group, the following strategy is proposed:

Step 1 – The number of biocontrol candidates can be pre-screened to select strains that produce the highest amount of lipopeptide biosurfactants that also produce the largest zone of oil displacement. This would reduce the number of biocontrol candidates to assess while selecting those with the highest number of “active” larvicidal compounds.

Step 2 – The identity of the strains should be identified using MLSA for reliable species affiliation. Molecular characterisation allows genes to be queried against curated databases to facilitated up-to-date species level identification which would also assist in the regulatory and registration process of the biocontrol agent. Gene sequences can also be deposited in online repositories, like GenBank, as a means to develop a patent or intellectual property of a strain.

Step 3 – Endospore treatments of the candidates should be assessed through lab scale larvicidal or insecticidal assays. Endospores are desired from a product formulation perspective because it offers longer shelf life, resilience to formulation processing methods and storage conditions when compared to vegetative cells.

Step 4 – The best performing strains from lab-scale bioassays should be further investigated though field-like conditions, such as manure trials.

Step 5 – The most effective biocontrol candidate from field-simulated evaluations should undergo toxicological screening using one or more model organisms (eg. zebrafish, stingless honey bees, etc) to assess its potential pathogenic and ecological risks. These studies should include drug resistance assays, toxicity to mammalian and avian cell lines to improve the comprehensiveness of the assessment. Genome mining was useful in acquiring information about pathogenic and toxin related genes that PHP1601 could poses, therefore, it should be included to detect and assess factors that could have gone unnoticed.

Sep 6 – The biocontrol candidate can be considered to be adequately assessed and can be patented or have its intellectual property secured. Also, it can be formally undergo further assessments for regulatory and registration purposes as part of the product development process.

7.3 Future investigations

It is important to remember that the increasing adoption biological pesticides is attributed to the continuous development and optimisation of biocontrol products and pest management programs, aligns with the increase in global population, increase in margins for sustained food security, decline of arable land and health concerns about chemcial pesticides (Smagghe et al. 2023). This positions biocontrol products as the future of pest control, especially in the agriculture and livestock sectors which constitute significantly as economic support pillars. Therefore, continual research, development and optimisation in biological pest control is required.

Through consultation of the literature, it was apparent that insect biocontrol applications of members of the *B. subtilis sensu lato* group are, in many regards, absent or relatively unexplored. Hence, the study could be expanded on various aspects in developing PHP1601 as an industrially viable biocontrol candidate against fly species. Areas for future work are proposed as follows:

- The previously used bottle fly traps were a cost-efficient and reusable means of capturing flies. By varying the bait, different flies could be captured and this would allow for the host range of PHP1601 to be investigated. Flies of the Nematocera have demonstrated

susceptibility to strains of *B. subtilis* and would be worth investigating to determine susceptibility and compare biocontrol efficacies (Sidahmed et al. 2017). Also, bottle traps could be used to develop a bait-and-kill fly biocontrol system to control fly infestations in confined areas.

- This study determined that PHP1601 has larvicidal properties warrenting further development as a biocontrol product. However, the lethality of its cells, endospores and biosurfactants towards fly eggs, different larvae instars and adults warranting further investigation. This would determine which developmental stages are most susceptible and possibly lead to the development of aerosolised or mist applications for adult fly control.
- Lipopeptide biosurfactants are the bioactive compounds responsible for the larvicidal effect of PHP1601, however, bioassays using mutants that lack the genes for surfactin, iturin or fengycin production or mutants that produce only one lipopeptide family would provide a more comprehensive idea on the dependency of these compounds for effective larvae antagonism (Wang et al. 2022; Wu et al. 2023). This has the potential to expose the role or microbial parasitic methods which could have otherwise gone by unnoticed.
- Bioassays testing cell, endospore, CFS and lipopeptide biosurfactant treatments produced larvae cadavers that displayed varying degrees of physiological distortion. Histopathological autopsies after varying exposure times would be beneficial in identifying organs and tissue that are most effected by the treatments (Mnif et al. 2013) and in relation to the level of discolouration observed in the infected larvae and cadavers. This would contribute to a better understanding of the larvicidal mechanism of PHP1601.
- Assessment of biosurfactants to the cuticle of the larvae should be investigated to explore lethality of topical treatments. This would contribute to building a model of larvicidal pathways.
- The fly larvae exhibited a dose-related behavioural response to the PHP1601 treatments. Initially, it was hypothesised that the larvae were repelled by the odours released by PHP1601 fermentation of the bioassay medium; however, it is evident that media fermented by PHP1601 is attractive to fly larvae. Assessment of chemical standards of compounds that constitute the scent of PHP1601 are required to better understand this effect. Additionally, the response of adult flies to these odours requires further

investigation. Consequently, the dose-related repulsion effect was presumed to be toxicity related rather than odour related. To assess this hypothesis, bioassays assessing the retention of larvae to media amended with different concentrations of cells, endospores, CFS and CLP are required.

- The upscale of endospore production is required for larger scale biocontrol evaluations. This prompts the need for medium development for cost-effective and high yield endospore production and formulation development. This generally involves using raw materials (e.g. sorghum grains) or agricultural by-products (e.g. sugarcane bagasse) as cheaper alternatives to lab-grade pre-mixes. Additionally, method development would be required to assess the media used and the formulation methods for their impacts on endospore robustness, endospore germination (%), levels of lipopeptide biosurfactant production, biocontrol efficiency and shelf life.
- Manure trials have demonstrated that endospore additions of PHP1601 were effective in antagonising various fly species. These trials were performed in summer when fly activity was more prevalent; however, trials need to be conducted over different seasons to determine the climatic impact on biocontrol performance. Additionally, large(r) scale field trials need to be conducted in a similar way that the strain would be applied as a biocontrol agent (e.g. as a spray-application in livestock pens).
- Pig manure trials explored the potential host range of PHP1601. Bioassays need to be conducted with larvae or flies from pure colonies to confirm its host range. This should ideally be done through control lab assays and assays conducted in a similar manner to the manure trial to confirm antagonism under naturally occurring environmental conditions.
- qPCR quantification was limited to vegetative cells as endospores were resistant to lysis treatments. Therefore, a protocol that can effectively lyse endospores, without compromising the quality of DNA, requires further investigation and development.
- Quantification of lipopeptides has typically been done via reverse phase high performance liquid chromatography (Dlamini et al. 2020). Although it allows for robust analysis, it comes with a caveat of high initial purchasing costs, expensive operational costs, long processing times and a requirement for relatively pure samples (Dlamini et al. 2020). Therefore, the development of, or, optimisation of a pre-existing technology (eg.

UV-vis, FT-NIR) to establish a cost-effective and sensitive means of lipopeptide quantification that from environmental sources is needed. This would allow the behaviour of the strain to be monitored in the environment it is applied in (Vigneshwaran et al. 2021; Meena et al. 2020).

- The ZET assays showed that lipopeptides produced by PHP1601 were slightly toxic and did not induce any developmental abnormalities; however, the study needs to be extended to evaluate the impact of CLP on the adult stage of the fish and monitor for potential developmental and behavioural changes. Additionally, the lethality of PHP1601 and its CLP should be assessed against other model organism such as honey or stingless bees (Hymenoptera: Apidae) *Melipona* spp. and free-living soil nematodes (Rhabditida: Rhabditidae) *Caenorhabditis elegans* for better assessment of ecological and soil environmental risk respectively (Lourencetti et al. 2023; Qu et al. 2023).
- The most effective biocontrol responses have been achieve through the integration of many control agents, chemical and biological, applied in a regimented plan. This allows the biological control agents to work in tandem with one another to reduce the delay or shortfalls in pest mortality that they can individually achieve. Therefore, the compatibility of PHP1601 with existing biocontrol and chemical control agents needs to be assessed in terms of viability of the control agent and cumulative pest control response. Additionally, dosage and application rates and types need to be explored to suppress and sustain a low-to-no pest pressure or population.

7.4 Conclusion

Aerobic endospore-forming bacteria have become increasingly more involved in plant growth promotion and microbial plant pathogen disease suppression applications. Many of these candidates, especially members of the *B. subtilis sensu lato* group, are favoured as promising biocontrol agents against plant-based pathogens and, until recently, have not been considered as potential biocontrol agents against macro pests such as flies. This investigation addressed this gap in the literature through a series of investigations where the biocontrol efficacy of *B. velezensis* PHP1601 against *L. cuprina* larvae was evaluated. Through this, the larvicidal mechanism and bioactive compounds responsible were elucidated, and, its mode of action is

proposed to be attributed to a membrane disruption mechanism of lipopeptides in the intestine of larvae. The findings of this study suggest that PHP1601 is a promising candidate for further investigation as a biocontrol agent based on lab-scale bioassays, manure trials and toxicological assessments.

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APPENDIX

Appendix A: First reported incidence of *Bacillus velezensis* exhibiting effective antagonism against a blowfly species, *Lucilia cuprina*

Table A1 Primers used for MLSA of PHP1601

Gene	Size (bp)	Primer sequence (5'- 3')	Reference
16S rRNA	~1380	F: AGAGTTTGATCCTGGCTC R: CGGGAACGTATTCACCG	Ström et al. (2002)
*dna*J	~788	F: GGGGTAGGTAAGAGCGCTTC R: CGGCATTTCGCAGTAAATATC	Connor et al. (2010)
*gyr*A	~1000	F: CAGTCAGGAAATGCGTACGTCCTT R: CAACGTAATGCTCCAGGCATTGCT	Roberts et al. (1994)
*rpo*B	~808	F: AGGTCAACTAGTTCAGTATGGACG R: GTCCTACATTGGCAAGATCGTATC	Roberts et al. (1994)

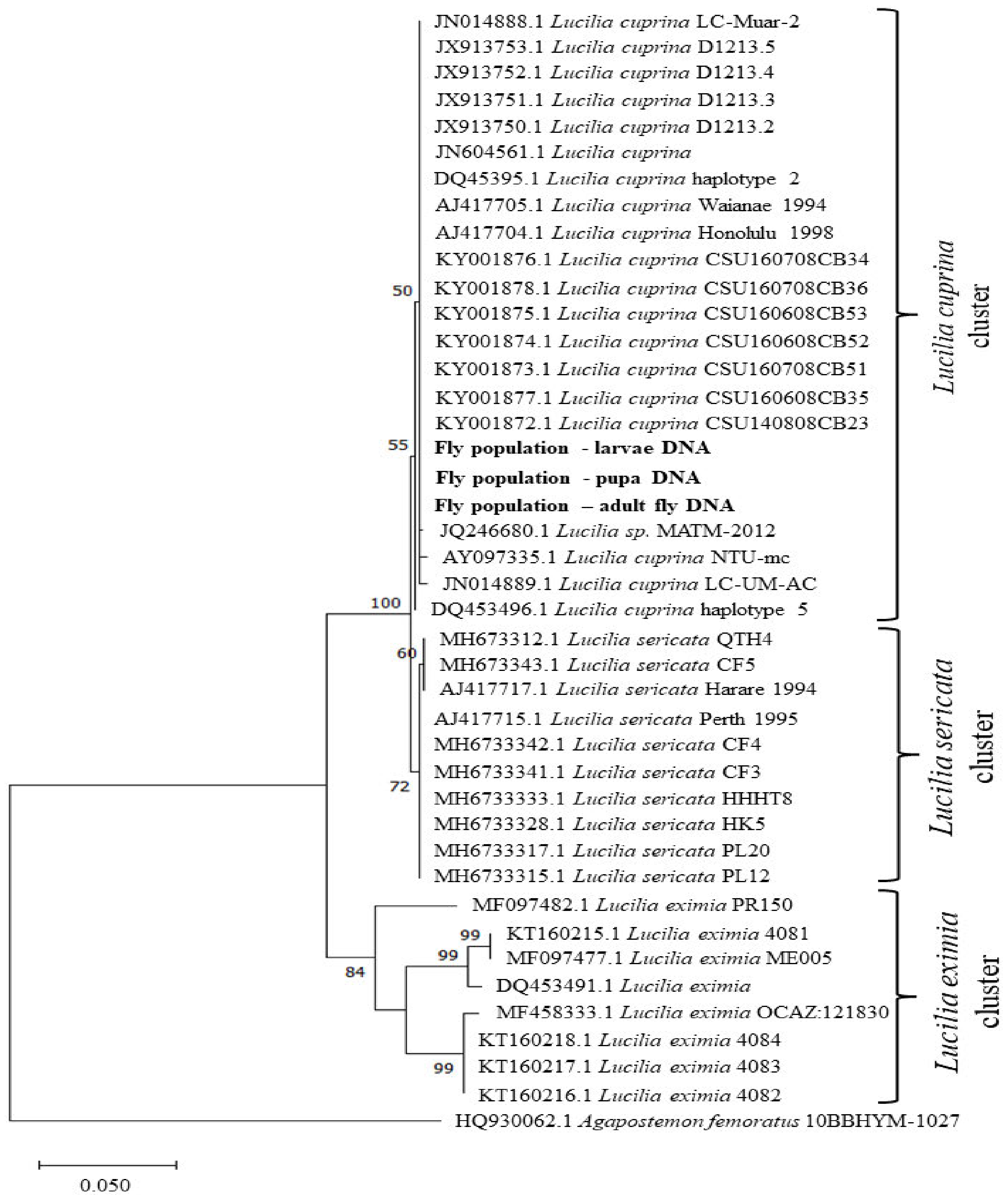

Fig. A1 Maximum likelihood phylogenetic tree inferring the evolutionary relationship analysis of cytochrome oxidase subunit 1 gene sequences of DNA from green bottle blowfly larvae, pupa and adult fly samples. This analysis involved 42 sequences with 648 nucleotide

positions in the final data set. Of the 1000 bootstrap replicates, only values ≥ 50% were included. The scale bar represents 0.050 substitutions per sequence position. The cytochrome oxidase subunit 1 gene sequences of closely related strains of *Lucilia sericata* and *L. eximia* were included for comparison.

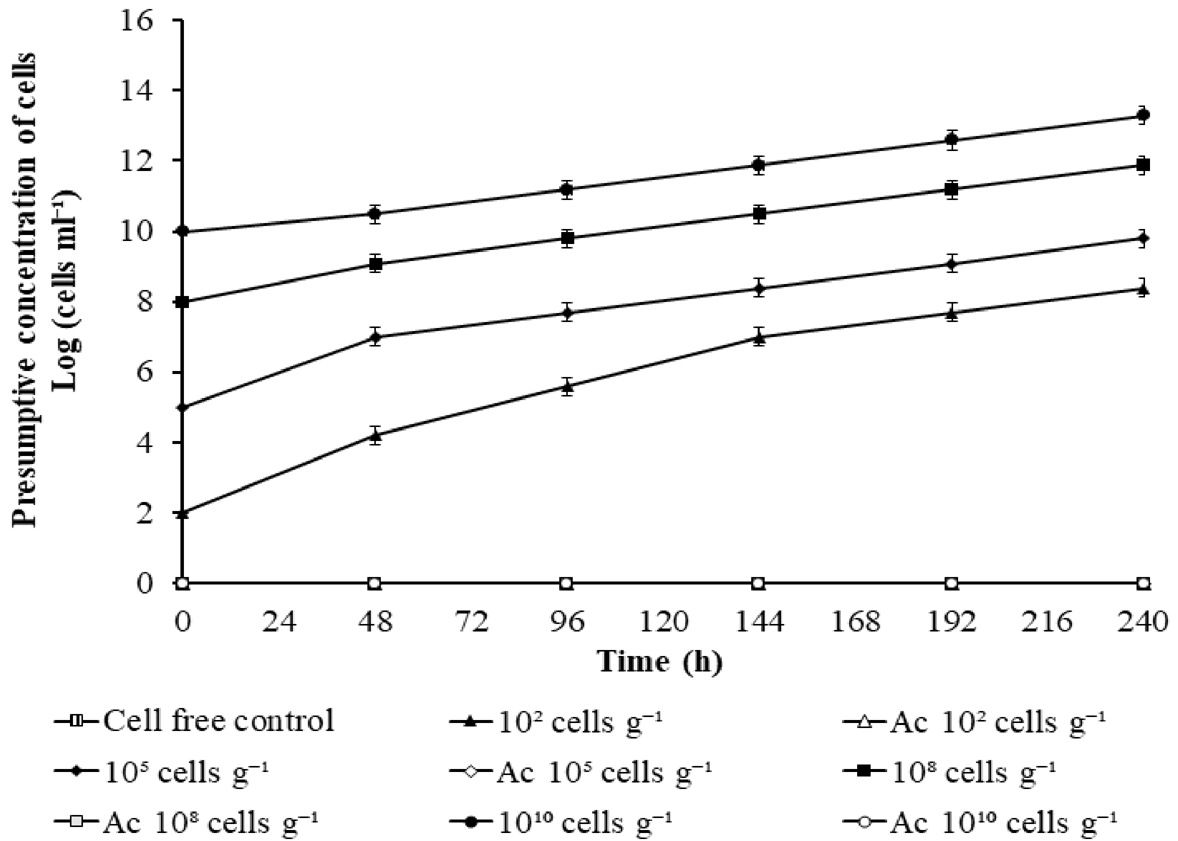

Fig. A2 The effect of various starting cell concentration of PHP1601 on its growth in MG agar over a 240-h study at 28ºC. The "Ac" sets represent the autoclave killed cell controls. Error bars represent the standard error of the average concentration of cells determined at tested time intervals.

Table A2 Tukey HSD pairwise comparison of larvae mortality (%) exhibited by the different starting concentrations of cells of PHP1601 at time intervals that corresponded to LT_{50}, LT_{90} and LT_{100} of the 10^5 cells g^{-1} treatment

Cell treatment (cells g^{-1}) - A	Cell treatment (cells g^{-1}) - B	LT_{50}		LT_{90}		LT_{100}	
		Difference in larvae mortality (%)	P-value	Difference in larvae mortality (%)	P-value	Difference in larvae mortality (%)	P-value
10^2	10^5	* -28.05	1.10×10^{-4}	* -37.77	3.00×10^{-6}	* -42.26	1.00×10^{-6}
	10^8	-14.50	3.30×10^{-2}	-11.77	9.60×10^{-2}	-16.67	1.80×10^{-2}
	10^{10}	* -19.11	5.00×10^{-3}	-15.28	2.30×10^{-2}	* -20.75	3.00×10^{-3}
10^5	10^2	* 28.05	1.10×10^{-4}	* 37.77	3.00×10^{-6}	* 42.26	1.00×10^{-6}
	10^8	13.55	4.90×10^{-2}	* 26.00	2.31×10^{-4}	* 25.59	4.76×10^{-4}
	10^{10}	8.94	2.69×10^{-1}	* 22.49	1.00×10^{-3}	* 21.51	2.00×10^{-3}
10^8	10^2	14.50	3.30×10^{-2}	11.77	9.60×10^{-2}	16.67	1.80×10^{-2}
	10^5	-13.55	4.90×10^{-2}	* -26.00	2.31×10^{-4}	* -25.59	4.76×10^{-4}
	10^{10}	-4.61	7.64×10^{-1}	-3.51	8.75×10^{-1}	-4.08	8.41×10^{-1}
10^{10}	10^2	* 19.11	5.00×10^{-3}	15.28	2.30×10^{-2}	* 20.75	3.00×10^{-3}
	10^5	-8.94	2.69×10^{-1}	* -22.49	1.00×10^{-3}	* -21.51	2.00×10^{-3}
	10^8	4.61	7.64×10^{-1}	3.51	8.75×10^{-1}	4.08	8.41×10^{-1}

Pairwise comparisons (A - B) were conducted to determine the difference in the average larvae mortality between the cell treatments at the specified LT intervals. The Tukey HSD test determined whether the differences in larvae mortality were significant (*) at $P\text{-value} < 0.01$.

Table A3 Tukey HSD comparison of larvae mortality (%) exhibited by the different starting concentrations of endospores of PHP1601 at time intervals that corresponded to LT_{50}, LT_{90} and LT_{100} of the 10^5 endospores g^{-1} treatment

		LT_{50}		LT_{90}		LT_{100}	
Endospore treatment – A (endospores g^{-1})	Endospore treatment – B (endospores g^{-1})	Difference in larvae mortality (%)	P-value	Difference in larvae mortality (%)	P-value	Difference in larvae mortality (%)	P-value
10^2	10^5	* -33.43	8.67×10^{-7}	* -34.65	1.12×10^{-4}	* -37.75	3.00×10^{-6}
	10^8	* -20.73	2.78×10^{-4}	-14.11	1.14×10^{-1}	-15.59	2.00×10^{-2}
	10^{10}	* -28.45	7.00×10^{-6}	-17.52	3.80×10^{-2}	* -19.39	4.00×10^{-3}
10^5	10^2	* 33.43	8.67×10^{-7}	* 34.65	1.12×10^{-4}	* 37.75	3.00×10^{-6}
	10^8	12.70	1.90×10^{-2}	20.54	1.40×10^{-2}	* 22.15	1.00×10^{-3}
	10^{10}	4.98	5.69×10^{-1}	17.12	4.30×10^{-2}	* 18.36	6.00×10^{-3}
10^8	10^2	* 20.73	2.78×10^{-4}	14.11	1.14×10^{-1}	15.59	2.00×10^{-2}
	10^5	-12.70	1.90×10^{-2}	-20.54	1.40×10^{-2}	* -22.15	1.00×10^{-3}
	10^{10}	-7.72	2.18×10^{-1}	-3.42	9.35×10^{-1}	-3.79	8.50×10^{-1}
10^{10}	10^2	* 28.45	7.00×10^{-6}	17.52	3.80×10^{-2}	* 19.39	4.00×10^{-3}
	10^5	-4.98	5.69×10^{-1}	-17.12	4.30×10^{-2}	*-18.36	6.00×10^{-3}
	10^8	7.72	2.18×10^{-1}	3.42	9.35×10^{-1}	3.79	8.50×10^{-1}

Pairwise comparisons (A - B) were conducted to determine the difference in the average larvae mortality between the endospore treatments at the specified LT intervals. The Tukey HSD test determined whether the differences in larvae mortality were significant (*) at $P < 0.01$.

Table A4 Tukey's HSD pairwise comparison larvae mortality (%) exhibited by the different concentrations of CFS at time intervals that corresponded to LT_{50}, LT_{90} and LT_{100} of the 10% (v w^{-1}) CFS treatment

CFS treatment (% v w^{-1}) - A	CFS treatment (% v w^{-1}) - B	LT_{50} Difference in larvae mortality (%)	P-value	LT_{90} Difference in larvae mortality (%)	P-value	LT_{100} Difference in larvae mortality (%)	P-value
1	5	-13.29	2.95×10^{-1}	-13.33	1.14×10^{-1}	* -14.55	7.00×10^{-3}
	10	* -33.44	1.00×10^{-3}	* -31.62	5.20×10^{-5}	* -28.55	2.00×10^{-6}
	20	* -42.92	2.22×10^{-5}	-18.28	1.60×10^{-2}	-8.28	2.15×10^{-1}
	30	* -44.60	1.30×10^{-5}	-13.43	1.10×10^{-1}	-6.69	4.07×10^{-1}
5	1	13.29	2.95×10^{-1}	13.33	1.14×10^{-1}	* 14.55	7.00×10^{-3}
	10	-20.14	4.40×10^{-2}	-18.29	1.60×10^{-2}	-14.00	1.00×10^{-2}
	20	* -29.62	2.00×10^{-3}	-4.95	8.72×10^{-1}	6.26	4.71×10^{-1}
	30	* -31.31	1.00×10^{-3}	-0.09	1.00	7.86	2.58×10^{-1}
10	1	* 33.44	1.00×10^{-3}	* 31.62	5.20×10^{-5}	* 28.55	2.20×10^{-6}
	5	20.14	4.40×10^{-2}	18.29	1.60×10^{-2}	14.00	1.00×10^{-2}
	20	-9.48	6.12×10^{-1}	13.33	1.14×10^{-1}	* 20.26	2.31×10^{-4}
	30	-11.17	4.60×10^{-1}	18.19	1.70×10^{-2}	* 21.86	9.00×10^{-5}
20	1	* 42.92	2.22×10^{-5}	18.28	1.60×10^{-2}	8.28	2.15×10^{-1}
	5	* 29.62	2.00×10^{-3}	4.95	8.72×10^{-1}	-6.26	4.71×10^{-1}
	10	9.48	6.12×10^{-1}	-13.33	1.14×10^{-1}	* -20.26	2.31×10^{-4}
	30	-1.69	9.99×10^{-1}	4.86	8.79×10^{-1}	1.60	9.93×10^{-1}
30	1	* 44.60	1.30×10^{-5}	13.43	1.10×10^{-1}	6.69	4.07×10^{-1}
	5	* 31.31	1.00×10^{-3}	0.09	1.00	-7.86	2.58×10^{-2}
	10	11.17	4.60×10^{-1}	-18.19	1.70×10^{-2}	* -21.86	9.00×10^{-5}
	20	1.69	9.99×10^{-1}	-4.86	8.79×10^{-1}	-1.60	9.93×10^{-1}

Pairwise comparisons (A - B) were conducted to determine the difference in the average larvae mortality between the CFS treatments at the specified LT intervals. The Tukey HSD test determined whether the differences in larvae mortality were significant (*) at P-value < 0.01.

Table A5 Tukey HSD pairwise comparison larvae mortality (%) exhibited by the different concentrations of CLP at intervals that corresponded to the LT_{50}, LT_{90} and LT_{100} of the 1000 μg g^{-1} CLP treatment

CLP treatment (μg g^{-1}) - A	CLP treatment (μg g^{-1}) - B	LT_{50} Difference in larvae mortality (%)	LT_{50} P-value	LT_{90} Difference in larvae mortality (%)	LT_{90} P-value	LT_{100} Difference in larvae mortality (%)	LT_{100} P-value
1000	100	* 0.81	2.91×10^{-3}	0.43	2.89×10^{-1}	0.45	3.70×10^{-1}
	20	* 1.39	3.98×10^{-6}	* 1.37	2.10×10^{-5}	* 1.36	1.61×10^{-4}
	10	* 2.08	5.81×10^{-9}	* 1.82	3.19×10^{-7}	* 1.78	4.30×10^{-6}
	5	* 2.57	1.40×10^{-10}	* 2.11	2.84×10^{-8}	* 2.52	1.73×10^{-8}
100	1000	* -.812	2.91×10^{-3}	-0.43	2.89×10^{-1}	-0.45	3.70×10^{-1}
	20	0.57	4.54×10^{-2}	* 0.94	2.00×10^{-3}	0.91	1.04×10^{-2}
	10	* 1.27	1.42×10^{-5}	* 1.39	1.70×10^{-5}	* 1.33	2.22×10^{-4}
	5	* 1.76	9.94×10^{-8}	* 1.68	1.00×10^{-6}	* 2.06	4.45×10^{-7}
20	1000	* -1.39	3.98×10^{-6}	* -1.37	2.10×10^{-5}	* -1.36	1.61×10^{-4}
	100	-0.57	4.54×10^{-2}	* -0.94	2.00×10^{-3}	-0.91	1.04×10^{-2}
	10	0.69	1.17×10^{-2}	0.45	2.47×10^{-1}	0.42	4.49×10^{-1}
	5	* 1.18	3.72×10^{-5}	0.74	1.70×10^{-2}	* 1.16	1.06×10^{-3}
10	1000	* -2.08	5.81×10^{-9}	* -1.82	3.19×10^{-7}	* -1.78	4.30×10^{-6}
	100	* -1.27	1.42×10^{-5}	* -1.39	1.70×10^{-5}	* -1.33	2.22×10^{-4}
	20	-0.69	1.17×10^{-2}	-0.45	2.47×10^{-1}	-0.42	4.49×10^{-1}
	5	0.49	1.12×10^{-1}	0.29	6.48×10^{-1}	0.74	4.63×10^{-2}
5	1000	* -2.57	1.40×10^{-10}	* -2.11	2.84×10^{-8}	* -2.52	1.73×10^{-8}
	100	* -1.76	9.94×10^{-8}	* -1.68	1.00×10^{-6}	* -2.06	4.45×10^{-7}
	20	* -1.18	3.72×10^{-5}	-0.74	1.70×10^{-2}	* -1.16	1.06×10^{-3}
	10	-0.49	1.12×10^{-1}	-0.29	6.48×10^{-1}	* -0.74	4.63×10^{-2}

Larvae mortality data were transformed ($\sqrt[3]{x}$) to correct for normality before conducting statistical tests. Pairwise comparisons (A - B) were conducted to determine the difference in the average larvae mortality between the CLP treatments at the specified LT intervals. The Tukey HSD test determined whether the differences in larvae mortality were significant (*) at P-value < 0.01.

Appendix B: Elucidation of lipopeptide biosurfactants responsible for the larvicidal activity of *Bacillus velezensis* PHP1601 towards *Lucilia cuprina* larvae

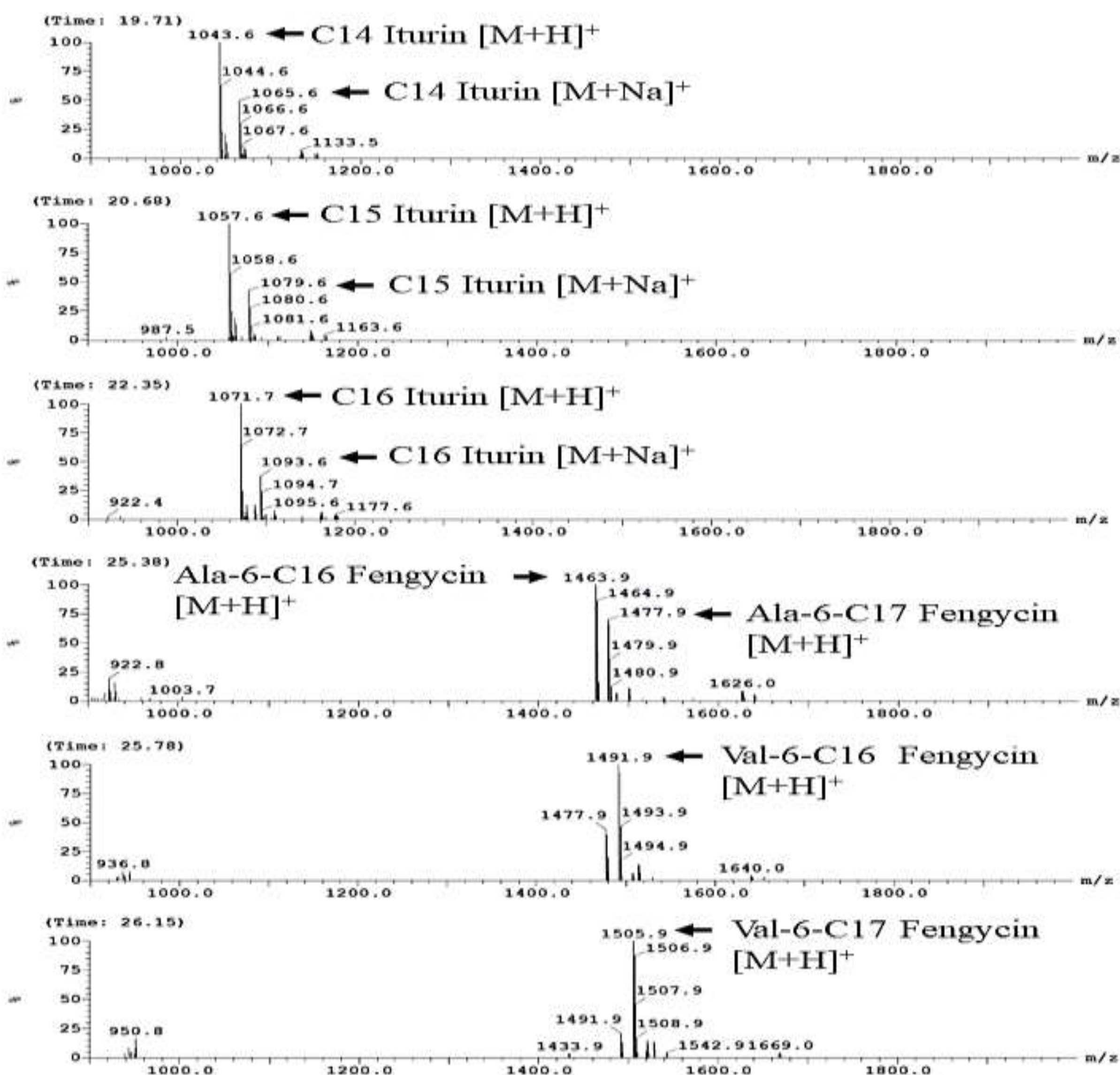

Fig. B1 Representative ESI-TOF MS mass spectra (m/z) of iturin and fengycin compounds detected in the CLP of PHP1601. Each chromatogram corresponds to the UPLC peak it was eluted from, as indicated by the time of elution (min). Compounds were characterised based on their mass (m/z) data which also allowed for detection of protonated [M+H]+ isoforms or those in association with sodium adducts [M+Na]+.

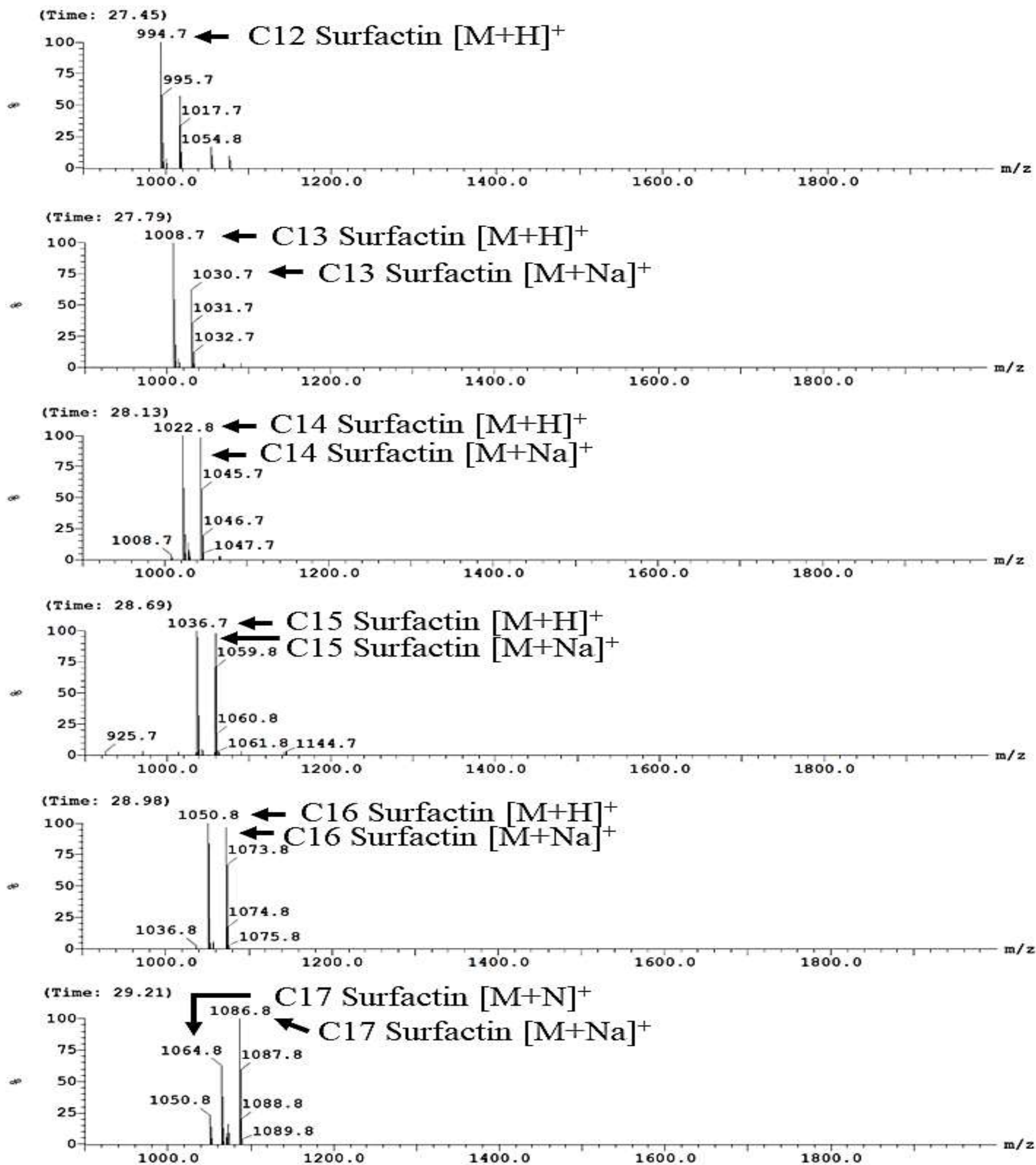

Fig. B2 Representative ESI-TOF MS mass spectra (m/z) of surfactin compounds detected in the CLP of PHP1601. Each chromatogram corresponds to the UPLC peak it was eluted from, as indicated by the time of elution (min). Compounds where characterised based on their mass (m/z) data which also allowed for detection of protonated $[M+H]^+$ isoforms or those in association with sodium adducts $[M+Na]^+$.

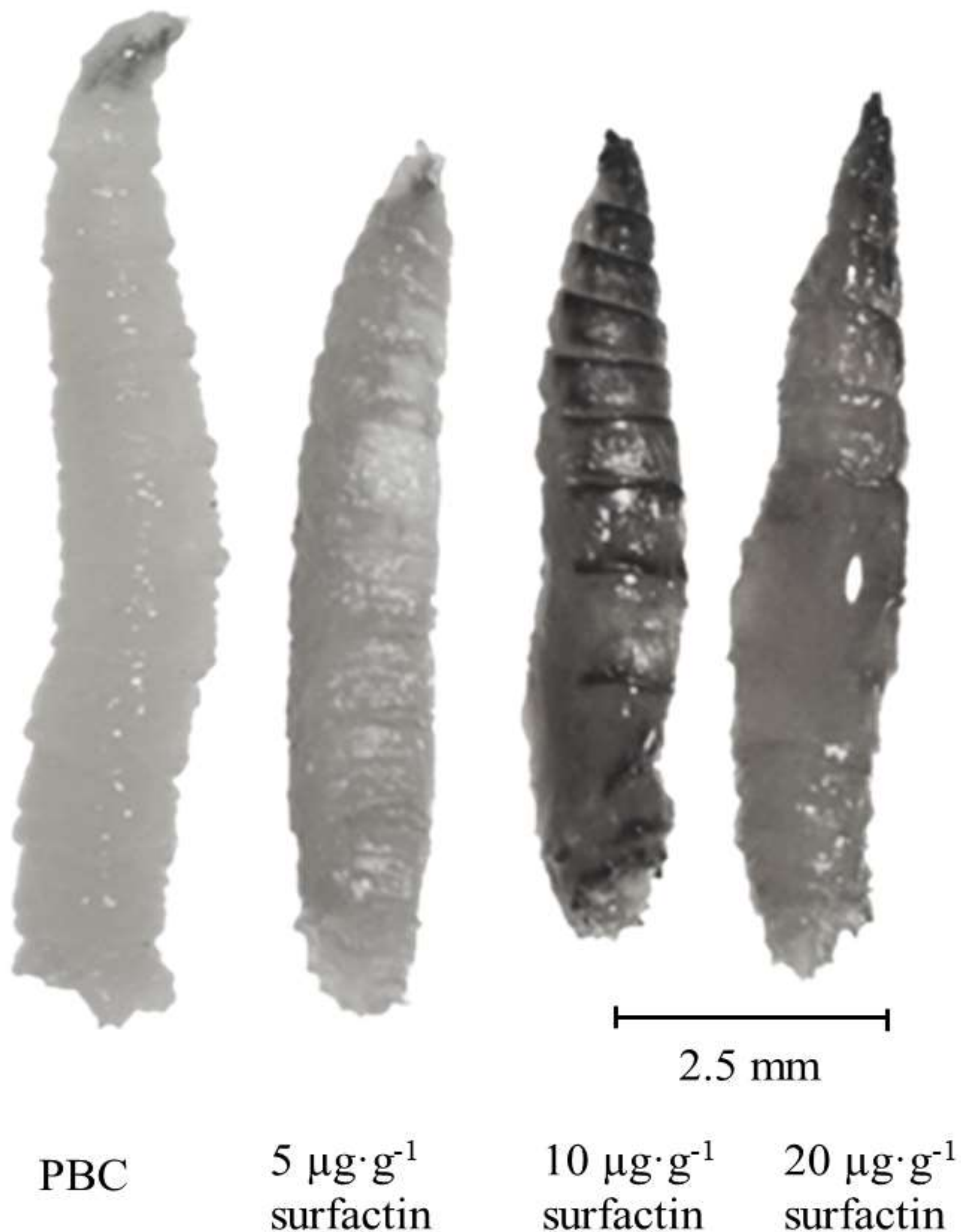

Fig. B3 Cadavers of larvae recovered from treatments of 5, 10 and 20 µg g^{-1} of surfactin which displayed severe physiological deformations which were not observed in the larvae in the controls. Larvae in the phosphate buffer control (PBC) were included as a healthy control. All larvae were collected at the 120 h bioassay interval.

Appendix C: Manure trial evaluation of *Bacillus velezensis* PHP1601 as a viable biocontrol candidate against nuisance fly species at an operational piggery

Fig. C1 Design of farm trial containers. Containers used were 12 cm tall × 10.5 cm wide. A 3 cm hole was drilled into each container lid and an inverted clear plastic bottle screw cap was attached and replaces. Plastic bottles were 14 cm tall × 4.5 cm wide and transparent to view fly emergence. The base of the bottles were removed and a piece of organza cloth was attached to allow for aeration and as a barrier preventing the entry or release of flies or larvae.

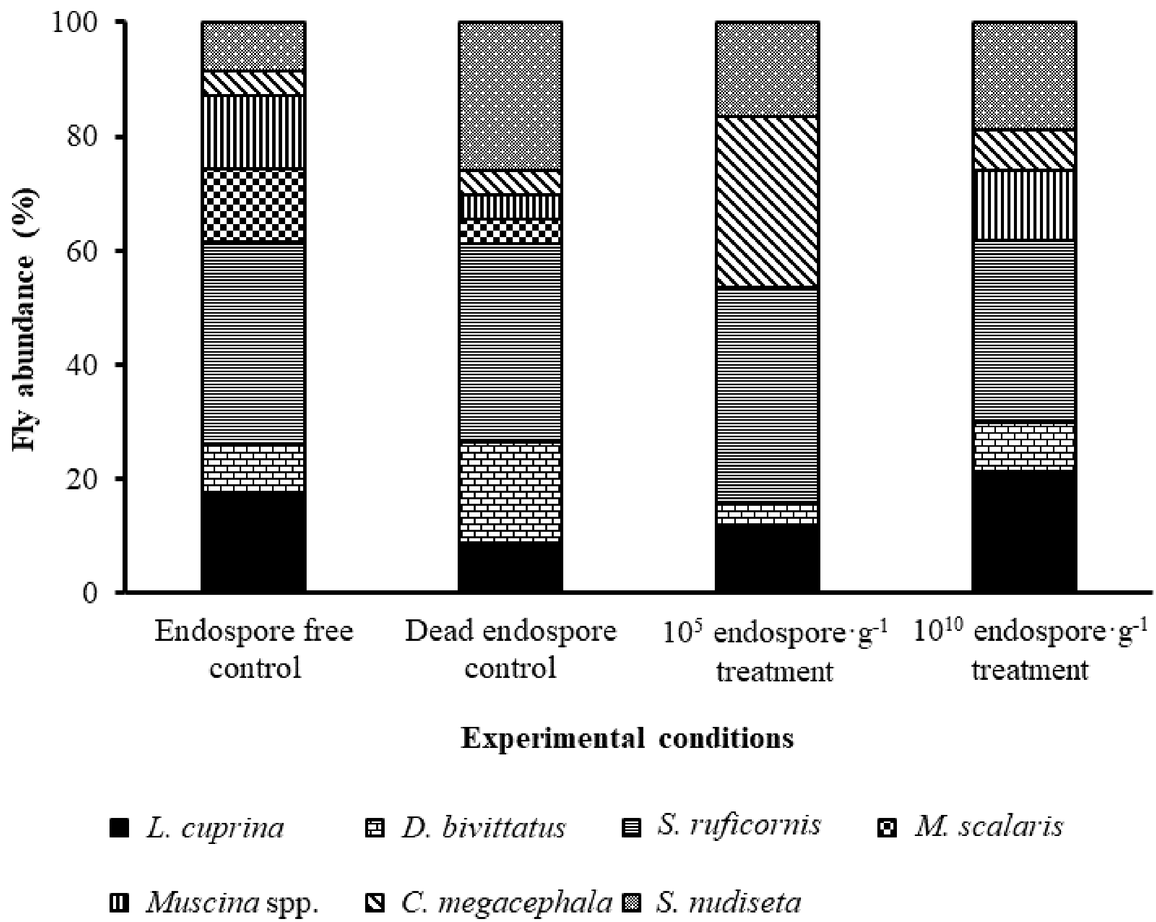

Fig. C2 Fly population observed during the first fly emergence event of the second farm trial. Percentage abundance was determined from the total number of flies that emerged from 9 – 24 d per treatment and control. The total number of flies counted in the endospore free control, dead endospore control, 10^5 endospore g^{-1} and 10^{10} endospore g^{-1} treatment was 295, 349, 84 and 165 flies respectively.

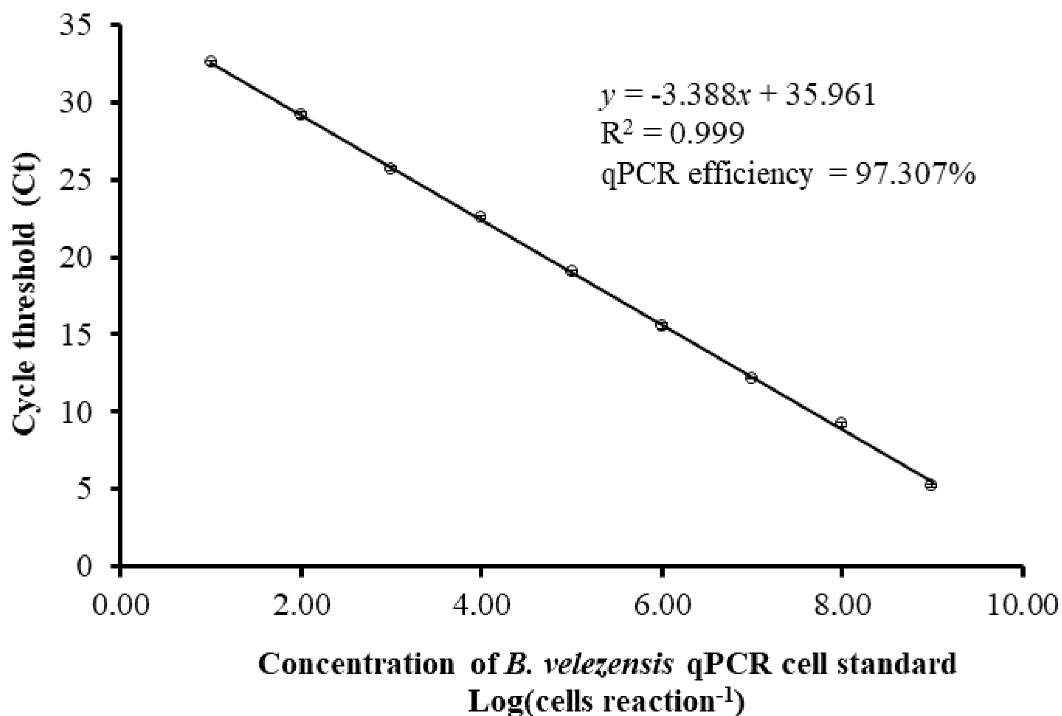

Fig. C3 qPCR standard curve correlating the cycle threshold (Ct) values to the concentration (10 – 10^9 cells reaction^{-1}) of *B. velezensis*. *Bacillus velezensis* qPCR standards, 10 – 10^9 cells reaction^{-1}, were comprised of genomic DNA of *B. velezensis* PHP1601 and were run in triplicate. The qPCR standard curve represents the cell concentration, Log(cells reaction^{-1}), on the *x*-axis and the copy threshold (Ct) values on the *y*-axis. All Ct values were determined using an amplification threshold of ΔRn = 0.04. The Ct values, amplification efficiency and standard curve data were acquired using the QuantStudio Scientific Design and Analysis software. Error bars represent SE.

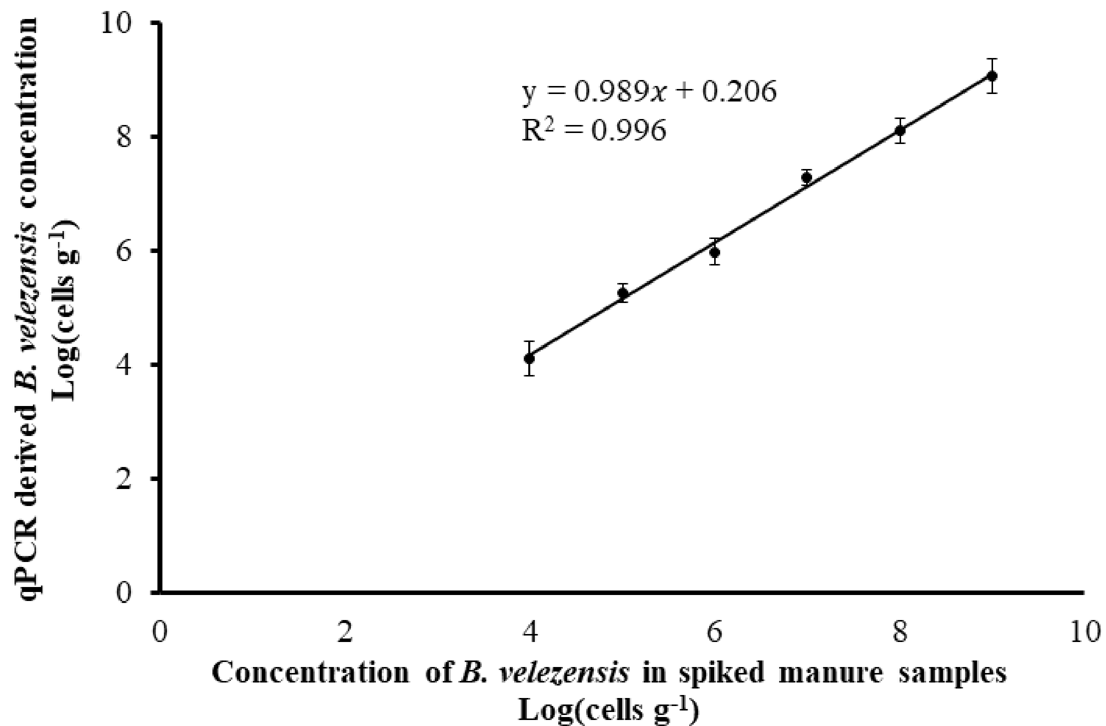

Fig. C4 qPCR derived concentration of *B. velezensis* (Log(cells g^{-1})) from *B. velezensis* spiked manure standards. *Bacillus velezensis* specific qPCR was used to detect and quantify *B. velezensis* species only. Pig manure samples were spiked with *B. velezensis* PHP1601 to achieve a concentration range of 1 – 10^9 cells g^{-1}. DNA extractions were done in duplicate. Error bars represent the standard error of the mean.

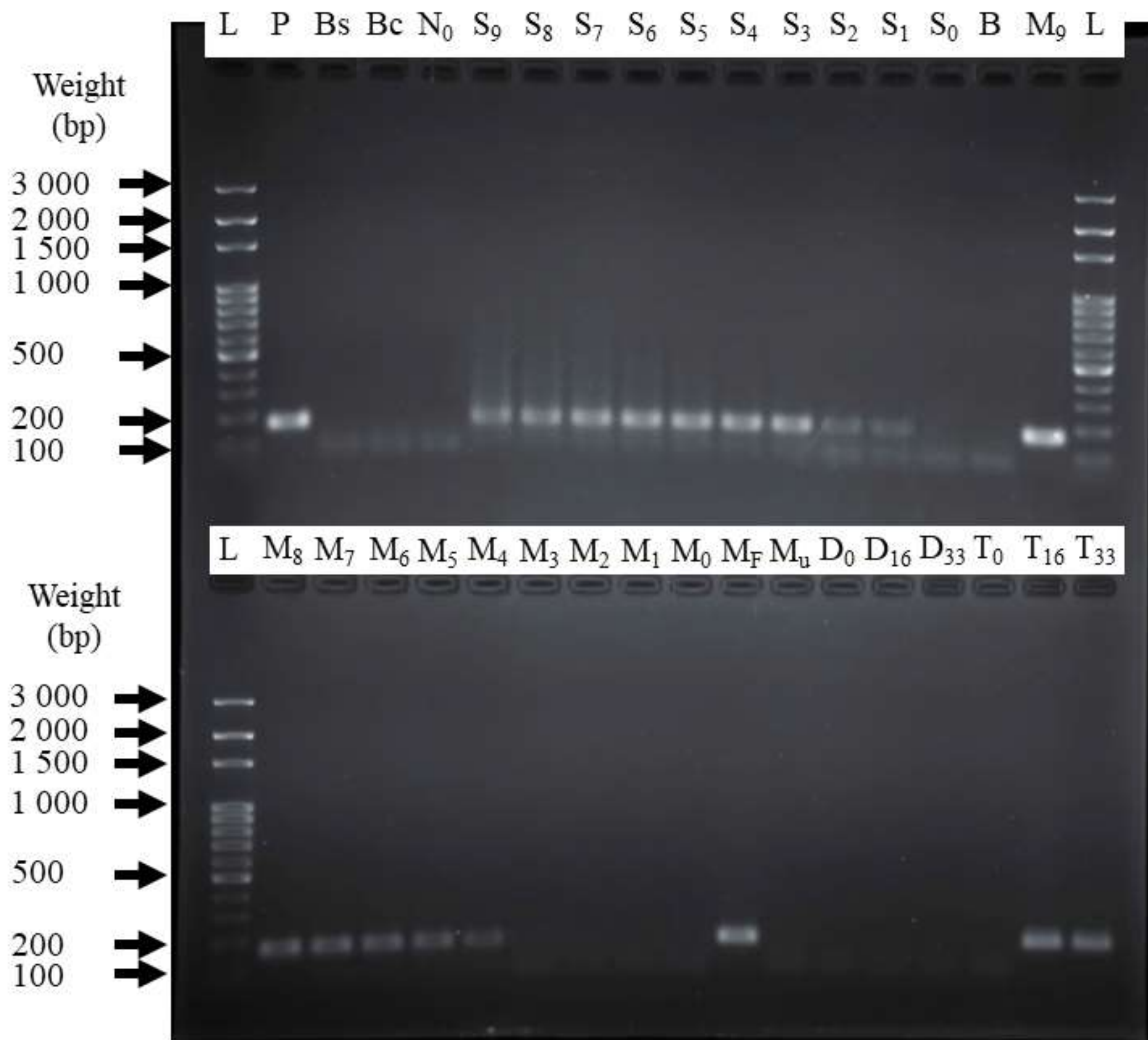

Fig. C5 Representative agarose (1.5% w v^{-1}) gel electrophoresis image of the products from the *B. velezensis* qPCR assay used to quantify *B. velezensis* in pig manure samples of in the second farm trial. The size of qPCR products were determined using the 1 kbp molecular weight ladder (L). A positive control (P) containing genomic DNA of *B. velezensis* PHP1601, non-specific amplification controls using genomic DNA of *B. subtilis* DSM10^T (Bs) and *B. cereus* DFbc (Bc) and a no template control (N_0) were used to assess for qPCR specificity and DNA contamination. *Bacillus velezensis* qPCR cell standards containing $10^9 – 10^0$ cell reaction^{-1} (S_9 – S_0) were used to facilitate qPCR quantification. Unamended manure (M_u), manure standards spiked with 10^9 – 10° cell g^{-1} (M_9 – M_0), manure spiked with 10^9 cells g^{-9} of *B. velezensis* FZB42^T (M_F) and the PowerSoil kit elution buffer (B) were tested to evaluate the

innate *B. velezensis* population, extraction efficiency of a the type *B. velezensis* strain, the limits of detection and potential kit contamination respectively. qPCR quantification of *B. velezensis* in the dead endospore (D) control and 10^5 endospore g^{-1} treatment (T) were conducted with manure samples from the 0, 16 and 33 d intervals of the manure trial.

Appendix D: Toxicological assessment of *Bacillus velezensis* PHP1601 based on zebrafish embryo toxicity assays and genome mining

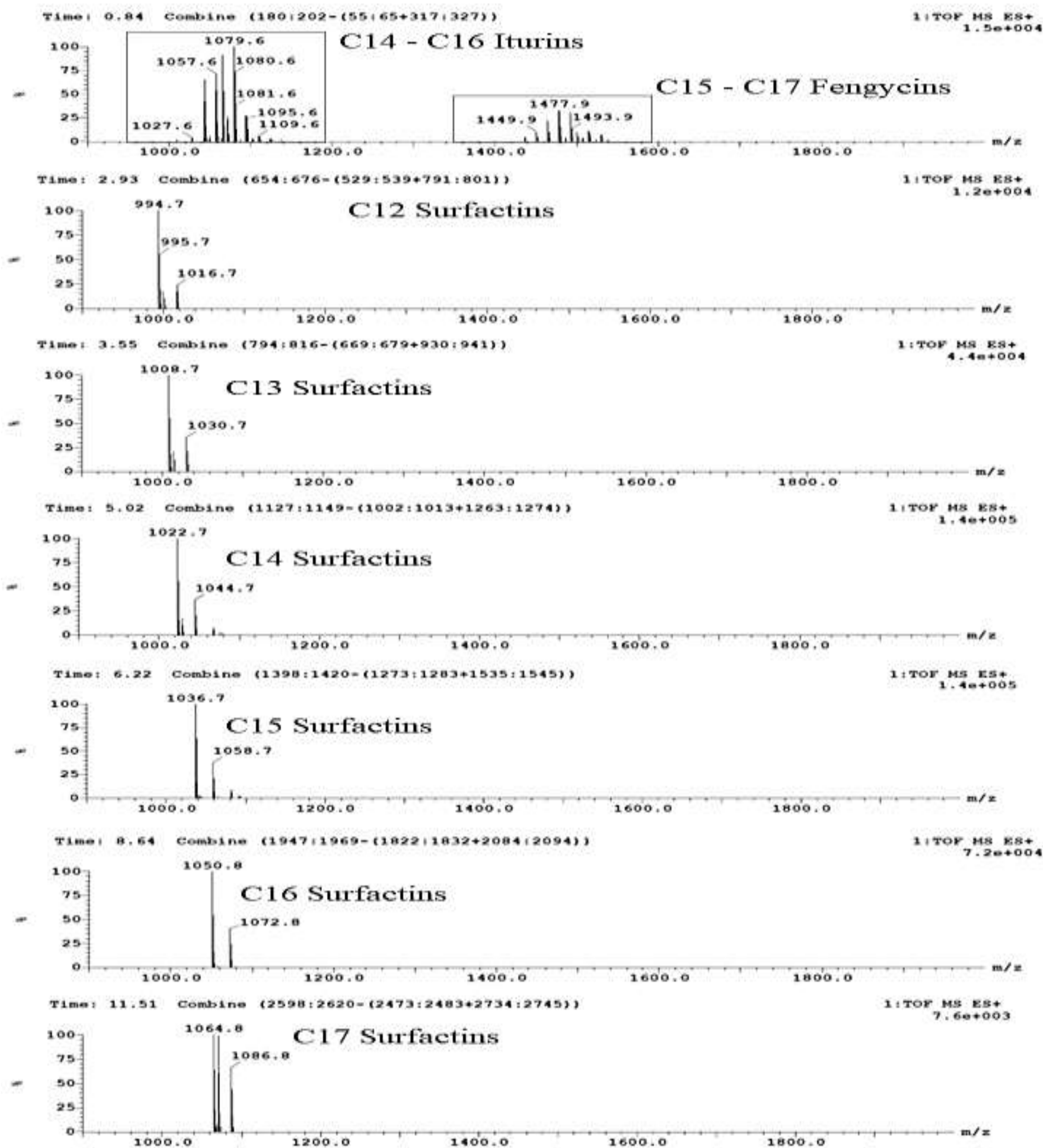

Fig. D1 UPLC chromatogram of indicating the lipopeptide biosurfactants eluted by chromatography and detected by ESI TOF MS. The mass peaks detected by ESI TOF MS were characterised by those present in the literature (Koumoutsi et al. 2004; Hunter 2016; Adu and Hunter 2021).

Table D1 Location (ORF) of virulence associated genes in the genome of *B. velezensis* PHP1601 detected by the VFDB.

Gene(s)	Gene location (ORF)	Direction
fbpA	+2385	+
*has*C	+2089	+
*cap*A	+537	+
*cap*B	+535	+
*cap*C	+536	+
*cap*D	+2066	+
*dhb*A	+977	+
*dhb*B	+980	+
*dhb*C	+978	+
*dhb*E	+979	+
*dhb*F	+981	+
*hly*III	+1925	+
*ctp*V	+798	+
*kat*A	+3099	+
*lsp*A	+2405	+

Genes were detected by the VFDB when the genome was analysed from the 5' - 3- direction (+) or the reverse (-). The location of the detected genes was described based on the location of their open reading frame (ORF).

Table D2 Location (bp) of AMR associated genes in the genome of *B. velezensis* PHP1601 detected by using CARD.

Gene	Gene location		
	Start (bp)	End (bp)	Direction
tet	1383338	1384714	+
*qac*J	2144003	2144317	+
*qac*J	2144335	2144688	+
*Bc*l	2776628	2777542	-
*qac*G	3353044	3353358	+
*qac*G	3467109	3467474	+
*clb*A	3397548	3398597	-

Putative AMR genes were detected by CARD when the genome of *B. velezensis* PHP1601 was analysed from the 5' - 3- direction (+) or the reverse (-). The location of the detected genes was described based on the location in base pairs (bp) on either the forward or reverse strand of the genome.

Table D3 Location (bp) of secondary metabolite gene clusters in the genome of *B. velezensis* PHP1601 detected by using AntiSMASH.

	Gene location		
Gene cluster	Start (bp)	End (bp)	Direction
Bacilysin	283925	343462	-
Difficidin	1556448	1648807	-
Bacillomycin D	1748383	1853458	-
Fengycin	1932370	2066680	-
Bacillaene	2140422	2240987	+
Macrolactin H	2460174	2548407	+
Surfactin	3544828	3610235	+

Complete gene clusters for secondary metabolite production were detected by AntiSMASH when the genome of B. *velezensis* PHP1601 was analysed from the 5' - 3- direction (+) or the reverse (-). The location of the detected genes was described based on the location in base pairs (bp) on either the forward or reverse strand of the genome.