Antimicrobial screening of secondary metabolites from Solanaceae

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This thesis was submitted for the degree of Doctor of Philosophy at Royal Holloway University of London, in September 2012

Candidate declaration

Candidate declaration

I declare that the work presented in this thesis is the original work of the author unless otherwise stated. Original material used in the creation of this thesis has not been previously submitted either in part or whole for a degree of any description from any institution.

Katarina Nice

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Katarina Nice

Abstract

Abstract

The aim of the project was to analyse the potential antimicrobial activity of extracted secondary metabolites from wild tomato species, and other Solanaceous plants. The wild tomato species such as *Solanum lycopersicum* cv M82, *Solanum cheesmaniae*, *Solanum chmielewskii*, *Solanum chilense*, *Solanum peruvianum*, *Solanum pimpinellifolium*, *Solanum pennellii*, *Solanum habrochaites*, and *Solanum neorickii* were the primary focus of the project which were analysed for antimicrobial compounds. The hypothesis that the wild tomato relatives could be antimicrobial is linked to the insecticidal and fungicidal properties of several wild tomato relatives. This research is further supported by the previous findings into the antimicrobial potential of certain Solanaceae species, where the leaf tissue was noted to have the highest general antimicrobial activity.

As part of this project the fully expanded undamaged leaves were examined for antimicrobial activity, after extracting the secondary metabolites in a range of solvents. Antimicrobial activity was determined through monitoring the plant extracts effect on the growth of gastroenteric bacteria *Salmonella*, *Escherichia coli*, and *Staphylococcus aureus* primarily through disc diffusion assays and growth curve analysis. The metabolomic profile of *Staphylococcus aureus* grown in the presence of identified antimicrobial *Solanum pennellii* extract was used to identify the compounds mode of action, and electron microscopy was used to view the effects of this extract upon the bacteria cell structure.

Further putative identification of selected antimicrobial extracts was achieved through the use of thin layer chromatography, and gas chromatrography-mass spectrometry analysis. The Solanaceae plants which were identified to have a significant antimicrobial activity were *Solanum habrochaites*, *Solanum pennellii*, and *Nicotiana rustica*. The trichomes of these plants were identified as the primary target site for the production of these antimicrobial compounds.

Acknowledgements

Acknowledgements

I would like to thank all my family and friends for their continued support and motivation during this period of my life. Particularly my best friend Emily Shell who is always there for me when I need her, my mother Jane Head for her enthusiastic encouragement, and my husband Sebastian Nice for his love and support.

I would like to thank all my colleagues at the AHVLA and the RHUL who have aided me in this research, and who have trained me in specific laboratory techniques. With special thanks to Mary Bagnall, Emma Kennedy, Chris Gerrish, Dr. Daniel Rickett, Dr. Laura Perez-Fons, Dr. Michael Goodfellow, Dr. Matthew Jones, Dr. Leticia Mora, and Dr. Tom Wells, for your help and support.

I would also like to thank Bill Cooley for performing the EM work, and I would like to thank Prof. Graham Seymour at the University of Nottingham for initially supplying plant material.

I could not have completed this project without the financial support and resources from the Animal Health and Veterinary Laboratories Agency and the Royal Holloway which included a Thomas Holloway Scholarship.

My supervisors have been fantastic, and I would like to thank them all sincerely; Prof. Roberto La Ragione for his sensible guidance, Prof. Peter Bramley for his advice and support, and particularly Dr. Paul Fraser for his confidence in me, and for allowing me to complete this research with creative freedom.

I thank you all.

Dedication

Dedication

This thesis is dedicated to my husband Sebastian Nice, because he has dedicated his life to me.

In memory of Anakin.

List of Abbreviations

Abs	Absorbance
ARB	Antibiotic resistant bacteria
ANOVA	Analysis of variance
AUC	Area under the curve
BCFAs	Branched-chain fatty acids
C	Carbon
c	Celsius
Ca	Chlorophyll a
Cb	Chlorophyll b
CDT	Cytolethal distending toxin
CBTols	Cembratrienols
CFU	Colony forming unit
CI	Confidence interval
CMV	Cucumber mosaic virus
cv	Cultivar
DPPC	Dipalmitoylphosphatidycholine
DHEC	Diarrhoea-associated haemolytic
DMS	Dimethyl sulfide
DMSO	Dimethyl sulfoxide
DAHP	3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase
DNA	Deoxyribonucleic acid
DW	Dry weight
EPEC	Enteropathogenic *E. coli*
ESBIs	Extended spectrum beta lactamases
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ETEC	Enterotoxigenic *E. coli*
EaggEC	Enteroaggregative
ECM	Extracellular matrix
EM	Electron microscopy
EPS	Exopolysaccharides
ELISA	Enzyme-linked immunosorbent assay
FW	Fresh weight
GC-MS	Gas chromatography-mass spectrometry
HCL	Hydrochloric acid
HPLC-PDA	High performance liquid chromatography-photodiode array
HPLC	High performance liquid chromatography
h	Hour
HUS	Haemolytic uremic syndrome
KPT	Potassium phosphotungstate
ILs	Introgression lines
L	Litre
LB	Luria broth
LPS	Lipopolysaccharide
LC-MS	Liquid chromatography-mass spectrometry
LC-PDA-TOFMS	Liquid chromatography-photodiode-array-time of flight-mass spectrometry
MDR	Multi drug resistant

MAS	Marker-assisted selection
MEOX	Methylhydroxylamine hydrochloride
MALDI	Matrix-assisted laser desorption/ionization
MF	Match factor
min	Minute
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSTFA	*N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide
NAM	*N*-acetylmuramic acid
NAG	*N*-acetylglucosamine
NCEs	New chemical entities
NIST	National Institute of Standards and Technology
OD	Optical density
PBS	Phosphate buffer saline
PCA	Principle component analysis
PPO	Polyphenol oxidase
QTL	Quantitative trait loci
R_f	Relative mobility factor
RT	Retention time
RTPCR	Reverse transcriptase polymerase chain reaction
rdar	Red dry and rough
RP	Reverse phase
RTPCR	Reverse transcription polymerase chain reaction
SEs	*Staphylococcal* enterotoxins
SEM	Standard error of the mean
SCAs	Sesquiterpene carboxylic acids
SC	Self-Compatible
SI	Self-Incompatible
spp	Species
SPIs	*Salmonella* pathogenicity islands
SPE	Solid phase extraction
StAPs	Aspartic proteases
STEC	Shiga toxin producing *E. coli*
TEM	Transmission electron microscopy
TLC	Thin layer chromatography
TMS	Trimethylsilyl group
TSST-1	Toxic shock *staphylococcal* toxin 1
TYLCV	Tomato yellow leaf curl virus
ToMoV	Tomato mottle virus
TMV	Tobacco mosaic virus
ToMV	Tomato mosaic virus
Tris	*Tris*-hydroxymethyl-aminomethane
UNKnp	Unknown from a non-polar extract
UNKp	Unknown from a polar extract
UV	Ultra violet
v	Volume
w	Weight
WT	Wild-type
ZIA	Zone of inhibition assay

List of Equations

1. % yield = Mass of extracted plant material /Mass of extracted plant residue x 100

2. R_f = distance of solvent migration (cm) / distance of compound (band) migration (cm)

3. µg g^{-1}DW=[Area / (mass (mg) of plant sample DW) x 1000] / [Area of IS / µg of IS]

4. X = (Abs x Y x1000) /absorbance coefficient x100)

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Chapter 1

Introduction

1.1 Aims and objectives

This project aimed to identify antimicrobial compounds produced by Solanaceae species. Whilst there have been previous studies into the antimicrobial potential of certain Solanaceae, there has been no extensive research into the potential antimicrobial activity of secondary metabolites extracted specifically from wild tomato species. The tomato species *S. lycopersicum* cv. M82, *S. cheesmaniae*, *S. chmielewskii*, *S. chilense*, *S. peruvianum*, *S. pimpinellifolium*, *S. pennellii*, *S. habrochaites*, and *S. neorickii* were the primary focus of the project, which were analysed for the presence of antimicrobial activity and candidate compounds conferring this activity. The hypothesis that the wild tomato relatives could be antimicrobial is linked to the insecticidal and fungicidal properties of several wild tomato relatives (Besser et al., 2009; Moco et al., 2006; Skaltsa et al., 2003). This study looks further into the bactericidal effects of these extracts through monitoring how the plant extracts affect the growth of clinically relevant enteric and non-enteric bacteria such as *Salmonella*, *E. coli*, and *S. aureus*, primarily through disk diffusion assays and growth curve analysis.

Once the antimicrobial Solanaceae species are identified, a further investigation into the active compounds is necessary so that the identified compounds can be purified. The specific plant organ and extraction procedure needs to be discovered for optimal recovery of the antimicrobial compounds from the plants. Putative identification of selected antimicrobial extracts can be achieved through the use of thin layer chromatography, HPLC, GC-MS, and LC-MS approaches, and comparisons of candidates with authentic chemical standards/extracts. The identification of antimicrobial secondary metabolites from Solanaceae species has several potentially valuable applications, such as using the compounds as antibacterial drugs, preservatives, or producing these compounds in other Solanaceae species to increase their resistance to pathogenic bacteria.

The mode of action of the identified antimicrobial compounds is also of value, due to the continuing need to find new target sites for drug discovery. Metabolomic analysis of bacteria grown in the presence of identified antimicrobial plant extract can indicate the effects the compounds have upon the metabolism of the cell, and electron

microscopy can be used to view the effects of this extract upon the bacteria cell structure. If the mode of action of the antimicrobial compounds is discovered this can help to indicate in which capacity the compounds can be utilised.

1.2 Solanaceae family

The Solanaceae species includes over 3000 species, such as tomato, potato, chilli pepper, nightshade, petunia, and tobacco (Zygadlo et al., 1994). These plants have a high economic value worldwide and are used for foods, spices, and medicines (Friedman, 2002). Many of these plants are rich in alkaloids, and examples of medicines which have been identified are atropine, hyoscine (Lee, 2007) and nicotine (Friedman, 2002). Although Solanaceae fruit and vegetables are widely consumed, the leaves of these plants can be toxic to humans due to the presence of alkaloids, such as *α*-tomatine in tomato leaves. The rich source of alkaloids and other secondary metabolites makes Solanaceae plant species have a high potential for drug discovery (Table 1-2).

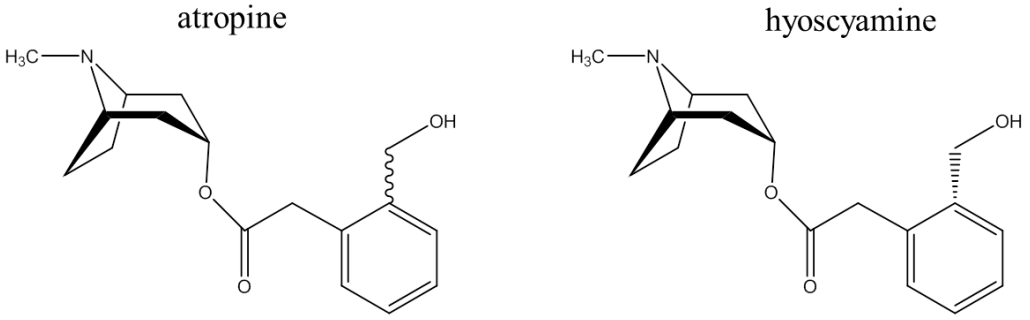

Figure 1-1. Structures of tropane alkaloids.

Structures represent hyoscyamine, and atropine. These are common tropane alkaloids produced within certain Solanaceae species, drawings adapted from Chemspider (www.chemspider.com) using ChemDraw Pro 13.0 (www.cambridgesoft.com)

Potato (*Solanum tuberosum*) is the world's fourth most important crop (Ducreux et al., 2005). Potatoes are a rich source of steroidal glycoalkaloids, such as *α*-solanine and *α*-chaconine, which are bitter and toxic to humans, and several breeding strategies have been used to try to reduce these compounds for improved human consumption (Kozukue et al., 1999). Tomato species also produce toxic glycoalkaloids, primarily α-tomatine within the leaves and the soluble cytoplasm phase of fruit cells. The green

organs of the plants are poisonous if consumed, and α-tomatine has an intravenous LD_{50} value equal to 18 mg mL^{-1} in mice (Friedman and Levin, 1995). Glycoalkaloids such as *α*-tomatine are believed to be produced to defend plants against insects and fungal plant pathogens, and act through disrupting cell membranes by lysing liposomes (Friedman and Levin, 1995).

Tomato is an economically important plant worldwide, and the fruit is known to be rich in vitamins and antioxidants, primarily carotenoids such as lycopene, *β*-carotene (pro-vitamin A) and a rich source of vitamin C. Tomatoes are an important source of vitamin C worldwide, and they provide around 10% of the total dietary intake of vitamin C within the USA (Gerrior and Bente, 2002). The tomato species which is consumed the most in fresh and processed food around the world is *S. lycopersicum* "cerasiforme", followed by the fruit of *S. pimpinellifolium*, and whilst some of the other wild tomato relatives are slightly edible, they are mainly reported to be used by the indigenous people of the Andean region for medicinal purposes (Schauer et al., 2005; Grandillo et al., 2011).

The larger fruit size of the domesticated tomato *S. lycopersicum* cv. M82 has been bred to meet the requirements of a high tomato yield, desirable ripening, optimal fruit size and colour, cultivated through selective breeding of the cherry tomato *S. lycopersicum* "cerasiforme" (Schauer et al., 2005). The domestication of this species of tomato for human consumption has led to an inevitable loss in genetic variance (Schauer et al., 2005), and allows the domesticated *S. lycopersicum* to be more susceptible to plant pathogens.

The genus *Capsicum* is also part of the Solanaceae family, and like the tomato relatives, originates from South America in the regions of Central America and the Andean region (Paran et al., 2007). *Capsicum* spp. have been domesticated and grown as vegetables. They are very important spices worldwide (Materska and Perucka, 2005) with the actively pungent alkaloid capsaicinoid compounds having antimicrobial activities, and medicinal properties (Cowan, 1999).

Figure 1-2. An example of jalapeño chilli pepper (*Capsicum annuum*).
The fruit development is shown from the flower to the ripe fruit. *Capsicum* spp. is an economically important Solanaceae crop species.

The aubergine (*Solanum melongena* L.) belongs to the Solanaceae family, originates from Eastern countries, and was domesticated as a vegetable, through breeding for larger fruit size, decreased bitterness, and removing plant prickliness (Frary et al., 2007). The plant bitterness is mainly due to the production of alkaloids within the fruit, and the production of alkaloids, phenolic acids and anthocyanins have led to the aubergine being used in traditional medicine (Frary et al., 2007).

1.2.1 Solanaceae species with known antimicrobial activities

Antimicrobial screening of Solanaceae plants has been specific to certain individual plant species, with little investigation or comparison performed between varieties (Table 1-1). Many Solanaceae species originate from South America, a region in which many antimicrobial plants have been previously discovered. For example, antimicrobial screening of essential oils from 29 Brazilian medicinal plants against *E. coli* has been investigated by Duarte et al., (2007) which included the plant families Asteraceae, Liliaceae, Boraginacea, Poaceae, Cyperaceae, Verbenaceae, Lamiaceae, Piperaceae, and Malvaceae, but not any Solanaceae. The Solanaceae family of plants has been a good source of medicinal compounds. There have been several investigations into the antimicrobial activities of Solanaceae species where evidence suggests that there could be antimicrobial potential, particularly against *E. coli* and *S. aureus* (Table 1-1).

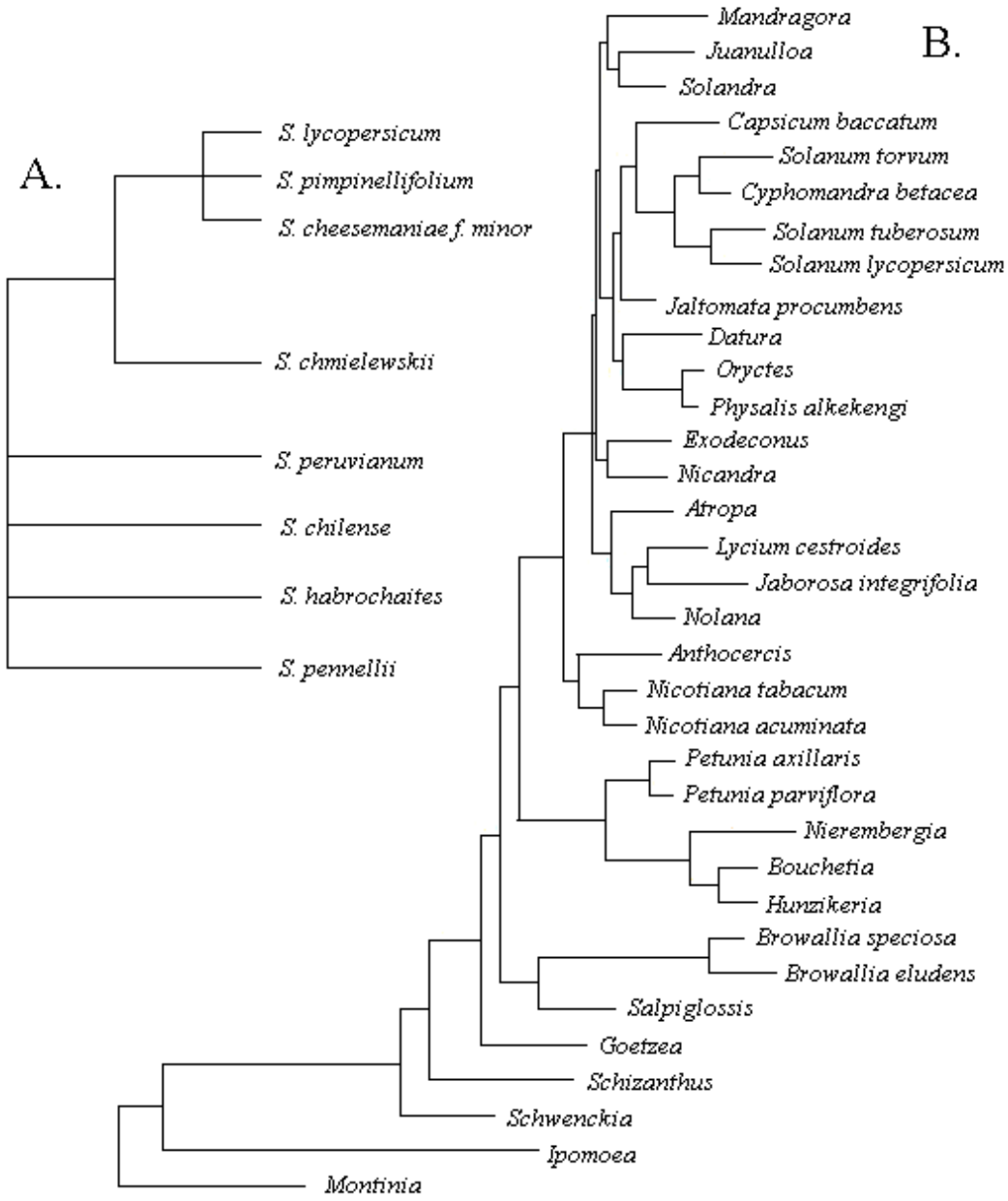

Figure 1-3: Solanaceae phylogenetic tree.

A. Phylogenetic tree adapted from the gene phylogeny determined using the granule-bound starch synthase (GBSSI) gene in wild tomato species by Peralta and Spooner (2001). B. Phylogenetic tree determined by chloroplast genes *rbcL* and *ndhF* by Olmstead et al. (1999).

Table 1-1: Solanaceae plants with antimicrobial compounds produced.

Species Name	Common name	Compounds	Extraction method	Antimicrobial activity	References
Fabiana broides	False heath	-	500 µg mL^{-1} leaf extract ethanol	MIC 50-100 µg mL^{-1} against *E. coli, Bacillus subtilis, Streptococcus* sp., *S. aureus, Salmonella* Typhi	Sheeba, 2010
Solanum palinacanthum	-	Rutin and 3,5-dicaffeoylquinic acid	Leaf material	*Aeromonas hydrophila, B. subtilis, S. aureus*, and *Aspergillus ochraceus*.	Pereira et al., 2008
Solanum pyracanthun	Porcupine tomato	Tropane alkaloids	Leaves, stems and fruit	Generally toxic, resistant to *Aphis gossypii Glov*.	Sambandam et al., 1970
Physalis alkengi	Chinese lantern	Physalins	Fruit	Used as an antiseptic	Kawai et al., 1992
Hydroscyamus niger	Henbane	Scopolamine	Methanol extracts of leaves	Antimicrobial against *A. niger, B. subtilis, E. coli, P. aeruginosa*, and *S. aureus*.	Khan et al., 1992
Physalis peruviana L.	Cape gooseberry	Withanolides	Aerial parts	Antioxidant, cytotoxic	Lan et al., 2009
Datura Stramonium	Jimson Weed	Saponins, tannins, alkaloids, and glycosides	Ethanol extract of leaves	Antimicrobial against *P. aeruginosa, Klebsiella pneumonia* and *E. coli*	Banso and Adeyemo, 2006.

Jimson weed (*Datura stramonium*) contains saponins, tannins, alkaloids and glycosides, and the ethanol extract has been found to be antimicrobial against *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Escherichia coli* (Banso and Adeyemo, 2006). The methanol extract of *Solanum palinacanthum* has antimicrobial activities against *S. aureus* ATCC 25923 with an MIC of 2500 µg mL^{-1}, and the isolated compounds rutin and 3,5-dicaffeoylquinic acid had MICs of >1000 µg mL^{-1} against *S. aureus* (Pereira et al., 2008). *Solanum tomentosum* showed antimicrobial activity from acetone extracts against Gram-negative bacteria and methanol extract against Gram-positive bacteria (Aliero and Afolayan, 2006). *Cestrum diurnum* is antimicrobial against *S. aureus* and *P. aeruginosa*, and is toxic to humans. Its leaves contain a calcinogenic glycoside 1, 25-dihydroxychlocalciferol which acts by increasing calcium ion Ca^{2+} leading to vitamin D toxicity, and the antimicrobial oil contains palmitic, stearic and oleic acid (Bhattacharjee et al., 2005). *Physalis* is a genus within the Solanaceae family mostly native to North and South America, and a physalin B from *Physalis angulata* was shown to inhibit *S. aureus* and *N. gonorrhoeae* (Silva et al., 2005).

Table 1-2: Secondary metabolites in tomato species which have been found to have pharmaceutical, antimicrobial, or antioxidant activities.

Tomato species	Class	Compound	Activity	Reference(s)
S. lycopersicum, in most organs, except ripe fruit.	**Alkaloids**	*α*-tomatine (*β*, *δ*, *γ*-tomatine)	Inhibits the growth of fungi through binding to 3*β*-hydroxy sterols in fungal membranes and cytotoxic to animal cell lines	Eltayeb and Roddick, 1984 Kozukue et al., 2004 Sandrock and VanEtten, 1998 Ono et al., 1997
S. lycopersicum, from ripe fruits pink colour type.		esculeoside A	Steroidal alkaloid glycosides	Fujiwara et al., 2004
S. lycopersicum, from ripe red colour type.		esculeoside B	Steroidal alkaloid glycosides	Fujiwara et al., 2004
S. lycopersicum		dehydrotomatine (tomatidenol-3-*β*-lycotetraose).	Cytotoxic to animal cell lines	Moco et al., 2006 Kozukue et al., 2004 Ono et al., 1997 Friedman et al., 1997
S. lycopersicum, isolated from the fruits and leaves.		lycoperoside (A, B, C, G, F, H)	Steroidal alkaloid glycoside	Moco et al., 2006 Yahara et al., 1996
		trigonelline	Extracts of trigonelline from coffee were shown to be antibacterial against several species including *E. coli* and *S. enterica*	Lòpez-Gresa et al., 2009 Almeida et al., 2006
S. habrochaites (LA1777)	**Terpenoids** *(Class I sesquiterpenes)*	germacrene B	Increased resistance to insects	Hoeven et al., 2000 Besser et al., 2009
S. habrochaites (LA1777)		germacrene D	Increased resistance to insects	Hoeven et al., 2000 Besser et al., 2009
S. lycopersicum, and *S. habrochaites (LA1777)*		*α*-humulene	Increased resistance to insects	Hoeven et al., 2000 Besser et al., 2009
S. lycopersicum and *S. habrochaites (LA1777)*		*β*-caryophyllene	Increased resistance to insects. Extracts from *Stachys* species have been shown to be antibacterial with an MIC for *E .coli* at 0.2 mg mL^{-1} and antifungal with an MIC at 0.09 mg $mL^{-1}$for *A. niger*	Besser et al., 2009 Skaltsa et al., 2003
S. lycopersicum, and *S. habrochaites (LA1777)*		*β*-elemene	Anti-cancer agent, with a low toxicity	Besser et al., 2009 Li et al., 2009
Trichomes of *S. habrochaites (LA1777)*		*γ*-elemene	Present in the antimicrobial essential oils of several plant species, such as *Salvia verbenaca*	Besser et al., 2009 Al-Howiriny, 2002
S. habrochaites (LA1777)	*(Class II sesquiterpenes)*	*α*-santalene	Increased resistance to insects	Hoeven et al., 2000
Trichomes of *S. habrochaites (LA1777)*		*α*-bergamotene	Increased resistance to insects	Hoeven et al., 2000 Besser et al., 2009

Trichomes of *S. habrochaites (LA1777)*	*Monoterpenes*	limonene	Used as an insecticide	Besser et al., 2009 Schie et al., 2007
Trichomes of *S. lycopersicum* cv. M82		*α*-pinene, perbenene, δ-2-carene, *β*-phellandrene	*α*-pinene from *Stachys* species has been shown to be antibacterial with an MIC for *E. coli* at 0.2 mg mL^{-1}, and antifungal with an MIC at 0.09 mg mL^{-1} for *A. niger*	Besser et al., 2009 Skalta et al., 2003
Trichomes of *S. habrochaites (LA1777)*	*Other*	*β*-cubebene	Present in the antimicrobial essential oils of several plant species, such as *Ballota pseudodictamnus*	Besser et al., 2009, Couladis et al., 2002
S. habrochaites L. Pl126449	*Methylketones*	2-undecanone	Insect and animal repellent	Fridman et al., 2005
Isolated from tomato fruit	**Phenolic Acids** *Benzoic acids*	protocatechuic acid	Antimicrobial (high activity on *Mucor* sp.) Extracts of protocatechuic acid from coffee were shown to be antibacterial against including *E. coli*, and *S. enterica*	Penna et al., 2001 Mattila and Kumpulainen, 2002; Moco et al., 2006 Adriana et al., 2006
Isolated from tomato fruit		*p*-hydroxybenzoic acid	A food preservative, which acts on the proton motive force in *E. coli* membrane vesicles, inhibiting the uptake of reducing substances	Moco et al., 2006 Eklund, 1985
S. lycopersicum leaves infected with *P. syringae*		linoleic acid	Antimicrobial properties such as shown against *Bacillus cereus,* and *S. aureus*	López-Gresa et al., 2009 Lee et al., 2002
S. lycopersicum cv. Rutgers infected with tomato mosaic virus		gentisic acid	Accumulates after infection with tomato mosaic virus	Bellés et al., 1999
		vanillic acid	Vanillic acid has an antimicrobial effect upon *Listeria spp.*	Moco et al., 2006 Delaquis et al., 2005
	Cinnamic acids	isobutyl cinnamate	Antimicrobial action against both Gram-positive and Gram-negative bacteria, and good antifungal activity. Lipophilic activity	Narasimhan et al., 2004 Moco et al., 2006
		dibromo cinnamic acid	Antimicrobial action against both Gram-positive and Gram-negative bacteria, and good antifungal activity. Lipophilic activity	Narasimhan et al., 2004 Moco et al., 2006
		m-coumaric acid	Active against *L. monocytogenes*. Toxic to potential herbivores	Moco et al., 2006 Wen et al., 2003 Dixon and Paiva, 1995
		p-coumaric acid	Anti-platelet and anti-inflammatory properties, making the plant toxic to potential herbivores. Active against *L. monocytogenes*	Mattila and Kumpulainen, 2002 Luceri et al., 2007 Wen et al., 2003 Dixon and Paiva, 1995
S. lycopersicum leaves infected with *P. syringae*.		caffeic acid	Active against *L. monocytogenes*. Extracts of caffeic acid from coffee were shown to be antibacterial against several species including *E. coli*, and *S. enterica*	Moco et al., 2006 Wen et al., 2003, Lòpez-Gresa et al., 2009

S. lycopersicum leaves infected with *P. syringae*		ferulic acid	Antimicrobial against Gram-positive and Gram-negative bacteria and fungi, strong activity against gastrointestinal micro-flora. Active against *L. monocytogenes*	Mattila and Kumpulainen, 2002; Ou and Kwok, 2004 Wen et al., 2003 Lòpez-Gresa et al., 2009
		salicylic acid	Down regulates virulence factors of *Pseudomonas aeruginosa*, and *S. aureus*	Prithiviraj et al., 2005 Kupferwasser et al., 2003
		sinapic acid	Bactericidal towards *Salmonella enterica* subsp. *enterica*. It has been found from mustard seeds that the two methoxyl groups and hydroxyl group in sinapic acid had antibacterial activity against *E. coli*, all the substituents of the benzene rings were effective against *S. enteritidis*, and the propenoic group was effective against *S. aureus*	Moco et al., 2006 Johnson et al., 2008 Tesaki et al., 1998
S. lycopersicum leaves infected with *P. syringae*		chlorogenic acid	Active against *L. monocytogenes*. Extracts of chlorogenic acid from coffee were shown to be antibacterial against *E. coli*, and *S. enterica*	Moco et al., 2006 Wen et al., 2003 Lòpez-Gresa et al., 2009 Adriana et al., 2006
		anthocyanins	Antioxidants, and may have some antiviral and antimicrobial activities	Sapir et al., 2008 Bongue-Bartelsman et al., 1994
Isolated from tomato skin		naringenin	Naringenin has antimicrobial activity against several bacterial and fungal species	Moco et al., 2006 Rauha et al., 2000
Isolated from tomato skin		naringenin chalcone	Anti-allergic, inhibits histamine release in humans	Moco et al., 2006
		kaempferol	Has shown to have a strong antimicrobial activity against *E. coli, B. subtilis*, *Klebsiella pneumoniae*, *Bacillus polyxyma* and *C. albicans*	Moco et al., 2006 Adeloye et al., 2007
		quercetin	Induces apoptosis in *Trypanosoma brucei*, and reduces the inflammation caused by activated human macrophages. Antimicrobial to bacterial and fungal species.	Moco et al., 2006; Mamani-Matsuda et al., 2004 Rauha et al., 2000
		myricetin	Inhibits *E. coli* DnaB helicase. It inhibits telomerases, kinases, helicases, DNA/RNA polymerases, reverse transcriptases.	Moco et al., 2006 Griep et al., 2007
		astragalin	Has anti-inflammatory, anti-allergenic and antimicrobial activity	Moco et al., 2006 Kovganko et al., 2004
		isoquercitrin	Strong inhibitory activity against *S. aureus*, *Proteus rettgeri*, *Candida lusitaniae*, *Candida tropicalis* and *Microsporum gypseum*. *Staphylococcus* sp.	Moco et al., 2006 Pepeljnjak et al., 2005
S. lycopersicum leaves infected with *P. syringae*		rutin	Strong inhibitory activity against *Staphylococcus aureus*, *Proteus rettgeri*, *Candida tropicalis* and *Microsporum gypseum*. *Staphylococcus* sp. (coagulase-negative). *Fusarium graminearum* also strongly inhibited. 35µg mL^{-1} MIC against *Aspergillus ochraceus*	Moco et al., 2006 Pepeljnjak et al., 2005 Lòpez-Gresa et al., 2009 Pereira et al., 2008
	Defensins	DEF2	Defensins are antimicrobial peptides, such as DEF2 which is expressed during early flower development, and protects against *Botrytis cinerea*	Stotz et al., 2009

1.2.2 Wild tomato relatives

Wild tomato plants are herbaceous species, although they are known to produce secondary growth within their native habitats, and they usually act as annuals in response to environmental effects such as frost or drought (Peralta and Spooner, 2000) The wild tomato relatives are a rich source of genes, and have different phenotypes based on the geographical location of their habitats, the wild tomato species are mainly native to South America, particularly across the regions of Peru, Chile, Ecuador, Colombia and the Galápagos Islands. The different geographical distribution of the wild tomato species is reflected in their genetic diversity, and physical barriers such as deserts and mountains have kept the wild tomato species genetically distinct (Grandillo et al., 2011).

Different breeding strategies and quantitative trait loci (QTL) mapping for various desirable traits have been explored, such as improving yield, stress tolerance, pathogen resistance, taste, and nutritional properties (Schauer et al., 2005; Panthee and Chen, 2010). This has been done using the wild tomato species to genetically improve the domesticated tomato which has a poor genetic diversity from inbreeding during the domestication of the tomato (Zhang et al., 2002). This has led to investigations into the secondary metabolites produced such as phenolic (insecticidal) properties of the wild tomato relatives (Antonious et al., 2003).

The fruit sizes of all wild species of tomato are smaller and usually stay green at maturity (with exception to red fruited *S. pimpinellifollum*) (Schauer et al., 2005). The use of wild tomato relatives for desirable traits comes with challenges, such as many of the wild tomato species have small green fruit, which is not a desirable phenotype to acquire by the domestic tomato, and they are also incompatible with the domestic species with the exception of *S. cheesmaniae, S. galapagense, and S. pimpinellifolium*.

The *Solanum* sect. *Lycopersicum* includes 13 species and subspecies of tomato: the domesticated *S. lycopersicum* ‘cerasiforme’, and the wild tomato species *S. arcanum, S. cheesmaniae, S. pimpinellifolium, S. chilense, S. chmielewskii, S. corneliomulleri, S. galapagense, S. habrochaites, S. huaylasense, S. neorickii, S. pennellii, and S. peruvianum* (Grandillo et al., 2011).

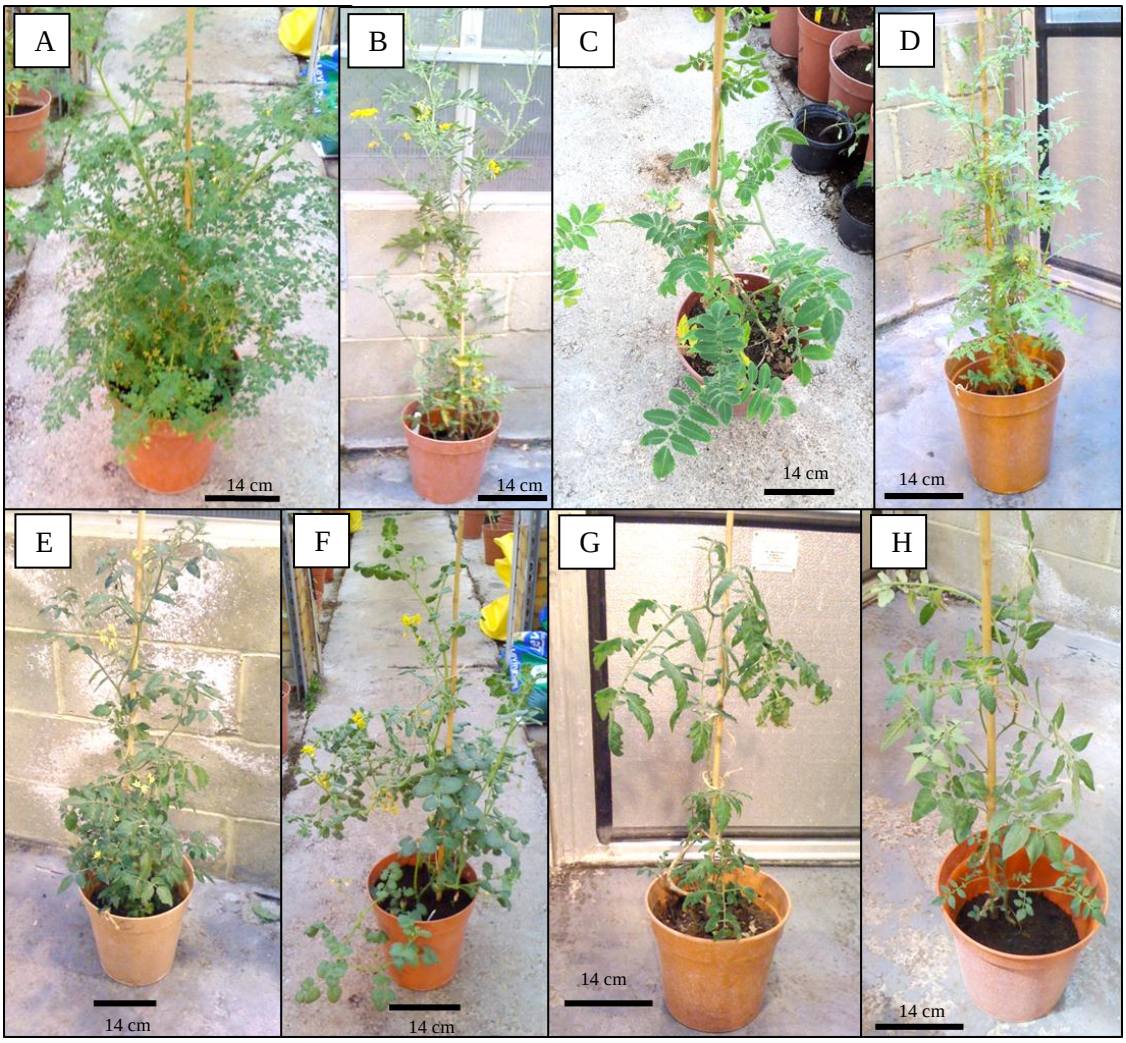

Figure 1-4: The mature plants from selected wild tomato species.

Mature plant species; A. *S. cheesmaniae* LA1984, B. *S. peruvianum* LA2744, C. *S. habrochaites* LA1777, D. *S. chilense* LA2759, E. *S. pimpinellifolium* LA0114, F. *S. pennellii* LA0716, G. *S. lycopersicum* cv. M82, H. *S. neorickii* LA2133. The plant species were grown in a controlled environment within a greenhouse, and pictures were taken at the flowering stage of each plant which all showed different development times. All the black scale bars represent 14 cm.

There are five main wild tomato species which are easily crossed with *S. lycopersicum*, these include *S. pennellii*, *S. habrochaites*, *S. pimpinellifolium*, *S. neorickii*, and *S. chmielewskii* (Schauer et al., 2005). *S. pennellii* originates from a dry habitat, and this tomato species has evolved to have greater salt and drought resistance (Tal et al., 1979). *S. habrochaites* originated from Southern Ecuador, with tolerance to cold temperatures, and produces pest control products such as the pesticide-2-tridecanone (Schauer et al., 2005). *S. pennellii* has a leaf metabolite profile which is highly similar to *S.*

lycopersicum, and second only to *S. pimpinellifolium* which is the closest wild species (Schauer et al., 2005). *S. habrochaites* has the most unique leaf metabolic profile compared to *S. lycopersicum*, however, it also has the closest fruit metabolic profile to *S. lycopersicum* (Schauer et al., 2005).

1.2.2.1 *S. lycopersicum* "cerasiforme" LA1511, LA2675, LA0114

S. lycopersicum "cerasiforme" is the cherry tomato and is the most distributed worldwide, mainly in the tropics and subtropics, in often weedy habitats with semi-erect sprawling plants, with red fruits of up to 2.5 cm (Grandillo et al., 2011). The *S. lycopersicum* "cerasiforme" LA2675 turning fruit is bright orange in colour, but the fruit of *S. lycopersicum* "cerasiforme" LA0134C is green for most of its growth into large mulitlocular fruits and then during tuning the fruits develop yellow stripes and turns red when ripe reaching up to 10 cm in length. *S. lycopersicum* "cerasiforme" LA1230 is resistant to powdery mildew (*Oidium neolycopersici*), and *S. lycopersicum* "cerasiforme" L285 is resistant to bacteria wilt (*Ralstonia solanacearum*) (Grandillo et al., 2011).

1.2.2.2 *S. pennellii* LA0716

S. pennellii species are usually self-incompatible (SI) although some are highly inbreed such as the accession LA0716 which are self-compatible (SC), and for this reason and its high homozygosity LA0716 is commonly used in genetics (Grandillo et al., 2011). *S. pennellii* is distributed from the coastal valleys of central to southern Peru (near sea level to 1920 m), in dry, sandy and rocky slopes. The plants are spreading shrubs, with broad, round sticky leaves and green fruit up to 1 cm. *S. pennellii* LA0716 is resistant to *Alternaria* stem canker (*Alternaria alternata* f. sp. *lycopersici*), *Fusarium* wilt (*Fusarium oxysporum* f. sp. *lycopersici* (race 1, 2 and 3) (Grandillo et al., 2011).

The *S. pennellii* fruit are green throughout growth and when ripe, are covered in many trichomes, producing its sticky secretions. The *S. pennellii* wild relative excretes a sticky substance from its glandular trichomes, and the plants grown as part of this research were covered in small black flies. The glandular trichomes produce mixtures of 2,3,4-tri-*O*-acylated glucose esters, the main fatty acids present in the mixture are 2-

methylbutanoic, *n*-decanoic and 8-methylnonanoic acids (Walters and Steffens, 1990). *S. pennellii* can produce glucose esters which total approximately 25% of leaf dry weight, equalling around 400 μg per cm^2 of glucose esters on the leaf surface (Walters and Steffens, 1990).

The large number of flies attached to the plant suggests that the flies may have been attracted to the plant, and shows a mechanism of insect defence. The flies which have landed on the *S. pennellii* plants have likely been accidentally trapped in the sticky secretions, because the main role of the glandular trichomes has been proposed to be a method for retaining water on leaves from plants from dry habitats (Nonomura et al., 2009). The water droplets can be absorbed though the trichome heads, and the when exudates combine with water, water droplets become amphiphilic and lays on the leaf surface in a thin layer (Nonomura et al., 2009). *S. pennellii* originates from a dry habitat (Tal et al., 1979), and therefore the main role of their trichomes most likely evolved to conserve the plants water. However, the increased trichome levels provide a phenotype which could be utilised for the collection of useful compounds from the exudates.

S. pennellii also produce other protective secondary metabolites, such as terpenes, including *α*-phellandrene as the main volatile, *α*-terpinene, *β*-phellandrene, and *γ*-terpinene (Schilmiller et al., 2009). *S. pennellii* LA0716 has been found to repel pest insects *Bemisia tabaci* (Gennadius) and the white fly (*Trialeurodes vaporariorum*) through the compounds *p*-cymene, *β*-myrcene, *γ*-terpinene, *α*-terpinene, and *α*-phellandrene (Bleeker et al., 2009).

1.2.2.3 *S. peruvianum* LA2744, LA1987, LA1937

S. peruvianum, is distributed mostly in coastal central and southern Peru (1000 to 3000 m). The plants originate from a dry to sandy environment, where they trail along the ground and have green-purple fruit up to 1 cm (Grandillo et al., 2011). The *S. peruvianum* LA2744 fruit are small reaching a maximum of 1 cm in size, they are white or light green in colour with black or purple stripes, the fruit looks very similar throughout ripening and they are ripe once the stripes appear. The *S. peruvianum* LA2744 leaves remained small throughout the development of the plant and had curled leaves. *S. peruvianum* s.1. $(ns)^g$ are resistant to tomato yellow leaf curl virus (TYLCV),

corky rot (*Pyrenochaeta lycopersici*), and *Fusarium* crown and root rot (*Fusarium oxysporum* f. sp. *radici-lycopersici*) (Grandillo et al., 2011).

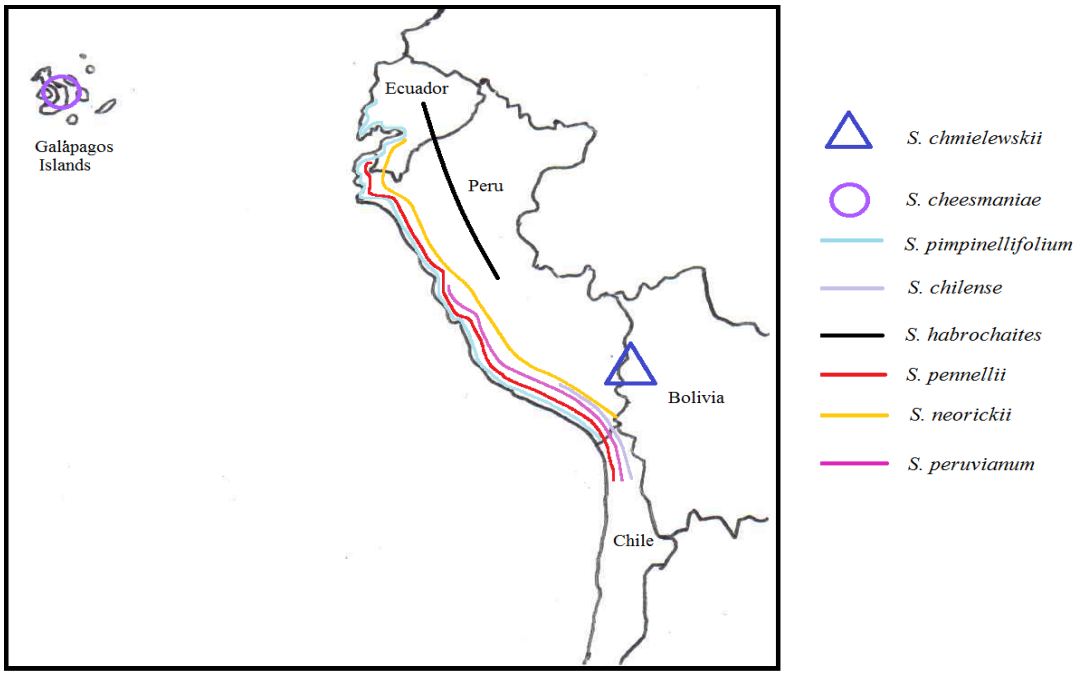

Figure 1-5. The approximate distribution patterns showing the native habitats of certain wild tomato relatives.

The wild tomato relatives are native to South America, the habitats include the Galápagos islands, Ecuador, Peru, Bolivia, and northern Chile, and they can be found within coastal regions, or within the rocky slopes of the mountain regions.

1.2.2.4 *S. pimpinellifolium* LA0114

S. pimpinellifolium, is a wild currant tomato from lowland Ecuador and coastal regions of Peru (mainly found below 1000 m), it grows in dry, sandy habitats close to water sources. The plants are semi-erect, sprawling, with red fruit up to 1 cm (Grandillo et al., 2011). The fruit from the wild relatives have different phenotypes; the fruit from *S. esculentum* LA2675 and *S. pimpinellifolium* LA0114 are very similar in size with only a slight difference in colour during ripening from green to red fruit. The ripening fruit of *S. pimpinellifolium* LA0114 is pink during turning stages. *S. pimpinellifolium* "hirsute INRA" is resistant to TYLCV, and *S. pimpinellifolium* PI 79532 is resistant to *Fusarium* wilt (*Fusarium oxysporum* f. sp. *lycopersici* (race 1), and grey leaf spot (*Stemphyllium* spp.) (Grandillo et al., 2011).

1.2.2.5 *S. chilense* LA2759

S. chilense is distributed from southern Peru, and northern Chile (from 80 to 3600 m elevation), from dry, saline, rocky slopes. The plants are erect, with purple green fruit up to 1 cm (Grandillo et al., 2011). *S. chilense* LA0422 is resistant to the cucumber mosaic virus (CMV), *S. chilense* LA2779, LA1932, and LA1938 are resistant to TYLCV and tomato mottle virus (ToMoV) (Grandillo et al., 2011).

1.2.2.6 *S. chmielewskii* LA2659 and *S. neorickii* LA2133

S. neorickii is a sister taxa with *S. chmielewskii*, which is distributed in the inter-Andean valleys from Cusco to central Ecuador (1500 to 2500 m). The habitat is well drained rocky slopes, and the plants are sprawling, with green fruit up to 1 cm. *S. neorickii* G1.1601is resistant to powdery mildew (*Oidium neolycopersici*) and grey mold (*Botrytis cinerea*) (Grandillo et al., 2011). *S. chmielewskii* is also distributed in the inter-Andean valleys of central and southern Peru (1600 to 3100 m). The habitat is well drained rocky slopes, with sprawling trailing plants, and green fruit up to 1.5 cm (Grandillo et al., 2011). The *S. chmielewskii* LA2695 leaves and *S. chilense* LA2879 leaves are large during early growth, and have a similar size to *S. peruvianum* LA2744 during flowering and fruit producing stages.

1.2.2.7 *S. habrochaites* LA1777 and LA1918

S. habrochaites is distributed in north-western and western central Peru, and western to southern Ecuador (40 to 3300 m), in varied habitats of slopes and stream banks. Its growth at high elevations is expected to be tolerant to low temperatures. The plants are sprawling shrubs, with green fruit with a dark stripe up to 1 cm. *S. habrochaites* has been known to be used to treat skin ailments, altitude sickness, and gastroenteric problems (Grandillo et al., 2011).

Table 1-3. Resistance mechanisms from selected Solanaceae species

Solanaceae species	Resistance	Responsible compounds	Reference
Nicotiana gossei Domin	Tobacco aphid *Myzus nicotianae* Blackman, white fly *Bemisia tabaci* (Gennadius)	Sugar esters	Severson et al., 1994
S. neorickii G1.1601	Powdery mildew (*Oidium neolycopersici*) and grey mould (*Botrytis cinerea*)	-	Grandillo et al., 2011
S. lycopersicoides	Grey mould	-	Guimaraes et al., 2004
S. habrochaites Humb. And Bonpl. accession PII26445, NC84173	Restistant to 14 out of the 16 tomato pests excluding only early and late blight	Methyl ketones such as 2-tridecanone	Zang et al., 2002 Schauer et al., 2005; Fridman et al., 2005
S. habrochiates f. *typicum* LA1363 and LA1927	Reistant to arthropod herbivores such as two-spotted spider mites (*Tetranychus urticae* Koch)	Trichome secretions of 2,3-dihydrofamesoic acid (3,7,11-trimethyl-6,10-dodecadienoic acid, farnesoic acid, and 16:0 and 18:0 fatty acids	Snyder et al., 1993
S. habrochaites LA1033 and LA2099	Late blight (*Phytophthora infestans*)	-	Grandillo et al., 2011
S. habrochaites LA1777	*Bemisia tabaci* and the white fly (*Trialeurodes vaporariorum*)	Zingiberene, and curcumene	Bleeker et al., 2009
S. pennellii LA0716	Powdery mildew, *Helicoverpa zea* (Boddie) and *Spodoptera exigua* (Hübner). *Alternaria* stem canker (*Alternaria alternate* f. sp. *lycopersici*) and Fusarium wilt (*Fusarium oxysporum* f. sp. *lycopersici*), *Bemisia tabaci*, and white fly	Type VI glandular trichome exudates, and *p*-cymene, *β*-myrcene, γ-terpinene, *α*-terpinene, and *α*-phellandrene	Nonomura et al., 2009; Juvik et al., 1994; Grandillo et al., 2011; Bleeker et al., 2009
S. lycopersicum "cerasiforme" LA1230	Powdery mildew	-	Grandillo et al., 2011
S. lycopersicum "cerasiforme" L285	Bacterial wilt (*Ralstonia solanacearum*)	-	Grandillo et al., 2011
S. peruvianum s.1. (ns)[g]	TYLCV, corky rot (*Pyrenochaeta lycopersici*), and Fusarium crown and root rot	-	Grandillo et al., 2011
S. pimpinellifolium "hirsute INRA"	TYLCV	-	Grandillo et al., 2011
S. pimpinellifolium PI 79532	Fusarium wilt and grey leaf spot (*Stemphyllium* spp.)	-	Grandillo et al., 2011
S. chilense LA0422	Cucumber mosaic virus (CMV)	-	Grandillo et al., 2011
S. chilense LA2779, LA1932, and LA1938	TYLCV and tomato mottle virus (ToMoV)	-	Grandillo et al., 2011
S. neorickii G1.1601	Powdery mildew and grey mould	-	Grandillo et al., 2011
S. cheesmaniae LA0422	Resistant to black mould (*Alternaria alternata*)	-	Grandillo et al., 2011

The *S. habrochaites* species contains 8-fold higher levels of terpenoids than the domesticated *S. lycopersicum* species (Besser et al., 2009). The wild tomato species *S. habrochaites* f. glabratum contains a rich source of methylketones, such as 2-undecanone and 2-tridecanone (Fridman et al., 2005; Schmiller et. al., 2010).The methylketone 2-tridecanone along with other allelochemicals is toxic towards the fruit worm *Heliothis zea* (Dimock et. al., 1982). *S. habrochaites* PI 126445 is resistant to the tobacco/tomato mosaic virus (TMV/ToMV) and early blight (*Alternaria solani*), *S. habrochaites* B6013 is resistant to TYLCV, *S. habrochaites* LA1033 and LA2099 is resistant to late blight (*Phytophthora infestans*) (Grandillo et al., 2011). *S. habrochaites* LA1777 has been found to repel pest insects *Bemisia tabaci* (Gennadius) and the white fly (*Trialeurodes vaporariorum*) through the compounds zingiberene, and curcumene (Bleeker et al., 2009).

1.2.2.8 *S. cheesmaniae*

S. cheesmaniae and *S. galapagense* are endemic to the Galápagos Islands (found from sea level to 1500 m) in a habitat of dry rocky slopes close to the sea. They are semi-tolerant to saline conditions, and the plants are semi-erect, sprawling, with small orange fruits up to 1.5 cm (Grandillo et al., 2011). *S. cheesmaniae* LA0422 is resistant to black mould (*Alternaria alternata*) (Grandillo et al., 2011).

1.2.2.9 Wild tomato and domesticated tomato introgression lines (ILs)

Investigations into the genetic diversity within the wild tomato relatives has been pursued through the development of crosses between the domesticated tomato varieties such as *S. lycopersicum* cv. M82, and wild tomato relatives such as *S. pennellii* LA0716, and subsequently the generation of introgression lines. Introgression lines are created through repeated backcrossing of an interspecific hybrid with a parent species. This method is useful because it does not involve genetic modification and utilises QTLs which can be used to improve the fruit quality of the domesticated tomato and to improve the resistance mechanism against pests, which would improve crop yield.

Introgression lines are available for the domesticated tomato with chromosome segments of wild tomato species integrated into their genomes, such as the *S. pennellii*

ILs; *Solanum pennellii* (LA0716) x *Solanum lycopersicum* cv. M82 (LA3475) (Figure 1-6), and the *S. habrochaites* ILs*; Solanum habrochaites* (LA1777) x *Solanum lycopersicum* cv. E6203 (LA4024) (Chetelat, 2006). These introgression lines have the potential to yield a range of new metabolic pathways, which may lead to favourable phenotypes, and novel compounds. Introgression lines which represent an entire genome can be an important genetic tool, as phenotypic variation can be directly linked to the introgressed genetic region (Fridman et al., 2004).This allows DNA to be quickly associated with the phenotypes of interest, and may lead to the identification of important genes, and their roles in tomato metabolism.

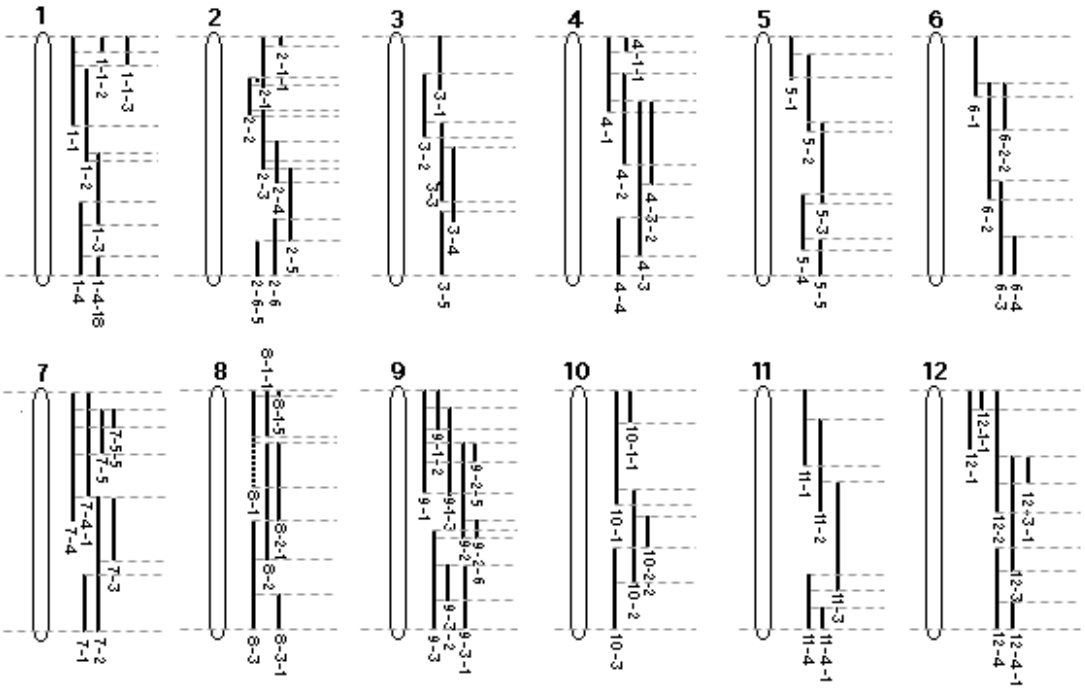

Figure 1-6: Map of *S. pennellii* introgression lines.

Each line is homozygous for a single chromosome segment introgressed from *S. pennellii* LA0716 into *S. lycopersicum* cv. M82 LA3475, the entire *S. pennellii* genome is represented by 50 overlapping lines developed by Eshed and Zamir (1995).

The development of DNA markers and molecular linkage maps has been an important resource for the discovery of QTLs. Marker assisted selection of genes associated with a specific QTL can be used to select for a desirable trait, and this reduces the incorporation of undesirable genes from the rest of the wild tomato species genetic background (Grandillo et al., 2011). It has already been discovered through mass spectrometry screening of trichome compounds from *S. pennellii* ILs that there is diversity in the production of secondary metabolites between the lines such as terpenes,

flavonoids and acyl sugars (Schilmiller et al., 2010). There can be a reduction in secondary metabolites compared to the parent species such as in *S. pennellii* ILs 6-2 and 6-2-2 which do not produce detectable amounts of sesquiterpenes, 10-2 has up to 90% reduced sesquiterpenes, and IL 2-2 has reduced levels of detectable terpenes (Schilmiller et al., 2010).

The ILs 1-3 and 1-4 both accumulate the monoterpene *α*-thujene but it was also found that a acetyl group on one of the major acyl sugar molecules was missing, and IL 8-1-1 shifts the acyl chain length of its major acyl sugars without changing the number of substitutions, with a higher proportion of isobutyl acyl chains (Schilmiller et al., 2010). These are just some of the examples of how the secondary metabolite profile of *S. pennellii* ILs is different from *S. lycopersicum* cv. M82 or *S. pennellii*. These differences could lead to the development of lines which have an increase in a desirable compound, or a decrease in a non-desirable compound, or the production of novel compounds such secondary metabolite isomers, such as an isomer of dehydrotomatine found to accumulate in ILs 1-1 and 1-1-3 (Schilmiller et al., 2010).

1.2.3 Plant Wounding

Phytoanticipins are antimicrobial compounds which are stored within the plant as a quick response to an invading pathogen. The plant also produces phytoalexins, synthesised in response to a pathogenic attack or a wounding event (Morrissey and Osbourn, 1999). It is known that the first enzyme of the shikimate pathway, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHP), is induced by plant wounding in potato and tomato tissue, where it is involved in the production of aromatic compounds, a response which may be linked to the supply of phenylpropanoid precursors for defensive secondary metabolites and lignin repair of wounds (Dyer et al., 1987). An extension of this work could be to test plant antimicrobial production in response to a specific plant pathogen, or to test for antimicrobial accumulation after inflicting a wound upon the plant tissue. This may enhance low levels of antimicrobial compounds, or promote the production of novel antimicrobial compounds which may not usually be produced under stress free conditions of the plants.

The tomato plants can also be infected with *Salmonella* spp. The bacteria enters the plant through open wounds, can enter via the stomata, and broken trichomes (Guo et al., 2002), and can overcome plant defences to survive within the intracellular cellular compartments (Schikora et al., 2008). The consumption of such infected plant produce has been known to cause human *Salmonella* infections (Schikora et al., 2008). *Arabidopsis thaliana* leaves infected with *Salmonella* die within five days, but infected apical meristems can survive the infection (Schikora et al., 2008). Tomato flowers have previously been infected with *Salmonella*, and showed that *Salmonella* can survive on or in the tomato fruit until fruit ripening (Guo et al., 2002).

1.2.4 Trichomes

Trichomes are epidermal hair like cellular structures found on most plants, which are usually glandular in *Solanum* spp., producing compound containing secretions (Fridman et al., 2005). The exudates from type VI glandular trichomes (Figure 1-7) of *S. pennellii* have recently been identified as a mechanism used by the plant to combat the tomato powdery mildew pathogen *Oidium neolycopersici*, via the accumulation of water on the leaf surface (Nonomura et al., 2009). Acylglucoses found in *S. pennellii* trichome secretions are also responsible for protecting the plant from pathogens, such as reducing the growth and development of the larvae of two tomato pests *Helicoverpa zea* (Boddie) and *Spodoptera exigua* (Hübner) (Juvik et al., 1994).

S. habrochaites contains two resistance genes for tomato powdery mildew, and although *S. pennellii* does not carry these genes, it still shows a high level of resistance (Nonomura et al., 2009), suggesting that chemicals produced from the *S. pennellii* glandular trichomes protect the plant from an initial pathogen invasion. Research into the glandular trichomes of *S. habrochaites* has discovered a positive correlation between the number of trichomes containing methylketones, and resistance to plant pathogens (Fridman et al., 2005). A comparison of secretions from the type VI trichomes of *S. habrochaites* collected directly using a capillary tube, and products recovered from dried leaf chloroform extraction method, revealed similar terpenoid compositions, suggesting that the majority of terpenoids produced are secreted by the type VI glandular trichomes in *S. habrochaites* (Besser et al., 2009).

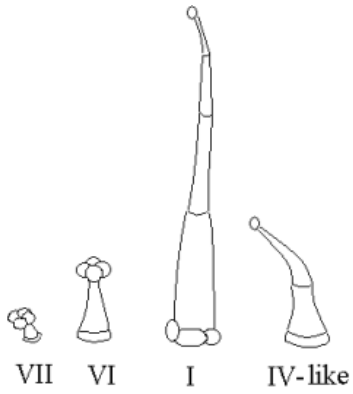

Figure 1-7: Trichome structures.

Trichome are hair like structures on the surface of leaf, stem and fruit, the diagram shows shows the main types (VII, VI, I and IV). Drawn from SEM and cryoscanning EM images of three week old *S. lycopersicum* plant trichomes by Kang et al. (2010).

1.2.5 Plant resistance compounds

Around 80% of developing countries use traditional medicine (Nascimento et al., 2000). Examples of Solanaceae species used as medicine by the Mapuche Amerindians of Chile have been compiled by Houghton and Manby, (1985), which includes *Datura stramonium* L. used to treat asthma, toothache, inflammation, cancers, and *Nicotiana tabacum* L. used to reduce inflammation. These plants are mainly utilised for their active compounds synthesised as part of their secondary metabolism, such as phenolic acids within their essential oils and tannins (Nascimento et al., 2000).

Within the Kingdom of plants over 100 000 low molecular mass non-essential secondary metabolites are produced, and most are derived from the isoprenoid, phenylpropanoid, alkaloid, fatty acid or polyketide pathways (Dixon, 2001). Some common antimicrobials in plants are phenols, phenolic acids, quinones, flavones, flavonoids, tannins, coumarins, terpenoids, alkaloids, and sugars. Phytoanticipins are synthesised and stored antimicrobials within the plant, phytoalexins are synthesised by the plant in response to microbial attack or in response to plant damage (VanEtten et al., 1994; Dixon, 2001).

Natural organic compounds commonly found in a wide range of plants have been investigated for antimicrobial activity against *S.* Typhimurium and *E. coli*, such as thyme (thymol), oregano (carvacrol), clove (eugenol) and cinnamon (cinnamic acid) all

of which have MICs ranging from 1 to 7.5 $mmoL^{-1}$ (Olasupo et al., 2003; Smith-Palmer at. al., 1998). After testing and discovering antimicrobial activities of *Cymbopogon martini* essential oil, geraniol was identified to have an MIC of 20 $\mu g\ mL^{-1}$ against *E. coli* (Duarte et al., 2007). Thymol and carvacrol are known to be antimicrobial through disintegrating the outer membranes of bacteria (Helander et al., 1998). However, when combined with nisin, a Gram-positive antimicrobial which is believed to be unable to penetrate the Gram-negative outer membrane unless subjected to chelators or pulse electric fields, these compounds failed to enhance the antimicrobial activity of nisin (Olasupo et al., 2003).

1.2.5.1 Phenylpropanoids

Phenolics include approximately 3,000 natural compounds and the term phenolics refers to simple phenols, phenolic acids, coumarins, flavonoids, stilbenes, tannins, lignans, and lignins (Stalikas, 2007). Phenols are linked to the health benefits of fresh plant foods, through acting as antioxidants, and their roles as secondary metabolites in plants are to assist wound healing, prevent herbivore attack and to protect the plant from damage caused by photosynthesis (Antonious et al., 2003). Phenolic phytochemicals are antioxidants due to their ability to donate hydrogen from hydroxyl groups along their aromatic ring (Shetty and Lin, 2005).

Dihydroxy phenolics can be oxidised to form quinones, which are a class of aromatic compounds which have antimicrobial properties through interacting with proteins to form melanoid polymers, they can cause loss of function to polypeptides in the cell membranes (Shetty and Lin, 2005). It has been suggested that hydrophobic phenolics may affect bacterial membrane stability, and that more polar phenolic acids may act through affecting the proton motive force across the plasma membrane, leading to energy depletion (Shetty and Lin, 2005).

1.2.5.2 Flavonoids

Flavonoids are phenolic aromatic molecules, containing one carbonyl group (Cowan, 1999). The flavonoids are classified into subgroups: chalcones, aurones, isoflavones, flavones, flavonols, flavandiols, anthocyanins, condensed tannins, and phlobaphenes (Winkel-Shirley, 2001). Flavonoids have several functions within plants; anthocyanins provide pigmentation to fruit, flowers and leaves (Winkel-Shirley, 2001), and they enhance protection from DNA damaging UV light, with their presence in the epidermal layers of the leaf where they effectively absorb UV-B wavelengths (Dixon and Paiva, 1995).

Flavonoids are also used as signalling molecules between plants and microbes (Winkel-Shirley, 2001), and have a role in plant-pathogen interactions (Bovy et al., 2007). They are synthesised in response to plant wounding (Cowan, 1999) as phytoalexins such as chlorogenic acid, and caffeic acid in potato. Evidence shows that many flavonoids have antimicrobial properties, and they are important defence compounds in plants, such as the inhibition of the rice pathogen *Xanthomonas* by naringenin (Padmavati et al., 1997). It is believed that their antimicrobial activity is conferred through binding to proteins, and some flavonoids may disrupt cell membranes (Cowan, 1999).

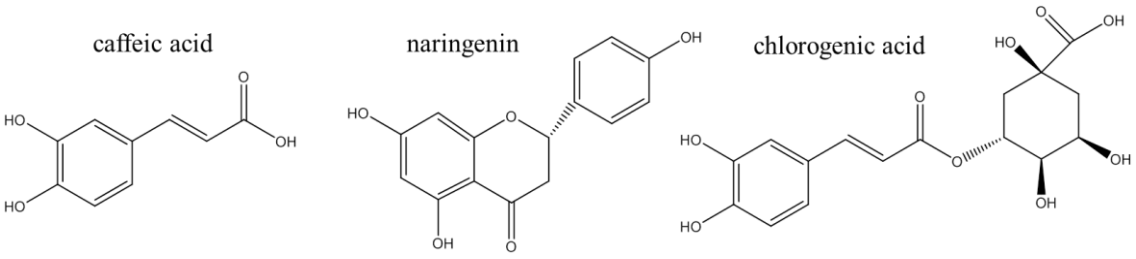

Figure 1-8: Structures of flavonids

Structures are; caffeic acid, naringenin and chlorogenic acid, common flavonoids in plants. The drawings were adapted from Chemspider using ChemDraw Pro 13.0.

Flavonols have high activities even after ingestion, e.g. isoflavones which can be ingested and have anticancer and antioxidant properties (Winkel-Shirley, 2001). Flavonoids can be enzymatically degraded, and samples containing flavonoids must be kept frozen, or dried (Andersen and Markham, 2005). Therefore, the raw plant material

was kept at -20^{o}C until ready to be freeze-dried for extraction. Flavonoid compounds have different chemical polarities, the less polar flavonoids are isoflavones, flavones, flavonols, and methylated flavones, and the more non-polar flavonoids are the flavonoid glycosides and aglycones (Andersen and Markham, 2005).

1.2.5.3 Phenols and phenolic acids

Phenolic acids are compounds which contain a phenolic ring and a carboxylic acid function, examples include cinnamic and caffeic acids. Caffeic acid can be found in herbs such as thyme, and is known to have antimicrobial properties (Cowan, 1999). Cinnamic acid is active (inhibition zone $\geq$7 mm) against *E. coli, S. aureus,* and *Enterobacter aerogenes* and benzoic acid is active against *E. coli* and *K. pneumonia* (Nascimento et al., 2000).

Many phenolic compounds with a highly oxidised state, such as caffeic acid, have antimicrobial activities, and this is seemingly through enzyme inhibition through reaction with sulphhydryl groups or protein interactions (Cowan, 1999). An example of a widely used antimicrobial is the phenolic amide capsaicin from chilli peppers, which inhibits *Helicobacter pylori* (Cowan, 1999).

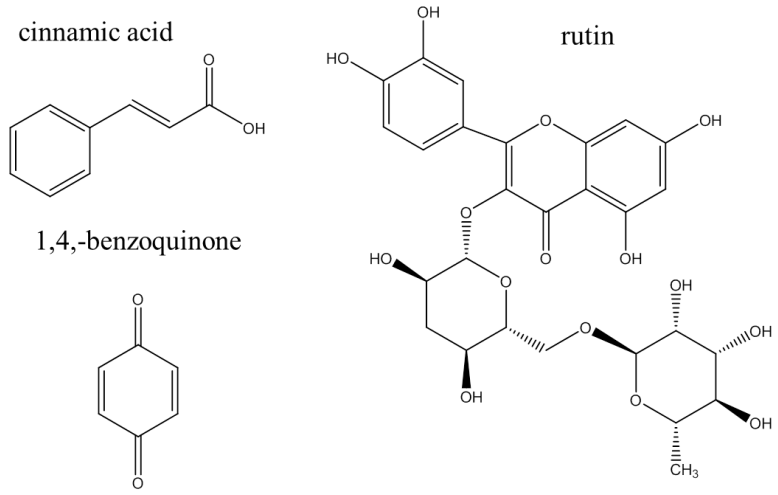

Figure 1-9. Structures of phenylpropanoids.

Structures are; cinnamic acid, rutin and 1,4-benzoquinone, showing the phenol ring structure present in each compound. The drawings were adapted from Chemspider using ChemDraw Pro 13.0.

1.2.5.4 Quinones, tannins and coumarins

Quinones are highly reactive coloured aromatic rings, which have a similar activity to flavonoids as they are able to inactivate proteins through binding irreversibly to its nucleophilic amino acids (Cowan, 1999). These chemicals can be antimicrobial through both direct damage to the micro-organisms proteins, such as the inactivation of adhesins, and membrane bound peptides (Cowan, 1999). Tannins are found in almost every plant organ and green teas containing them can be beneficial to health (Cowan, 1999). Coumarins such as Warfarin are anti-inflammatory; they also inhibit *Candida albicans*, and relations such as hydroxycinnamic acids are antibacterial towards Gram-positive bacteria (Cowan, 1999).

1.2.5.5 Glycoalkaloids

Glycoalkaloids are usually toxic, and are highly common in the Solanaceae family. The glycoalkaloids from the Solanaceae species include compounds such as solanine, which is composed of a sugar (oligosaccharides) and an alkaloid portion (aglycone). The aglycones are usually solanidine or diosgenin (Gubarev et al., 1998). Tomatoes contain a steroidal glycoalkaloid called α-tomatine, a secondary metabolite involved in plant defence, with green tomatoes known to contain up to 500 mg of α-tomatine per kg (Friedman, 2002). α-Tomatine is cytotoxic and has antifungal activities through binding to the 3β-hydroxy sterols in the fungal membrane (Sandrock and VanEtten, 1997), leading to the formation of trans-membrane pores and a loss of integrity. Glycoalkaloid concentrations declines as tomato fruit ripens and lycopene develops (Eltayeb and Roddick, 1984). Although tomatine is known to decrease as tomato fruit ripens, recent studies have found that alkaloids are still present when the fruit is ripe, such as the spirosolane-type glycoside esculeoside A, which has been isolated from the fruits of cherry tomato (*S. lycopersicum* "cerasiforme" ALEF) (Fujiwara et al., 2004). Solamargine is another poisonous glycoalkaloid present in the Solanaceae family. Tobacco species are known to produce nicotine and other alkaloids and under stress conditions can acylate nornicotine with C_{10} to C_{14} fatty acids which are secreted from its trichomes as defensive compounds (Schilmiller et al., 2008).

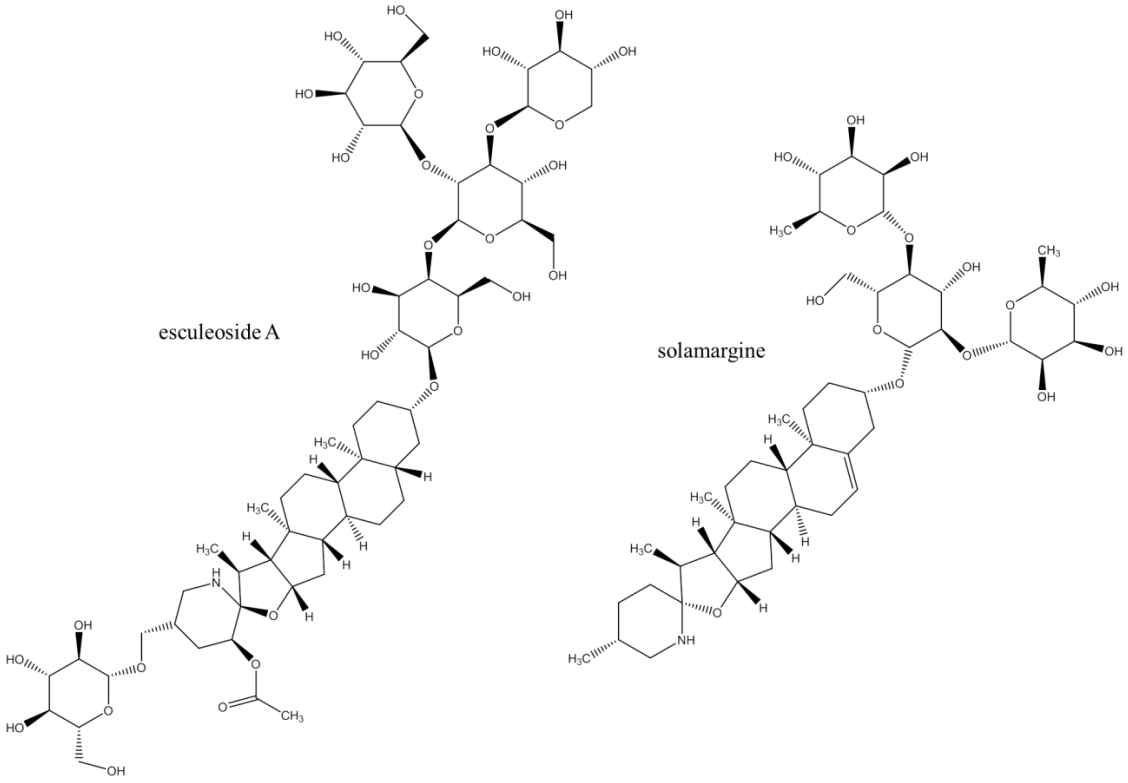

Figure 1-10. Structures of glycoalkaloids.

Structures of solamargine, and esculeoside A, showing the attached sugars (oligosaccharides) and an alkaloid portion (aglycon). The drawings were adapted from Chemspider using ChemDraw Pro 13.0.

1.2.5.6 Terpenoids, fatty acids and waxes

Vitamin E (α-tocopherol) and carotenoids such as β-carotene are lipophilic compounds which can act as antioxidants through reacting with free radicals such as singlet molecular oxygen; antioxidants are associated with reducing DNA damage, and lowering lipid peroxidation (Sies and Stahl, 1995). These compounds are classified as hydrocarbons specifically terpenoids (isoprenoids). Terpenoids are widely synthesised by many organisms, but the mono-(C_{10}), sesqui-(C_{15}) and the di-(C_{20}) terpenoids are highly common in plants, and volatile terpenoids are used by plants to attract pollinators (Schilmiller et al., 2009).

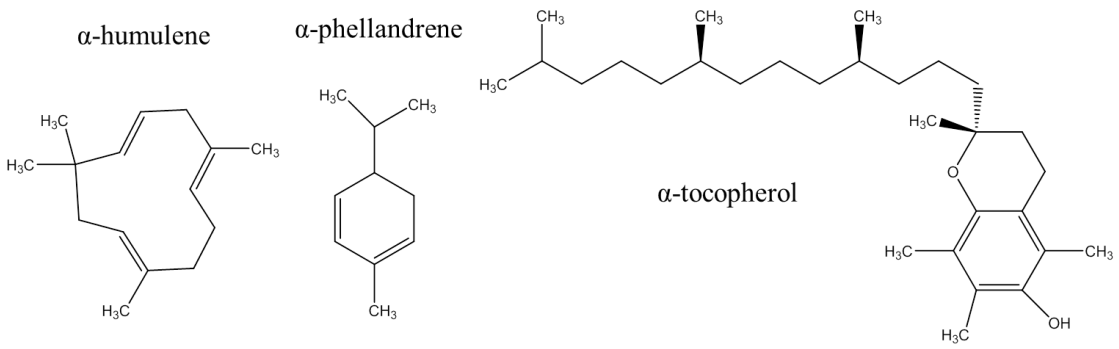

Figure 1-11. Structures of terpenoids.

Structures of *α*-humulene, *α*-phellandrene, and *α*-tocopherol, these were selected as examples of terpenoids found within Solanaceae species. The drawings were adapted from Chemspider using ChemDraw Pro 13.0.

The essential oils in plants are secondary metabolites with an isoprene structure, with similar origins to fatty acids (Cowan, 1999). Terpenoids have an antimicrobial effect upon bacteria such as *Listeria monocytogenes*, fungi, viruses, and protozoa (Cowan, 1999). Terpenoids also have insecticidal properties through modification of the class II sesquiterpenes compounds to *α*-santalene, *α*-bergamonotene and *β*-bergamonotene (Besser et al., 2009).

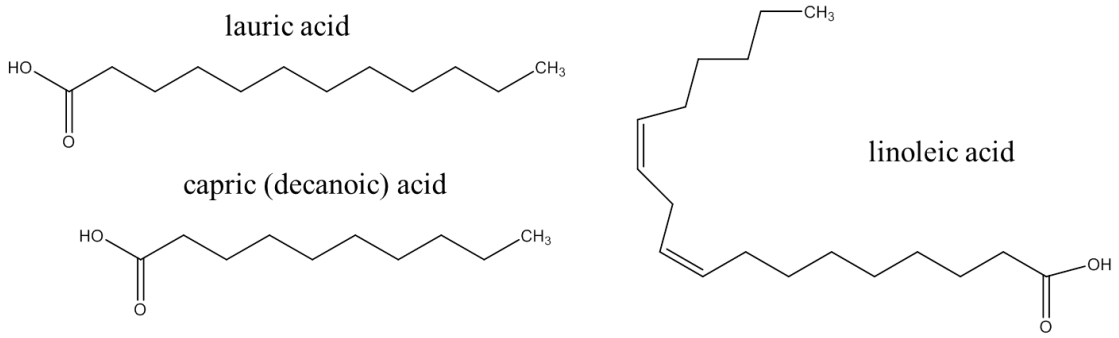

Figure 1-12. Structures of fatty acids.

Structures of lauric acid, capric (decanoic acid), and linoleic acid, these were selected as examples of terpenoids found within Solanaceae species. The drawings were adapted from Chemspider using ChemDraw Pro 13.0.

The glandular trichomes (Type VI) of *S. lycopersicum* contain a mixture of monoterpenes and sesquiterpenes (Schilmiller et al., 2009). Monoterpenes make up the majority of the terpenoids present in *S. lycopersicum*, such as *δ*-2-carene, *α*-pinene,

verbenene, α-phellandrene and β-phellandrene (Besser et al., 2009; Schilmiller et al., 2009), although a small amount of sesquiterpenes can also be found such as; β-caryophyllene, α-humulene and δ-elemene (Schilmiller et al., 2009). The main type of terpenoid present in *S. habrochaites* LA1777 are sesquiterpenes, such as γ-elemene, germacrene B and α-santalene (Schilmiller et al., 2009). The main volatile in *S. lycopersicum* cv. M82 is β-phellandrene, compared to the main volatile in *S. pennellii* LA0716 which is α-phellandrene, another difference is that *S. pennellii* does not produce sesquiterpenes (Schilmiller et al., 2009).

Fatty acids from milk and their monoacylglycerols have been found to inhibit *S. aureus.* These include lauric acid, glycerol monolaurate, linoleic acid, *cis*-9-trans-11-conjugated linoleic acid, *trans*-10-*cis*-12-conjugated linoleic acid, capric acid (decanoic acid), and myristic acid (Kelsey et. al., 2006).

1.2.5.7 Peptides and lectins

Although most research focuses on the secondary metabolites produced by plant trichomes, specific proteins produced by the trichomes can influence how the trichomes function as defensive organs. Leaves washed with water and concentrated have been found to contain only a few dominant proteins, which suggest a selective accumulation of specific potentially antimicrobial proteins (Shepherd and Wagner, 2006). Antimicrobial peptides have been known to have a wide range of activity, inhibiting fungi, Gram-positive and Gram-negative bacteria (Ohh et al., 2009). The antimicrobial small, cysteine-rich peptides in plants are called defensins (Portieles et al., 2006), which typically act through forming pores in microbial membranes (Shepherd and Wagner, 2006).They are usually expressed during early development such as DEF2 produced in early flower development (Stotz et al., 2009) and AT2 produced in the shoot apex (Brandstader et al., 1996).

Trichomes have also been known to produce significant amounts of proteinase inhibitor proteins which act through inhibiting the digestive proteinases of insect and animal herbivores (Schilmiller et al., 2009). The peptides which are often antimicrobial have been found to often include a positive charge and disulfide bonds (Cowan, 1999). Many *Solanum* species such as the wild potato *S. berthaultii* are known to produce significant

concentrations of the oxidative enzyme polyphenol oxidase which can oxidise phenolics into reactive quinone compounds, and acts as a defence compound (Yu et al., 1992).

The potato (*S. tuberosum*) is also known to produce aspartic proteases (StAPs) under stress conditions, and these StAPs have antimicrobial activity against the spores of *Fusarium solani* and *Phytophthora infestans*, with a membrane permeabilization mode of action (Mendieta et al., 2006). Studies of broiler chickens feed with potato protein such as Potamin-1from Gogu valley tubers showed that coliform bacteria are reduced in the excreta and cecum (Ohh et al., 2009).

1.2.5.8 Sterols and sapogenins

The leaves of several *Solanum* species show a high level of glycosylated sterols, particularly acylated sterol glycosides. The average total sterol content of Solanaceae species ranges between 0.1-0.2% total DW. The steryl glycosides (SG) and acyl sterol glycosides (ASG) are the most abundant sterol types produced by the *Solanum* genus, such as *S. lycopersicum* L. where ASG is 56% and SG is 26% of total sterols. *S. melongena* L. produces similar quantities and the ASG is 45% and SG 22% of total sterols. *Nicotiana tabacum* L. has an opposite sterol composition to the *Solanum* genus, with the free sterols (FS) produced at 50%, and esterified sterols (ES) at 34% of total sterols (Duperon et al., 1984).

The sterols produced by the Solanaceae family include sitosterol, campesterol, isofucosterol, cholesterol, stigmasterol, 24-methylenecholesterol, and brassicasterol. Many *Solanum* species are known to produce steroidal saponins such as digitogenin, and steroidal alkaloids such as tomatidine. *S. lycopersicum* L., *S. melongene* L., and *N. tabacum* L. produce sitosterol, stigmasterol, cholesterol, and campesterol as their principal sterols (Duperon et al., 1984). The composition of SG and ASG produced by tomato are monoglycosides of sterols due to the release of galactose and glucose after acid hydrolysis and the primary fatty acid in the ASG is palmitic acid (Duperon et al., 1984).

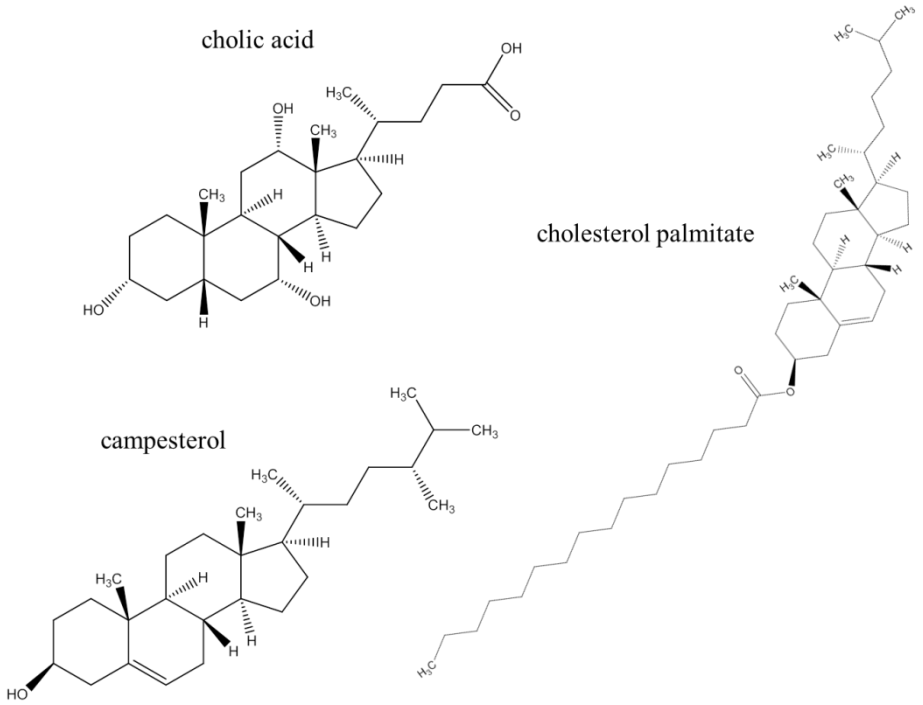

Figure 1-13. Structures of sterol and sterol esters.

Structures of the plant sterols cholic acid, campesterol, and a sterol attached to a fatty acid chain (cholesterol palmitate), the drawings were adapted from Chemspider using ChemSketch.

1.2.5.9 Glycosylated esters

Glycosylated esters are commonly produced by the Solanaceae, and consist of sugars attached to fatty acids, with up to 5 glycoside bonds per sugar molecule, and the fatty acid acyl chains of varying lengths. *S. pennellii* is known to produce a significant amount of glucose esters in the form of 2,3,4-tri-*O*-acyl glucoses bearing short chain fatty acids such as 2-methyl propanoic acid, and medium length fatty acids such as *n*-decanoic acid (Walters and Steffens, 1990). The glucose esters of different *S. pennellii* accessions vary with geographical distribution, with *S. pennellii* LA0716 expressing the highest total acyl sugars at 439 µg cm^{-2}, and the accession LA1809 producing the lowest total acyl sugars of 155 µg cm^{-2}, both these accessions have the highest difference in acyl sugar production, and there is the largest distance between the collection sites with 1749 km between the sites along the coast of Peru (Shapiro et al., 1994).

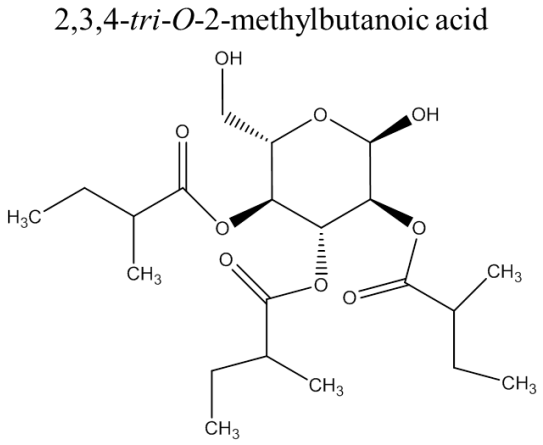

Figure 1-14. Structures of a typical glucose ester from *S. pennellii.*

Structure of 2,3,4-tri-*O*-2-methylbutanoic acid glucose, which is produced by *S. pennellii.* The drawing was adapted from Chemspider using ChemSketch.

The sugar esters of *S. pennellii* accessions vary between producing primarily acyl sucroses to primarily acyl glucoses, *S. pennellii* LA0716 produces mainly acyl glucoses, whilst *S. pennellii* LA1809 produces mainly acyl sucroses (Shapiro et al., 1994). There are several other Solanaceae species which have been found to also produce either acyl glucoses or acyl sucroses, which include plants within *Solanum, Nicotiana, Petunia* and *Datura* genus (Shapiro et al., 1994).

1.3 Enteric bacterial pathogens

The gut micro-flora are typically seperated in three primary regions; the first is the stomach environment with low pH contains a low bacteria load of facultative anaerobes such as streptococci, yeast, and lactobacilli (approximately 100 CFU mL^{-1}) (Rastall, 2004). The second environment is the small intestine which contains facultative anaerobes such as streptococci, lactobacilli, and enterobacteria, and the anaerobes include *Bifidobacterium* spp., clostridia, and *Bacteroides* spp. (approximately 10^4 to 10^8 CFU mL^{-1}) (Rastall, 2004). The third region is the colon which contains the highest bacterial load consisting of mainly obligate anaerobes such as *Bacteroides* spp., clostridia, *Bifidobacterium* spp., peptococci, and *Atopobium* spp. (approximately 10^{11} to 10^{12} CFU mL^{-1}), and some facultative anaerobes such as lactobacilli, enterococci,

streptococci, and *Enterobacteriaceae* (approximately 10^8 to 10^9 CFU mL^{-1}) (Rastall, 2004).

Some strains of pathogenic enteric bacteria such as *E. coli*, *Campylobacter*, and *Salmonella*, cause zoonotic infections such as gastroenteritis. *Campylobacter* are Gram-negative S-shaped microaerophilic bacteria and they are responsible for a large proportion of human gastroenteritis (Baserisalehi and Bahador, 2008). The average weekly number of reported cases of gastrointestinal infections in England and Wales (calculated from weeks 5-8 in 2013) caused by *Campylobacter* is 892, *E. coli* O157:H7 is 6, and *Salmonella* is 49 (HPA, 2013). Therefore, the development of a cheap drug to defend against or treat infections would be of high scientific and practical value. There is an urgent requirement for the development of new drugs, due to the increasing resistances of many bacteria to current antibiotics.

Through testing plant extracts for inhibition against enteric bacteria such as *Salmonella* and *E. coli*, novel inhibitory compounds may be found. These compounds could be used to treat and prevent gastroenteritis in humans and animals. If the compounds can be produced within the tomato or a Solanaceae host species, these compounds could become an inexpensive naturally defensive food source or preservative against enteric bacteria. This is especially important as most enteric bacterial infections are acquired from consuming poorly prepared food. If these antimicrobial compounds could be used to reduce zoonotic enteric bacterial infections, it may also reduce the emergence of antibiotic resistant bacterial strains, which can be cultivated through the excessive and improper use of antimicrobial drugs (Spika et al., 1987).

1.3.1 *Salmonella*

Salmonella are Gram-negative facultative anaerobes, which are typically pathogenic, and contain multiple virulence factors clustered within *Salmonella* pathogenicity islands (SPIs) (Soto et al., 2006). *Salmonella* Typhimurium is responsible for human gastroenteritis, cases of which were reported to be 9,133 infections for England and Wales in 2010 (HPA, 2011[a]). The SPI1 and SPI2 encode functionally different type III secretion systems, which are used to inject effector proteins into the host cells (Soto et

al., 2006). Enteric pathogenic bacteria can produce a range of harmful components to aid them in the infection of the host organism. Lipopolysaccharides (LPS) are found in the cell membranes of Gram-negative bacteria such as *Salmonella*, and these molecules are used as part of bacterial identification, and play a role in the immune response of the animal host. The gastroenteritis caused by *Salmonella* is usually a self-limiting infection, but bacteria can spread past the intestine and cause systemic infections involving other organs (Soto et al., 2006).

Salmonella are the second leading cause of bacterial foodborne illness in the USA. Within the USA the annual number of Salmonellosis infections ranges from 800,000 to 4,000,000, with a medical and productivity costs which reach into billions of pounds (Soto et al., 2006). Human salmonellosis is caused by *Salmonella* Typhimurium and *Salmonella* Enteritidis, often when infected animal food products are consumed (Humphrey et al., 1988). The main *Salmonella* species used within this study was *S.* Typhimurium LT2 10248, which is a *Salmonella* strain, with smooth cell surface LPS. The other species used in the antimicrobial analysis was *Salmonella enterica* serovar Enteritidis S1400 (see Chapter 2 Table 2-2). *S.* Enteritidis is linked to the human salmonellosis caused by the consumption of contaminated eggs (Guard-Petter, 1998), and there are a approximatly 80 strains which cause gastroenteritis or bacteraemia (Soto et al., 2006). *S.* Enteritidis is commonly associated with infections from poultry products, where as *S.* Typhimurium is more often associated with cattle and their meat products (Huehn et al., 2010). In England and Wales there were a total of 391 *Salmonella* infections recorden in January 2013, of which 78 were caused by *S.* Enteritidis and 86 were caused by *S.* Typhimurium (HPA, 2013).

The most prevelant *Salmonella* serovars found in Europe are; *S.* Typhimurium, *S.* Enteritidis, *S.* Infantis, *S.* Virchow, and *S.* Hadar (Huehn et al., 2010). *S.* Typhimurium 10248 and *S.* Enteritidis S1400 were selected to study in this research, because *S.* Typhimurium and *S.* Enteritidis are the serovars which cause the most infections in human salmonellosis (approximately 80%) (Huehn et al., 2010). *Salmonella* infections are consistently higher within children aged 4 and under, with levels of infection of 79 per 100,000 of the population under 1 years, 42 for those between 1-4 years, compared to an average of 13 for ages 5-75+ in 2010 (HPA, 2011[c]).

Salmonella have a protective outer membrane forming the outermost layer of the cell envelope which acts as a defensive shield against a range of hydrophobic solutes and macromolecules (Olasupo et al., 2003). *Salmonella* can survive the organic acids, and low pH of the stomach, they have many stress response genes, and can produce acid shock proteins, regulated by the RpoS σ-factor, PhoPQ and Fur proteins (Foley el al., 2008). Most *Salmonella* strains possess both *fliC* and *fliB* genes which code for flagellins (Kilger and Grimont, 1993). They can switch between these genes to be either Phase I or Phase II, and this allows the bacteria to periodically switch their antigens presumably helping them to evade the host's immune system. These resistance mechanisms are developed from acquiring resistance genes through horizontal gene transfer and this is increased with the selection pressures from antibiotic substances, which increase the transfer of transposons and integrons (Huehn et al., 2010).

Many *Salmonella* strains are antibiotic resistant; especially to ampicillin, streptomycin, sulphonamides, and tetracyclines (20 to 15% from 1996 to 2003), and a low level of resistance was found to ciprofloxacin and ceftriaxone (>1% from 1996 to 2003) tested by the National Antimicrobial Resistance Monitoring System (Foley et al., 2008). The most common multidrug resistance phenotype reported in 2006 by the U.S. Food and Drug Administration (FDA) was to ampicillin, chloramphenicol, streptomycin, sulphonamides, and tetracyclines (ACSSuT) (Foley et al., 2008). *S.* Typhimurium strain SL1344 is resistant to streptomycin (Antunes et al., 2010). The mechanisms of *Salmonella* antimicrobial resistance include the production of enzymes to degrade, or modify the structure of antibiotics, the reduced uptake of antibiotics into the cell, the active removal of antibiotics using efflux pumps, and modification of the drug target (Foley et al., 2008). The production of *β*-lactamase enzymes is an important mechanism for *β*-lactam resistance. Aminoglycosides are modified by aminoglycoside phosphotransferases, acetyltransferases, and adenyltransferases. Quinolone and fluoroquinolone resistance involves gene mutations in the topoisoimerase enzymes preventing antimicrobial binding to the topoisomerase targets.

S. Typhimurium resistance genes provide protection against ampicillin, ceftiofur, ceftriaxone, cephalosporins, and penicillins. *β*-Lactamase enzymes degrade the chemical structures of the antibiotics (Foley et al., 2008; Aarestrup et al., 2004). Antibacterial resistance enzymes aminoglycoside phosphotransferase (APH) produced

from the *aph*A gene, aminoglycoside acetyltransferase from *aac*C, and aminoglycoside adenyltransferases from *aad*A and *aad*B, confer resistance to kanamycin, gentamicin, and streptomycin, respectively. Mutations in the genes involved in DNA replication can also lead to quinolone and fluoroquinolone resistance. Efflux pumps encoded by *tet*, *flo*R or *cml* confer tetracycline and chloramphenicol. Chloramphenicol resistance is also produced from drug target modification by chloramphenicol acetyltransferase (cat genes). Trimethoprim and sulphonamides resistance is achieved using folic acid biosynthetic enzymes encoded by *dhfr sul*I, *sul*II genes (Foley et al., 2008; Aarestrup et al., 2004).

The drugs used for *Salmonella* septicaemia are typically fluoroquinolones or ceftriaxone (Foley et al., 2008). The most common *Salmonella* human infections are from the serovars Typhimurium, Enteritidis, Newport which are responsible for a third of the *Salmonella* cases in the USA, and *Salmonella* Enteritidis is the most predominant within the European Union (Foley et al., 2008). *Salmonella* Typhimurium is commonly known to be resistant to at least 5 antimicrobial agents, and *Salmonella* Newport strains are often resistant to at least 7 (MDR-AmpC) (CDC, 2011).

1.3.2 *E. coli*

E. coli O157:H7 is a Gram-negative, highly virulent, food-borne pathogen. There are seven classes of enteric *E. coli*, enteropathogenic (EPEC), enterohaemorrhagic (EHEC), enteroinvasive (EIEC), enterotoxigenic (ETEC), enteroaggregative (EaggEC), diarrhoea-associated haemolytic (DHEC) and cytolethal distending toxin (CDT)-producing *E. coli* (Duarte et al., 2007). ETEC are currently considered to cause the majority of *E. coli* outbreaks worldwide, and produce at least one heat stable enterotoxin (ST) or heat labile enterotoxins (LT) (Clarke, 2001). *E. coli* O157:H7 evolved from a strain of EPEC and it has acquired the shiga toxin gene, with the O referring to the somatic antigen and the H referring to the flagella antigen (Besser, 1999). Similarly to *Salmonella* EHEC also posess a type III secretion system, which has an important role in the infection of the host cells, through promoting attatchment and the transfer of effector proteins (Tree et al., 2011).

E. coli O157:H7 is responsible for causing outbreaks of haemorrhagic colitis, which occasionally leads to fatal kidney failure (Perna et al., 2001). *E. coli* O157:H7 causes haemolytic uremic syndrome (HUS), the most common cause of acute renal failure in children, which is linked to shiga toxin (Stx; Vero cytotoxin) produced by the bacteria which causes the destruction of red blood cells and damages the kidneys, the symptoms can include diarrhea, anemia, vomiting and kidney failure. Additional to renal failure, there is also a risk of death from cerebral involvement (Gerber et al., 2002).

HUS occurs in approximately 6% of infections, with death occurring in 0.6% of patients, with the highest death rate in those over 60 years of age, and is particularly harmful to children (Gould et al., 2009; Burt et al., 2007). The shiga toxin producing *E. coli* (STEC) can be acquired from infected food products such as milk and meat, but also from other sources such as water (Gerber et al., 2002). The shiga toxin is associated with the bacterial SOS stress resonses, and its production can be triggered through antibiotics which cause DNA damage. Certain antibiotics are avoided when treating STEC infections due to their role in the pathogenesis of the STEC disease, these antibiotics include quinolones, trimethoprim, and furazolidone (Kimmitt et al., 2000).

There were a total of 40,819 reported cases of *E. coli* bacteraemia within England between July 2011 and September 2012 (HPA, 2011[b]). Studies within US hospitals have shown that patients infected with *E. coli* O157:H7 usually have severe bloody diarrhoea and abdominal cramps, with fever in only 40%, and with fewer than half the patients showing signs of fever mistaken diagnoses can occur particularly from children and the elderly (Besser, 1999). *E. coli* O157:H7 strains collected from 1985 to 2000 were tested for antimicrobial activity and it was found that 61% were susceptible to all 13 tested antimicrobials, however 27% were resistant to tetracycline, 26% resistant to sulfamethoxazole and 17% resistant to cephalothin (Schroeder et al., 2002). Certain strains are highly resistant to antibiotics, with resistance mechanisms such as extended spectrum β-lactamases (ESBLs) particularly CTX-M (Woodford et al., 2004), which confers resistant to penicillins and cephalosporins. Other resistance genes include *qnr* which confers quinolone resistance (Jacoby et al., 2005) and VIM-2 and KPC-1 resistance to carbapenems (Navon-Venezia et al., 2006).

The *E. coli* O157:H7 NCTC 12900 bacterial strain used within these studies is a non-pathogenic strain which is phenotypically similar to the toxigenic strain of *E. coli* O157:H7, but it does not produce verotoxins (Tremoulet et al., 2002). An additional strain, *E. coli* ATCC 25922 was also used as a control bacterial strain which is an antibiotic susceptible strain (Fass and Barnishan, 1979).

1.3.3 *S. aureus*

Staphylococcus aureus is a Gram-positive bacterium which is commonly carried on the human skin as a commensal organism. *S. aureus* is a pathogen associated with serious community acquired nosocomial disease (Enright et al., 2000). Pig farming has also been identified as a risk for increasing the nasal colonisation of farmers with *S. aureus*, from pigs suffering with exudative epidermitis caused by MRSA, the bacteria can be further spread through the sale of pigs to different breeding lines (van Duijkeren et al., 2007).

The pathogen can cause mild skin infections such as impetigo, and serious infections are responsible for bacteremia, pneumonia, endocarditis, septic arthritis, osteomyelitis, and the formation of deep abscess (Enright et al., 2000). The virulence factors expressed by pathogenic *S. aureus* are responsible for causing the more harmful infections, such as toxic shock syndrome and staphylococcal scarlet fever, which are both caused by the toxin 1 (TSST-1) and staphylococcal enterotoxins (SEs) (Jarraud et al., 2002). *S. aureus* can cause nosocomial blood stream infections, which are a major cause of death, often acquired from hospital environments, where patients in intensive care have a high mortality (80% in the USA) (Wisplinghoff et al., 2004). The number of MRSA bacteraemia counts was reported by English NHS acute Trust hospitals to be a total of 1,248 between July 2011 to September 2012, and within the same time period MSSA counts were reported to be 10,935 (HPA, 2011[b]).

S. aureus can also cause staphylococcal food poisoning from its SEs, and the molecular basis of these virulence factors produced by *S. aureus* are related to their cell wall proteins, with expression controlled by the agr locus (Jarraud et al., 2002). Before the introduction of penicillin in 1959 most invasive *S. aureus* was untreatable, and yet as

early as the 1960s in Europe *S. aureus* became resistant to penicillin G and V, and continues to find resistance to subsequent antibiotics such as the semisynthetic penicillin methicillin (MRSA), and many MRSA strains are now becoming resistant to other antibiotics, including glycopeptides (glycopeptide-intermediate MRSA) (Enright et al., 2000). Methicillin resistant strains of *S. aureus* are able to produce a penicillin-binding protein (PBP2a) from the *mec*A gene (Murakami et al., 1991). The methicillin resistance is also dependent on the culture conditions, such as temperature, pH and concentrations of NaCL (Murakami et al., 1991).

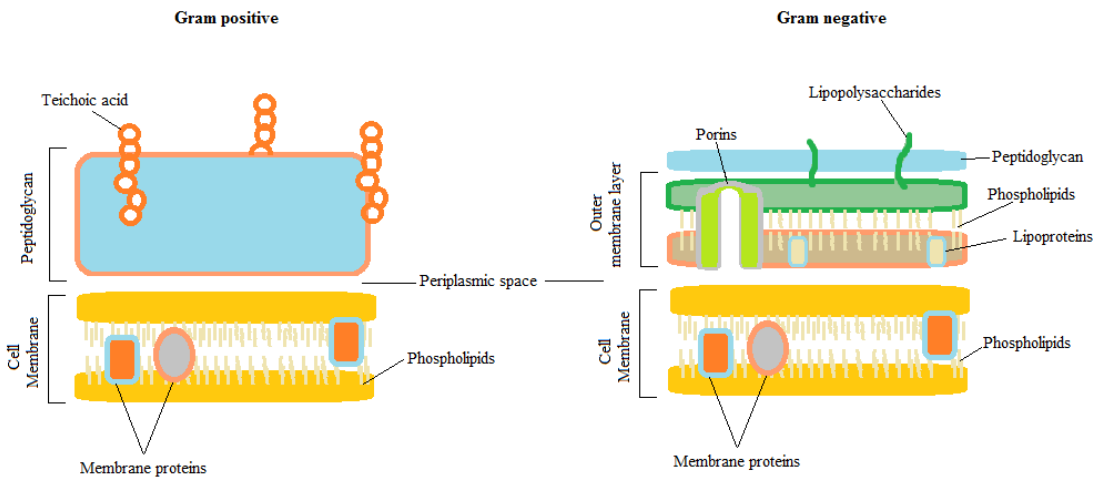

Figure 1-15.The cell walls of Gram-positive (left) and Gram-negative (right) bacteria cells.

The diagrams show the structural differences, representing a cross section of the bacterial cell wall. The main differences between the cell walls are a thicker peptidoglycan layer in the Gram-positive bacteria and a double phospholipid bilayer in the Gram-negative cell wall.

The *S. aureus* bacteria used within this study was *S. aureus* ATCC 25923, which is a control strain which does not have the *mec*A gene (Gibbons et al., 2003). There was a direct correlation between the increase in bacterial resistance and the proportion of *S. aureus* cases within the UK. The emergence of two epidemic strains MRSA (EMRSA-15 and EMRSA-16), coincided with a dramatic increase in *S. aureus* bacteraemia from 1% to 2% between 1990-1992, and then a vast increase to approximately 40% in 2000 (Johnson et al., 2001).

1.3.4 Bacterial plant infection

The phyllosphere is the total above ground surface of a plant to be utilised as habitat for microorganisms. Epiphytic is a term used to describe the microorganisms which colonise the plant leaf surface. Bacteria colonise the leaf surface in numbers close to 10^7 cells per cm^2 of leaf (10^8 cells g^{-1}) (Lindow and Brandl, 2003). Leaves are a habitat for a range of microorganisms including bacteria, filamentous fungi, yeast, algae, nematodes, and protozoa. Pigmented bacteria such as *Pseudomonas syringae* colonise the leaf surface (Lindow and Brandl, 2003). It is becoming increasingly apparent that the spread of food-borne human pathogens through fruit and vegetables is a common problem, such as norovirus outbreaks from fresh foods contaminated through workers hands (Berger et al., 2010).

Metabolomic analysis of plant secondary metabololites of *Brassica rapa* post infection with several bacteria species shows that Gram-positive and Gram-negative species have different effects upon the metabolomic plant profile (Jahangir et al., 2008).The research showed that additional to the characteristic increase in phenolic acids which occurred in response to all the tested bacteria, Gram-negative bacteria *E. coli* and *S.* Typhimurium increased histidine, feruloyl-malate and caffeoyl-malate, with Gram-positive *S. aureus* and *B. subtilis* causing an increase in coumaroyl-malate and fumarate within *Brassica rapa* (Jahangir et al., 2008).

Studies show that *S. enterica* and *E. coli* can colonise plants especially in humid conditions. They fail to grow in dry conditions, but can remain dormant and grow again when the plants become wet again. Under such conditions infected plant products can act as vectors for the spread of gastroenteric bacteria which can occur due to the low number of cells *Salmonella* organisms required for infection varies from 30 to more than 10^9 bacteria cells (Foley et al., 2008). Attachment to the leaf surface by *E. coli* O157 occurs due to the filamentous type III secretion system, and *Salmonella* is also linked to outbreaks from fresh produce, and the association with fresh produce is serovar-specific involving the flagella, curli, cellulose and O antigen capsule (Berger et al., 2010).

The link between contaminated vegetables with pathogenic bacteria present in the manure is an increasing concern. The UK government Environmental Agency suggests that fresh solid manures and slurries should not be applied to land within 12 months of harvest which includes a 6 month period before planting (Environmental Agency, 2012). Studies have shown that detectable levels of pathogenic bacteria such as *E. coli* and *S.* Typhimurium can be present depending on the weather, as freeze thaw cycles are required to eliminate most of the bacteria (Natvig et al., 2002). Washing vegetables before cooking has shown to reduce the levels of bacteria which can be spread from just handling the food prior to cooking and it is strongly recommended (Natvig et al., 2002).

1.3.5 Antibiotic resistance

There will always be a need for continuing antimicrobial drug development, as all new antibiotics have a limited period of time before evolution takes place and selects for bacteria with either intrinsic or acquired resistance, allowing them to thrive due to the termination of their predecessors (Walsh, 2003). Antimicrobial resistance has occurred for every major class of antibiotic (Lambert, 2005). Within the UK resistance to antibiotics is particularly increasing in Gram-negative hospital acquired bacteria. This is shown through asn increased resistance of *E. coli* bloodstream infections from 2% resistance to cephalosporins and 4% to ciprofloxacin in 2000, to current resistance of 11% and 21% respectively (HPA, 2012). The cost to human health of antibiotic resistant bacteria relates to the increasing number of hospital acquired infections from opportunistic pathogens, increasing severity of infections and treatment failures (Aarestrup, 2005).

It is widely believed that the overuse of antibiotics can increase the development of novel antibiotic resistance mechanisms; this overuse is seen most prominently in human and veterinary medicine. The distribution of antibiotic use varies between human and veterinary medicine; with the relative distribution of antibiotic usage in dosages for humans in Denmark are penicillins (71%), tetracyclines (4%), macrolides (9%), sulfa/TMP (8%), other (6%) and cephalosporin (2%) (DANMAP, 2004). The antibiotic usage in humans is primarily penicillin, but the antibiotic used in veterinary medicine has a more even range across the antibiotics, for example the relative antibiotic dosages

in Denmark pigs include the use of macrolides (37%), tetracyclines (21%), other (17%), penicillins (12%), aminoglyco-nucleosides (10%), and sulfa/TMP (3%) (DANMAP, 2004).

The EU ban on antibiotics as growth promoters in animal feed from 2006 has led to a change in the way antibiotics are used by EU farmers. However, a large amount of antibiotics are still being used to treat and prevent disease, which creates favourable conditions for the emergence of new antibiotic resistant strains (Aarestrup, 2005). It seems however, that even after these measures were implemented the emergence and threat of ARB has continued. Antibiotic resistant strains can be passed from livestock to humans through environmental food contamination, and these strains can be passed onto animals from human excrement contamination of the environment, this has also been exacerbated through the worldwide trade of food and animals (Aarestrup, 2005). These antibiotic resistant strains need to be controlled to reduce the transfer of antibiotic resistance genes to other pathogenic bacteria. Cefquinome is a common antibiotic used to treat mastitis infections in milking cows. There has been increasing concerns over the levels of antibiotics in milk, and recent scientific studies show that the cephalosporin antibiotic cefquinome has been found in up to 21% of waste cow milk (Horton et al., 2012).

Current farming measures used to decrease the enteric pathogen load include the use of organic acids in animal feed such as fumaric acid, propionic acid, sorbic acid and tartaric acid; these exert their antimicrobial action both in the feed and in the GI-tract of the animal (Canibe et al., 2002). These organic acids replace some of the antimicrobial activity which has been lost from the removal of routine antibiotics as feed additives, and it performs a similar role in reducing bacterial load. The use of probiotics in animal feed needs to be additional to a mixed feed, and the primary bacteria used are *Enterococcus faecium*, *Bacillus* spp., and *Saccharomyces cerevisiae* (Ortwin, 2005).

There is a wider problem which looms over the antibiotic development industry, which is that the available antimicrobial target sites are reducing, and the newly produced antibiotics are adapted from the scaffolds of antibiotics of which many bacteria already have developed resistance mechanisms, such as second and third generation cephalosporins (Walsh, 2003). Antimicrobial drug development started in 1936 with the

discovery of sulfa drugs (eg. trimethoprim) which targets folate metabolism, an in 1940 β-lactams (e.g ampicillin) were introduced which target envelope synthesis (Walsh, 2003). Over the following three years several advances were made with antibiotics which target ribosomes including polyketides (e.g tetracycline) and phenyl propanoids (e.g. chloramphenicol) in 1949, aminoglycosides (e.g. gentamicin) in 1950, and macrolides (e.g. erythromycin) (Walsh, 2003). In 1958 glycopeptides (e.g. vancomycin) were discovered which act through targeting envelope synthesis, and quinolones (e.g. ciprofloxacin) which target nucleic-acid replication were found in 1962, along with streptogramins (e.g. Synercid) which target ribosomes (Walsh, 2003). The next leap in antimicrobial discovery did not occur until almost four decades later with the discovery of oxazolidinones (e.g. linezolid) which act through targeting ribosomes (Walsh, 2003).

A combination of the down turn in novel antibiotic production, and the realisation that antibiotic effectiveness expires over time through bacterial antibiotic resistances, has led to an increased awareness of the importance of the development of new antibiotics. The use of plants as antibiotics has been marginal since the 1950s due to the increased effectiveness of the antibiotics at that time (Cowan, 1999). However, with increasing bacterial resistances to current antibiotics, plants antimicrobial compounds create a new source of chemical structures and bacterial target sites to be explored. The fact that they have not been highly used as antimicrobial agents to date within the developed world is a useful circumstance. The lack of bacterial exposure to plant compounds suggests that the bacteria may not have developed high resistance mechanisms for these compounds.

1.4 Experimental strategy

The hypothesis that potentially antimicrobial secondary metabolites are produced by Solanaceae species, with a focus on wild tomato relatives, was designed with different stages of scientific discovery in mind, the first was to extract the secondary metabolites from the plant material which involved growing the chosen plant species under controlled conditions and then isolating a specific plant organ to harvest for further analysis. The plant material was then extracted using solvents and various separation techniques such as thin layer chromatography (TLC) and solid phase extraction (SPE). Methods of targeted and non-targeted extraction were used within the initial stages of

the project, where the identification of a general extraction method with the potential for extracting the optimum range and concentration of secondary metabolites from a range of alternative plant species was desired.

The second focus of the project involved identifying the antimicrobial plant species and isolating an active fraction. The identification of antimicrobial extracts was achieved using a plate method (disc diffusion assay) and a liquid broth method (growth curve analysis). The mode of action of these extracts was investigated using electron microscopy (EM) and metabolomics. This part of the project was linked to identification of the antimicrobial extracts through investigating the effects upon the bacteria metabolomics and the physical effects comparisons can be made with other bacteria responses to its environment. The use of selected bacteria, *E. coli*, *Salmonella* and *S. aureus* also allows comparisons to be made between known compounds which are effective at inhibiting either Gram-positive or Gram-negative bacteria.

The identification of the antimicrobial secondary metabolites within the crude extracts involved separating the compounds through TLC and SPE, and then subjecting the extracts to HPLC and GC-MS analysis. The investigations into the antimicrobial properties from pure compounds, herb species, and other Solanaceae species, were used to make comparisons with the wild tomato species. This helped to identify the compound class of the secondary metabolites responsible for the antimicrobial activity.

Chapter 2

Materials and methods

2.1 Plant cultivation and collection

Plants were grown in a greenhouse with supplementary lighting; a daytime temperature of 20 to 25^{o}C with 16 h of light, and a night temperature of 15^{o}C with 8 h of darkness was used. Plants were grown in 30 cm diameter pots containing M2 professional growing medium (Scotts Levington®). Plants were grown in yearly cycles with autumn and spring crops and each crop was grown from a first generation seed stock. Plant material collection occurred on a continuous basis once the plants reached a height of >30 cm, and included expanded fully developed undamaged leaves, with fruit samples also taken at both green and red ripening stages. The leaves were typically 5 to 15 cm long depending on the plant species.

Leaf material was pooled on a regular basis to maintain stocks of three plants for each species. All healthy plant material was taken and immediately stored at -20^{o}C. The plant material was flash frozen in liquid nitrogen, freeze dried over a two day period, and then returned to -20^{o}C until further analysis. Seed stocks were collected from ripe fruit, the collection method included fresh fruit cut into quarters, and covered in water:HCL (30:70 v/v) for 1 h, the seed was then collected using a sieve.

2.1.1 Plant species

2.1.1.1 Wild tomato species

The wild tomato relatives and the domesticated tomato control *S. lycopersicum* cv. M82 were grown from seed in triplicate. The wild tomato relatives included *S. cheesmaniae, S. chmielewskii, S. chilense, S. peruvianum, S. pimpinellifolium, S. pennellii*, and *S. habrochaites.* All wild tomato relatives and *S. pennellii* IL seeds were obtained from the Tomato Genetics Resource Centre in California from the University of California Davis. Additional plant material was collected from *S. lycopersicum* cv. M82, and introgression lines (ILs) *Solanum pennellii* (LA0716) x *Solanum lycopersicum* cv. M82 (LA3475) (Chetelat, 2006). These plants were collected from Nottingham University.

Table 2-1: Tomato plant species analysed, including wild relatives, domesticated tomato M82, and introgression lines

Species name	Accession number	Background/Origin	Reference(s)
S. lycopersicum "cerasiforme"	LA2675 LA1511 LA0134C	Casahuiri (Sangaban) Puno, Peru. Siete Lagoas, Minas Gerais, Brasil. Ayacuchomercado, Ayacucho, Peru.	TGRC report 2006, TGRC*
S. cheesmaniae	LA1137	Rabida N side, Galapagos Islands, Ecuador.	TGRC
S. chmielewskii	LA2695	Chihuanpampa, Cusco, Peru.	TGRC
S. chilense	LA2759	Mamina, Tarapaca, Chile.	TGRC
S. chilense	LA2879	San Roque de Peine, Antofagasta, Chile.	
S. peruvianum	LA2744	Sobraya, Tarapaca, Chile.	TGRC
S. pimpinellifolium	LA0114	Pacasmayo, La Libertad, Peru.	TGRC
S. pennellii	LA0716	West slopes of the Andes, Peru.	Burke et. al., 1987
S. habrochaites	LA1779 LA1777 LA1918	Puente moche, La. Libertad, Peru.	TGRC
S. lycopersicum cv. M82	M82	Domesticated tomato.	TGRC
S. neorickii	LA2133	Ona, Azuay, Ecuador	TGRC
S. pennellii ILs	71 lines	Introgression line *S. pennellii* (LA0716) in a *S. lycopersicum* cv. M82 (LA3475) background.	TGRC report 2006

*TGRC; Tomato Genetics Resource Center (http://tgrc.ucdavis.edu/)

2.1.1.2 Herb species and pure compounds

Herb species with known antimicrobial properties, specifically towards *E. coli* O157:H7, *E. coli* 25922, *S.* Enteritidis S1400, *S.* Typhimurium 10248, and *S. aureus* 25923 were also tested to validate the antimicrobial assay methods. The plant material was obtained from the supermarkets Tesco and Sainsbury, and from the retailer Holland and Barratt in various dried, or fresh forms. Pure compounds of at least 99% purity were also purchased from either Sigma Aldrich, or Extrasynthese.

2.1.1.3 Other Solanaceae species

In addition to the wild tomato relatives, other Solanaceous species were grown in three biological replicates. *Physalis peruviana* (Dwarf cape gooseberry*), Solanum melongena*

(Aubergine), *Nicandra physaloides* (Apple of Peru), *Capsicum chinense* (Scotch bonnet), *Solanum laciniatum* (Kangaroo apple) were purchased from kitchengarden seeds. *Solanum sisymbriifolium* (Sticky nightshade), *Solanum pyracanthon* (Porupine tomato*), Fabiana imbricata, Hyoscyamus niger, Physalis alkekengi* (Chinese lantern), *Nicotiana rustica* (Mapacho) from Chiltern Seeds. Scotch bonnet and bird eye chilli peppers were bought from a local supermarket where the seeds were collected from the fruit and grown and goji berries were bought from Holland and Barrett.

2.2 Plant extraction

The dry weight of plant material is usually 10% of its original fresh weight. This is defined as the percentage dry matter (pdm), which is the percentage dry matter to total plant fresh weight (0 to 100) (Greenwood et al., 1980). Plant material was mainly dried before extraction except for experiments which involved direct extraction from the leaf trichomes with chloroform. After extraction the samples were dried using an EZ-2 plus centrifugal evaporator from Genevac SP Scientific and stored at -20^{o}C until analysed. The solvents used for extraction were purchased from Fisher Scientific unless otherwise stated.

2.2.1 Biphasic plant extraction

Plants were rapidly frozen using liquid nitrogen, and then freeze dried. They were then reduced to powder via a pestle and mortar, and stored a -20^{o}C. Extraction of polar metabolites was performed using 50:50 (v/v) methanol (1.5 mL) and water (1.5 mL), and non-polar metabolites were dissolved in chloroform (2.5 mL). The extracts were centrifuged for phase separation at 3,500 rpm in a Hettich Rotofix 32A bench-top centrifuge; the phases were separated, evaporated on a Genevac evaporater using HPLC fraction setting for the methanol and water fraction, and the low BP setting was used for the chloroform fraction. The dry extracts were weighed and stored at -20^{o}C. Extractions were initially prepared from 200 mg of dried plant material, and this was increased to 600 mg to increase the concentration of compounds within the samples to be tested.

2.2.2 Compound specific extraction

There were several types of procedures used for the extraction of different classes of compounds. A phenolic extraction method was used with methanol, where dried plant material (20 to 30 mg) was boiled at 80^{o}C in 100% methanol (1 mL) for 1 h in a Techne Driblock DB-3 heat block. The solvent was removed from the debris by centrifugation at 3,500 rpm for 5 min and dried on a Genevac evaporator on low BP setting overnight before storage at -20^{o}C. Water extracts were prepared by grinding fresh plant material using a pestle and mortar in distilled water (10 mL) and then the remaining pulp was left to stand at room temperature for 1 h, and the liquid phase (5 to 6 mL) was dried in a Genevac evaporator on HPLC setting. A steroidal alkaloid glycoside extraction method was performed on dried plant material (150 mg), extracted with methanol and water 80:20 (v/v) (10 mL) containing HCL (1% v/v) in an ultrasonic bath for 20 min as described by Stobiecki (2003). The suspensions were filtered and concentrated under vacuum in a Genevac evaporator on HPLC setting overnight before storage at -20^{o}C.

2.2.3 Solvent specific extraction

The amount of solvent used to extract the plant compounds was an important consideration, the solvent volume to plant material ratio was maintained at around 1:10. The crude residues extracted were weighed and the percentage yield of the extracts was determined from using the equation:

% yield = Mass of extracted plant material / Mass of extracted plant residue x 100

This percentage yield was used to quantify the crude extract during analysis, and it was used to determine the level of reproducibility of the extractions.

Antimicrobial analysis of dried leaf and fruit extracts of several Solanaceae and herb species was performed, using eight solvents: water, methanol, ethanol, ethyl acetate, isopropanol, chloroform, petroleum ether, and hexane. Dried plant material (100 mg) was extracted in solvent (2 mL), dried and re-suspended in DMSO (200 µL) for disc diffusion analysis (10 µL disc^{-1}).

2.2.4 Solid phase extraction (SPE)

An alkaloid extraction method was performed which was specific for collecting α-solanine and α-chalconine from potato products (Schußeler, 2000). The method included extracting dried tomato leaves (1 g) in methanol (50 mL), and aliquots (5 mL) of this extract were added to water (8 mL). A C_{18}-E 55 um, 70A, 3 mL, 500 mg SPE cartridge (Strata, Phenomenex) was conditioned with methanol (3 mL) and then water (3 mL). Aliquots (0.5 mL) of extract were pushed through the column, washed with (6: 4 v/v) water: methanol (5 mL) and eluted with methanol (15 mL).

The SPE technique was used to collect fractions from *S. pennellii* trichome extracts, extracted with chloroform (5 mL g^{-1}). The extract (5 mL) was dried under vacuum in a Genevac evaporator on low BP setting, and then re-suspended in hexane (1 mL). A silica gel SEP-PAK® cartridge (3 ml, 500 mg, Millipore) was conditioned with methanol (3 mL) and then hexane (3 mL). The hexane plant sample was slowly pushed through the column, and the sample was eluted with solvents of increasing polar nature (1 mL). The solvent fractions were collected separately in order as; hexane, hexane:chloroform (90:10 v/v), hexane:chloroform (80:20 v/v), hexane:chloroform (70:30 v/v), hexane:chloroform (60:40 v/v), hexane:chloroform (50:50 v/v), chloroform, chloroform:ethyl acetate (50:50 v/v), ethyl acetate, ethyl acetate:ethanol (50:50 v/v), ethanol, methanol, and water. These samples were dried again as described previously, and used in disk diffusion assays and for GC-MS analysis.

2.2.5 Thin layer Chromatography (TLC)

Thin layer chromatography was used to separate the plant extracts into purified fractions, where they could be tested in isolation in antimicrobial tests. This method separates the compounds on a silica based plate using solvent. Silica gel (20 x 20 cm) TLC plates (Aldrich) were identified as appropriate plates for separating the compounds into identifiable bands. Plant extracts aliquots (1 or 5 mL) were spotted onto the bottom of the plates, and developed in glass chamber containing a solvent mixture of hexane:chloroform:methanol (50:50:2 v/v). The plates were dried and bands were removed for further analysis. Compounds were recovered from the silica gel through re-suspending the powder in 1 mL of chloroform:methanol (90:10 v/v), and centrifuging

for 5 min at 1,500 rpm to recover the supernatant. Bands were visualised through colour, or iodine staining after 20 min within a glass chamber containing iodine vapours. The R_f (relative mobility factor) values were determined for each plant extract using the following calculation:

R_f = distance of solvent migration (cm) / distance of compound (band) migration (cm)

2.2.6 Extraction of compounds from plant trichomes

Instead of directly removing the trichomes before extraction, the trichome exudate can be extracted directly from fresh whole leaf and stem material. Chloroform extraction of sucrose esters from the glandular trichomes of wild *Lycopersicum* species was performed on fresh leaf material which was extracted in chloroform (5 mL g^{-1}), soaked for 5 min, the chloroform was removed through centrifugal evaporation, re-suspended in acetone (4 mL), and filtered through Whatman number 1 filter paper at 0^{o}C before further solvent evaporation to yield an extract of the sugar esters (King and Calhoun, 1988; King et al, 1990). TLC was used to separate the trichome extracts (1 mL per plate) on silica gel (Aldrich). The solvent system used was a mixture of hexane:diethyl ether:glacial acetic acid (60:40:2 v/v) as described by Mudd et al., (1988), where the hydrocarbons should have a R_f value of 0.9, terpenoids R_f of 0.5, flavonoids R_f of 0.3, and glycolipids R_f of 0.15. Primulin (malvidine-3-galactoside chloride) was used as a non-destructive dye to visualise the bands at 340 nm, where lipids are bright yellow on a clear background (King and Calhoun, 1988; King et al., 1990).

The trichome chloroform extracts were further separated into a hydrocarbon, sugar ester fraction through re-suspending the crude extract in hexane (1 mL) and (80:20 v/v) methanol and water (1 mL). The hexane fraction was recovered and the residual polar compounds were removed though adding (75:25 v/v) methanol and water (1 mL) to the hexane fraction, this was repeated three times and the methanol and water fractions were recovered. The residual non-polar molecules were removed from the methanol and water fraction using hexane (1 mL) and then the hexane fraction was re-combined to the original fraction (Fobes et al., 1988). The fractions recovered were coloured, the hexane fraction was green and the methanol and water fraction was yellow.

Trichome extract samples were analysed after saponification which was achieved through adding NaOH (10% w/v 20 µL) to methanol (100 µL) re-suspended dried trichome extract (1:1 w/v) for 5 min, and then the saponified extract was acid neutrilised with HCL (5% v/v 30 µL) for 5 min. Hexane (100 µL) was added for phase separation at room temperature for 15 min and then the hexane phase was taken for analysis.

2.3 Bacteria species

The bacteria used as the initial micro-organisms to test for antimicrobial properties of each plant line were; *E. coli* ATCC 25922, *E. coli* O157:H7 NCTC 12900, *S.* Typhimurium SL1344, *S.* Typhimurium 10248, and *S.* Enteritidis S1400. They were grown from the Animal Health and Veterinary Laboratory Agancy (AHVLA) bead stocks in pre-prepared Luria-Bertani (LB) broth from Oxoid (Basingstoke), incubated aerobically for 16 h at 37^{o}C, this provided 1x10^{9} CFU mL^{-1} which was diluted to 1x10^{5} CFU mL^{-1} for antimicrobial tests. Due to the practical limitations associated with the large number of testable plant species and extracts, the number of bacterial species was reduced after optimisation to one of each species, with a view to use different strains of similar species for comparison at a later stage in the screens. The chosen bacteria for the final plant screens were *E. coli* O157:H7 NCTC 12900, *S.* Typhimurium 10248, and a Gram-positive bacteria *S. aureus* 25923.

Table 2-2. Bacterial species used in antimicrobial analysis of plant extracts.

Bacteria species	Strain number	Genotype	Reference(s)
Salmonella enterica serovar Typhimurium	SL1344	Wild type, Xyl, hisG, rpsL Human pathogen with high virulence	Gilberthorpe et al., 2007
Salmonella enterica serovar Typhimurium	LT2 10248	Wild type	Vereecke and Janssens, 2012
Salmonella enterica serovar Enteritidis	S1400	Wild type PT4, poultry associated	Woodward et al., 2000
Escherichia coli	ATCC 25922	Wild type, serotype O, produces α-hemolysin, does not produce verotoxin	Vereecke and Janssens, 2012
Escherichia coli	NCTC 12900 (O157:H7)	Wild type, non-toxigenic, serotype O157:H7	LeJeune et al., 2004 Vereecke and Janssens, 2012
Staphylococcus aureus subsp. aureus	ATCC 25923	Methicillin suceptible	Vereecke and Janssens, 2012

2.4 Antimicrobial assays

2.4.1 Growth conditions

Minimal media, LB, and Iso-sensitest media was used during the growth and antimicrobial testing of the bacteria. Minimal media was prepared using x10 MOPS (3-(*N*-morpholino)propanesulfonic acid) buffer (10 mL), 0.132 M K_2HPO_4 (1 mL), water (87 mL), and 2% w/v glucose (2 mL) and thiamine (0.25 µg mL^{-1}). Sterilisation, where necessary, was achieved via filtration through a 0.2 µm membrane filter (Millipore). All the bacteria were grown at 37^{o}C, and all inoculations were started initially with 10^5 CFU mL^{-1} of bacteria. All of the culture media reagents were obtained from Oxoid (Basingstoke) unless specified and prepared using distilled water (dH_20). Sterilisation was carried out by autoclaving at 121^{o}C at 15 p.s.i. for 15 min unless specified.

2.4.2 Zone of inhibition assays

Isosensitest agar is the recommended agar for disc diffusion assays by The British Society for Antimicrobial Chemotherapy (Andrews, 2012). It is more suitable due to better defined ingredients, with a stabilised mineral content which avoids antagonism between antimicrobial compounds and metal ions. Pre-prepared isosensitest agar plates from Oxoid (Basingstoke) were used for zone of inhibition assays. Zone of inhibition assays were conducted with 6 mm filter paper discs. Bacterial susceptibility was determined by measurement of halos equal to or greater than 7 mm which were considered to be susceptible to the extracts (Nascimento et al, 2000).

Zone of inhibition assays were conducted according to the British Society for Antimicrobial Chemotherapy (BACS) antimicrobial susceptibility testing methods (Andrews, 2012). Isosensitest agar plates were prepared using cultures containing of *E. coli* ATCC 25922, *E. coli* O157:H7 NCTC 12900, *S.* Typhimurium SL1344, *S.* Typhimurium 10248, and *S.* Enteritidis S1400 at 10^5 CFU mL^{-1} (50 µL per plate) lawn-spread onto the plates. Ciprofloxacin was the antibiotic suggested by BACS for tests against gastroenteric bacteria (Andrews, 2012). Ciprofloxacin was used as a control. It was prepared as 200 µg dissolved in distilled water (1 mL), and then 5 µL was added per disc to give a final concentration of 1 µL $disc^{-1}$. Six 6 mm diameter filter discs were

placed onto each plate; a solvent and a blank disc control were also used. Extracted samples were weighed and then re-suspended in a chosen solvent (usually DMSO). From each plant extraction, 10 µL was added to a filter disc for each bacteria strain, and the plates were then inverted and incubated at 37^{o}C aerobically for 16 h.

Motility assays were also conducted which vary from the zone of inhibiton assays because instead of measuring the zone of inhibition diameter, the zone of motility is measured. Cultures of *E. coli* ATCC 25922, *E. coli* O157:H7 NCTC 12900, *S.* Typhimurium 10248, *S.* Enteritidis S1400, and *S. aureus* 25923 were grown at 37^{o}C aerobically for 16 h within the presence of *S. pennellii* trichome extract (50, 25 and 0.78 mg mL^{-1}), with LB and DMSO controls. The plates were spotted with three droplets of culture at 10^5 CFU mL^{-1} (10 µL per plate), which were not inverted and were incubated at 37^{o}C aerobically for 16 h. The zones of motility were measured from the point of inoculation to the furthest ripple in the motility agar.

2.4.3 FLUOstar growth curves

The FLUOstar is a convenient way to measure the growth in multiple small liquid cultures of 200 µL per well. Each well starts with 2 µL of a 10^7 bacteria culture into 198 µl of either LB or x10 MOPS minimal media. It also allows for technical replicates to be incorporated into the experiment, which are particularly important when using such small volumes. There were three technical repeats for each bacteria strain under each growth condition. Within a single run on the FLUOstar the 96 well plates (IWAKI) can be designed to analyse different combinations of bacteria and samples, such as three bacteria strains and seven growth conditions, or four bacteria strains under six growth conditions. Each FLUOstar run included a minimal media, solvent, and an antibiotic control (ciprofloxacin), and wells free of bacteria were used as blanks. The raw data was blank corrected, which allowed the cultures to be compared through removing the interference of the compounds added. The FLUOstar settings included a positioning delay of 0.5 seconds, one kinetic window, 121 cycles (24 h total, 15 min per cycle), 10 flashes per well, 900 cycle time, orbital well scanning, additional 5 seconds of shaking before cycle, 3 mm well diameter, 37^{o}C temperature, 600 excitation filter and an empty emission filter.

Growth curve analyses were achieved through measuring the optical densities (OD) of bacteria grown in the presence of an extract, compared to controls, using a FLUOstar OPTIMA (BMG LABTECH). Data from bacterial cultures containing test extracts were blanked with non-inoculated broth containing test extracts, so that the OD of tested plant material and pure standards was removed as a variable for data analsyis. MICs were determined by measuring the optical density of bacteria grown within the presence of pure standards and plant extracts serially diluted within cultures, and identifying the lowest concentration at which the highest bacterial growth inhibition was observed; a method often used to quantify the MIC of compounds (Nascimento et al., 2000). This was followed by colony counts from wells which had visually no bacteria growth. Colony counts were prepared by serially diluting 20 µL of culture in 180 µL of 0.7 pH phosphate buffer saline (PBS), and then spotting 10 µL in triplicate on to LB agar and incubating overnight at 37^{o}C. The FLUOstar was used to measure the optical densities at 600 nm of bacteria grown in a 96 well plate at 37^{o}C, for 16 to 24 h. Data was investigated using the MARS Data Analysis Software package (BMG LABTECH).

2.5 Analyses of compounds

2.5.1 Investigating the phenolic compounds within plant extracts (HPLC-DAD)

Freeze-dried leaf material was ground with a pestle and mortar into a fine powder (20 mg) and was extracted as described by Davuluri et al., (2005), using methanol (1 mL) containing salicylic acid (20 µg mL^{-1}) internal standard. The samples were incubated for 1 h at 80 °C, before cooling on ice for 20 min, and then centrifuged at 3,500 rpm for 5 min. The methanol was removed and dried on a Genevac evaporator on low BP setting for 1 h. The samples were passed through a syringe (1 mL) through 0.2 µm nylon filters. Samples were frozen at -20°C for a maximum of four days before analysis.

The water and methanol extracts from wild tomato leaves were analysed for their phenolic acid content by high performance liquid chromatography using a diode array detector (HPLC-DAD) using an Agilent 1100 Series. The system was composed of a G1313A ALS autosampler, G1322A degasser, G1311A QuatPump, and G1315B DAD

units. The stationary phase included a 5 μm reverse phase C_{18} column (Hichrom Ltd, 4.6 x 300 mm, 16 % carbon loading) and guard column (4.6 x 25 mm).

Compounds were quantified from methanol extracts following the procedure of Melendez-Martinez et al., (2010) with slight modification. Extracts (1 mL) were placed into 1.5 mL Agilent sample vials and analysed using HPLC-DAD. Volumes of sample (25 μL) were injected, and a mobile phase flow rate of 1 mL min^{-1} was used. The mobile phase consisted of (A) water (HPLC grade) containing 2 % (v/v) methanol and 0.1 % (v/v) 6 M hydrochloric acid, which when combined was 0.2 μm filtered, and (B) acetonitrile. A linear gradient of 5 to 40 % solvent B for 20 min followed by 40 to 60 % solvent B for 15 min was used. A conditioning phase of 5 min returned the column to starting conditions. Elution from the column was monitored continuously by the on-line unit.

Analysis of the spectra was completed using Agilent Chemstation software (v. A.10.02), where chromatographic components were integrated and compared to compound libraries. The phenolic components were identified using an in-house library verified by authenticated standards. The measurement of compounds within samples was determined through near accurate quantification (not absolute as the response factors were not included) and quantification was carried out by comparison to the internal standard of salicylic acid (20 μg mL^{-1}), using the following equation (IS; internal standard):

μg g^{-1} DW = [Area / (mass (mg) of plant sample DW) x 1000] / [Area of IS / μg of IS]

2.5.2 Carotenoid and chlorophyll identification and quantification

The carotenoid concentration was calculated through comparisons with the absorbance readings of known concentrations of carotenoids. The absorption coefficients of most carotenoids at 1% in a 1 cm path-width cuvette have been published (Britton et al., 2009) and the following equation can be used to determine the carotenoid concentration of a sample where X= weight in mg of carotenoid, Y in mL of solution:

X = (Abs x Y x1000) /absorbance coefficient x100)

Carotenoids and chlorophylls were purified through TLC separation of chloroform leaf extracts (see section 2.2.5) on silica gel with a solvent system of hexane:chloroform:methanol (5:5:0.7 v/v). The coloured bands were re-suspended in HPLC grade ethyl acetate (100 µL) vortexed, centrifuged at 1,300 rpm for 5 min, the supernatant (10 µL) was added to ethyl acetate (40 µL) and placed into a glass insert for analysis by high performance liquid chromatography with photo diode array (Waters, HPLC-PDA) Alliance.

HPLC separations were performed using a C_{30} reverse-phase (RP) 5 µm column (150 x 4.6 mm) coupled to a C_{30} guard column (YMC Inc.). The mobile phases were methanol (A), water:methanol (20:80 v/v), containing 0.2% ammonium acetate (B), and tert-methyl butyl ether (C). The gradient was 95% A: 5% B, isocratically for 2 min, stepped to 80%A: 5% B: 15% C, from which a linear gradient to 30% A: 5% B: 65% C, over 23 min was performed. A Waters Alliance HPLC system was used (Waters 600S controller, Waters 610 pump, Waters 996 photodiode array detector and Waters 717 plus auto-sampler). The column temperature was maintained at 24°C during screening and 12°C during detailed analysis, with a Jones Chromatography column heater/cooler. Detection was performed continuously from 220 to 700 nm with an online photodiode array detector. Carotenoids were analysed at wavelengths of 286, 350 and 450 nm. Carotenoid identification was performed through comparison of their spectrum shape and their peak absorbances (λmax) with the reference spectra recorded in the literature (Britton et al., 2004; Fraser et al., 2000) and compared to natural standards.

The carotenoids were quantified using a Beckman Coulter DU®800 spectrometer to record the absorbance. Yellow/red bands were recorded at approximately 450 nm, and green chlorophyll bands absorbance was measured at 648 and 666 nm. An aliquot (10 µL) was added to chloroform (1 mL) for the red/yellow bands, and to petroleum ether for green bands, and blanked with the corresponding solvent. The absorbance coefficient of 2500 was used to estimate the concentrations of samples with unpublished absorbance coefficients. Chlorophylls were quantified in a similar way to carotenoids, and were determined using the following information, calculating chlorophyll a (*Ca*) and chlorophyll b (*Cb*) with λmax of C*a* and C*b* (666 nm and 648 nm respectively). Chlorophyll *a* (C*a*) = 10.91 Abs666 – 1.2 Abs648 and Chlorophyll *b* (C*b*) = 16.38 Abs648 – 4.57 Abs666 (Wellburn, 1994).

2.5.3 GC-MS analysis

Polar and non-polar metabolites were extracted using different solvents and analysed by GC-MS (Agilent 5975C Series GC-MSD), after derivatisation. A mixture of *n*-alkanes ranging from 8 to 32 carbons was used to generate external calibration retention indexes. An aliquot (100 to 500 µL depending on concentration) was taken from each extract or spent media, internal standards were added and dried in a Genevac evaporator. Samples were derivatised to their methoxylated and silylated forms as described by Halket et al., (2005). First, methoxyamine hydrochloride (MEOX) (30 µL) in pyridine anhydrous (20 mg mL w/v) was added to samples and incubated at 40°C for 1 h. Following this reaction, samples were treated with *N*-methy-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA, 70 µL) and heated at 40°C for 2 h. The final solution (1 µL) was injected in split mode (1:10) into a 7890A gas chromatograph on-line with an Agilent 5975C mass spectrometer. Metabolites were separated in a J&W Scientific DB-5MS 30 m x 250 µm x 0.25 µm column equipped with a 10 m guard column and helium was employed as the carrier gas.

The parameters used for the bacteria metabolomic samples was a temperature gradient ranging from 70° to 320°C (held at 320°C for 1 min) at 5°C min^{-1} with a total run time of 55 min and the flow rate was set at 0.5 mL min^{-1}. The inlet was heated to 280°C and the mass spectrometer transfer line to 250°C. Samples were injected as 10 µL and split in a ratio of 10:1. The control parameters for plant extracts analysed by GC-MS were a temperature gradient ranging from 70°C to 320°C (held at 320°C for 10 min) at 4°C min^{-1} with a total run time of 77.5 min and the flow rate was set at 1 mL min^{-1}. Inlet was heated to 280°C and the mass spectrometer transfer line to 250°C. Samples were injected 10 µL on a split-less setting.

2.5.3.1 Metabolomics

2.5.3.1.1 Preparation of cultures for metabolomic analysis

Cultures of LB (20 mL) were inoculated with *Salmonella* and grown overnight at 37°C in a shaking incubator, the cultures were diluted to approximately 10^7 CFU mL^{-1} by adding 100 µL into LB (10 mL), and then inoculated into Duran bottles containing LB

(100 mL) to a final concentration of approximately 10^5 CFU mL^{-1}. The cultures were grown for 20 h at 37^{o}C in a shaking incubator. Chloroform leaf trichome extracts from a pool of three *S. pennellii* LA0716 plant replicates were re-suspened in DMSO and added to the LB cultures to a final concentration of 0.78 mg mL^{-1} during mid-exponential phase of bacterial growth (6 h). At this point an aliquot (8 mL) of culture was taken and then the test sample was added followed by a second aliquot (8 mL) taken after 30 min of challenge, this was followed by a final aliquot (8 mL) taken at early stationary phase (8 h). The metabolomic cultures included controls of LB broth, LB broth (9 mL) with solvent (1 mL), and LB broth (9 mL) with solvent (1 mL) containing plant extract or purified compounds.

2.5.3.1.2 Quenching method used to prepared cultures for metabolomic analysis

Quenching solution was prepared (60% v/v methanol, 40% v/v of a 0.9% v/v NaCl solution) pre-cooled to -20^{o}C and the metabolomics bacterial cultures (8 mL) were added to aliquots (30 mL) of the quenching solution. The solutions were immediately centrifuged at -20^{o}C for 5 min at 4,500 rpm, the liquid was removed and the cellular material was frozen at -80^{o}C overnight. The biomass was freeze-dried for 24 h, weighed and stored at -20^{o}C prior to extraction. A sample of the supernatant (200 µL) was also stored at -20^{o}C after centrifugation for analysis to ensure that the leakage of metabolites into the quenching solution was not too high.

2.5.3.1.3 Extraction of bacteria for metabolomic analysis

After storing the samples at -80^{o}C, the pellets were re-suspended in methanol (250 µL) and water (250 µL), sonicated for 15 min and then chloroform (1 mL) was added. The mixture was centrifuged to achieve phase separation at 700 rpm for 5 min, and the upper polar layer was taken for analysis. Subsequently the lower non-polar layer was taken for analysis. The remaining central pellet was subjected to further saponification for fatty acid extraction using NaOH (1 mL, 10% w/v) in a sonicator bath for 10 min. The samples were centrifuged at 1,300 rpm for 5 min, and the NaOH was removed, the remaining pellet was re-extracted using tris buffer (300 µL, 50 mM, pH 7, NaCl 1 M), chloroform (600 µL) left at room temperature for 15 min. This was centrifuged for

phase separation and the bottom layer was pooled into the first non-polar extract. To analyse the polar extract on the GC-MS the extract (200 µL) was placed into a glass vial with the internal standard d4-succinic acid (10 µL) in methanol (1 mg mL^{-1} w/v), for the non-polar samples the whole extract was dried along with the internal standard d27-myristic acid (10 µL) in chloroform (1 mg mL^{-1} w/v). The dried polar and non-polar samples were derivatized by adding MEOX (30 µL) and incubating for 1 h at 40^{o}C, then adding MSTFA (70 µL) and incubated for 2 h at 40^{o}C.

2.5.3.1.4 Metabolomic data analysis

Metabolites were analysed by GC-MS (Agilent), corrected for using the dried weight of biomass and quantified relative to the internal standard. Compounds generating multiple peaks in the chromatogram as a consequence of the methoxylation and silylation reactions were quantified by combining the areas of the different compound derivatives. A typical examples of this are amino acids and compounds which contain amine ($–NH_2$) groups show multiple peaks which have different TMS groups and the derivatisation of the keto groups (–C=O) into methoxyme ($–C=N–O–CH_3$), which generates geometric isomers displayed as two peaks in the chromatogram. AMDIS (v.2.69) software was used for peak deconvolution and establishing libraries for polar and non-polar metabolites. Identification of metabolites for library construction was done through comparing mass spectra and retention indexes to NIST version 2.0 (2008) and Golm Metabolome (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/msri/gmd_sspq.html) mass spectral databases and where possible confirmed with authentic standards from Sigma Aldrich. Those compounds not identified were named as UNK followed the sub index *p* or *np* for polar or non-polar extracts, respectively, and by its corresponding retention time.

The overall reproducibility of the measurements was determined through calculation of the standard error of the six biological replicates, and the metabolomic cultures were compared through multivariate PCA analysis. Data matrixes were transformed by using the pareto-scaled method (van den Berg et al., 2006) and multivariate analysis performed using SIMCA-P+ 12.0 (Umetrics). Principal component analysis (PCA) has been previously used in metabolomic analysis to classify the mode of action of natural

products according to their similarities with antibiotics with known mechanisms (Biao-Yi et al., 2008). Pathway diagrams were created using the in-house developed software BioSynlab© (Royal Holloway, University of London, http://www.rhul.ac.uk).

2.5.4 LC-PDA-TOFMS (MAXIS) analysis

The plant trichome extracts were separated into fractions using either TLC (see section 2.2.5) or SPE (see section 2.2.4). The extracts were dried, and re-suspended in 1 mL of methanol. Analysis of active fractions from plant extracts were carried out using the high resolution Q-TOF mass spectrometer UHR-MAXIS (Bruker Daltonics) on-line with a UHPLC UltiMate 3000 equipped with a PDA detector (Dionex Softron). Chromatographic separation was performed in a RP C_{18} 2 µm column (100 x 2 mm i.d.) coupled to a 10 x 2 mm C_{18} guard column (YMC Inc.,). The mobile phase was comprised of (A) 0.05% formic acid in water (v/v) and (B) acetonitrile containing 0.05% formic acid (v/v). These solvents were used in a gradient mode starting at 95% (A) then stepped to 60% (A) for 10 min and followed by a linear gradient over 7 min to 40% (A). This last condition was kept for 3 min in isocratic mode and after that initial conditions (95% A) were restored for 1 min. The column was then re-equilibrated for 9 min. The flow rate used was 0.2 mL min^{-1} and the injection volume was 5 µL. Electrospray ionisation (ESI) operating in negative mode was employed. Dry gas (nitrogen) and nebulizer were at 6 L min^{-1} and 0.8 bar respectively and temperature of ion source was set at 190°C.

A full MS scan was performed from 50 to 1500 *m/z* and quadrupole's transfer and pre-pulse storage time were set at 55 and 10 µs respectively. Collision energy ramp from 20 to 25 eV was applied for MS/MS fragmentation of target masses between 200 and 300 *m/z*. Instrument calibration was performed externally prior to each sequence with sodium formate solution prepared by dissolving NaOH in 50% 2-isopropanol containing 0.2% formic acid to a final concentration of 10 mM. In addition, automated post-run internal calibration was also performed by injecting the same sodium formate calibrant solution at the end of each sample run via a six port divert valve equipped with a 20 µL loop. All solvents were LC-MS grade and purchased from Fischer Scientific Ltd.

2.5.5 Electron microscopy (EM)

Preparation of test cultures (10 mL) included a LB control, a LB culture containing DMSO solvent control, and LB culture containing *S. pennellii* trichome chloroform leaf dip extract. The plant extract sub-inhibitory MIC was determined to be 25 mg mL^{-1} for cultures of *E. coli* O157:H7 and *S.* Typhimurium 10248, and was 0.78 mg mL^{-1} for *S. aureus* 25923. These sub-inhibitory MICs were determined from previous disc diffusion assay, and growth curve results, and from the visual growth of the bacteria cultures. The cultures were grown for 24 h in LB at 37^{o}C.

Bacterial cultures were centrifuged (10 mL), for 15 min at 3,500 rpm at room temperature for a pellet. The pellets were re-suspended in PBS (200 to 1000 µL) or until the solutions were milky white depending on pellet size. The grids were prepared by placing PBS bacteria cultures (50 µL) on to dental wax which will form droplets. This was repeated with potassium phosphotungstate (KPT) stain (50 µL). The forceps were sterilised using alcohol-flame, and then they were used to remove the EM grids, and to blot dry the edges using filter paper. EM grids were placed on top of the droplets of bacteria cultures silver side down, and left for five min. The forceps were sterilised again, and used to place the EM grids on top of the KPT stain droplets silver side down. The forceps were immediately sterilised again, and used to remove the EM grids from the KPT stain droplets, and used to blot the EM grids on their edge. The grids were stored silver side up on an EM pad within a petri dish for viewing by EM (transmission electron microscope).

2.6 Statistics

Student's *t*-tests determined significant differences between pair-wise comparisons of similarly treated samples. Student's *t*-tests, means, and standard error of the means (SEM) were calculated using Excel software (Microsoft) or GraphPad Prism 6. The significant difference between the control and the test samples were accepted when *t*-tests returned a *P* value of <0.05. The significance was further classified into different levels of significance such as, * $P<0.05$, ** $P<0.005$, and *** $P<0.0005$. Man-Whitney U tests were performed when comparing data which did not confirm to a normal distribution, and was used to compare medians at a 95% confidence limit 2-tailed. One-

way ANOVA was used for identifying significant differences between the area under the curve (AUC) means for bacterial growth curves in the presence of phytochemicals. Tukey's multiple comparisons test was used to compare all groups to each other, Dunnetts multiple comparisons was used when only comparing to a control and two-way ANOVA was used when comparing multiple fractions of multiple plants.

Chapter 3

Antimicrobial wild tomato relatives

3.1 Introduction

The tradition of using plants as medicine is an important practice world-wide with the World Health Organisation (WHO) suggesting that approximately 80% of people living in developing countries use traditional medicine (Eloff, 1998). Investigations into the use of plants in traditional medicine has shown that approximately 28% of plants have been used, of which circa 121 prescription drugs are derived from plants, 45 of these being used in the USA (Farnsworth, 1984). The number of new chemical entities (NCEs) which include natural products derived from plants has been limited to only 3 (1981-2010), and were identified as antiallergic, anticancer, and antipsoriatic (Newman and Cragg, 2012). This suggests that research into plants as sources of antibacterial NCEs needs to be improved. Investigations into the antimicrobial potential of plant species, such as the wild tomato relatives, may highlight novel antimicrobial secondary metabolites which have alternative inhibitory modes of action to the ones currently utilised by antibiotics against bacteria (Farnsworth, 1984).

The wild tomato relatives which were investigated as part of this research include the domesticated *S. lycopersicum* 'cerasiforme' LA2675, LA1511, LA0134C, *S. lycopersicum* cv. M82 and the wild tomato species *S. cheesmaniae* LA1984, *S. pimpinellifolium* LA0114, *S. chilense* LA2759, *S. chmielewskii* LA2695, *S. habrochaites* LA1777, *S. neorickii* LA2133, *S. pennellii* LA0716, *and S. peruvianum* LA2744 (Grandillo et al., 2011). Selected *S. pennellii* ILs were also tested, however, investigations into the complete IL collection for *S. pennellii* was not continued as a major aspect of this research. This was because it is important to determine the extent of the antimicrobial potential of the wild tomato relatives such as *S. pennellii*, before moving onto further analysis of the IL collections.

This chapter explores the antimicrobial potential of wild tomato relatives through selected antimicrobial tests using; *E. coli*, *Salmonella*, and *S. aureus* as model organisms. Two main methods were employed to test the antimicrobial properties of the extracted plants. These were 1: zone of inhibition assays and 2: liquid broth growth curve analysis through measuring OD (600 nm) using the FLUOstar. The zone of inhibition assays measures the area of inhibition of bacteria after selected plant extracts have been placed onto filter discs and incubated for 16 to 24 h. The growth curves OD

results were confirmed through determining the CFU mL^{-1} of the individual cultures using colony counts.

The aim of this research was to identify wild tomato relatives which have antimicrobial potential, and to utilise the secondary metabolites produced by them as potential classes of novel antimicrobial compounds. The plant material investigated included analysis of whole, dried ripe and unripe fruit. However, the analysis of fully expanded mature leaf material was the primary focus of this research due to the increased trichome numbers, and higher concentrations of certain secondary metabolites such as glycoalkaloids. It is important to identify the plant organs which are involved in the antimicrobial activity, as antimicrobial compounds can be found in different organs depending on the plant species, such as within the fruit, leaves, roots, stems or seeds (Cowan, 1999).

It is also important to use the appropriate extraction method, as specific plant organs can produce alternative secondary metabolites, which will have different properties such as solubility, polarity, volatility, and stability. The properties of potentially antimicrobial compounds were an aspect which was closely considered as part of this research, and influenced the approaches to method developments for plant extraction and storage of plant material and extracts. This chapter explores several approaches to identifying antimicrobial secondary metabolites from wild tomato relatives using different targeted and non-targeted solvent extraction techniques.

3.2 Results

3.2.1 Disc diffusion assays of wild tomato relatives

The screen of nine tomato relatives showed that *S. pennellii* LA0716 has antimicrobial activities against *S. aureus* 25923 and *S.* Typhimurium 10248, with no inhibition observed against *E. coli* O157:H7. *S. habrochaites* LA1777 was also inhibitory to the growth of *S. aureus* 25923 and *S.* Typhimurium 10248, with petroleum ether (0.418 mg $disc^{-1}$) and hexane (0.335 mg $disc^{-1}$) acting as the highest inhibitory extracts (Figure 3-1). The plant material was extracted in eight solvents ranging in polarity from water to hexane, the inhibitory compounds from *S. pennellii* was soluble in all eight solvents, with a slightly lower inhibitory effect seen in water extracts (2.2 mg $disc^{-1}$). The water

extracts re-suspended in DMSO showed less inhibitory effects than the DMSO solvent control against *E. coli* O157:H7 (NCTC 12900) which was usually inhibited, with an average zone of inhibtion diameter of 6 to 7 mm, showing that secondary metabolites extracted in water have a protective effect against the inhibitory solvent.

Table 3-1. Average dry weights of extracts from wild tomato relatives extracted using in eight solvents (100 mg 2mL^{-1}) and the concentrations of the stock solutions for the antimicrobial assays.

	S. pennellii LA0716				*S. lycopersicum* cv. M82				*S. chilense* LA2759			
	mg DW (Mean±SEM)			*mg mL^{-1}	mg DW (Mean±SEM)			*mg mL^{-1}	mg DW (Mean±SEM)			*mg mL^{-1}
Water	44.40	±	2.40	222.00	30.13	±	2.58	150.67	30.33	±	0.37	151.67
Methanol	24.33	±	1.47	121.67	28.47	±	3.05	142.33	30.07	±	0.83	150.33
Ethanol	26.37	±	4.91	131.83	17.73	±	8.03	88.67	9.83	±	0.53	49.17
Isopropanol	27.27	±	4.17	136.33	17.63	±	3.84	88.17	13.83	±	1.37	69.17
Ethyl acetate	25.43	±	5.18	127.17	10.23	±	1.68	51.17	10.53	±	1.97	52.67
Chloroform	34.33	±	7.98	171.67	19.40	±	2.97	97.00	13.60	±	1.50	68.00
Petroleum ether	32.07	±	2.17	160.33	10.67	±	2.19	53.33	16.50	±	1.30	82.50
Hexane	29.97	±	4.55	149.83	15.17	±	4.07	75.83	8.30	±	1.50	41.50
	S. chmielewskii LA2695				*S. pimpinelifollium* LA0114				*S. peruvianum* LA2744			
	mg DW (Mean±SEM)			*mg mL^{-1}	mg DW (Mean±SEM)			*mg mL^{-1}	mg DW (Mean±SEM)			*mg mL^{-1}
Water	20.00	±	3.98	100.00	36.80	±	3.56	184.00	29.73	±	3.47	148.67
Methanol	19.90	±	2.79	99.50	24.30	±	1.91	121.50	18.90	±	0.50	94.50
Ethanol	6.80	±	3.56	34.00	12.57	±	2.45	62.83	12.43	±	3.17	62.17
Isopropanol	6.27	±	2.99	31.33	15.67	±	0.63	78.33	17.23	±	3.07	86.17
Ethyl acetate	11.83	±	1.36	59.17	12.40	±	3.45	62.00	10.13	±	1.17	50.67
Chloroform	19.23	±	4.07	96.17	17.20	±	2.23	86.00	21.93	±	1.67	109.67
Petroleum ether	9.90	±	2.32	49.50	11.37	±	3.13	56.83	18.13	±	2.07	90.67
Hexane	9.10	±	4.50	45.50	10.13	±	1.88	50.67	9.27	±	2.63	46.33
	S. neorickii LA2133				*S. habrochaites* LA1777				*S. cheesmaniae* LA1984			
	mg DW (Mean±SEM)			*mg mL^{-1}	mg DW (Mean±SEM)			*mg mL^{-1}	mg DW (Mean±SEM)			*mg mL^{-1}
Water	13.70	±	6.36	68.50	10.40	±	0.96	52.00	13.80	±	3.70	69.00
Methanol	12.23	±	1.70	61.17	12.80	±	2.61	64.00	16.40	±	1.63	82.00
Ethanol	5.77	±	0.71	28.83	10.47	±	3.76	52.33	7.97	±	3.95	39.83
Isopropanol	5.10	±	1.20	25.50	10.00	±	5.55	50.00	6.13	±	0.84	30.67
Ethyl acetate	5.27	±	2.56	26.33	9.10	±	5.05	45.50	9.70	±	6.07	48.50
Chloroform	2.90	±	1.57	14.50	9.47	±	3.83	47.33	10.30	±	2.32	51.50
Petroleum ether	3.63	±	2.81	18.17	6.70	±	3.37	33.50	7.83	±	0.90	39.17
Hexane	3.93	±	0.87	19.67	8.37	±	2.05	41.83	4.93	±	0.47	24.67

The data shows the mean extract (DW) from three biological plant replicates of 100 mg of ground plant material extracted in 2 mL of solvent, with the standard error of the mean. The final concentrations (*mg mL^{-1}) are also shown and represent the concentration of the stock solutions of DW extract dissolved in 200 µL of DMSO used to test against the bacteria in the FLUOstar and disc diffusion assays.

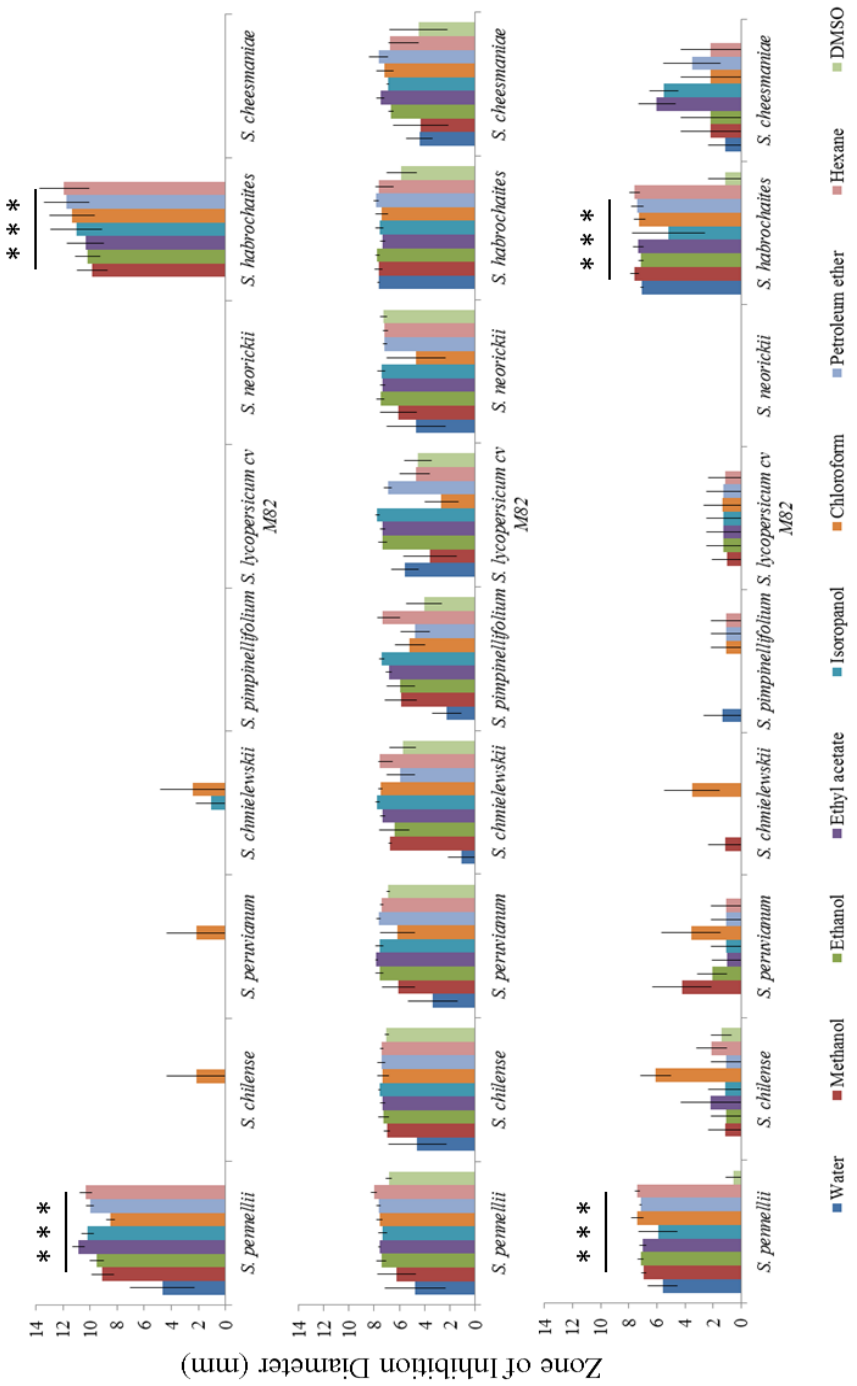

Figure 3-1.Wild tomato disc diffusion assay screen.

Nine wild tomato relative leaf extracts were tested against *S. aureus* 25923, *E. coli* O157:H7, and *S.* Typhimurium 10248. Each tested plant extract contained varying concentrations of crude DW of extract (see Table 3-1) because all the extracts were re-suspended in 200µL DMSO to a stock solution of which 10 µL was added per 6 mm disc. The antimicrobial activities are represented by the diameter of the zone of inhibition (mm), standard error bars are from three biological replicates and two technical replicates of each. Statistical analysis was performed comparing the results from the inhibitory effect of the solvent extracts of wild tomato relatives compared to *S. lycopersicum* cv. M82 (Man Whitney non-parametric, 95% confidence interval, 2-tailed (***P<0.0005).

The inhibitory effects of DMSO against the Gram-negative bacteria *E. coli* O157:H7 and *S.* Typhimurium 10248 were blanked against the final results. The chloroform extracts were consistently inhibitory, even in plant species where all other solvent extractions had no inhibitory effect, such as *S. chilense*, and *S. chmielewskii* (Figure 3-1). The chloroform extractions had the highest inhibitory activity, so these extracts were tested for antimicrobial activity in liquid broth. *S. cheesmaniae* also had slight antimicrobial activity against *S.* Typhimurium 10248, particularly from extracts of ethyl acetate and isopropanol.

Statistical analysis (Man Whitney non-parametric, 95% CI, 2-tailed) of the wild tomato relative disc diffusion assays confirm that the median zones of inhibition (mm) of *S. aureus* 25923 in the presence of the solvent extracts of *S. pennellii* is statistically different from *S. lycopersicum* cv. M82 (***P<0.0002), but not statistically different from *S. habrochaites* (P<0.104). The median zones of inhibition (mm) of *E. coli* O157:H7 in the presence of the solvent extracts of *S. pennellii* is not significantly different from *S. lycopersicum* cv. M82 (P<0.13) or *S. habrochaites* (P<0.44). The median zones of inhibition (mm) of *S.* Typhimurium 10248 in the presence of the solvent extracts of *S. pennellii* is significantly different from *S. lycopersicum* cv. M82 (*** P<0.0002), and *S. habrochaites* (***P<0.0002).

The dry weights of extracted plant compounds from the wild tomato relatives ranged depending on the plant species and the extracting solvent, for example, a hexane extract from *S. neorickii* LA2133 was on average 3.93 mg DW, compared to a hexane extract of *S. pennellii* LA0716 which was on average 29.97 mg DW (Table 3-1). These differences in the weights of the dry extracts contributed to different concentrations used from each solvent and plant extract in the antimicrobial experiments. This could have been corrected through standardisation of the extracts so they could be tested at the same concentration, however, it was decided that due to the large number of plant species and the different solvent extractions to be tested, that a non-standardised approach would be initially used.

3.2.1.1 FLUOstar assay results of nine wild tomato relative leaf material, against *E. coli* O157:H7, *S.* Typimurium 10248 and *S. aureus* 25923

Statistical analysis (one-way ANOVA, 95% CI) was used to compare the mean area under the curve (AUC) of all the plant species, and further analysis was performed with Tukey's multiple comparisons test, used to compare the individual groups to each other. The one-way ANOVA analysis showed that at least two of the mean AUC for *E. coli* O157:H7 grown in the presence of the wild tomato relatives were statistically different from each other ($P<0.01$). *E. coli* O157:H7 was significantly inhibited ($P<0.01$) by *S. pennellii* compared to the LB control but it was not significantly inhibited compared to the DMSO control. The *S. pennellii* extract extended the lag phase and growth was delayed until after 6 h for *E. coli* O157:H7. All other tested wild tomato relatives were shown not to have a significant inhibitory effect, as seen when comparing the growth curves with the LB and DMSO solvent control. There was a significant difference between the AUCs for *S. neorickii*, *S. pimpinellifolium* and *S. peruvianum* compared to *S. pennellii* ($P<0.05$) (Figure 3-2).

One-way ANOVA showed that at least two of the AUC means were significantly different for the *S. aureus* 25923 grown in the presence of the wild tomato relatives ($P<0.001$). Multiple pairwise comparisons showed that the AUC for *S. pennellii and S. habrochaites* were significantly below the LB control ($P<0.05$), but not compared to the DMSO control. *S. habrochaites* completely inhibits the growth of *S. aureus*, and *S. pennellii* also inhibits growth and then recovers after 13.5 h. There were significant differences between the AUCs for both *S. pennellii* ($P<0.05$) and *S. habrochaites* ($P<0.01$) when compared to *S. lycopersicum* cv. M82, *S. neorickii*, *S. pimpinellifolium*, *S. peruvianum*, *S. chilense*, and *S. chmielewskii* (Figure 3-3).

One-way ANOVA showed that at least two of the AUC means were significantly different for *S.* Typhimurium 10248 grown in the presence of the wild tomato relatives ($P<0.001$). *S.* Typhimurium 10248 AUC was also significantly different for *S. pennellii* compared to LB ($P<0.01$) and DMSO ($P<0.05$), where growth did not commence until after 8 h. The AUC for *S. neorickii* and *S. peruvianum* were also significantly different when compared to *S. pennellii* ($P<0.05$) (Figure 3-4). Colony counts were determined at 0 and 24 h confirm the patterns of growth seen from the OD readings.

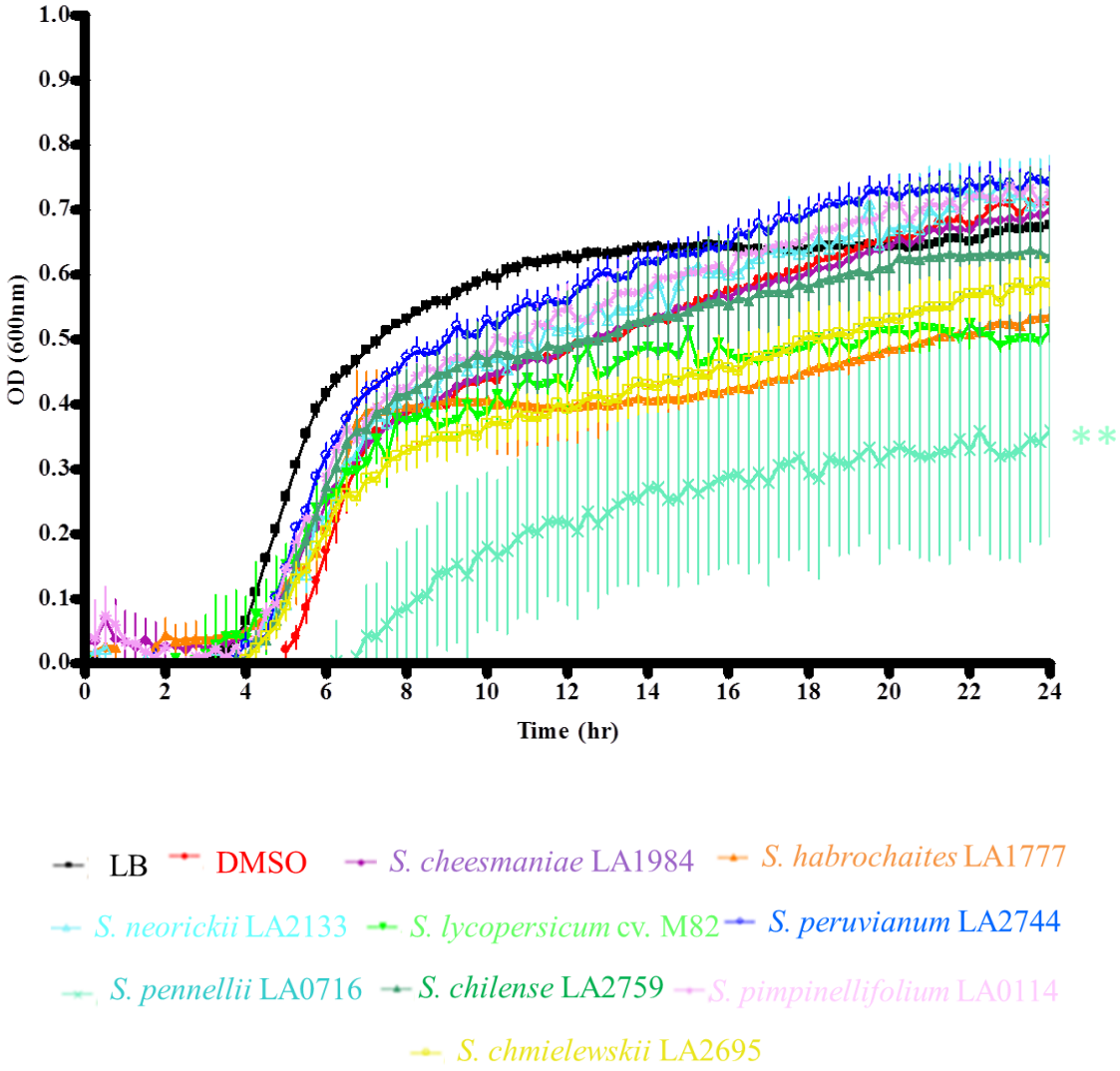

Figure 3-2. FLUOstar growth curve of *E. coli* O157:H7 in the presence of wild tomato leaf extracts. Bacteria was grown in LB control, 5% (v/v) DMSO control and 5% (v/v) of DMSO re-suspended plant extracts. *S. cheesmaniae* LA1984 (purple, 2.5 mg mL^{-1}), *S. habrochaites* LA1777 (orange, 2.3 mg mL^{-1}), *S. neorickii* LA2133 (light blue, 0.7 mg mL^{-1}), *S. lycopersicum* cv. M82 (light green, 4.8 mg mL^{-1}), *S. peruvianum* LA2744 (blue, 5.4 mg mL^{-1}), *S. pennellii* LA0716 (turquoise, 8.5 mg mL^{-1}), *S. chilense* LA2759 (dark green, 3.4 mg mL^{-1}), *S. pimpinellifolium* LA0114 (pink, 4.3 mg mL^{-1}), *S. chmielewskii* LA2695 (yellow, 4.8 mg mL^{-1}). Error bars represent the standard error from three biological replicates, and two technical replicates. The asterisk (* *= $P<0.01$) represents the significant difference between the area under the *E. coli* O157:H7 growth curves grown in the presence of the wild tomato relatives compared to the LB control, using one-way ANOVA Tukey's multiple comparisons test (95% CI).

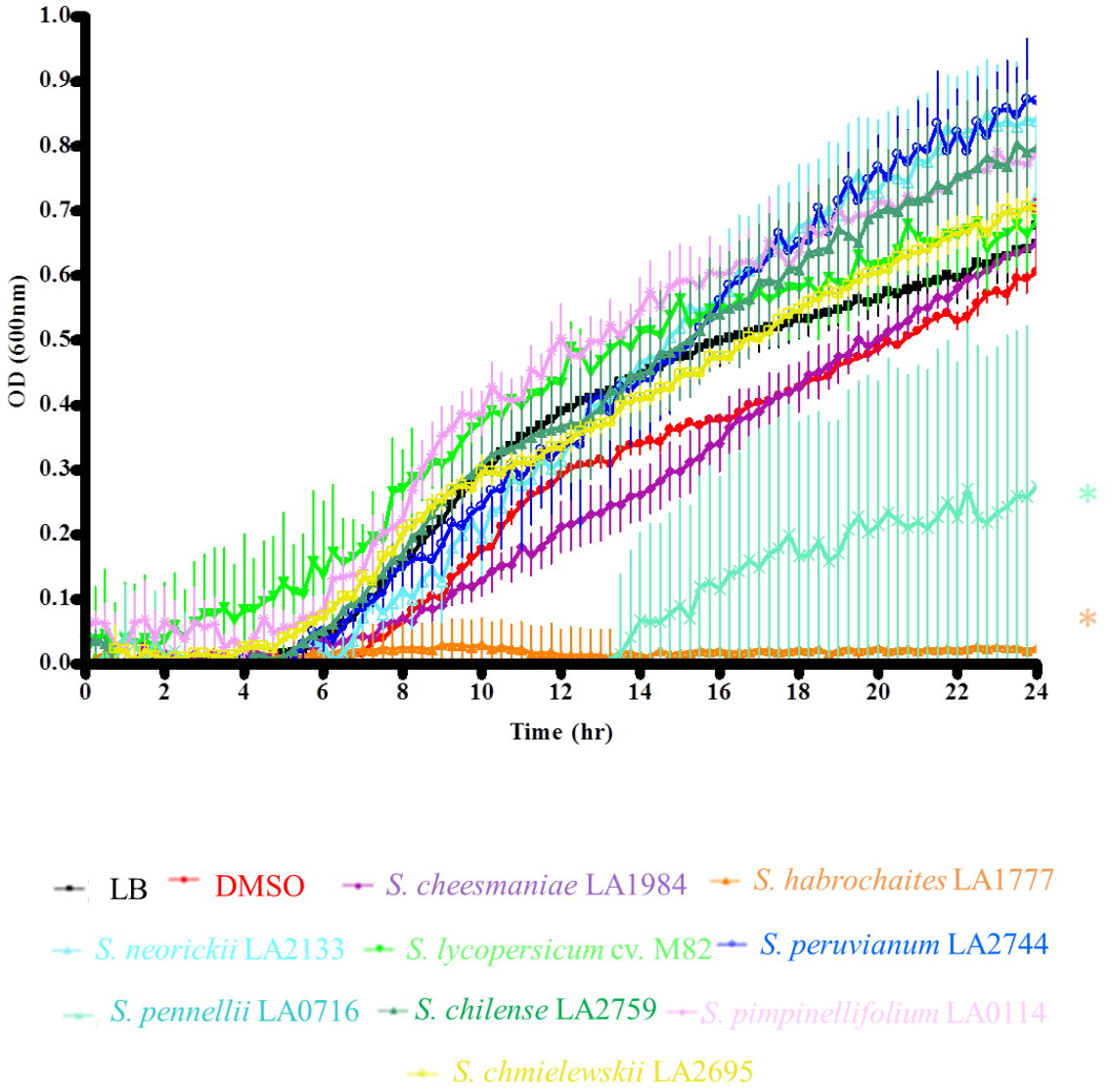

Figure 3-3. FLUOstar growth curve of *S. aureus* 25923 in the presence of wild tomato leaf extracts. Bacteria was grown in LB control, 5% (v/v) DMSO control and 5% (v/v) of DMSO re-suspended plant extracts. *S. cheesmaniae* LA1984 (purple, 2.5 mg mL^{-1}), *S. habrochaites* LA1777 (orange, 2.3 mg mL^{-1}), *S. neorickii* LA2133 (light blue, 0.7 mg mL^{-1}), *S. lycopersicum* cv. M82 (light green, 4.8 mg mL^{-1}), *S. peruvianum* LA2744 (blue, 5.4 mg mL^{-1}), *S. pennellii* LA0716 (turquoise, 8.5 mg mL^{-1}), *S. chilense* LA2759 (dark green, 3.4 mg mL^{-1}), *S. pimpinellifolium* LA0114 (pink, 4.3 mg mL^{-1}), *S. chmielewskii* LA2695 (yellow, 4.8 mg mL^{-1}). Error bars represent the standard error from three biological replicates, and two technical replicates for each. The asterisk (* = $P<0.05$) represents the significant difference between the area under the *S. aureus* 25923 growth curves grown in the presence of the wild tomato relatives compared to the LB control, using one-way ANOVA Tukey's multiple comparisons test (95% CI).

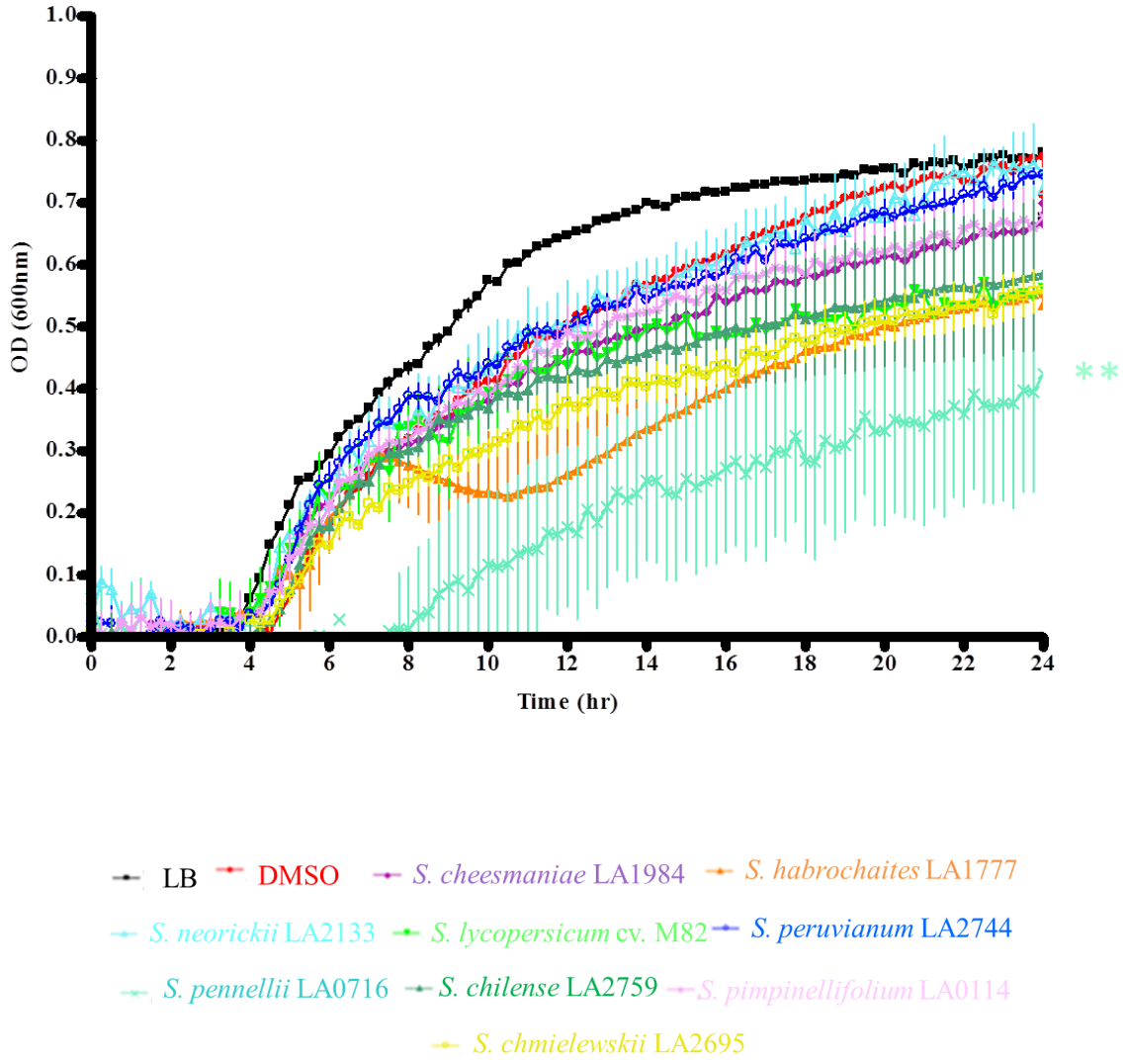

Figure 3-4: FLUOstar growth curve *S.* Typhimurium 10248 in the presence of wild tomato leaf extracts.

Bacteria was grown in LB control, 5% (v/v) DMSO control and 5% (v/v) of DMSO re-suspended plant extracts. *S. cheesmaniae* LA1984 (purple, 2.5 mg mL^{-1}), *S. habrochaites* LA1777 (orange, 2.3 mg mL^{-1}), *S. neorickii* LA2133 (light blue, 0.7 mg mL^{-1}), *S. lycopersicum* cv. M82 (light green, 4.8 mg mL^{-1}), *S. peruvianum* LA2744 (blue, 5.4 mg mL^{-1}), *S. pennellii* LA0716 (turquoise, 8.5 mg mL^{-1}), *S. chilense* LA2759 (dark green, 3.4 mg mL^{-1}), *S. pimpinellifolium* LA0114 (pink, 4.3 mg mL^{-1}), *S. chmielewskii* LA2695 (yellow, 4.8 mg mL^{-1}). Error bars represent the standard error from three biological replicates, and two technical replicates for each. The asterisk (** = $P<0.01$) represents the significant difference between the area under the *S.* Typhimurium 10248 growth curves grown in the presence of the wild tomato relatives compared to the LB control, using one-way ANOVA Tukey's multiple comparisons test (95% CI).

3.2.2 Extraction of secondary metabolites from the fruit of selected wild tomato relatives

Ripe and unripe fruit from several wild tomato species were extracted with water, and their antimicrobial activity was investigated using FLUOstar growth curve analysis, at concentrations of approximately 3 mg mL^{-1}. One-way ANOVA showed that there was no significant difference between the mean AUCs of *E. coli* O157:H7 and *S.* Enteritidis S1400 grown in the presence of the tested ripe fruit of wild tomato relatives. At least two of the AUC means were significantly different for *E. coli* 25922 (P<0.0001) and *S.* Typhimurium 10248 (P<0.02) grown in the presence of the tested ripe fruit.

Further analysis was performed with Tukey's multiple comparisons test, used to compare the individual groups to each other. Significant differences were seen from *S.* Typhimurium 10248 grown in the presence of *S. peruvianum* LA2744 compared to the minimal media control. *S. peruvianum* LA2744 (P<0.001) also showed a significant difference between the AUCs of *E. coli* 25922 compared to the water control. In several instances, the growth of the bacteria in these plant extracts was increased with a longer exponential phase; this is seen most from *S. lycopersicum* "cerasiforme" LA1511with a significant difference in the AUC compared to the water control for *E. coli* 25922 (P<0.01) (Figure 3-6A).

The unripe fruit extracted in water had similar growth patterns to the extracted ripe fruit (Figure 3-6 and Figure 3-7). One-way ANOVA showed that there were significant differences between the at least two mean AUCs from all of the tested bacteria grown in the presence of the unripe fruit (P<0.01). Comparisons between the AUC for bacteria grown in the presence of unripe fruit compared to the controls showed the *S. chmielewskii* LA2695 to be significantly different to the minimal media control for *S.* Typhimurium 10248 (P<0.01), *E. coli* O157:H7 (P<0.0001), and *E. coli* 25922 (P<0.01). The *S. chmielewskii* LA2695 AUC was also significantly different to *S. lycopersicum* "cerasiforme" LA1511 for *S.* Typhimurium 10248 (P<0.001), *S.* Enteritidis S1400 (P<0.05), and *E. coli* 25922 (P<0.05).

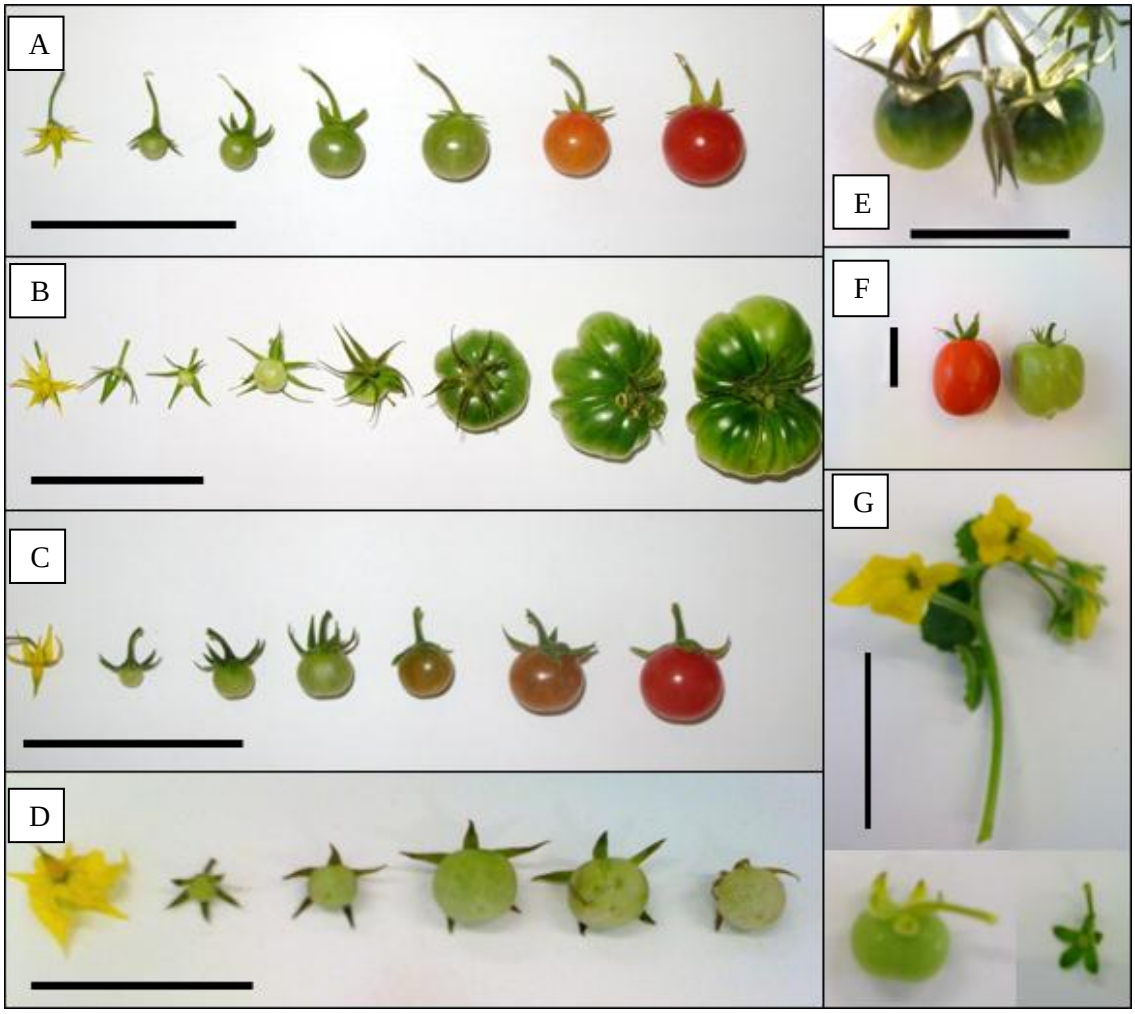

Figure 3-5: Developmental stages of fruit from wild tomato relatives.

A. *S. lycopersicum* "esculentum" LA2675, B. *S. lycopersicum* "esculentum" LA0134C, C. *S. pimpinellifolium,* LA0114, D. *S. peruvianum* LA2744, E. *S. lycopersicum* "esculentum" LA1511, F. *S. lycopersicum* cv. M82, and G. *S. pennellii* LA0716, black scale bars represent 5 cm.

The AUC for *S. peruvianum* LA2744 was significantly different compared to the minimal media control for *E. coli* 25922 ($P<0.001$), *S.* Enteritidis S1400 ($P<0.05$), and *E. coli* O157:H7 ($P<0.01$). It was also significantly different from the water control for *S.* Enteritidis S1400 ($P<0.01$), and was significantly different to *S. lycopersicum* "cerasiforme" LA2675 from *S.* Typhimurium 10248 ($P<0.05$), *E. coli* 25922 ($P<0.01$), and *S.* Enteritidis S1400 ($P<0.001$). *E. coli* O157:H7 AUC when grown in the presence of both *S. lycopersicum* "cerasiforme" LA1511 and *S. lycopersicum* "cerasiforme" LA2675 was significantly different to the water control (Figure 3-7B).

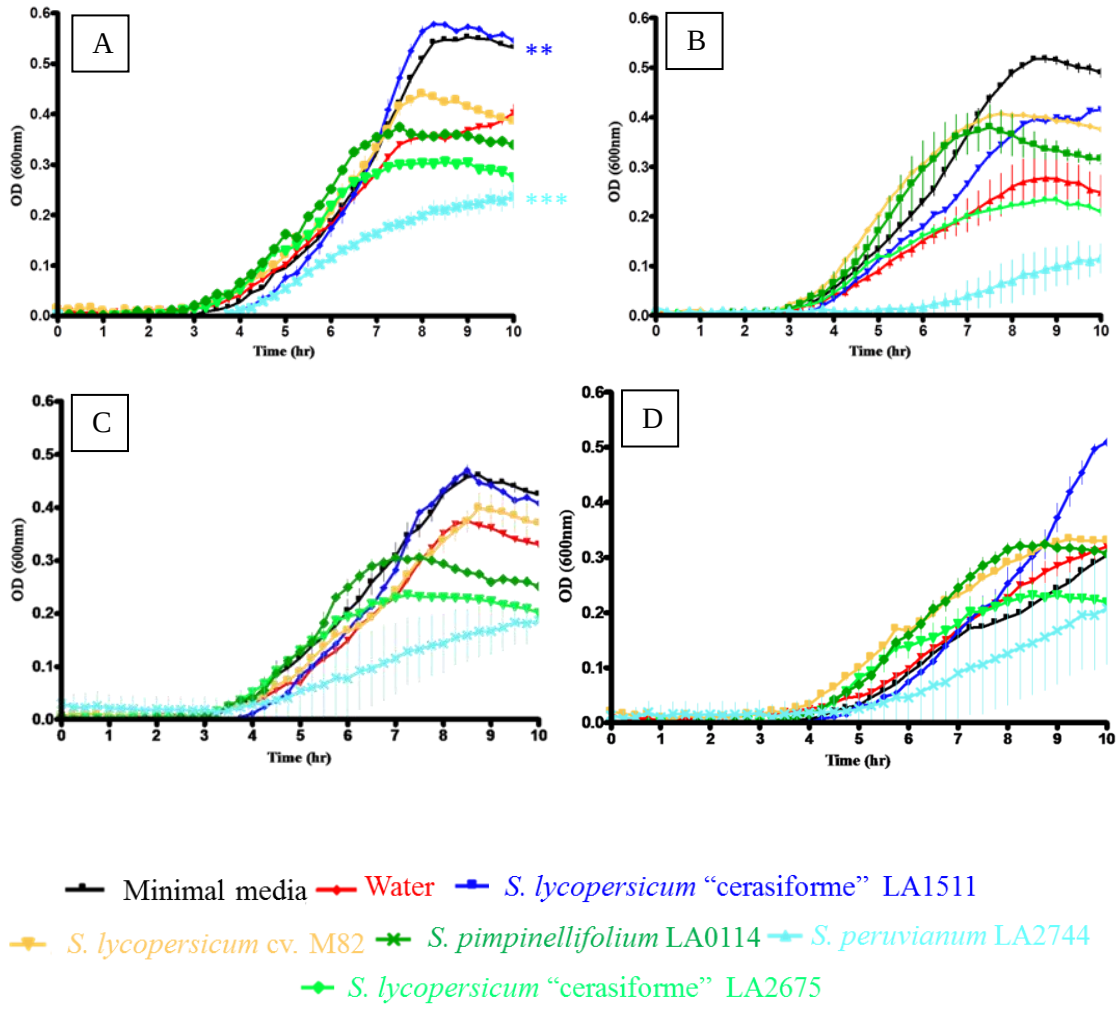

Figure 3-6: FLUOstar growth curves of bacteria grown in the presence of ripe fruit from tomato relatives extracted with water.

A. *E. coli* 25922, B. *E. coli* 12900, C. *S.* Typhimurium 10248, D. *S.* Enteritidis S1400. Freeze dried fruit were extracted in water and then added to the minimal media culture at 20% (v/v). Cultures included a minimal media control (black), water control (red), *S. lycopersicum* "cerasiforme" LA1511 (blue, 2.8 mg mL^{-1}), *S. lycopersicum* cv. M82 (yellow, 3.1 mg mL^{-1}), *S. pimpinellifolium* LA0114 (dark green, 2.6 mg mL^{-1}), *S. peruvianum* LA2744 (light blue, 3 mg mL^{-1}), and *S. lycopersicum* "cerasiforme" LA2675 (light green, 2.6 mg mL^{-1}). Error bars represent the standard error from three technical bacterial culture replicates of two pooled biological plant extract replicates. The asterisk (**=P<0.01, and ***=P<0.001) represents the significant difference between the area under the growth curves of bacteria grown in the presence of the wild tomato relatives compared to the water control, using one-way ANOVA Tukey's multiple comparisons test (95% CI).

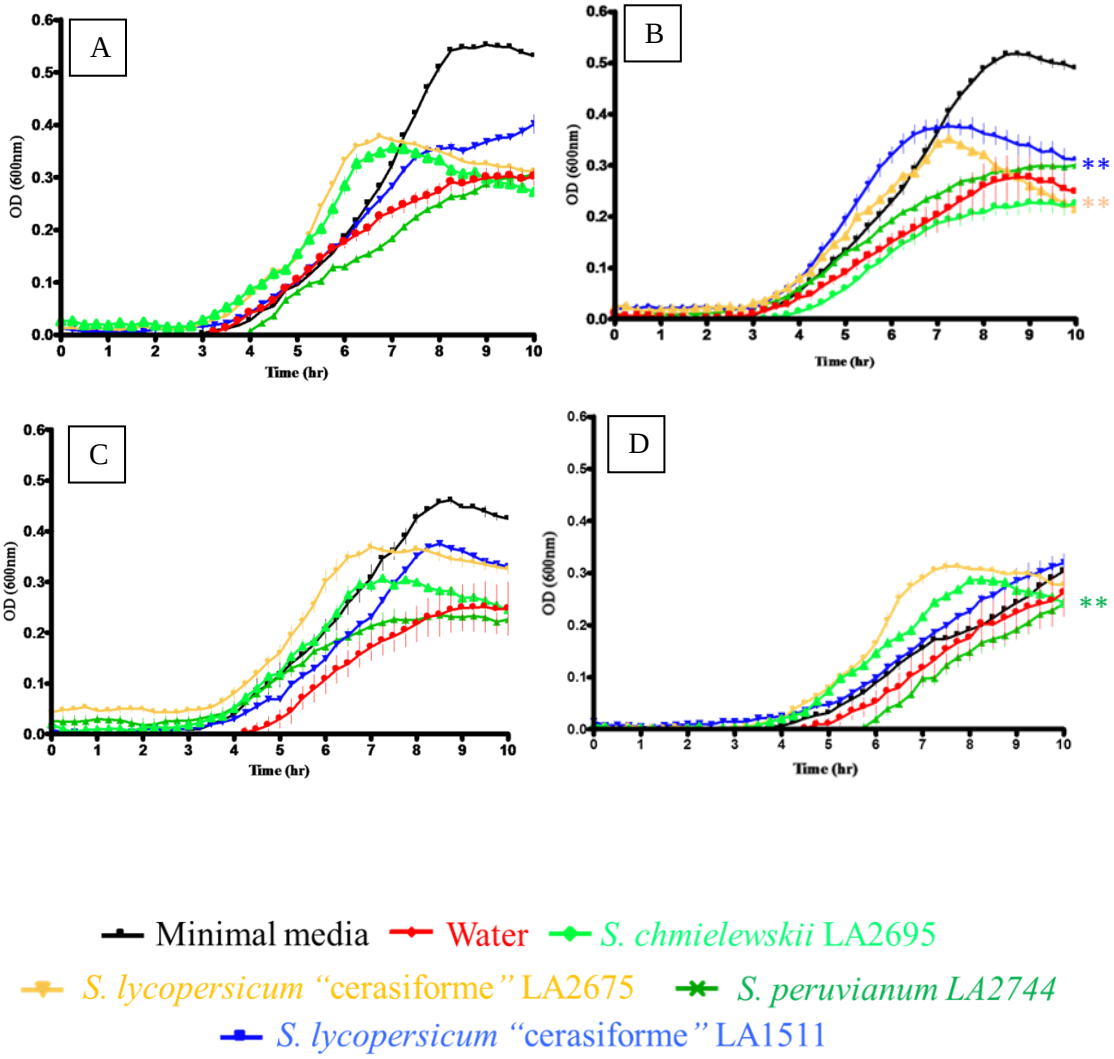

Figure 3-7. FLUOstar growth curves of bacteria grown in the presence of unripe fruit from tomato relatives extracted with water.

A. *E. coli* 25922, B. *E. coli* 12900, C. *S.* Typhimurium 10248, D. *S.* Enteritidis S1400. Freeze dried fruit were extracted in water and then added to the minimal media culture at 20% (v/v). Cultures included a minimal media control (black), water control (red), *S. lycopersicum* "cerasiforme" LA1511 (blue, 3 mg mL^{-1}), *S. lycopersicum* "cerasiforme" LA2675 (yellow, 3.2 mg mL^{-1}), *S. peruvianum* LA2744 (dark green, 3.5 mg mL^{-1}), and *S. chmielewskii* LA2695 (light green, 3.2 mg mL^{-1}). Error bars represent the standard error from three technical bacterial culture replicates of two pooled biological plant extract replicates. The asterisk (**=P<0.01) represents the significant difference between the area under the growth curves of bacteria grown in the presence of the wild tomato relatives compared to the water control, using one-way ANOVA Tukey's multiple comparisons test (95% CI).

3.2.3 *S. pennellii* ILs

The secondary metabolites can be stored within the extracellular space or remain on the leaf surface as epidermal secretions (Schilmiller et al., 2010). The trichome metabolites from *S. pennellii* introgression lines have been analysed by Schilmiller et al., (2010) who have identified the IL2-2 to have a decreased production of terpene levels, IL 10-3 has decreased sesquiterpenes, and ILs 5-3 and 11-3 have a reduction in acyl sugars compared to levels in *S. lycopersicum* cv. M82. There were some increases observed however, such as the production of the monoterpene α-thujene in the ILs 1-4 and 1-3, and their data show approximately a 2 fold increase in total acyl sugar levels for ILs 3-2 and 3-3. The biosynthesis of acyl sugars involves enzymes such as acyltransferases which use glucose as a substrate, which is produced in lower levels in the M82 compared to the *S. pennellii* leaf trichomes. The production of acyl sugars within M82 requires multiple, unlinked QTLs, which makes it difficult to create isogenic lines which produce these increased acyl sugars (Schilmiller et al., 2010).

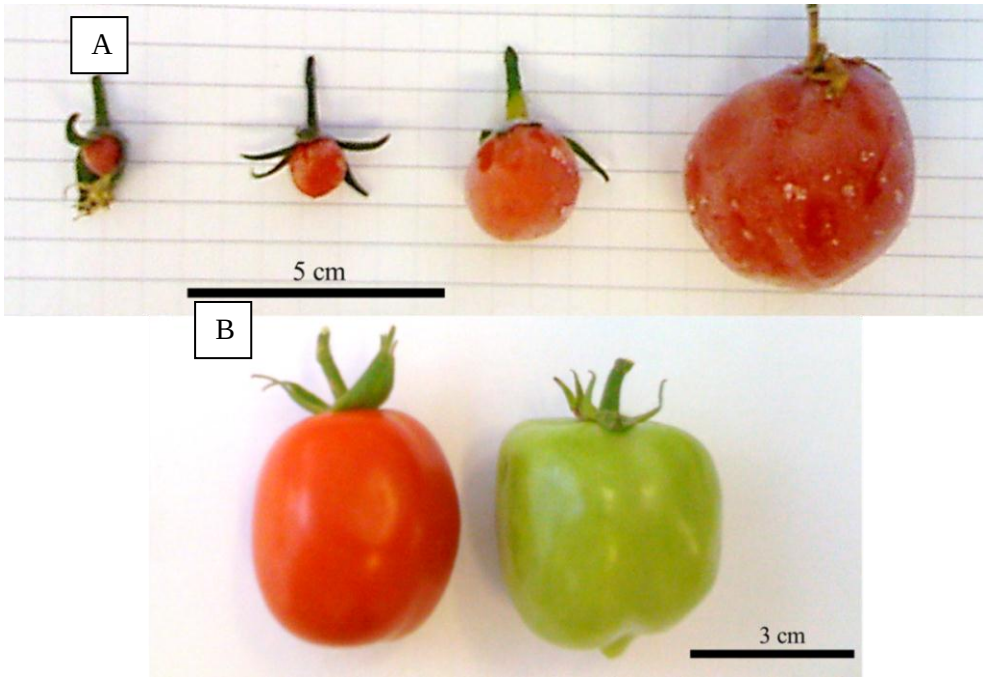

Figure 3-8. The fruit development in a selected *S. pennellii* IL compared with the domesticated tomato.

A. *S. pennellii* IL 4-3-2 (scale bar 5 cm), B. *S. lycopersicum* cv. M82 (scale bar 3 cm).

The *S. pennellii* leaves were the most different in shape compared to all the tested tomato relatives, with much thicker and rounder leaves. The growth of the wild tomato *S. pennellii* was more successful than the *S. lycopersicum* cv. M82, or the introgression lines. During collection of the plant material several lines of both *S. pennellii* and *S. habrochaites* ILs showed interesting phenotypes, indicating changes in the metabolite profile within the plant tissue. A tomato fruit goes through several pigment stages during development which are named breaker, turning, pink and red (Moco et al., 2007). However, the *S. pennellii* IL 4-3-2 has high levels of red pigment throughout all developmental stages of its fruit. When the fruit of *S. pennellii* IL 4-3-2 is compared with the fruit of *S. pennellii* IL 4-3, there is a clear difference in appearance. The phenotype of *S. pennellii* IL 4-3-2 is interesting because there is a visual indication of a metabolic change, which could involve acceleration of the process of chloroplasts in young green fruit developing into chromoplasts containing carotenoids (Cookson et al., 2003). Other *S. pennellii* ILs with striking visual phenotypes are IL 7-4-1 which has a stunted height, with large orange and green tomatoes, and IL 7-4 has thicker stems and sepals, a comparison can be made with IL 2-4 which still holds a strong resemblance to *S. lycopersicum* cv. M82. These phenotypes have been previously seen and comparisons between the fruit and leaves of the *S. pennellii* IL and other tomato introgression lines can be found on the Sol Genomic Network website (solgenomics.net).

3.2.3.1 Disc diffusion assays of *S. pennellii* ILs

Fresh *S. pennellii* IL leaf material was extracted using chloroform, and the antimicrobial activities of the extracts were verified using disc diffusion assays. Two concentrations were tested, 10 mg disc^{-1} was used because this was the maximum re-suspendable concentration of extract which could be applied per disc, and 0.312 mg disc^{-1} was selected as the minimum concentration, chosen from the MIC tests of *S. pennellii* (Figure 3-14). The results show, that the *S. pennellii* ILs do have antimicrobial activity from extracts of fresh leaf trichomes. The antimicrobial activity from trichome extracts from *S. pennellii*, *S. lycopersicum*, and *N. rustica* were also investigated. This showed that *S. pennellii* and *N. rustica* were antimicrobial against *S. aureus* 25923 and *S.* Typhimurium 10248 from both concentrations at 10 mg disc^{-1} and 0.312 mg disc^{-1}.

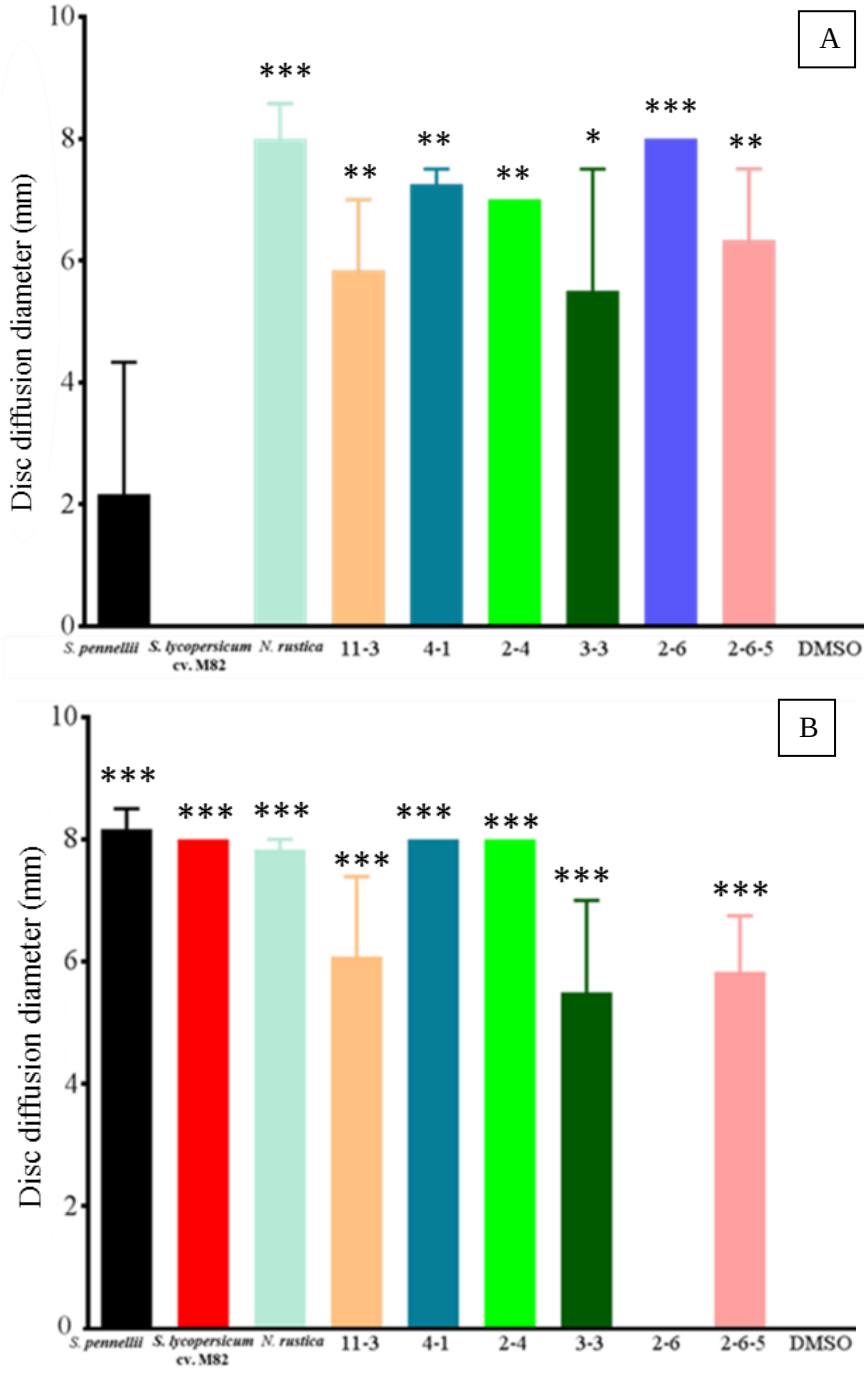

Figure. 3-9. Disc diffusion assay results of *S.* Typhimurium 10248 and *S. aureus* 25923 grown in the presence of *S. pennellii* ILs.

Chloroform extracts from the trichomes of fresh leaf material from *S. pennellii* ILs 11-3, 4-1, 2-4, 3-3, 2-6 and 2-6-7. *S. pennellii*, *S. lycopersicum* cv. M82, and *N. rustica* were also tested for comparison. The extracts were re-suspended in DMSO 1:1 (v/v) 10 mg disc^{-1}, A. *S.* Typhimurium 10248, B. *S. aureus* 25923. Error bars represent the standard error from three technical bacterial culture replicates of two biological plant extract replicates. The asterisk (*=P<0.05, **=P<0.01,***=P<0.001) represents the significant difference between the area under the growth curves of bacteria grown in the presence of the extract compared to the DMSO control, using one-way ANOVA Dunnetts multiple comparisons test (95% CI).

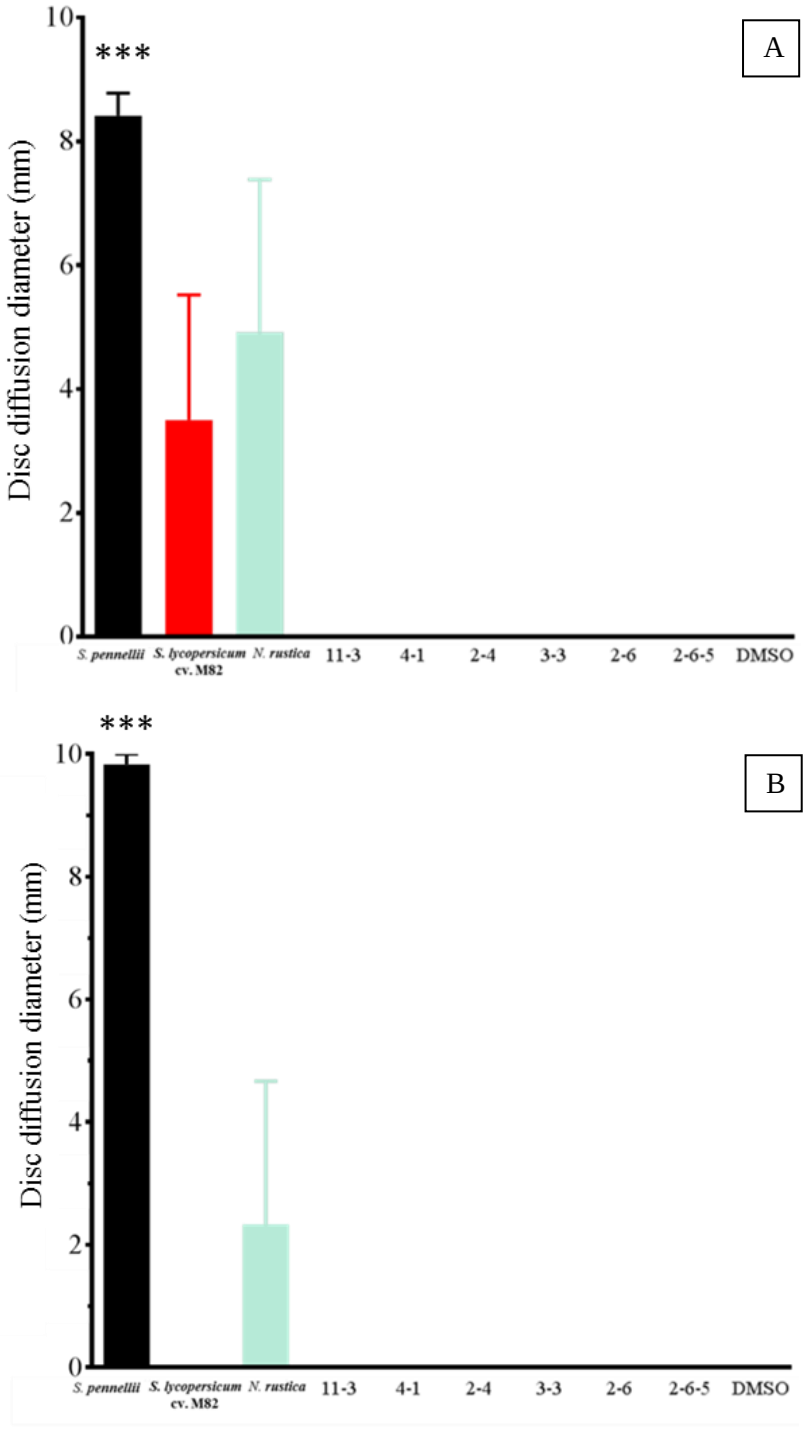

Figure. 3-10. Disc diffusion assay results of *S.* Typhimurium 10248 and *S. aureus* 25923 grown in the presence of *S. pennellii* ILs .

Chloroform extracts from the trichomes of fresh leaf material from *S. pennellii* ILs 11-3, 4-1, 2-4, 3-3, 2-6 and 2-6-7. *S. pennellii*, *S. lycopersicum* cv. M82, and *N. rustica* were also tested for comparison. The extracts were re-suspended in DMSO 1:32 (v/v) 0.312 mg disc^{-1}, A. *S.* Typhimurium 10248, B. *S. aureus*. Error bars represent the standard error from three technical bacterial culture replicates of two biological plant extract replicates. The asterisk (***=P<0.001) represents the significant difference between the area under the growth curves of bacteria grown in the presence of the extract compared to the DMSO control, using one-way ANOVA Dunnetts multiple comparisons test (95% CI).

S. lycopersicum cv. M82 showed slight antimicrobial activity from the more diluted sample (0.312 mg $disc^{-1}$) against *S.* Typhimurium 10248, however, this activity was reversed at the higher concentration (10 mg $disc^{-1}$) where no activity was achieved against *S.* Typhimurium 10248, but a high activity was observed against *S. aureus* 25923. The *S. pennellii* ILs showed a more consistent antimicrobial activity against both bacteria species (except IL 2-6 which only inhibited *S.* Typhimurium), where antimicrobial activity was only seen at the higher concentration (10 mg $disc^{-1}$).

Statistical analysis (one-way ANOVA Dunnetts multiple comparisons test, 95% CI) showed that the significantly different zone of inhibition diameters compared to the DMSO solvent control at 10 mg $disc^{-1}$ for *S. aureus* 25923 were; *S. pennellii*, *S. lycopersicum* cv. M82, *N. rustica*, 2-6-5, 3-3, 2-4, 4-1 and 11-3 (***P<0.001). The significantly differernt *S.* Typhimurium 10248 zones of inhibition diameters compared to the DMSO solvent control were; *N. rustica*, 2-6 (***P<0.001), 11-3, 4-1, 2-4, 2-6-5 (**P<0.01), and 3-3 (*P<0.05) (Figure 3-9). The zone of inhibition diameters were significantly different from the DMSO control from *S. aureus* 25923 and *S.* Typhimurium 10248, when grown in the presence of *S. pennellii* at the concentration of 0.312 mg $disc^{-1}$ (***P<0.001) (Figure 3-10).

3.2.3.2 Growth curves of *S. pennellii* ILs

S. pennellii ILs were selected to represent a wide coverage of the chromosomes, the selected ILs were 1-1-2, 4-3, 6-1, 7-5-5, 10-3, and 12-1. The leaf material was dried, and extracted in acidified methanol and water (80:20 v/v). The extracts were re-suspended in DMSO and tested for antimicrobial activity against bacteria using growth curve analysis. The extracts were dissolved in minimal media broth 5% (v/v) to a concentration of 0.22 mg mL^{-1}. The results showed through one-way ANOVA Dunnetts multiple comparisons test (95% CI) that the 12-1, 10-3, 7-5-5, 1-1-2, and 6-1 AUCs were significantly different to the DMSO control (P<0.001) for *E. coli* O157:H7. This difference was due to an increase in bacterial growth from the *S. pennellii* IL extracts compared to the DMSO control (Figure 3-11).

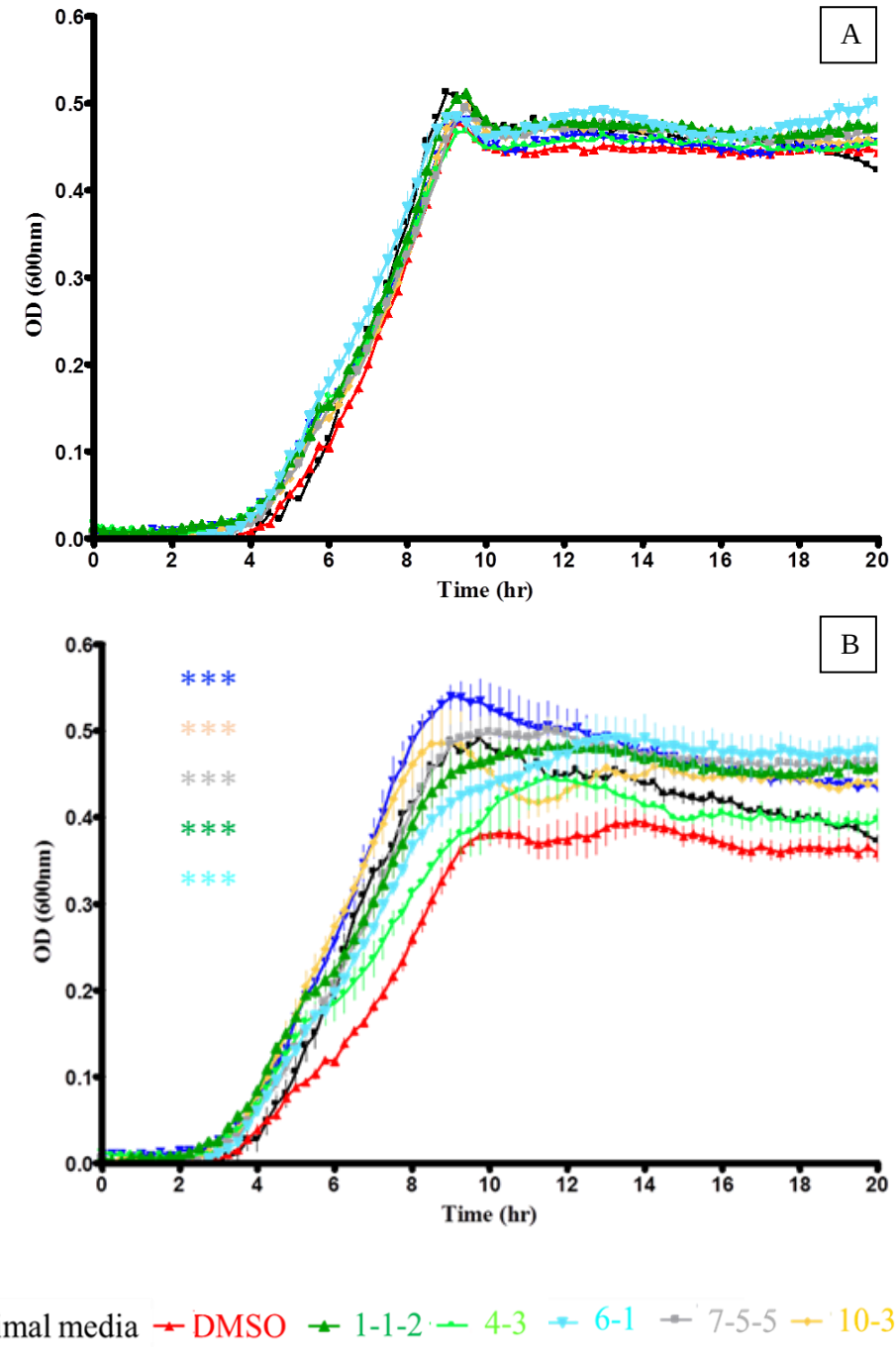

Figure 3-11. FLUOstar growth curves of bacteria grown in the presence of *S. pennellii* ILs.

S. pennellii ILs 1-1-2, 4-3, 6-1, 7-5-5, 10-3 and 12-2 were extracted in water:methanol (20:80 v/v), HCL (1% v/v) and tested against bacteria; A. *S.* Typhimurium 10248, B. *E. coli* O157:H7 at a concentration of 3% (v/v) 0.22 mg mL^{-1}. Error bars represent three technical bacterial culture replicates and one tested pool of three biological plant replicates. The asterisk (***=P<0.001) represents the significant difference between the area under the growth curves of bacteria grown in the presence of the extract compared to the DMSO control, using one-way ANOVA Dunnetts multiple comparisons test (95% CI).

3.2.4 Methanol and water mixtures of *S. pennellii*

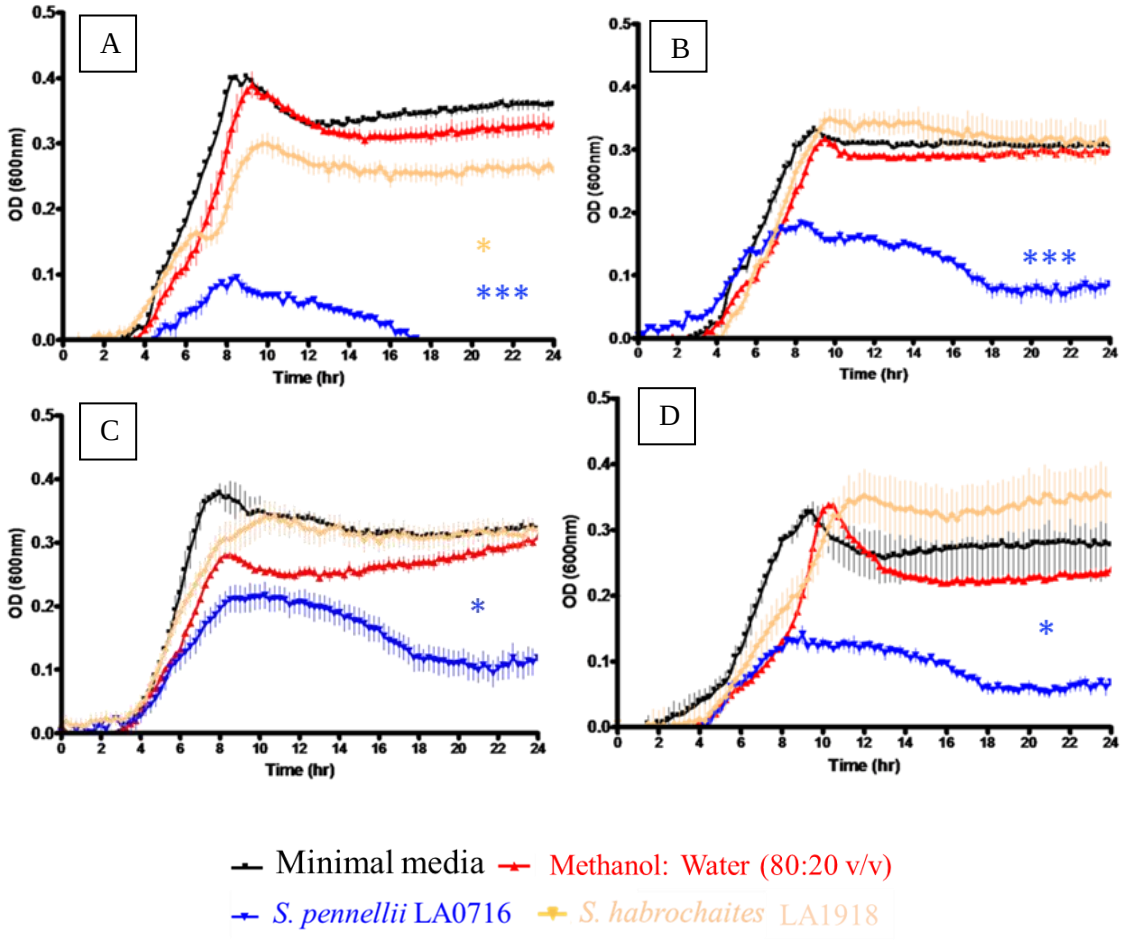

Figure 3-12. FLUOstar growth curves showing the effects of *S. pennellii* and *S. habrochaites* acidified polar extracts upon bacterial growth.

E. coli 25922, B. *S.* Typhimurium 10248, C. *E. coli* O157:H7, D. *S.* Enteritidis S1400, were grown in the presence of *S. pennellii* LA0716 and *S. habrochaites* LA1918 leaf material extracted in methanol:water (80:20 v/v) and HCL (1% v/v) , and tested at a concentration of 3% (v/v) 0.22 mg mL^{-1}. Error bars represent three technical bacterial culture replicates and one tested pool of three biological plant replicates. The asterisk (*=P<0.05 and ***=P<0.001) represents the significant difference between the area under the growth curves of bacteria grown in the presence of the extract compared to the methanol:water (80:20 v/v) control, using one-way ANOVA Dunnetts multiple comparisons test (95% CI).

S. pennellii LA0716 and *S. habrochaites* LA1918 were extracted in acidified methanol and water (80:20 v/v), and growth curve analysis of bacteria grown in minimal media broth containing 3% (v/v) of extract re-suspended in methanol and water (80:20 v/v) to a concentration of 0.22 mg mL^{-1}. The results showed that *S. pennellii* LA0716 produced

partial inhibition to the tested Gram-negative bacteria. Statistical analysis (one-way ANOVA 95% CI) of the AUCs, comparing to the methanol and water (80:20 v/v) control showed that the area under the *S. pennellii* LA0716 growth curve was significantly different than the methanol control for all the tested bacteria; *E. coli* 25922 (P<0.001), *E. coli* O157:H7 (P<0.05), *S.* Typhimurium 10248 (P<0.001), and *S.* Enteritidis (P<0.05). The AUCs of *S. habrochaites* LA1918 were significantly different to the methanol and water (80:20 v/v) control for only *E. coli* 25922 (P<0.05).

3.2.5 Disc diffusion assay of trichome extracts

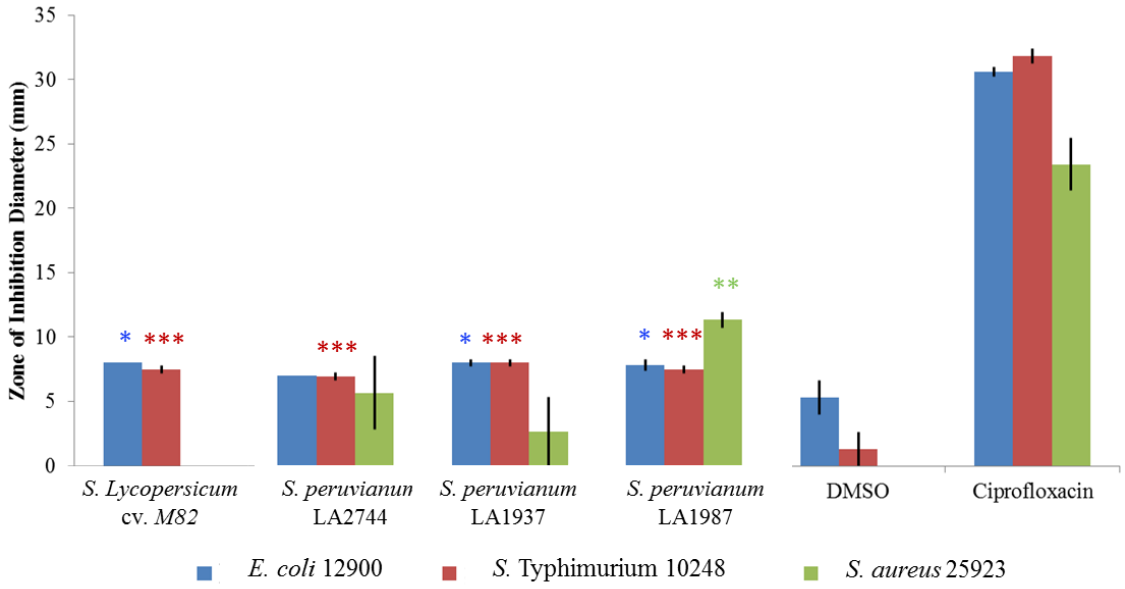

Figure 3-13. Disc diffusion assay results which show the activity of *S. peruvianum* accessions and *S. lycopersicum* cv. M82 against bacterial growth.

E. coli O157:H7 (Blue), *S.* Typhimurium (Red), and *S. aureus* 25923 (Green), showing the zone of inhibition from trichome extracts of *S. lycopersicum* cv. M82, *S. peruvianum* LA2744, LA1937, and LA1987. Error bars represent the standard error of three biological plant replicates each with two technical disc replicates. The asterisk (*=P<0.05, **=P<0.01 and ***=P<0.001) represents the significant difference between the area under the growth curves of bacteria grown in the presence of the extract compared to the DMSO control, using one-way ANOVA Dunnetts multiple comparisons test (95% CI).

Three *S. peruvianum* accessions, LA2744, LA1918, and LA1987, were tested for antimicrobial activity through disc diffusion assay and compared to *S. lycopersicum* cv. M82 and a DMSO control. Chloroform extracts of fresh plant material were dried and

re-suspended in DMSO, and tested against *E. coli* O157:H7, *S.* Typhimurium 10248, and *S. aureus* 25923. The results show that when compared to the DMSO solvent control there are significant differences in inhibition from *S.* Typhimurium 10248 (P<0.001) from all the *S. peruvianum* accessions and *S. lycopersicum* cv. M82. *E. coli* O157:H7 was significantly inhibited by *S. lycopersicum* cv. M82, *S. peruvianum* LA1937, and *S. peruvianum* LA1987 (P<0.05). Compared to the DMSO control *S. aureus* 25923 was also significantly inhibited by *S. peruvianum* LA1987 (P<0.01).

3.2.6 *S. pennellii* LA0716 and *S. habrochaites* LA1777 MIC

FLUOstar growth curves of serial dilutions of *S. pennellii* and *S. habrochaites* trichome extracts were tested to determine the minimal inhibitory concentration. The plant extracts were diluted to a maximum concentration of 50 mg mL^{-1}, which was due to the high viscosity of the extract dissolved in DMSO, which was best diluted at a concentration of 1:1 (v/v). This dilution was adequate for *S. aureus,* as both plant extracts showed a high level of inhibitory activity against *S. aureus* 25923. The results, however, show that the concentration of the extract needs to be higher, or further purified to have a stronger antimicrobial effect upon the Gram-negative bacteria. Chloroform extracts of trichomes from *S. habrochaites* LA1777 and *S. pennellii* LA0716 were tested for antimicrobial activity in growth curve assays, where the trichome extract was serially diluted so that an MIC could be determined.

The statistical analysis (one-way ANOVA Dunnetts multiple comparisons test 95% CI) of the AUC of *S. aureus* 25923 showed that all the *S. pennellii* LA0716 concentrations ranging from 50 mg mL^{-1} to 0.195 mg mL^{-1} were all statistically significant from the area under the curve of the DMSO control (P <0.001). The concentrations 25 mg mL^{-1} (P<0.001), 12.5 mg mL^{-1} (P<0.05), and 6.25 mg mL^{-1} (P<0.01) tested against *S.* Typhimurium 10248 were significantly different from the AUC of the DMSO control. The concentrations 50 mg mL^{-1} (P<0.05) and 25 mg mL^{-1} (P<0.05) tested against *E. coli* O157:H7 were also significantly different to the DMSO control (Figure 3-14).

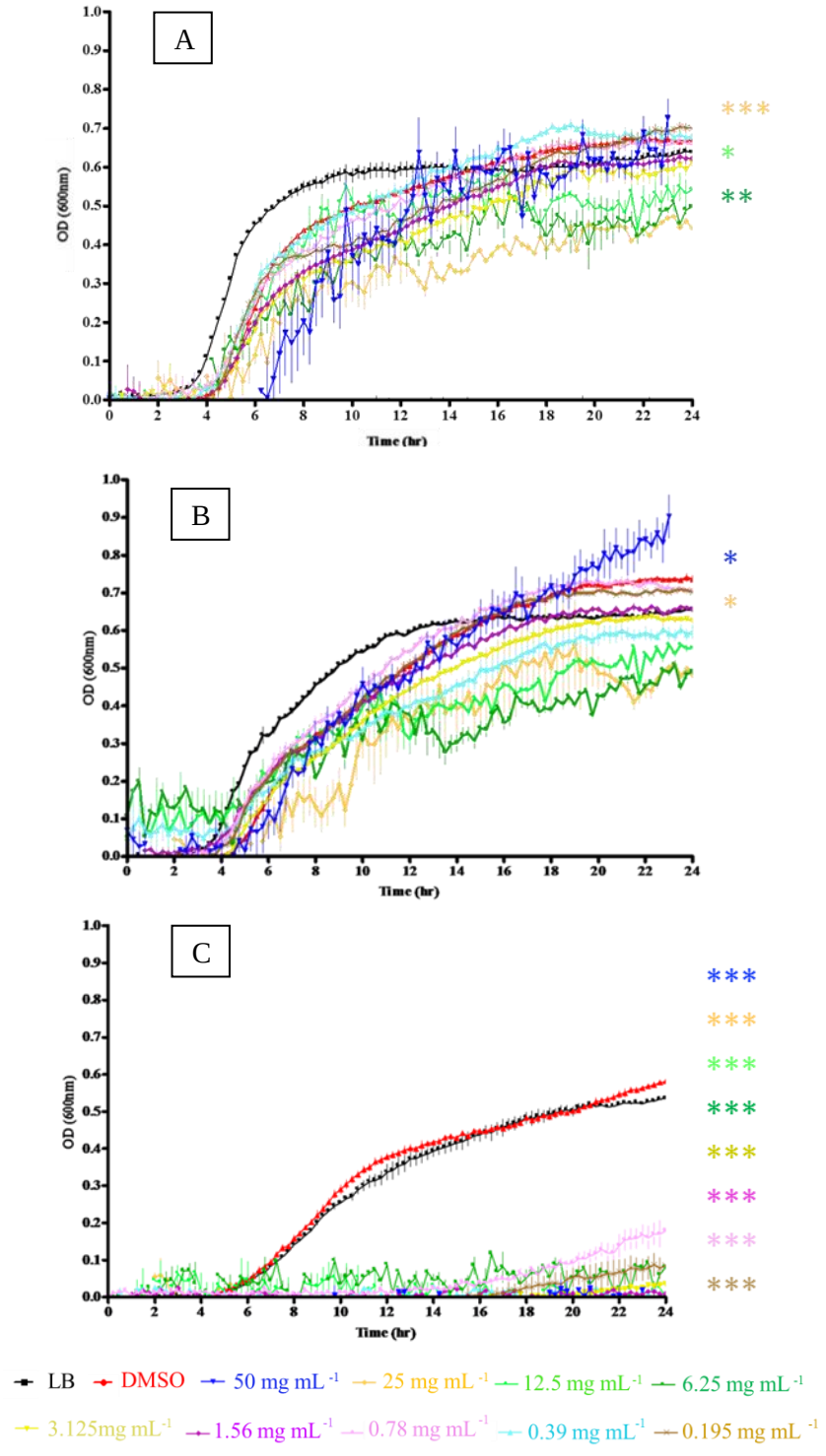

Figure 3-14. FLUOstar growth curves showing the MIC of *S. pennellii* trichome extract.

S. pennellii LA0716 chloroform leaf extracts serially diluted to determine MICs, plant extracts were dissolved in DMSO solvent and tested in LB (5% v/v). Growth curve analysis included; A. *S.* Typhimurium, B. *E. coli* O157:H7, and C. *S. aureus* 25923. Error bars represent two technical bacterial replicates and the extracts were obtained from a pooled sample of three biological plant replicates. The asterisk (*=P<0.05, **=P<0.01 and ***=P<0.001) represents the significant difference between the area under the growth curves of bacteria grown in the presence of the extract compared to the DMSO control, using one-way ANOVA Dunnetts multiple comparisons test (95% CI).

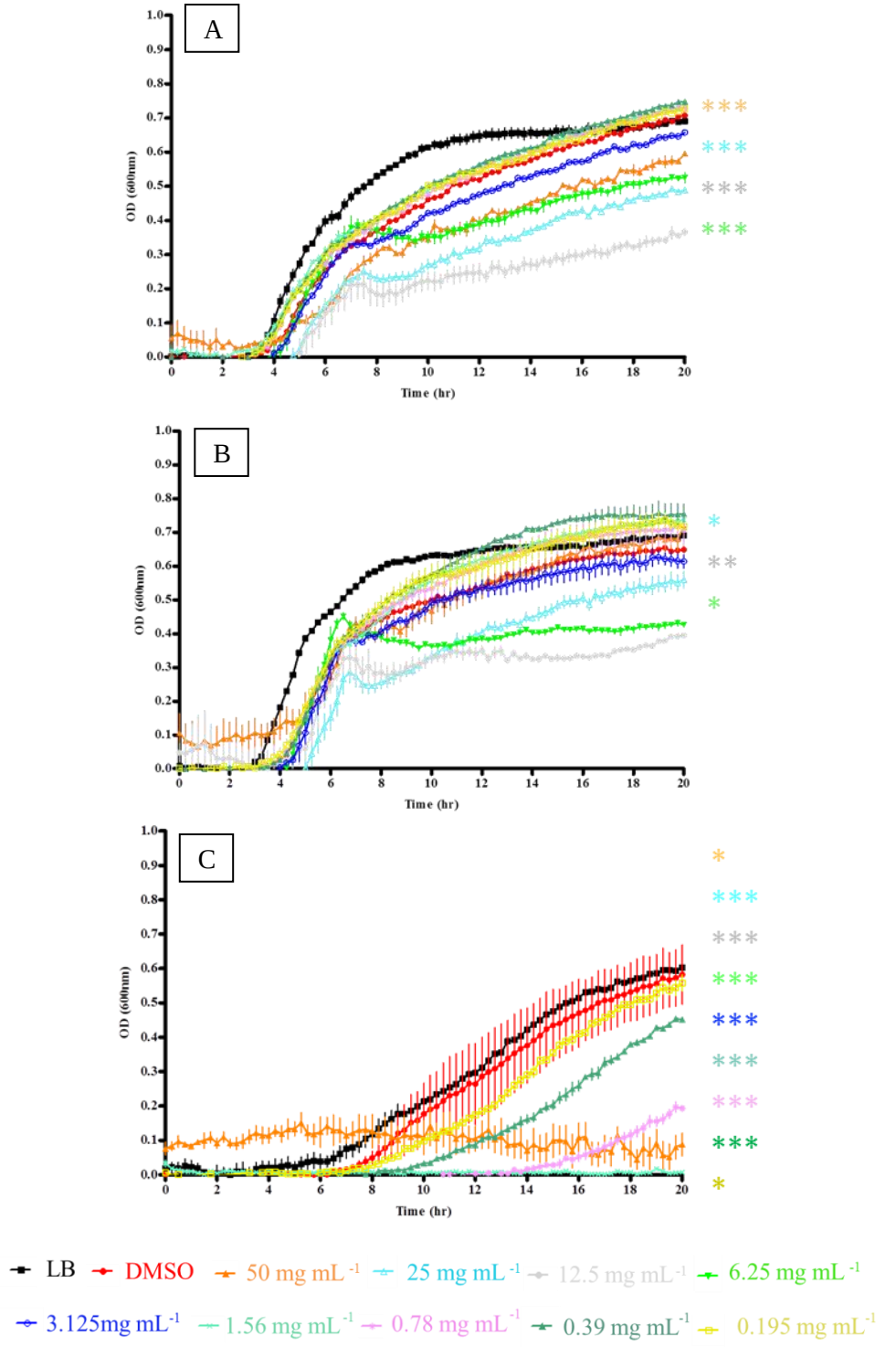

Figure 3-15. FLUOstar growth curves showing the MIC of *S. habrochaites* trichome extract.

S. habrochaites LA1777 chloroform trichome extracts serially diluted to determine MICs, plant extracts were dissolved in DMSO solvent and tested in LB (5% v/v). Growth curve analysis included; A. *S.* Typhimurium, B. *E. coli* O157:H7, and C. *S. aureus* 25923. Error bars represent two technical bacterial replicates and the extracts were obtained from a pooled sample of three biological plant replicates. The asterisk (*=P<0.05, **=P<0.01 and ***=P<0.001) represents the significant difference between the area under the growth curves of bacteria grown in the presence of the extract compared to the DMSO control, using one-way ANOVA Dunnetts multiple comparisons test (95% CI).

The activity of *S. habrochaites* LA1777 was also significantly inhibitory against *S. aureus* 25923, with an MIC of 1.56 mg mL^{-1}. This compared to MIC of 12.5 mg mL^{-1} for *S.* Typhimurium 10248, and 25 mg mL^{-1} for *E. coli* O157:H7 (Figure 3-15). The AUC compared to the DMSO control for *E. coli* O157:H7 showed the concentrations 25 mg mL^{-1}($P<0.05$), 12.5 mg mL^{-1}($P<0.01$), and 6.25 mg mL^{-1} ($P<0.05$) to be significantly different to the DMSO control. It was unexpected that the highest concentration tested 50 mg mL^{-1} was not as inhibitory as the lower concentrations of 12.5 mg mL^{-1} and 6.25 mg mL^{-1} against *E. coli* O157:H7 (Figure 3-15). The concentrations 50 mg mL^{-1} to 6.25 mg mL^{-1} ($P<0.001$) were significantly different to the DMSO control for *S.* Typhimurium 10248. All of the concentrations except the lowest (0.195 mg mL^{-1}) tested against *S. aureus* 25923 showed significant differences compared to the DMSO control ($P<0.001$), with 50 mg mL^{-1} and 0.39 mg mL^{-1} showing a lower significance value ($P<0.05$) (Figure 3-15).

3.3 Discussion

3.3.1 Efficiency of the extraction protocols

Wild tomato relatives were tested for antimicrobial activity through extracting their primary and secondary metabolites using various solvents which ranged in polarity, including water, methanol, ethanol, ethyl acetate, isopropanol, acetone, chloroform, petroleum ether, and hexane. Previous research has shown that finely ground plant material extracted for 5 min yields as many compounds as unground plant material extracted for 24 h (Eloff, 1998). Based on these findings the tested plant material was coarsely ground in a pestle and mortar and solvent extractions were performed at room temperature in the dark for 24 h.

There are conflicting discoveries of the best solvent to be used for the extraction of antimicrobial compounds. It appears that although solvent extractions can be tailored to target different classes of compounds, the compounds which are antimicrobial could be present in any of the solvent systems. The right solvent for extracting antimicrobial compounds depends on the plant species, although water is often regarded as a universal solvent for extracting compunds with antimicrobial activity. Most publications report

that active compounds are optimally extracted by one particular solvent, such as methanol, ethanol, chloroform, or water.

Solvents typically used for the extraction of antimicrobial compounds have been compared based on their individual properties and the choice of solvent depends on the research focus. Eloff (1998) made comparisons between these properties, and the conclusions are that whilst methanol and methanol:chloroform:water (12:5:3 v/v) give a high quantity of compounds extracted, they are also highly toxic to the microorganisms. A water (aqueous) based extraction procedure typically has a lower quantity of extracted compounds and is a difficult solvent to remove, although it does not have toxic effects upon the bioassay. Acetone yields a low quantity of extracted product, but a high level of individual compounds extracted, as it dissolves both hydrophilic and lipophilic compounds, a low toxicity level in the bioassay, and it is volatile so it is easily removed from the sample (Eloff, 1998).

Due to the varied properties of different solvents, eight solvents were chosen in this research to initially screen the wild tomato relatives, which included water, methanol, ethanol, isopropanol, ethyl acetate, chloroform, petroleum ether, and hexane. After initial tests chloroform was selected as the primary solvent for extracting antimicrobial secondary metabolites from plant material, due to its high volatility which made it easy to remove, and its ability to dissolve a range of polar and non-polar compounds. Additional targeted extractions were also performed such as the extraction of highly polar compounds from fruit extracting with water (Figure 3-6). After the plant material has been dried, weighed and extracted, DMSO is widely used as a re-suspending solvent to introduce the plant extract into selected medium for analysis (Pereira et al., 2008; Mellou et al., 2005). DMSO was usually used as the final plant re-suspending solvent for testing against bacteria. DMSO is often used due to its low antimicrobial activity, however, it can be antimicrobial and the appropriate controls and dosage was selected to be between 3 to 5% (v/v) of total culture.

3.3.2 Antimicrobial screen of wild tomato relatives

The bacteria used to test the antimicrobial properties of the selected plant extracts initially included gastro-enteric bacteria such as *E. coli* O157:H7, *E. coli* 25922, *S.*

Typhimurium 10248, *S.* Enteritidis S1400, and *S. aureus* 25923. These bacterial species cover both Gram-negative and Gram-positive bacteria, and include bacteria commonly used as antibiotic susceptible controls. Ciprofloxacin is a quinolone-carboxylic acid derivative with a broad range of antibacterial activity, inhibiting anaerobic, aerobic cocci and bacilli which have an MIC of 0.005 to 0.8 µg mL^{-1} against 90% of the family *Enterobacteriaceae*, specifically 0.01 to 0.02 µg mL^{-1} against *S.* Typhi and *E. coli*, 0.1 to 0.8 µg mL^{-1} against *S. aureus* (Chin and Neu, 1984).

Quinolone compounds are known to interfere with DNA gyrase and DNA nicking-closing enzymes (Chin and Neu, 1984). Ciprofloxacin was chosen as the positive antibiotic control, and was used to determine that the bacteria tested were suitably antibiotic susceptible and not antibiotic resistant strains, therefore ensuring that the tested bacteria should be susceptible to antimicrobial secondary metabolites within the tested plant extracts. Within all the antimicrobial tests, the ciprofloxacin control showed that the bacteria were consistently susceptible to the antibiotic, showing that the effects observed from the plant extracts were true for antibiotic susceptible strains (Figure 3-13).

The zone of inhibition screen of wild tomato extracts identifed two main relatives with antimicrobial activity: *S. pennellii* LA0716 and *S. habrochaites* LA1777 (Figure 3-1). *S.habrochaites* inhibited *S. aureus* 25923 and *S.* Typhimurium 10248 on agar and inhibited *S. aureus* 25923 in liquid culture (Figure 3-1, and Figure 3-3). It was also shown to be antimicrobial from methanol to hexane solvent extracts, but antimicrobial activity was not observed from the water extracts. *S. pennellii* was also active against *S. aureus* 25923 and *E. coli* O157:H7 on agar, and inhibited all the tested bacteria in liquid culture (Figure 3-1, Figure 3-2, Figure 3-3, and Figure 3-4). *S. habrochaites* had a higher inhibitory effect against *S. aureus* and the extract was shown to completely stop growth. *S. pennellii* had a lower activity, but had a broader range against both Gram-positive and Gram-negative bacteria.

There was some additional activity from the chloroform extracts of *S. chilense*, *S. peruvianum*, and *S. chmielewskii* against *S. aureus* 25923, and *S.* Typhimurium 10248 (Figure 3-1). *E. coli* O157:H7 was not shown to be significantly inhibited from any of the plant species from any type of solvent extract, once the effects of the DMSO solvent

inhibition were taken into account. The two antimicrobial wild tomato species visually have a high trichome load, suggesting that the antimicrobial compounds are likely to be produced by the trichomes, as a response to plant pathogens (Juvik et al., 1994).

The antimicrobial tests used were chosen as the most suitable for testing the plant extracts based on preliminary results, however, the limitations of antimicrobial tests as a whole should be considered to prevent the identification of false results. There are several limitations to the current antimicrobial assays, such as paradoxical effects of antimicrobial compounds which it has been discovered in antibiotics such as aminoglycosides against Gram-negative bacteria and from β-lactam antibiotics against Gram-positive bacteria, where bacteria growth is increased at concentrations 10 times the MIC (Holm et al., 1990). There can also be occasions where bacterial tolerance and persistence occurs from a few colonies, which may provide a false negative result and does not represent the population as a whole. These limitations have been avoided through the use of two antimicrobial screening methods which included solid and liquid media, with the disc diffusion assay method, and the growth curve analysis. Biological and technical replicates also provided additional assurance to the accuracy of the observed results.

The antimicrobial screen of the wild tomato relatives has identifed two significantly antimicrobial plants, however the specific secondary metabolites responsible for the observed activity are unknown, and this is further confounded by the range of antimicrobial activity observed from methanol to hexane from both the plants. This could suggest that the compounds involved are a mixture of synergistic elements, or that the active compounds within the plants are amphiphilic. The highest antimicrobial activity was observed against *S. aureus* 25923, which is susceptible to a range of secondary metabolites known to be produced by plants, such as polyhydroxylated flavones which are known to inhibit the growth of MRSA (Tsuchiya et al., 1996).

3.3.3 Solvent effects of DMSO

Dimethyl sulfide (DMS) is known to be oxidised to dimethyl sulfoxide (DMSO) in the presence of lyophilized algae and plant extracts containing pigments, which is believed to have occurred during assays because all the solvents used except water extracted

pigment from the plant material. DMSO can be modified by micro-organisms or degraded by photo oxidation by photosensitizers; this reaction is photodynamic and causes damage to micro-organisms amino acids, DNA and lipids through the production of singlet oxygen (Fuse et al., 1997). DMS is biochemically oxidized to DMSO which may, in turn, be biologically reduced back to DMS. These inter-conversions occur in both oxic and anoxic habitats. Under anaerobic conditions where light is present, DMS may be oxidized to DMSO by phototrophic bacteria. DMSO is an electron acceptor which supports the anaerobic metabolism or growth of a variety of micro-organisms, with DMS as the reduced end product (Taylor et al., 1989).

A typical strategy to reduce the antimicrobial effects of DMSO is to use <1% (v/v) of the solvent in the media (Mellou et al., 2005; Mitscher et al., 1972). Within this study tests showed that DMSO had a very low antimicrobial activity against *S.* Typhimurium and *S. aureus*. Therefore, a higher concentration was used up to a threshold which could still differentiate between the antimicrobial effects of the solvent and the potential antimicrobial effects of the plant extract. This was between 1 to 6% (v/v) in the media.

DMSO was inhibitory to *E. coli* at much lower concentrations compared to *S.* Typhimurium and *S. aureus*, so to counteract these effects DMSO controls were used alongside experiments so that the inhibitory effects could be blanked against the total inhibition observed. The reduced effects seen towards *S.* Typhimurium could be explained through *S.* Typhimurium encoding several DMSO reductases, and it has been suggested that DMS is used as environmental cue, as micro-organisms within the human gastrointestinal tract are known to produce DMS. Dimethyl sulfoxide (DMSO) inhibits *Salmonella hilA* and *Salmonella* pathogenicity island 1 (SPI-1) associated gene expression, and inhibits invasion of cultured epithelial cells, because DMSO can be reduced to DMS during anaerobic growth (Antunes et al., 2010). The significance of the regulation of *hilA* in response to environmental DMSO is currently unknown; however, the ability to respire DMSO suggests there must be a selective advantage (Antunes et al., 2010).

3.3.4 Analysis of growth curves with wild tomato relative leaf material

During the growth of the tested bacteria within liquid broth in the presence of certain plant secondary metabolites, there were mixtures of positive and negative responses depending on concentration of sample, concentration of solvent, sample type, and bacterial species. There were typically s-shaped growth curves, with exceptions to wells which had been contaminated (and subsequently disregarded), or wells with increased particulate matter which occurs in some cases from solvent evaporation during incubation at 37^{o}C within the FLUOstar. The results of the growth curves of bacteria grown in the presence of chloroform leaf extract of wild tomato relatives showed that *E. coli* O157:H7 was significantly inhibited by *S. pennellii, and S. lycopersicum* cv. M82 (P<0.05), *S. habrochaites* (P<0.05) and *S. chmielewskii* (P<0.05). *S. aureus* 25923 was significantly inhibited by *S. pennellii* (P<0.0005), *S. habrochaites* (P<0.0005) and *S. cheesmaniae* (P<0.05). *S.* Typhimurium 10248 growth was affected by the wild tomato relatives and was inhibited significantly by *S. pennellii* (P<0.0005), *S. chilense* (P<0.05), *S. lycopersicum* cv. M82 (P<0.05), and *S. chmielewskii* (P<0.05) (Figure 3-2, Figure 3-3, and Figure 3-4).

The antimicrobial plant extracts, and pure compounds were occasionally found to delay the bacteria growth, but the bacteria would usually recover with a stationary phase level of growth higher than the positive media control, such as the effect of 2-undecanone upon *S.* Enteritidis S1400 (Figure 4-10). This could be explained by the phenomenon referred to as hormesis, where a toxin in low doses gives the opposite effect of a large dose, often seen as a J shaped or U shaped curve (Calabrese and Baldwin, 1999). This hormesis response is not understood very well, but it is believed that the low concentrations are too low to cause serious damage but high enough to induce repair mechanisms which initially cause a delay in growth, but is preceded by bacteria which are healthier. This response has been documented during the introduction of yeast to small concentrations of poisons, and the Arndt-Schulz law which states that highly diluted poisons (with exceptions) enhance life processes, while strong concentrations inhibit life processes (Oberbaum and Cambar, 1996).

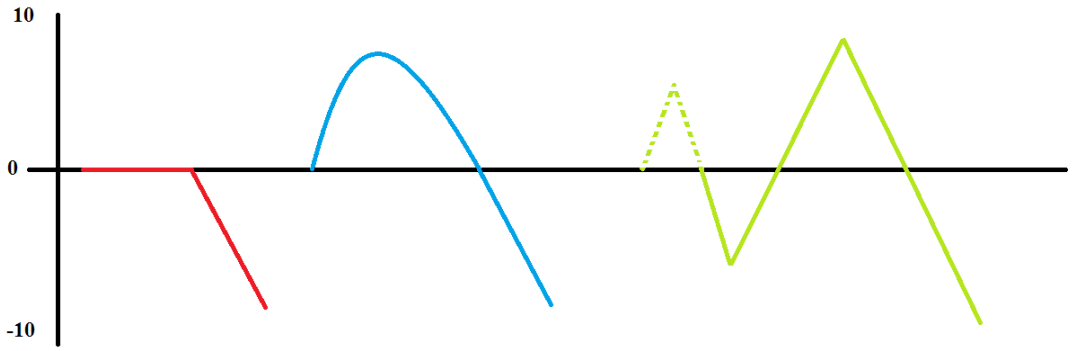

Figure 3-16. Typical hormesis growth curves showing the effects of subtoxic compounds.
Adapted from Oberbaum and Cambar (1996). Typical hormesis growth curves the red line shows no low or subtoxic effects upon growth, the blue line shows a peak of stimulation from a subtoxic concentration, and the green line shows the more rarely observed double spikes in inhibition and stimulation at different subtoxic concentrations.

Although hormesis could be one explanation for the effects seen, there is an alternative interpretation of the results, where due to the complexity of the crude plant extract, the biphasic effects observed could alternatively be in response to more than one effector, or one effector which has split into two. The biphasic responses are often found within complex solutions and natural extracts where although hormesis is possible, the growth response observed should not always be viewed as the effects of only one component (Murado and Vazquez, 2010).

3.3.4.1 Antimicrobial assays of wild tomato fruit extracts

Extracting plant material with water is a common method used when looking for antimicrobial polar compounds; it is a useful solvent to extract with as the residues can be easily re-suspended in water for the antimicrobial bioassays, and the residues extracted typically weigh more than when the plant material is extracted using other solvents. The fruit from several wild tomato species were extracted in water, from ripe fruit (*S. lycopersicum* cv. M82, *S. pimpinellifolium* LA0114, *S. lycopersicum* "cerasiforme" LA2675, *S. lycopersicum* "cerasiforme" LA2511 and *S. peruvianum* LA2744) and unripe fruit (*S. lycopersicum* "cerasiforme" LA2675, *S. lycopersicum* "cerasiforme" LA2511, *S. chmielewskii* LA2695, and *S. peruvianum* LA2744). These extracts were used to investigate whether the polar compounds in fruit are antimicrobial,

and the water extract was the only solvent tested because this is typically the way secondary metabolites would be released from the fruit, for example during cooking.

There was a mixture of effects seen from the tested ripe tomato fruit where no significant differences or inhibitory effects were observed towards *E. coli* O157:H7 and *S.* Enteritidis S1400. Significant inhibition was observed from *S. peruvianum* LA2744 ripe fruit against *S.* Typimurium 10248 and *E. coli* 25922. The unripe fruit from *S. peruvianum* LA2744 was also significantly inhibitory against *S.* Enteritidis S1400. This suggests that there is some antimicrobial potential from fruit extracts of *S. peruvianum* LA2744. Although some antimicrobial potential was found, the bacterial growth was generally significantly increased from fruit extracts, particularly from *S. lycopersicum* "cerasiforme" LA1511 (Figure 3-6A and D). This increase in growth of certain bacteria within the presence of the water extract of fruit is hypothesised to be related to the soluble sugar content and other metabolites which can be utilised by the bacteria when grown in minimal media.

3.3.4.2 Introgression lines of *S. pennellii*

The *S. pennellii* ILs are a large resource, which were only briefly investigated as part of this project. Further investigations into the *S. pennellii* ILs were discontinued as a major element of this research. This was because it was highlighted that the identification of antimicrobial compounds from the wild tomato relatives should be established first, and that the antimicrobial investigations into wild tomato ILs should be a succeeding stage in the research. The modest range of *S. pennellii* ILs which were investigated covered represented regions from within most of the 12 tomato chromosomes, these included 1-1-2, 2-1, 2-3, 2-2, 4-3, 4-4, 5-3, 6-1, 7-2, 8-1, 8-2, 8-2-1, 9-1-3, 9-1-2, 11-4, 12-1-1, and 12-4. The *S. pennellii* ILs which were selected for trichome extraction contained chromosome regions of interest such as the fragments IL 2-3, which has been linked to the *woolly* (*Wo*) gene responsible multicellular trichome initiation in *S. lycopersicum* (Yang et al., 2011). These examples were chosen due to the large numbers of around 72 ILs, and these plant leaf samples were extracted for polar and non-polar compounds using a biphasic extraction technique. The initial results from the disc diffusion assays showed no inhibitory activity from any of the extracted *S. pennellii* ILs plants for both

polar (water: methanol) and non-polar (chloroform). The *S. pennellii* ILs are a large resource, which have only been briefly investigated as part of this project. Further investigations into the *S. pennellii* ILs were discontinued as a major element of this research because it was

S. pennellii ILs 1-1-2, 4-3, 6-1, 7-5-5, 10-3, and 12-1 leaf material were extracted using acidified methanol and water (80:20 v/v), using 5% (v/v) of the dried extract in growth curve analysis after re-suspending the residue in 200 µL of DMSO. The results showed that there was no significant inhibition of *S.* Typhimurium 10248 or *E. coli* O157:H7 from any of the extracts, as all the growth curves were above the DMSO control (Figure 3-11). These results, combined with the initial zone of inhibition assay results, show that extraction of the dried leaf material of selected *S. pennellii* ILs using MCW (1.5:2.5:1.5 methanol:chloroform:water v/v), or acidified methanol and water (80:20 v/v) shows no antimicrobial activity. Therefore either the correct ILs have not been selected or the extraction method is inadequate, or there is no antimicrobial activity within these *S. pennellii* ILs.

Due to the lack of antimicrobial activity from extractions of dried *S. pennellii* IL leaf material, extractions were continued from selected *S. pennellii* trichomes using chloroform leaf dips. The extracts were diluted to either 1:1 or 1:32 (v/v) with DMSO and then 10 µL was inoculated onto each disk for analysis. The results showed that the *S. pennellii* ILs do have antimicrobial activity at the higher concentration (1:1 v/v) against both *S. aureus* 25923 and *S.* Typhimurium 10248. When these results are compared to the *S. pennellii* extract, which was also still antimicrobial at the dilution of 1:32 (v/v), it suggests that the concentrations of active compounds within these tested *S. pennellii* ILs are not as high as the wild tomato *S. pennellii*.

There are many *S. pennellii* introgression lines which have been found to have a persistent increase in specific compounds compared to the domesticated tomato. The *S. pennellii* introgression lines show that chromosomes 2, 6, 7 and 10 play a significant role in the production of secondary metabolites. There are increased compounds above the threshold of *S. lycopersicum* cv. M82 found in ILs 2-1, 2-1-1, 2-2, 2-3, 2-5 and 2-6. Chromosome 7 has also been highlighted due to increased compounds from ILs 7-1, 7-2, 7-3, 7-4-1, and 7-5, chromosome 6 has increased compounds in ILs 6-1, 6-3, and 6-4,

and chromosome 10 also has high compound levels in IL 10-1, 10-1-1, 10-2-2, and 10-3. Analysis of these lines with a significant increase in specific secondary metabolites could highlight these metabolites as antimicrobial if they are the highest compound concentrated within the sample. The introgression lines also have the potential to yield novel secondary metabolites due to their genetic differences, and can be used as genetic maps to antimicrobial QTLs. These marker assisted breeding programs are currently used to identify pest resistance QTLs (Lawson et al., 1997).

3.3.5 *S. habrochaites*

Extracts of *S. habrochaites* LA1777 were found to be antimicrobial to *S. aureus* 25923 and *S.* Typhimurium 10248 in disc diffusion assays with the highest activity against *S. aureus* 25923 from the extracts of dried leaf material in either petroleum ether or hexane. The growth curves showed that *S. habrochaites* was antimicrobial against *S. aureus* 25923, and caused a slight decrease in growth against *S.* Typhimurium 10248. The antimicrobial activity was primarily against *S. aureus* 25923, suggesting that the active compounds are effective against Gram-positive bacteria. The most active extracts were from dried leaf material extracted in chloroform, petroleum ether, or hexane, and this indicates that the active compounds are non-polar (Figure 3-1).

The MIC for *S. habrochaites* LA1777 was difficult to determine for the Gram-negative bacteria due to the high viscosity of the trichome extracts. It was shown for all the bacterial species that the 50 mg mL^{-1} extract was less inhibitory than several lower concentrations of the trichome extract, and this is likely to be linked to the solubility of the active compounds within the plant extract, where a 1:1 (v/v) diluted stock solution of trichome extract, dissolved with DMSO, was possibly too viscous and unable to dissolve well within the LB, which would cause the active compounds to dissolve less within the media and therefore be at a lower concentration compared to the extracts which contain less extract. The solubility of non-polar compounds may also have had an effect upon the zone of inhibition results, where solubility was reduced for non-polar comounds across the agar, and therefore, the inhibitory effects can not be accurately measured.

Despite the difficulties with the extract dissolving in the media there were significantly inhibited bacterial cultures from seleted concentrations of *S. habrochaites* trichome extract obtained for each of the tested bacterial species. The Gram-negative bacteria were slightly inhibited with an MIC of 12.5 mg mL^{-1}, and *S. aureus* 25923 was the most succeptible with an MIC of 1.56 mg mL^{-1}. The growth was able to recover in *S. aureus* at 0.78 mg mL^{-1}, however, this growth was still lower than the controls and at the concentration of 0.195 mg mL^{-1} the *S. habrochaites* LA1777 trichome extract was shown to have no effect upon *S. aureus* 25923 which had a growth pattern similar to the controls.

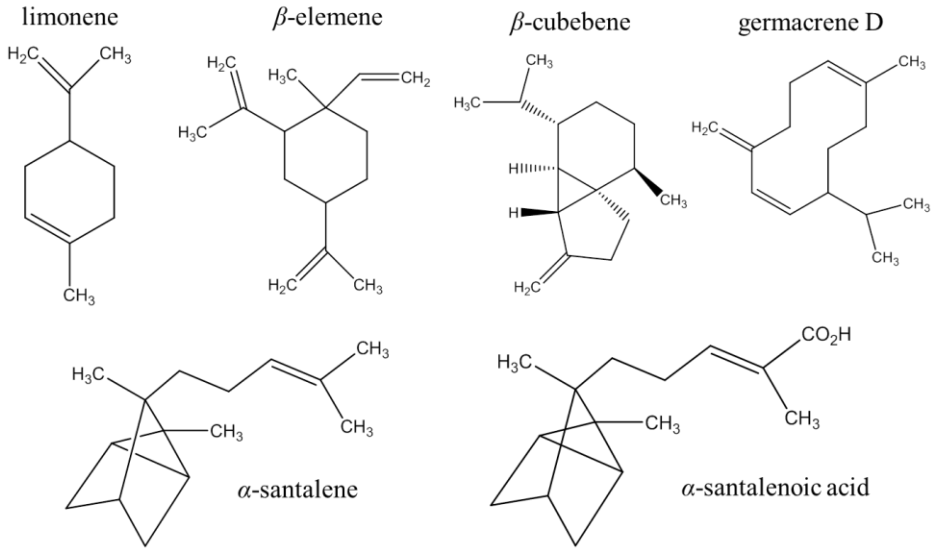

Figure 3-17. Structures of monoterpenes, sesquiterpenes, and sesquiterpene carboxylic acids (SCAs).

Structures of limonene, *β*-elemene, *β*-cubebene, germacrene D, *α*-santalene, and *α*-santalenoic acid, produced from the class VI glandular trichomes of *S. habrochaites* LA1777, the drawings were adapted from Chemspider using ChemDraw Pro 13.0.

Given that the active extracts from *S. habrochaites* LA1777 are likely to be non-polar, we can infer that the previously identified non-polar compounds produced by *S. habrochaites* trichomes are likely to be the antimicrobial compounds. The type VI trichomes of *S. habrochaites* are known to produce insecticidal fatty acid derivatives in the form of methyl ketones such as 2-undecanone (C_{11}), 2-tridecanone (C_{13}) and 2-pentadecanone (C_{15}) (Schmiller et al., 2008), in concentrations from >80 µg g^{-1} (Fridman et al., 2005). The insecticidal properties of the methyl ketones produced by *S. habrochaites* has been well documented and correlations have been found between the

levels of methyl ketones and the resistance to insect pests such as tobacco hornworm (*Manduca sexta*), spotted spider mite (*Tetranuchus urticae*), green peach aphids (Myzus persicae), Colorado potato beetle (*Leptinotarsa decemlineata*) and maize earworm (*Heliothis zea*) (Fridman et al., 2005). Although methyl ketones are usually produced in high quantities by *S. habrochaites* the methyl ketones produced by *S. habrochaites* LA1777 have been previously reported as produced in non-detectable amounts, and that the major compounds were identified as terpenes, primarily sesquiterpenes (Fridman et al., 2005). Therefore although *S. habrochaites* have insecticidal activities, the antimicrobial activities from LA1777 are likely to be an alternative class of compounds.

S. habrochaites LA1777 produce terpenoids from their type VI trichomes, which are primarily in the form of class II sesquiterpenes (Besser et al., 2009). The terpenoid profile of *S. lycopersicum* cv. M82 is different from LA1777 and is primarily composed of the monoterpenes α-pinene, verbenene, δ-2-carene, and β-phellandrene, and low levels of class I sesquiterpenes β-elemene, β-caryophyllene, and α-humulene (Figure 3-18). *S. lycopersicum* cv. M82 is not an antimicrobial plant suggesting that the terpenoids present do not exhibit antimicrobial properties, and that the terpenoids produced in the highest quantities by *S. habrochaites* LA1777 such as α-santalenoic acid, and β-*cis*-bergamotenoic acid (Besser et al., 2009) which could be responsible for the antimicrobial activity.

S. habrochaites LA1777 was found to be antimicrobial in both growth curves, and disc diffusion assays against several bacterial species (Figure 3-1, Figure 3-2, Figure 3-3, and Figure 3-4), however, when an alternative accession line *S. habrochaites* LA1918 was assumed to also have antimicrobial activity it was found that when extracted in methanol:water (80:20 v/v) there was only significant antimicrobial activity observed against *E. coli* 25922 (Figure 3-12A). This highlights the importance of how genetic divergence can influence the production of secondary metabolites within plant species, and that care should be taken when selecting plant species for antimicrobial analysis, because close relatives can be genetically distinct enough to produce significantly different results. Another example of this can be seen when three accessions of *S. peruvianum* trichome extracts were tested for antimicrobial activity, and there were differences between these relatives where *S. peruvianum* LA1987 had the highest antimicrobial activity against *S. aureus* 25923 (Figure 3-13).

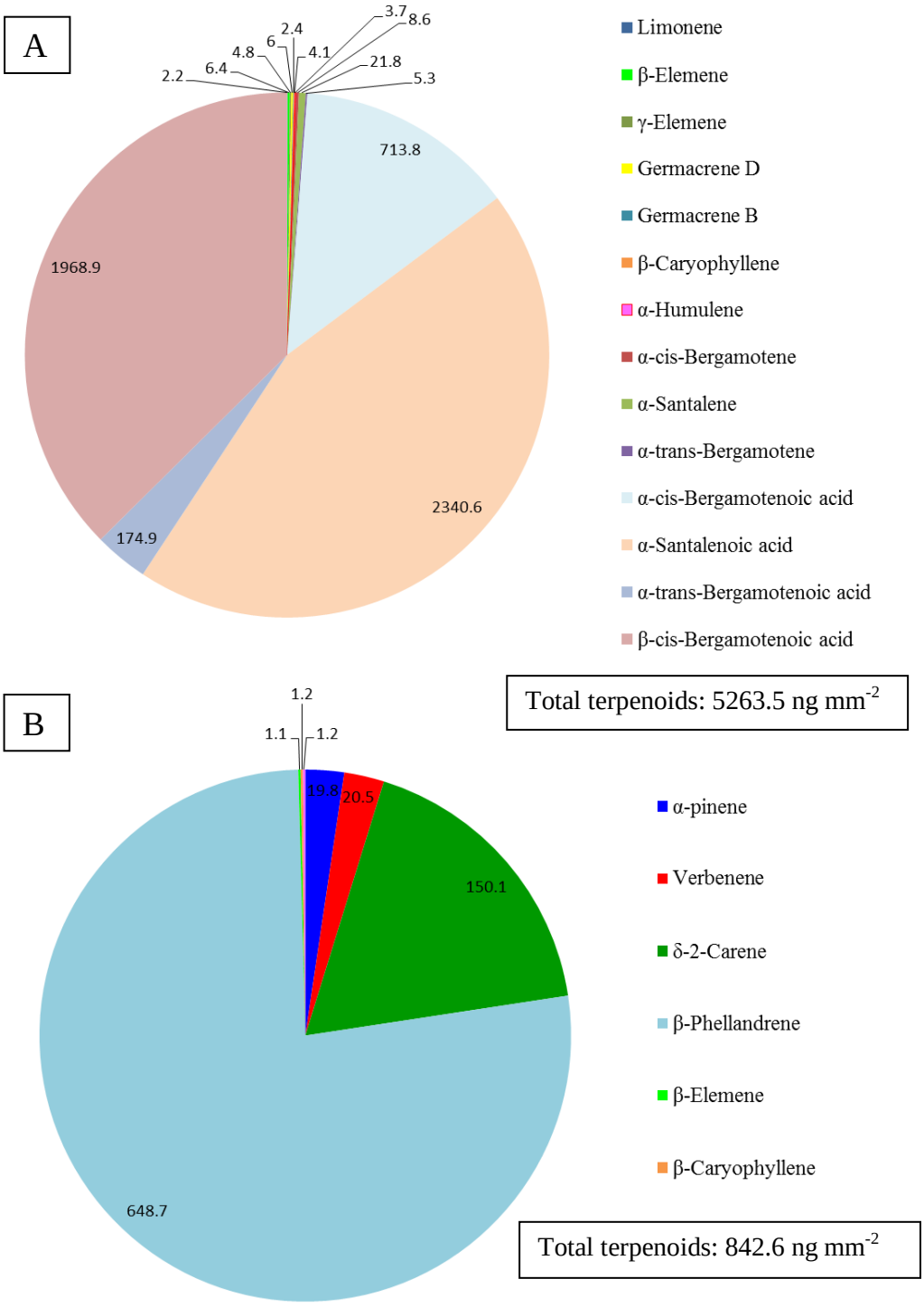

Figure 3-18. Terpenoids (ng mm^{-2}) produced from the trichomes of A. *S. habrochaites* LA1777, and B. *S. lycopersicum* cv. M82.

Data presentation adapted from the research completed by Besser et al., (2009) on terpenoid metabolism in the trichomes of tomato species (Besser et al., 2009).

3.3.6 *S. pennellii*

S. pennellii was antimicrobial against *S. aureus* 25923 and *S.* Typhimurium 10248 from all eight solvent extractions of dried plant material used in disc diffusion assays, and the antimicrobial effects were still seen in liquid broth growth curves (Figure 3-1, Figure 3-2, Figure 3-3, and Figure 3-4). The MICs were determined for *S. pennellii* LA0716 trichome extracts and showed that *S. aureus* 25923 was the most susceptible with an MIC of 1.56 mg mL^{-1}. The growth of *S.* Typhimurium 10248 and *E. coli* O157:H7 was not completely inhibited by *S. pennellii* trichome extracts, but it was inhibited to a minimum level by an average concentration of 25 mg mL^{-1}.

An extraction method specific for alkaloid glycosides extracted from *S. pennellii* leaves was shown to be significantly inhibitory against *E. coli* 25922, *E. coli* 12900, *S.* Typhimurium 10248, and *S.* Enteritidis S1400 (Figure 3-12). There are several steroidal glycoalkaloids which have been detected within tomato species. Steroidal glycoalkaloids are known to be inhibitory to, fungi and cytotoxic to animal cells, such as α-tomatine (Kozukue et al. 2004) and dehydrotomatine (Ono et al., 1997). There is also evidence that tomato steroidal glycoalkaloids are inhibitory to bacteria, such as trigonelline, which is antibacterial against *E. coli* and *S.* Enterica (Almeida et al., 2006). Further analysis of the secondary metabolite content from the *S. pennellii* leaf material and purification of the glycosides for further antimicrobial profiling will be discussed in Chapter 5. The extraction method specific for alkaloid glycosides would also extract other glycosides such as glucose esters. Therefore, alkaloid glycosides and glucose esters will be treated as potential compound classes responsible for the antimicrobial activity of *S. pennellii*.

Studies by Ermakov (1980) show that *S. pennellii* produces lipids composed of 15 fatty acids, the most dominant one being capric acid (C_{10}:0). A typical epicuticular extract from *S. pennellii* is composed of 89 to 95% glycolipids, 5 to 10% alkenes, and 1% terpenoids (Mudd et al., 1988). The non-polar alkenes include normal isomers and anteisomers of C_{27} to C_{34} (Mudd et al., 1988). The fatty acid esters which complete the sugar esters are found in the following patterns 1. 3x 2-methylpropanoic acid, 2. 2x 2-methylpropanoic acid, 2-methylbutanoic acid, 3. 2x 2-methylpropanoic acid, 3-methylbutanoic acid, 4. 2x 2-methylpropanoic acid, 8-methylnonanoic acid, 5. 2x 2-

methylpropanoic acid, decanoic acid, 6. 2-methylpropanoic acid, 3-methylbutanoic acid, 8-methylnonanoic acid, 7. 2-methylpropanoic acid, 3-methylbutanoic acid, 8-methylnonanoic acid, 8. 2-methylpropanoic acid, 2-methylbutanoic acid, decanoic acid, and 9. 2-methylpropanoic acid, 3-methylbutanoic acid, decanoic acid (Mudd et al., 1988).

The *S. pennellii* sugar esters have been tested in laboratory animals for oral toxicity, which revealed the sugar esters to be non-toxic (Mudd et al., 1988). This is important because should the sugar esters of *S. pennellii* be discovered to be the active compounds within the antimicrobial leaf extracts, the identification of antimicrobial compounds which are also non-toxic to mammalian cells can lead to useful drug discovery, and the production of a drug which has no toxic side effects upon the patient, and the ability to use the crude extract orally with no toxic effects.

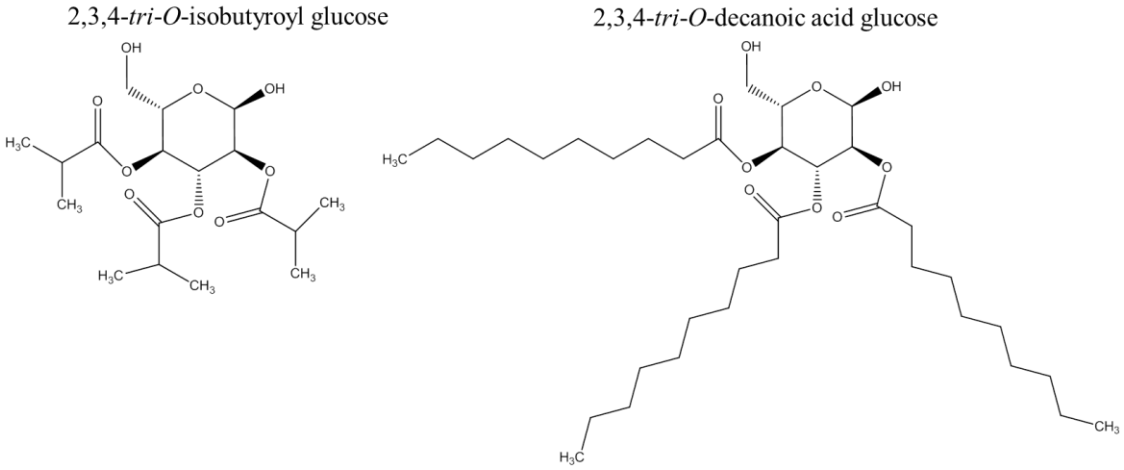

Figure 3-19. Examples of structures of glucose fatty acid methyl esters
2,3,4-*tri*-*O*-isobutyroyl glucose and 2,3,4-*tri*-*O*-decanoic acid glucose produced by the trichomes of *S. pennellii* LA0716, structures drawn using ChemDraw Pro 13.0 and Chemspider.

3.3.7 Antimicrobial potential of sugar esters

The glandular trichomes of *S. pennellii* produce mixtures of 2,3,4-tri-*O*-acylated glucose esters, and the fatty acids present in the mixture are 2-methylbutanoic, *n*-decanoic and 8-methylnonanoic acids (Walters and Steffens, 1990). Naturally produced sugar esters are increasingly recognised as useful chemicals within industry; they are currently used as surfactants, in cosmetics, the food industry and have antibiotic potential (Slocombe et

al., 2008; Chortyk et al., 1993). Recent literature suggests that, whilst there has been a heavy research focus on the insecticidal properties of exudates of wild tomato relative trichomes, there has been no antimicrobial research into the sugar esters produced from wild tomato relatives.

The only published data for antimicrobial sugar esters from Solanaceous species is from Chortyk et. al., (1993) where antibiotic sugar esters from *Nicotiana* species such as *N. tabacum* and *N. glutinosa* were identified. The sugar esters occur as 2,3,4-tri-*O*-acyl-sucrose (acyl represents C_3 to C_9 aliphatic acids), and 2,3,4-tri-*O*-acyl-glucose (Chortyk et al., 1993). *Nicotiana* sugar esters were tested for antimicrobial activity against a range of Gram-positive and Gram-negative bacteria, and *N. plumbaginifolia*, *N. gossei*, *N. palmerii*, *N. cavicola*, *N. amplexicaulis*, *N. langsdorffii*, and *N. glutinosa* were found to be the most active against the tested bacteria which included *B. subtilis*, *B. cereus*, *Microbacterium thermosphactum*, *Enterobacter cloacae*, and *Citrobacter freundii* (Chortyk et al., 1993). *Nicotiana tabacum* L. leaf trichomes were also found to produce bacteria inhibiting compounds such as α-duvatriene-diol and a 3-methylvalerate sucrose ester which inhibit *Bacillus subtilis* and *B. cereus* but not Gram-negative bacteria. Although it was also shown that 3-methylvalerate glucose and methylvaleric acid had no biological activity (Cutler et al., 1986). It was also observed that sugar esters with higher carbon number acyl groups had the highest inhibitory, particularly glucose esters with C_7 and C_8 acyl groups such as 8-methylnonanoic, and n-decanoic acids (Chortyk et al., 1993).

S. pennellii can produce glucose esters which account for approximately 25% of leaf dry weight, equalling around 400 μg per cm^2 of glucose esters on the leaf surface (Walters and Steffens, 1990). The positive antimicrobial results from the disc diffusion assay screen of eight solvent extractions of *S. pennellii* and from the glycoside extracted leaf material suggest that amphiphilic sugar esters are a likely candidate responsible for these antimicrobial activities.

The Solanaceae family hosts a number of plant species which produce glucose and sucrose esters in exudates on their leaves, usually containing a short to medium chain fatty acyl (C_2 to C_5) (Dembitsky, 2004). Sugar esters from the glandular trichomes of *L. hirsutum* and *L. hirsutum* f. sp. *glabratum* were identified as 2,3,4,1'-*tetra*-*O*- and 2,3,4-

tri-O-acylated sucrose esters, respectively, with ester groups of 2-methylbutyryl, 3-methylbutyryl, *iso*-undecanoyl and dodecanoyl (King et al., 1990). *L. peruvianum* var. *glandulosum* was found to produce a single 2,3,1-*tri-O*-acylated sucrose ester of fatty acids (SEFAs) in small quantities, with 2-methylbutyryl and dodecanoyl groups (King et al., 1990; Dembitsky, 2004). 2,3-*di-O*- and 1,2,3-*tri-O*-acylated glucose esters with ester groups of pentanoyl, hexanoyl, heptanoyl, octanoyl, and undecanoyl were identified from the glandular trichomes of *Datura metel* L. (King and Calhoun, 1988). The sugar ester chemical compositions have been widely elucidated after extraction of leaf material with chloroform (King et al., 1990).

Macrolactone glycosides are an important natural class of antibiotics, widely used in clinical and veterinary medicine (Dembitsky, 2004), including erythromycin and pikromycin (Akey et al., 2006). The attachment of a sugar molecule to a compound greatly increases its solubility, and therefore its ability to reach target sites. Further evidence to suggest that sugar esters are responsible for the antimicrobial activity can be inferred from the selective activity of *S. pennellii* against *S.* Typhimurium, and *S. aureus* 25923, while the antimicrobial effects are resisted in *E. coli* O157:H7, and preliminary studies show resistance from *S.* Enteritidis S1400. This is important because *E. coli* O157:H7 is known to have many antimicrobial resistance mechanisms, such as resistance to macrolactone glycosides such as erythromycin.

Due to the identified antimicrobial potential within the two wild tomato relatives *S. pennellii* and *S. habrochaites* there is reason to believe that there will be other unidentified Solanaceae species which produce antimicrobial secondary metabolites. The investigation of other plant extracts with known and unknown antimicrobial potential against the selected bacteria might provide correlations between the major compound classes known to be produced by the plants and the antimicrobial potential of the Solanaceae family as a whole. The Solanaceae species are known to produce specific compounds such as glyco-alkaloids, and the antimicrobial analysis of pure compound examples could provide information about what compounds inhibit the bacteria and to what extent they may be responsible for the antimicrobial activity observed within the crude extracts of the identified antimicrobial *S. pennellii* and *S. habrochaites*.

Chapter 4

Antimicrobial Solanaceae, herbs and chemical standards

4.1 Introduction

The use of plants such as herbs as natural food preservative has been a tradition practiced by many cultures around the world dating back to pre-history (Cowan, 1999), such as a wide range of antimicrobial oils with both Gram-positive and Gram-negative antimicrobial activities. In many cases in plant medicine, the exact antimicrobial compounds responsible for the antimicrobial activity are unknown, or linked putatively to a class of compounds, yet plants such as herbs are generally considered safe due to their use within food or medicine over many years (Cowan, 1999).

This chapter describes studies on the antimicrobial potential of certain herb plants, selected Solanaceae species and chemical standards. Various herb species were tested to validate the extraction and antimicrobial assays and to look for suitable control plants for antimicrobial comparisons. Through comparing their effects against the gastro-enteric bacteria chosen in this research project, with the effects of the antimicrobial wild tomato relatives, the antimicrobial class of compounds can be putatively identified.

Herb plants were extracted and tested for antimicrobial activity because it is important to determine to what extent the bacteria chosen are susceptible to different plant extracts. Furthermore, correlations can be made between the known compounds produced by the herbs and the secondary metabolites produced by the antimicrobial Solanaceae species. The methods used to determine antimicrobial activity included zone of inhibition assays, and growth curve analysis of bacteria cultured in the presence of extracted plant material or dissolved chemical standards and as described in Chapter 2 sections 2.4.2 and 2.4.3.

Additional Solanaceae were also tested for antimicrobial activity, due to the antimicrobial activity found within *S. pennellii* LA0716 and *S. habrochaites* LA1777. The wild tomato relatives with a high trichome load showed the most antimicrobial potential, therefore species with a high trichome load such as *Solanum melongena* (aubergine) and *Nicotiana rustica* (wild tobacco) were selected to study. Other Solanaceae such as chilli peppers, were also chosen to act as a positive Solanaceae controls, because the hot chilli pepper fruit such as the Scotch bonnet contains high

levels of the antimicrobial compound capsaicin (Cowan, 1999). The isolated antimicrobial fractions from Solanaceae are diverse in their polarity; therefore the method of extracting with eight solvents was continued for the selected herb and Solanaceae species.

Table 4-1. Herb species showing the antimicrobial activities of their phytochemicals.

Species Name	Common name	Compounds	Plant organ	Antimicrobial activity	References
Rosmarinus officinalis	Rosemary	Diterpenoids Carnosic acid	Leaves and stem, Essential oil	General antimicrobial activity	Cowan, 1999
Catharanthus roseus	Madagascar Periwinkle	-	Leaf, stem, flower and root material	Inhibits *E. coli* and *Salmonella* Typhi	Raza et al., 2009
Lycium barbarum, and *L. chinense*	Gogi berries	Proteoglycans, polysaccharides, essential oils, glycerogalactolipids.	Fruit	Antioxidant	Potterat, 2010
Aloe barbedensis	*Aloe vera*	Saponins, salicylic acid, anthraquinones-aloin, and emodin	Juice	*E. coli, S. aureus and Salmonella*	Lorenzetti et al., 1964
Vaccinium spp.	Blueberry and Blackberry	Monosaccharide Fructose	Fruit	*E. coli*	Cowan, 1999
Thymus vulgaris	Thyme	Terpenoids (Caffeic acid), Phenolic acids (Tannins) and Tannins (Polyphenols)	Leaf	*P. aeruginosa, C. albicans*	Nascimento et al. 2000
Cichorium intybus	Chicory	Sesquiterpene lactones-Lactucin and lactucopicrin. Aesculetin, Aesculin, Cichoriin, Umbelliferone, Scopoletin and 6.7-Dihydrocoumarin. Volatile oils. Bitter substances	Leaves and root.	Antifungal, against *Cladosporium herbarum Pyricuraia oryzae, Pellicularia sasakii,* and *Alternaria kikuchiana.*	Nishimura et al., 2000 Nishimura and Satoh, 2006
Allium sativum	Garlic	Sulfoxide, Allicin, Ajoene	Roots	Aqueous and 40% hydro-alcoholic active against *S. aureus*	Cowan, 1999
Punica granatum	Pomegranate	Ellagitannins α-nor-hydrolapachone and α-nor-lapachone	Fruit-Seed, juice, and skin.	Ethanol extract against MRSA and *S. aureus, P. aeruginosa* and *B. subtilis*	Nascimento et al. 2000 and Braga et al., 2005
Allium schoenoprasum	Chives	Diallyl sulfide	Essential oil	*E. coli* O157:H7, *S. enterica, B. cereus, C. jejuni, C. botulinum, V. cholerae* and *S. aureus*	Rattanachaikunsopon, and Phumkhachorn, 2008
Citrus paradisa	Grapefruit	Terpenoids	Fruit skin	Antifungal	Cowan, 1999

Table 4-2. Solanaceae species showing the antimicrobial activities of their phytochemicals.

Species Name	Common name	Compounds	Plant organ	Antimicrobial activity	References
Solanum surattense Burm. F.	-	-	500µg mL^{-1} leaf extract ethanol	MIC 50 to 100µg mL^{-1} against *E. coli*, *B. subtilis*, *Streptococcus* spp, *S. aureus*, *S.* Typhi	Sheeba, 2010,
Solanum macrocarpum L.	African Eggplant	Saponins and steroids from the root bark and unripe fruits	Methanol extract High alkaloid content from leaves	Inhibition of *E. coli*, *B. subtilis*, and *C. albicans* 50 to 100 µg mL^{-1}	Ajaiyeoba1, E. O., 1999
Solanum torvum	Turkeyberry	Steroidal alkaloids and glycosides from the roots	Methanol extract High alkaloid content from leaves	*E. coli* and *S. aureus*, *C. albicans* inhibition at 50 to 100 µg mL^{-1}	Ajaiyeoba1, E. O., 1999
Cestrum diurnum (L.)	Day Jasmine	Palmitic acid Stearic acid and oleic acid	Toxic to humans and livestock leaves	Inhibited *S. aureus* and *P. aeruginosa* at 100 to 600 mg L^{-1}	Bhattacharjee et al., 2005
Capsicum annum	Chilli pepper	Capsaicin	Terpenoid	Bacteria	Cowan, 1999
Solanum tuberosum	Potato	α-Solanine	Leaves, and tuba	Bacteria and fungi	Cowan, 1999
Solanum Sisymbrifolium	Sticky Nightshade	Solasodine	Leaves Small edible fruits	Root-knot nematodes	Dias et al., 2012
Nicotiana rustica	Mapacho	MAOI *β*-carbolines	High concentration of nicotine	Resistant to *P. infestans*	Kamoun et al., 1998
Solanum melongena L.	Aubergine	Nicotinoid alkaloids	Roots, and fruit	Pathogenic microorganisms	Al-Janabi, and Al-Rubeey, 2010
Capsicum spp.	Chile peppers	Capsaicin and dihydrocapsaicin	Fruit	*B. cereus*, *B. subtilis*, *Clostridium sporogenes*, *Clostridium tetani*, and *Streptococcus pyrogenes*	Cichewicz et al., 1995

4.2 Results

Dried leaf material was extracted using eight solvents and the antimicrobial activity was determined through comparisons of the diameter of the zones of inhibition which ranged from 6.5 mm as the minimum recorded evidence of inhibition to the antibiotic ciprofloxacin positive control which could reach up to 32 mm. The results in the disc diffusion tables represent the zone of inhibition diameter minus 6 mm which equals the diameter of the filter pad used to hold the plant extract during the tests (Table 4-3 and Table 4-4).

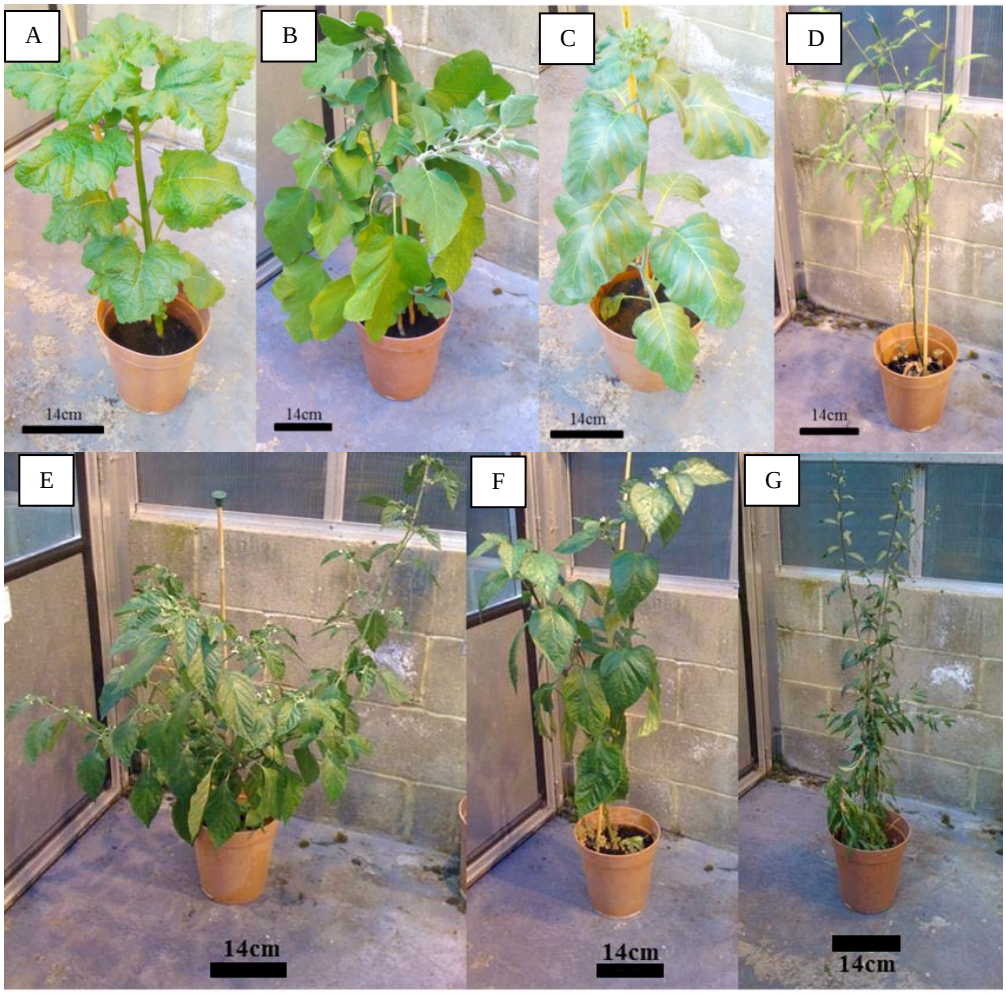

Figure 4-1 Mature Solanaceae plant species.

Solanceae species grown to the flowing stage of plant development, A. Apple of Peru (*Nicandra physalodes*), B. Aubergine (*Solanum melongena)*, C. Thuoc Loa (*Nicotiana rustica*), D. Bird eye chillies (*Capsicum chinense*), E. Scotch bonnet (*Capsicum chinense*), F. Sweet bell pepper (*Capsicum annuum*), G. Goji berry (*Lycium barbarum*). Scale bars all represent 14 cm.

4.2.1 Solanaceae plant antimicrobial disc diffusion assay results

A chloroform extract from the leaf of Apple of Peru was only antimicrobial against *S. aureus* 25923. The fruit extract from chilli peppers, finger chillies and Scotch bonnet were antimicrobial against *S.* Typhimurium 10248 and *S. aureus* 25923, ranging from polar water to non-polar hexane. Some slight antimicrobial activity was seen from the green fruit of sweet pepper and the leaf material of *N. rustica* against *E. coli* O157:H7. There were no antimicrobial activities seen from any of the solvent extracts from goji berry fruit (Table 4-3).

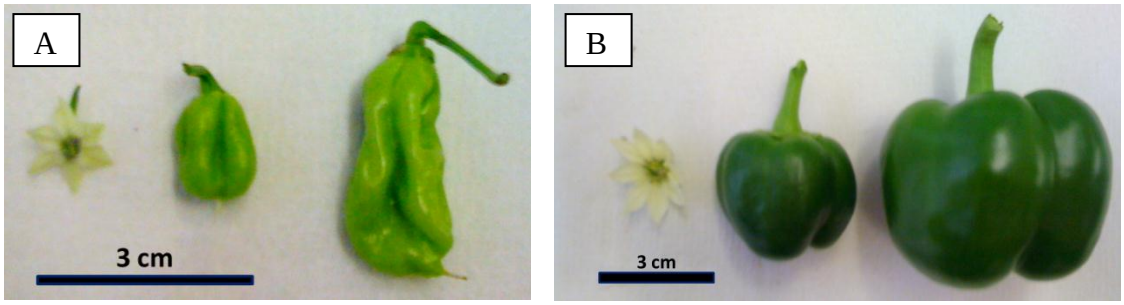

Figure 4-2. Developmental stages of *Capsicum* spp.

Images of the green fruit and flowers from A. Scotch bonnet and B. Sweet bell pepper, scale bars represents 3 cm Scotch bonnet have a concaving shape to its fruit cavities, compared to the Sweet bell pepper, which has a more convex shape to its fruit. The number of petals on the flower of Scotch bonnet is six compared to eight on Sweet bell pepper.

The main positive results are that the *Capsicum* spp., such as green finger chilies and red Scotch bonnet, are inhibitory from all solvent extractions, compared to the green sweet pepper. *E. coli* O157:H7 was the most resistant bacteria to all of the Solanaceae, with smaller zone of inhibitions of approximately 1 to 2 mm, which is within the margin of error for the effects of the DMSO. The antimicrobial effects of the DMSO solvent were subtracted against the final results. Therefore, the results shown are the additional effects of the plant extracts, or any synergistic effects between the solvent and extract.

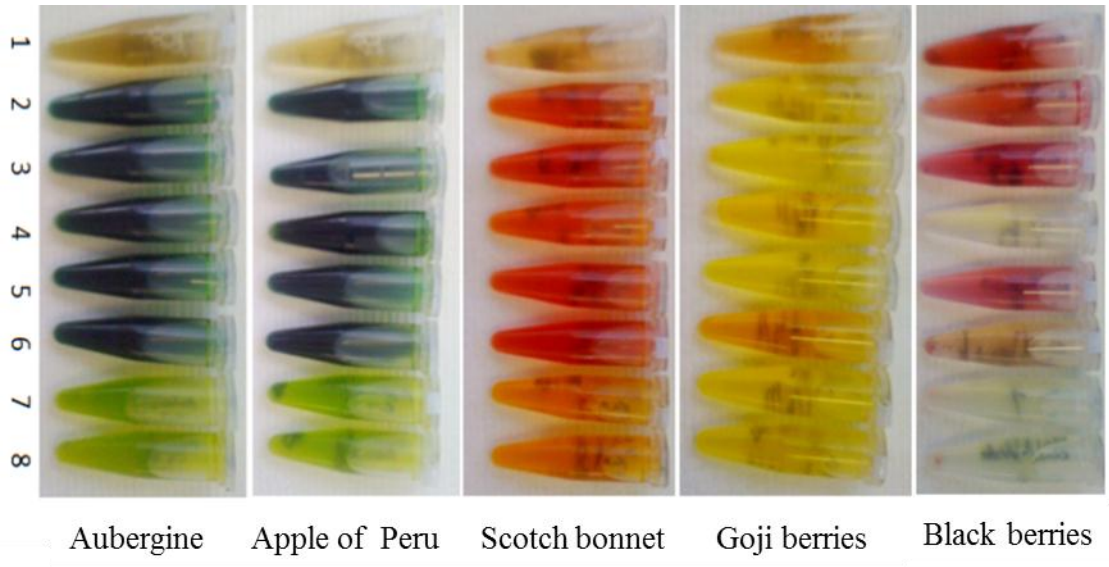

Figure 4-3. Solvent extracts of leaf and fruit from plant species.

Where 100 mg of plant material was extracted in 2 ml of; 1. water, 2. methanol, 3. ethanol, 4. isopropanol, 5. acetone, 6.chloroform, 7. petroleum ether, 8. hexane.

Table 4-3. Disc diffusion assay results from Solanaceae extracts.

Solanaceae		Apple of Peru	Green sweet pepper	Red scotch bonnet	Finger chillies	Goji berries	Aubergine	*N. rustica*
		Leaves	Fruit	Fruit	Fruit	Fruit	Leaves	Leaves
S. Typhimurium 10234	Water	-	-	+++	+++	-	-	-
	Methanol	-	-	+++	++	-	-	-
	Ethanol	-	-	+++	+++	-	-	-
	Ethyl acetate	-	-	+++	+++	-	-	-
	Isopropanol	-	-	+++	+++	-	-	-
	Chloroform	-	-	+++	++	-	-	-
	Petroleum ether	-	-	+++	++	-	-	-
	Hexane	-	-	+++	++	-	-	-
E. coli O157:H7	Water	-	-	-	-	-	-	-
	Methanol	-	-	-	-	-	-	-
	Ethanol	-	-	-	-	-	-	-
	Ethyl acetate	-	-	+	+	-	-	-
	Isopropanol	-	+	-	-	-	-	-
	Chloroform	-	-	-	-	-	+	+
	Petroleum ether	-	+	+	-	-	-	-
	Hexane	-	-	+	-	-	-	+
S. aureus 25923	Water	-	-	-	++	-	-	-
	Methanol	-	-	+++	-	-	-	-
	Ethanol	-	-	-	+	-	-	-
	Ethyl acetate	-	-	-	+++	-	-	-
	Isopropanol	-	-	+++	++	-	-	-
	Chloroform	+++	-	-	++	-	-	-
	Petroleum ether	-	-	-	++	-	-	-
	Hexane	-	-	-	+++	-	-	-

Plant material was extracted and tested against bacteria in disc diffusion assays. The solvents used were; 1. water, 2. methanol, 3. ethanol, 4. ethyl acetate, 5. isopropanol, 6. chloroform, 7. petroleum ether, 8. hexane. The extracts were evaporated and re-suspended in 200 µL DMSO (10 µL per 6 mm disc). The antimicrobial activities are summarised as; -: no inhibition, +: 1 to 2 mm, ++: 2 to 6 mm, +++ : >6 mm, zones represent the radius from the disc edge to outer zone.

4.2.2 Disc diffusion assay results from herb species

Aliquots of dried and ground plant material (100 mg) were each extracted with various solvents (2 mL), and the extracted product was dried and re-suspended to a stock

solution using 200 µL of DMSO for analysis. Water, methanol and ethanol extracts of pomegranate skin (10 µL of stock solution per disk) were highly inhibitory to *S. aureus* 25923 (Table 4-4). The initial herb tests showed *Aloe vera*, rosemary and pomegranate skin to have the largest antimicrobial activity, especially against *S. aureus* 25923.

Table 4-4: Disc diffusion assay results from herb extracts.

Herbs		Thyme	Blackberry	Pomegranate	Pomegranate	Rosemary	*Aloe vera* L.
		Leaves	Fruit	Seed and Juice	Skin	Leaves	Leaves
S. Typhimurium 10234	Water	-	+++	-	-	-	-
	Methanol	-	+++	-	-	++	+++
	Ethanol	-	+++	-	-	-	+++
	Ethyl acetate	-	-	-	-	-	+++
	Isopropanol	-	-	-	-	-	+++
	Chloroform	-	-	-	-	-	+++
	Petroleum ether	-	-	-	-	++	-
	Hexane	-	-	-	-	++	+++
E. coli O157:H7	Water	-	-	-	-	++	-
	Methanol	-	-	-	-	++	+
	Ethanol	+	-	-	-	-	+
	Ethyl acetate	-	-	-	-	-	-
	Isopropanol	-	-	-	-	-	-
	Chloroform	+	-	-	-	-	-
	Petroleum ether	-	-	-	-	++	+
	Hexane	+	-	-	-	++	+
S. aureus 25923	Water	-	-	+++	+++	+++	-
	Methanol	-	-	-	+++	+++	+++
	Ethanol	-	-	-	+++	+++	+++
	Ethyl acetate	-	-	++	-	+++	+++
	Isopropanol	-	-	+++	-	+++	+++
	Chloroform	-	-	+++	-	+++	+++
	Petroleum ether	-	-	-	-	+++	-
	Hexane	-	-	-	-	+++	-

Plant material was extracted and tested against bacteria in disc diffusion assays. The solvents used were; 1. water, 2. methanol, 3. ethanol, 4. ethyl acetate, 5. isopropanol, 6. chloroform, 7. petroleum ether, 8. hexane. The extracts were evaporated and re-suspended in 200 µL DMSO (10 µL per 6 mm disc). The antimicrobial activities are summarised as; -: no inhibition, +: 1-2 mm, ++: 2-6 mm, +++ : >6 mm, zones represent the radius from the disc edge to outer zone.

There was antimicrobial activity from majority of the plant extracts against *E. coli* O157:H7, but this activity was not significant enough to be seen as a positive antimicrobial effect. The results are more likely due to slight variations in the DMSO inhibition which *E. coli* is most susceptible to, and to which the samples were compared against. Blackberry fruit water, methanol and ethanol extracts were inhibitory against *S.* Typhimurium 10248, pomegranate seed/juice ethyl acetate, isopropanol and chloroform extract, and Apple of Peru leaf chloroform extract was inhibitory against *S. aureus* 25923.

Rosemary leaf water, methanol, petroleum ether and hexane extracts had the highest antimicrobial activity towards *S. aureus* 25922, with slight activity against *S.* Typhimurium and *E. coli* O157:H7. *Aloe vera* leaf extracts showed broad spectrum antimicrobial activity through inhibiting *S.* Typhimurium 10248 (>6 mm), *E. coli* O157:H7 (>1 to 2 mm) and *S. aureus* 25923 (>6 mm), but no activity occurred from the water extracts.

4.2.3 Inhibitory effects of pure compounds

The pure standards were tested for antimicrobial activity which included selected terpenoids; 2-tridecanone, 2-undecanone, *trans*-caryophyllene, and *α*-pinene, due to their presence within the trichome exudates of wild tomato species such as *S. habrochaites* LA1777 and *S. lycopersicum* cv. M82 (Besser et al., 2009). Glycoalkaloids which are produced by many Solanaceae species were also tested which included; *α*-solanine, *α*-tomatine and *α*-chaconine. The phenolic acids chosen were; vanillic acid, chlorogenic acid, *p*-coumaric acid, salicylic acid, sinapic acid, caffeic acid and ferulic acid. The other compounds tested were sterols such as; cholesterol, *β*-sitosterol, stigmasterol and 6-keto-cholesterol. Through identifying pure antimicrobial compounds, which are known to be present in Solanaceae species, potential synergistic effects can be ascertained, and correlations can be formed between the antimicrobial potential of different classes of compounds.

4.2.3.1 Phenolic acids

Phenolic acids present in tomato plants were dissolved directly in the minimal media at a concentration of 500 mg mL^{-1} and tested for antimicrobial activity by measuring the growth curves in a FLUOstar. The phenolic acids with activity below the minimal media control curve were chlorogenic acid, caffeic acid, salicylic acid, and *p*-coumaric acid, for both *S.* Enteritidis S1400 and *E. coli* ATCC 25922 (non-toxigenic) (Figure 4-5). It is also interesting to see that ferulic acid and vanillic acid maintained exponential growth for longer than the minimal media control, particularly in *S.* Enteritidis S1400 (Figure 4-5B).

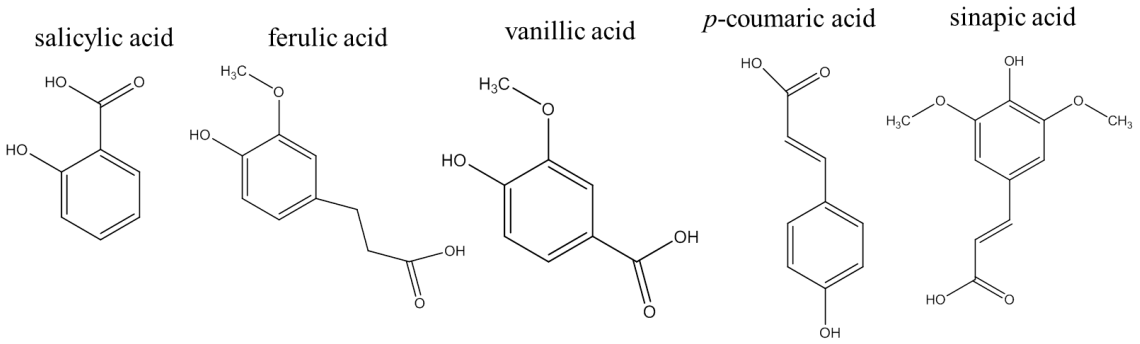

Figure 4-4. Structures of phenolic acids.

Structures which contain a phenolic ring with a carboxylic acid function; chlorogenic acid, ferulic acid, vanillic acid, sinapic acid, salicylic acid, caffeic acid, and *p*-coumaric acid. These compounds were selected for antimicrobial analysis using FLUOstar growth curves of bacteria cultured within their presence. The drawings were adapted from Chemspider using ChemDraw Pro 13.0.

The AUC for *S.* Enteritidis S1400 and *E. coli* ATCC 25922 was compared to the minimal media control and showed that chlorogenic acid, sinapic acid, caffeic acid, and ferulic acid was significantly different ($P<0.001$) (one-way ANOVA Dunnetts multiple comparisons test, 95**%** CI). The AUC for *S. aureus* 25923 also shows significant difference from the control for theall the tested compounds ($P<0.001$), except *p*-coumaric acid which was not significantly different. Caffeic acid completely prevented growth, and chlorogenic acid treated cells showed limited growth (Figure 4-5 C).

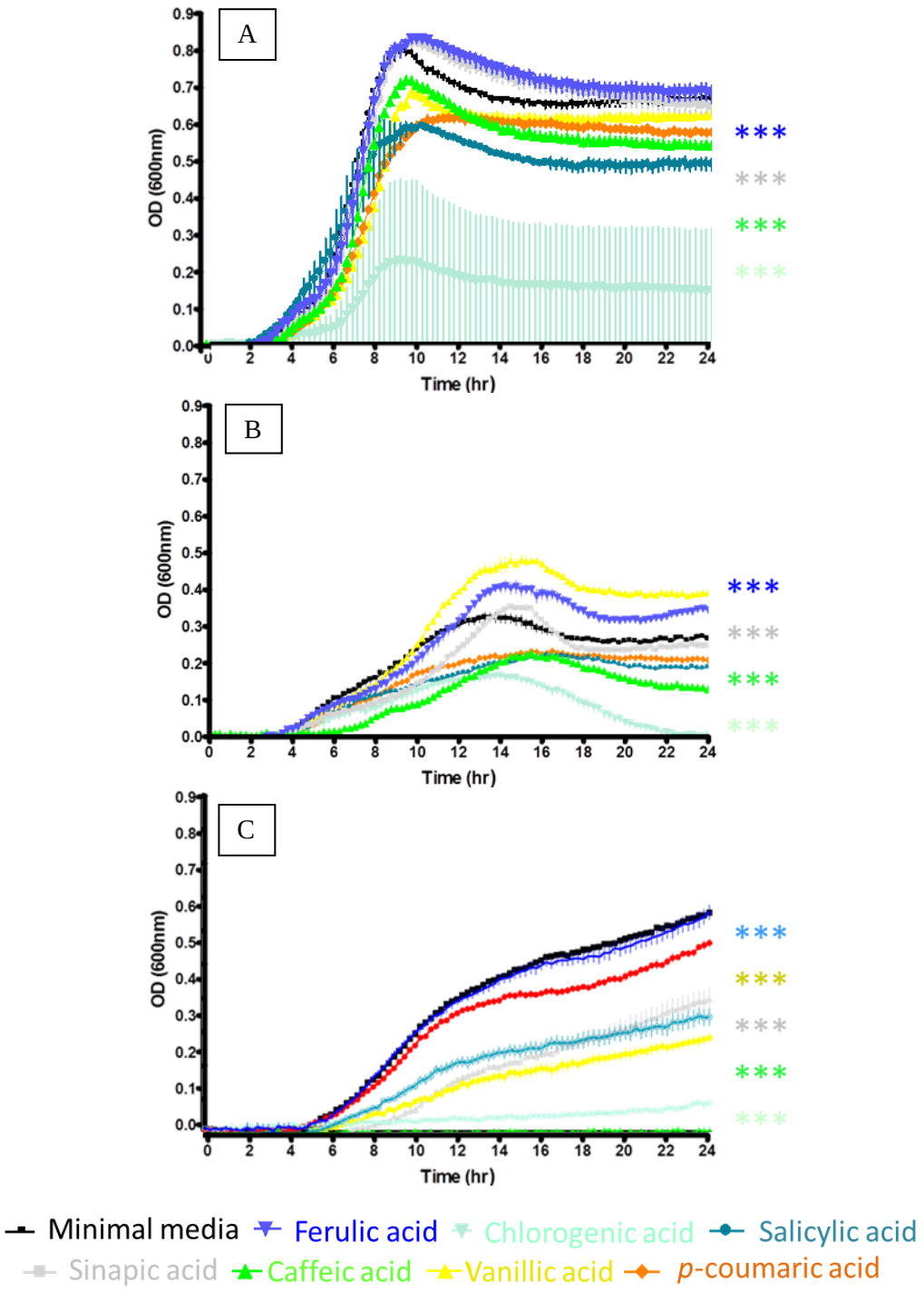

Figure 4-5: FLUOstar growth curves of bacteria grown with pure phenolic acid standards.

Pure phenolic compounds vanillic acid, chlorogenic acid, *p*-coumaric acid, salicylic acid, sinapic acid, caffeic acid and ferulic acid were re-suspended directly into minimal media cultures for growth curves (500 mg mL^{-1}), against A. *E. coli* ATCC 25923, B. *S.* Enteritidis S1400 and C. *S. aureus* ATCC 25923. Error bars represent the standard error, and three biological replicates of the bacteria cultures. The asterisk (***=P<0.001) represents the significant difference between the area under the growth curves of bacteria grown in the presence of the extract compared to the DMSO control, using one-way ANOVA Dunnetts multiple comparisons test (95% CI).

4.2.3.2 Glycoalkaloids

Some of the glycoalkaloids widely available within the Solanaceae plant family are *α*-solanine, *α*-tomatine, and *α*-chaconine. The antimicrobial properties of these glycoalkaloids were tested through measuring the growth curves of *Salmonella* sp. and *E. coli* sp. grown in their presence measured in a FLUOstar. The glycoalkaloids were dissolved in DMSO and added to a concentration of 3% (v/v) in minimal media with a total concentration of 10 μg mL^{-1}.

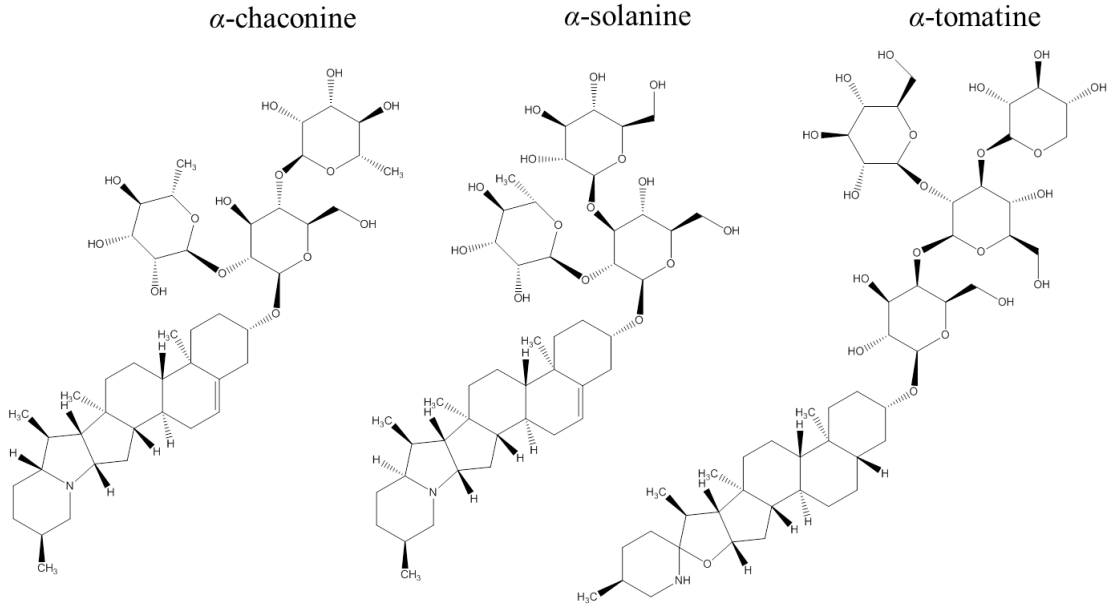

Figure 4-6. Structures of glycoalkaloids.

Structures show; *α*-chaconine (solanidane-type aglycone, with rhaminose and glucose moieties), *α*-solanine (solanidane-type aglycone, with rhaminose, glucose, and galactose moieties) which are produced by potato species and *α*-tomatine (spirosolane-type aglycone, with xylose, glucose and galactose), which is produced by tomato species (Roddick and Melchers, 1985). These compounds were selected for antimicrobial analysis using FLUOstar growth curves of bacteria cultured within their presence. The drawings were adapted from Chemspider using ChemDraw Pro 13.0.

A significant inhibitory difference was found compared to the AUC of the DMSO control with *α*-solanine ($P<0.001$), *α*-tomatine ($P<0.05$) and *α*-chaconine ($P<0.05$) against *E. coli* 25922 (Figure 4-7 B). This significant difference was also seen for *α*-solanine ($P<0.001$), and *α*-tomatine ($P<0.05$) for *E. coli* O157:H7 (Figure 4-7 A). For the both of the *Salmonella* species tested there were significantly different AUC compared to the DMSO control for *α*-solanine ($P<0.001$). *S.* Typhimurium 10248 also

had significant differences from *α*-tomatine ($P<0.001$) and *α*-chaconine ($P<0.001$) (Figure 4-8). The growth of *S. aureus* 25923 in the presence of these compounds was not significantly different to the growth maintained in the LB and DMSO controls (Figure 4-8 C).

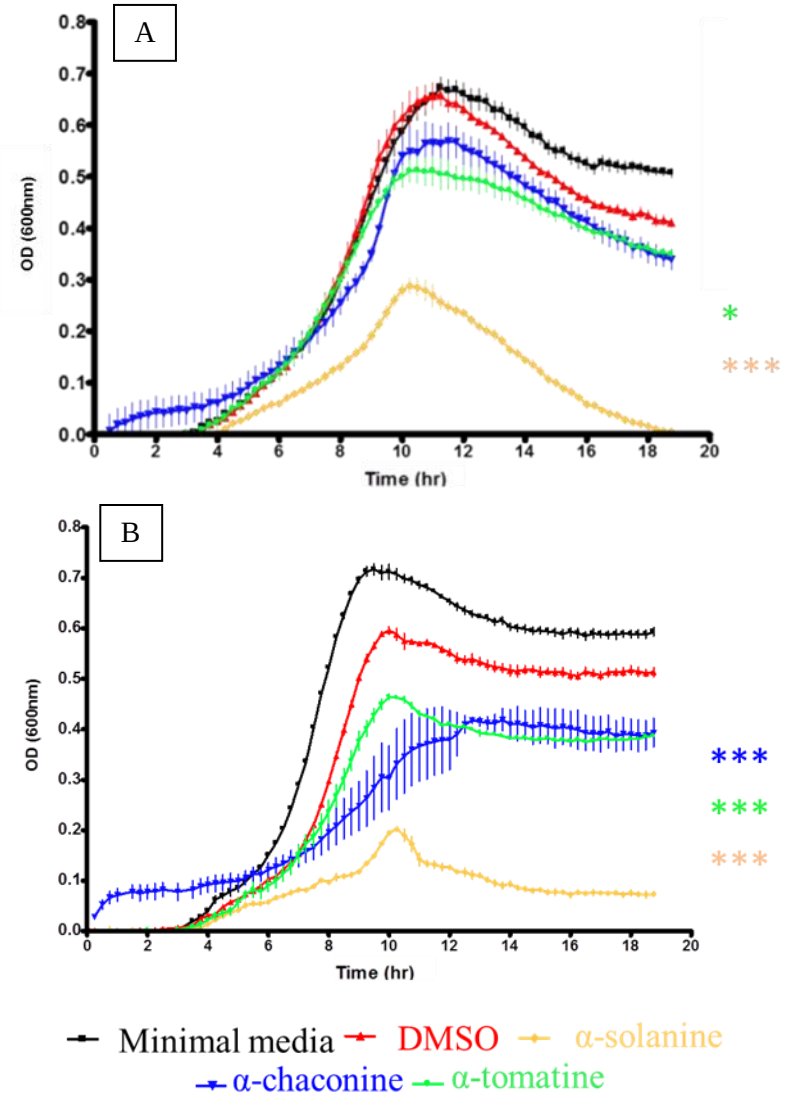

Figure 4-7: FLUOstar growth curves of bacteria grown with pure glycoalkaloids standards.

Pure glycoalkaloids (*α*-solanine, *α*-tomatine and *α*-chaconine) were re-suspended in DMSO into minimal media cultures for growth curves (10 µg mL^{-1}). A. *E. coli* O157:H7, B. *E. coli* 25922. Error bars represent the standard error, and three biological replicates of the bacteria cultures. The asterisk (*=$P<0.05$, ***=$P<0.001$) represents the significant difference between the area under the growth curves of bacteria grown in the presence of the extract compared to the DMSO control, using one-way ANOVA Dunnetts multiple comparisons test (95% CI).

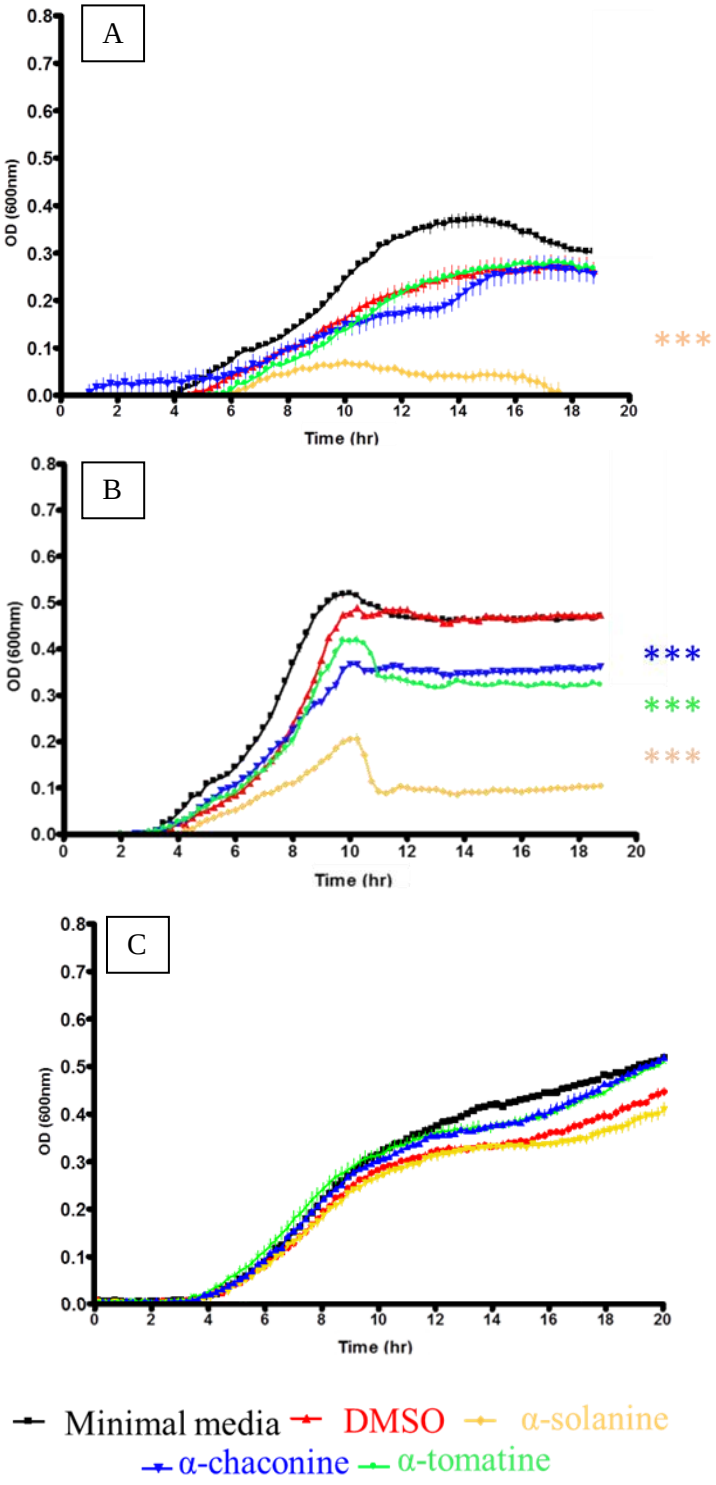

Figure 4-8: FLUOstar growth curves of bacteria grown with pure glycoalkaloids standards.

Pure glycoalkaloids (*α*-solanine, *α*-tomatine and *α*-chaconine) were re-suspended in DMSO into minimal media cultures for growth curves (10 µg mL^{-1}). A. *S.* Enteritidis S1400, B. *S.* Typhimurium 10248, and C. *S. aureus* 25923. Error bars represent the standard error, and three biological replicates of the bacteria cultures. The asterisk (***=P<0.001) represents the significant difference between the area under the growth curves of bacteria grown in the presence of the extract compared to the DMSO control, using one-way ANOVA Dunnetts multiple comparisons test (95% CI).

4.2.3.3 Terpenoids

The terpenoids *α*-pinene, *trans*-caryophyllene, 2-tridecanone and 2-undecanone (Figure 4-9) were tested for antimicrobial activity, using the FLUOstar to measure growth curves. The terpenoid *α*-pinene was chosen as a positive control because it has been previously found to be antimicrobial (Skaltsa et al., 2003) and can be found within *S. lycopersicum* cv. M82 (Besser et al., 2009). The terpenoids 2-tridecanone and 2-undecanone are found in the trichomes of the tomato wild relative *S. habrochaites* f. glabratum (Fridman et al., 2005; Schmiller et al., 2010). The FLUOstar growth curves showed that *α*-pinene (3% v/v) lowered the number of cells during stationary phase for *E. coli* O157:H7 (Figure 4-11 A), and increased the lag phase during the growth of the *S.* Enteritidis S1400, and *S.* Typhimurium 10248 compared to the controls (Figure 4-10 A and B). Comparisons between the AUC of the acetone control and *α*-pinene showed a significant difference for all the tested bacteria ($P<0.001$).

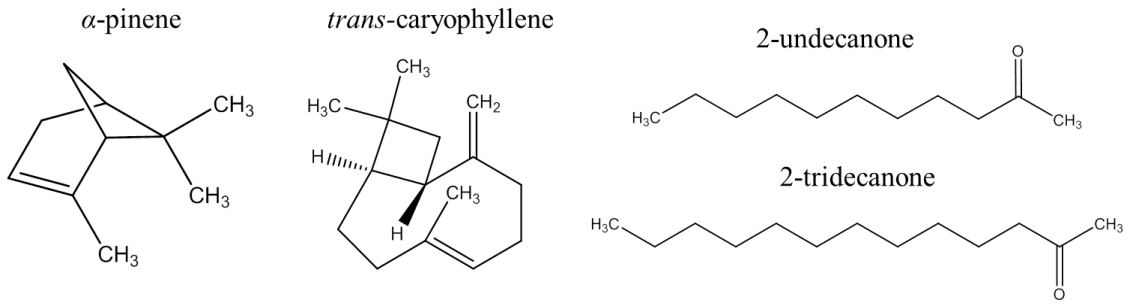

Figure 4-9. Structures of terpenoids.

Structures of *α*-pinene (four-membered ring structure), 2-tridecanone (methylketone), 2-undecanone (methylketone), and *trans*-caryophyllene (bicyclic sesquiterpene), which are a range of terpenoids selected for antimicrobial analysis using FLUOstar growth curves of bacteria cultured within their presence. The drawings were adapted from Chemspider using ChemDraw Pro 13.0.

The terpenoid 2-undecanone did not have a significant inhibitory effect compared to the acetone control, but did increase the lag phase in *S.* Enteritidis S1400. Growth was also slightly increased above the acetone control for the two *Salmonella* strains, and for *E. coli* O157:H7. When compared to the AUC of the acetone control 2-undecanone was significantly different for *S.* Typhimurium 10248 and *S. aureus* 25923, although growth was only inhibited for *S. aureus* 25923.

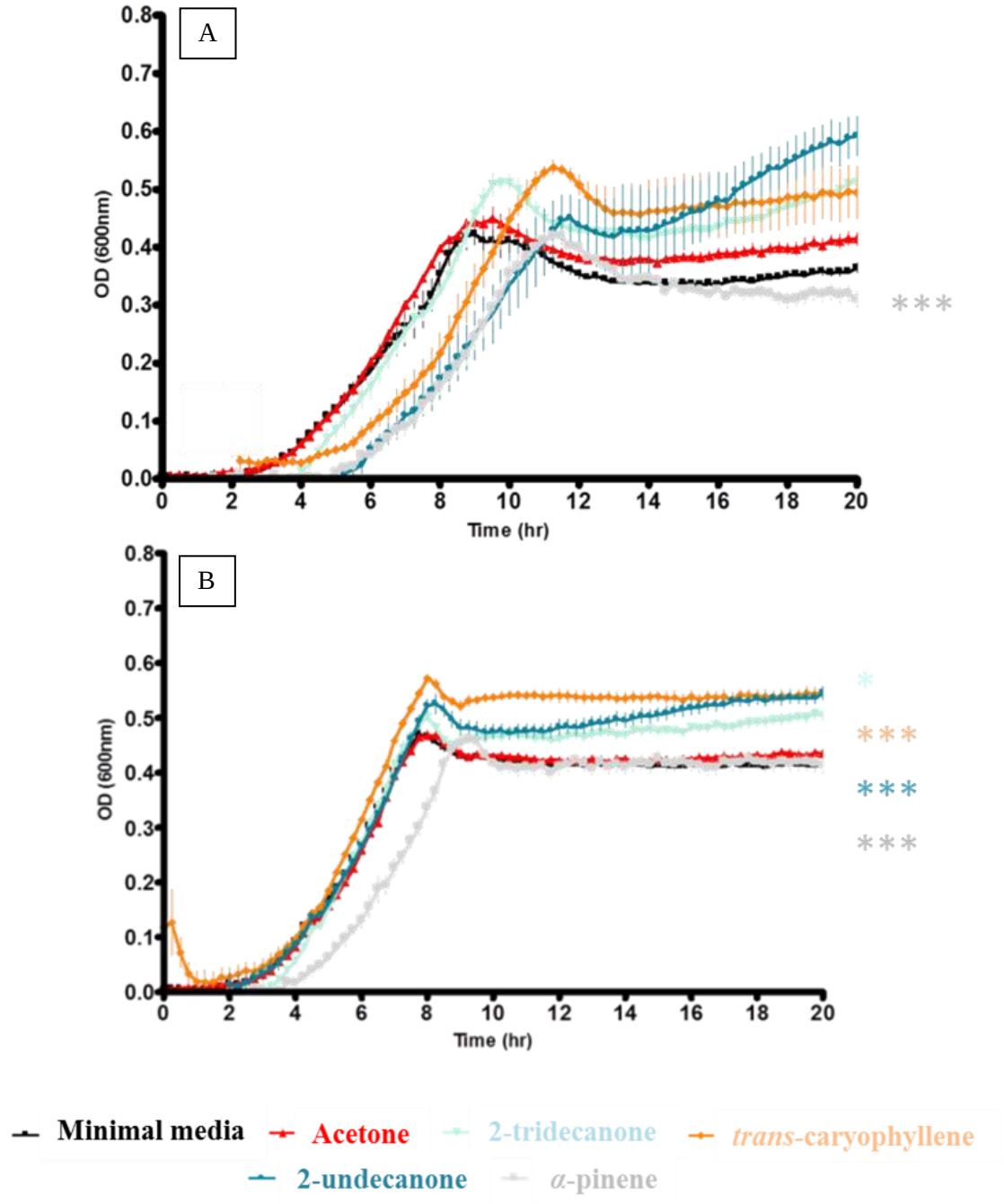

Figure 4-10: FLUOstar growth curves of bacteria grown with pure terpenoid standards.

2-tridecanone, 2-undecanone, *trans*-caryophyllene, and α-pinene were re-suspended in acetone and disolved in minimal media cultures for growth curves in a concentration of 3% (v/v) 15 µL mL^{-1}, against; A. *S.* Enteritidis S1400, and B. *S.* Typhimurium 10248. Error bars represent the standard error, and three biological replicates of the bacteria cultures. The asterisk (***=*P*<0.001) represents the significant difference between the area under the growth curves of bacteria grown in the presence of the extract compared to the acetone control, using one-way ANOVA Dunnetts multiple comparisons test (95% CI).

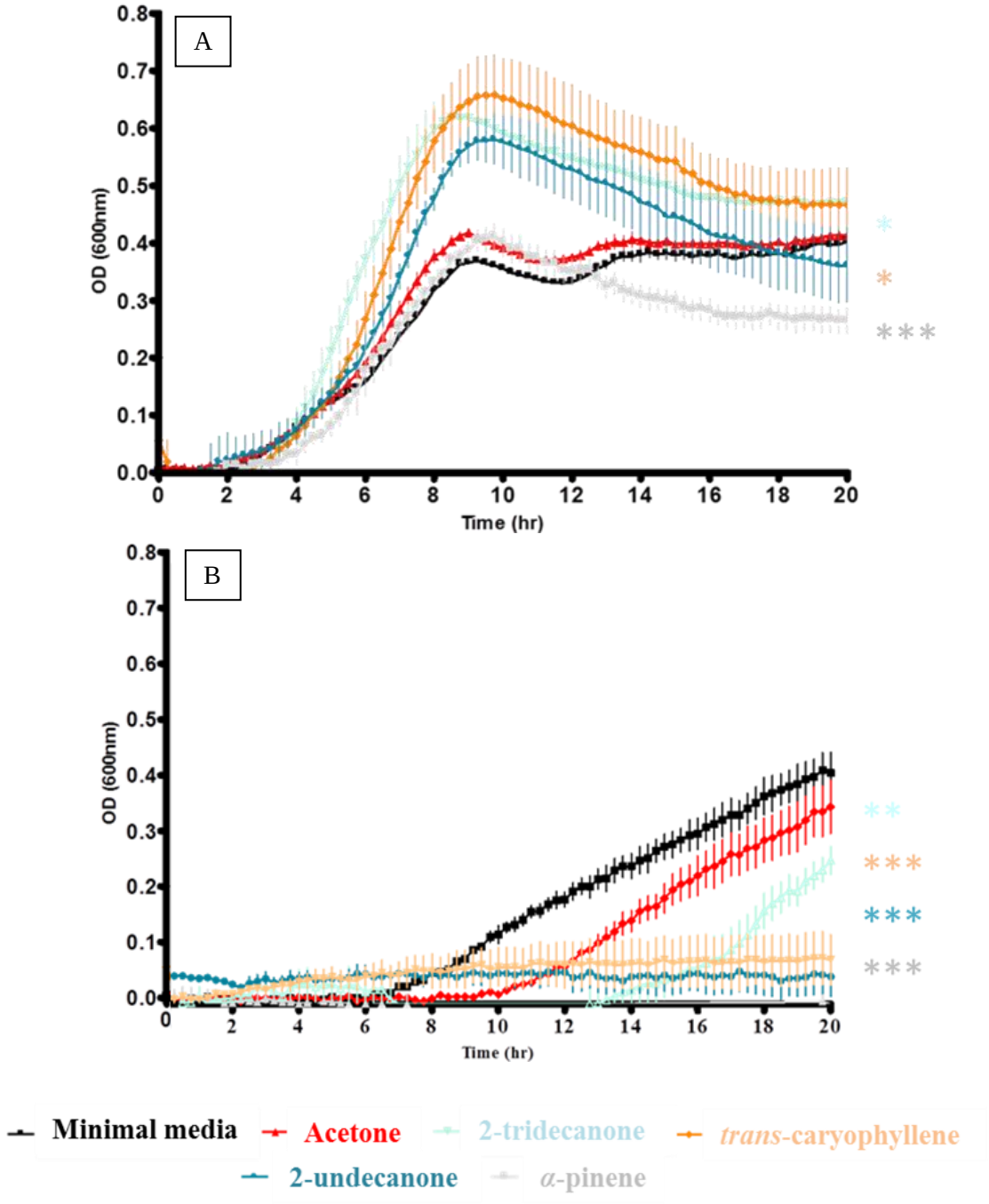

Figure 4-11: FLUOstar growth curves of bacteria grown in the presence of pure terpenoid standards.

2-tridecanone, 2-undecanone, *trans*-caryophyllene, and α-pinene were re-suspended in acetone and disolved in minimal media cultures for growth curves in a concentration of 3% (v/v) 15 µL mL^{-1}. A. *E. coli* O157:H7 and B. *S. aureus* 25923. Error bars represent the standard error, and three biological replicates of the bacteria cultures. The asterisk (***=P<0.001) represents the significant difference between the area under the growth curves of bacteria grown in the presence of the extract compared to the acetone control, using one-way ANOVA Dunnetts multiple comparisons test (95% CI).

There was an increase in growth with 2-tridecanone, 2-undecanone and *trans*-caryophyllene with all the Gram-negative bacteria tested. There was significant difference compared to the acetone control for 2-tridecanone and *trans*-caryophyllene for *S.* Typhimurium 10258 ($P<0.05$ and $P<0.001$), *E. coli* O157:H7 ($P<0.05$) and *S. aureus* 25923 ($P<0.001$). The terpenoids only had inhibitory effects upon *S. aureus* 25923, where growth was completely inhibited from 2-undecanone, *α*-pinene, and *trans*-caryophyllene ($P<0.001$), and there was also significant inhibition from 2-tridecanone ($P<0.01$) (Figure 4-11 B).

4.2.3.4 Sterols

Stigmasterol, *β*-sitosterol, cholesterol and 6-keto-cholesterol were tested against the selected bacteria, and none were found to be significantly antimicrobial against *S. aureus* 25923, *E. coli* O157:H7 or *S.* Typhimurium 10248. *S. aureus* 25923 showed significant differences compared to the DMSO control for all the tested sterols ($P<0.001$), but this difference was due to a positive effect on growth. Antimicrobial activity can be seen from stigmasterol with both *S.* Typhimurium 10248 and *E. coli* O157:H7. This difference in growth is most prominent from *S.* Typhimurium 10248, where the maximum OD after 20 h reached 0.7 for *S.* Typhimurium grown in LB and only 0.4 for *S.* Typhimurium grown in stigmasterol (Figure 4-13).

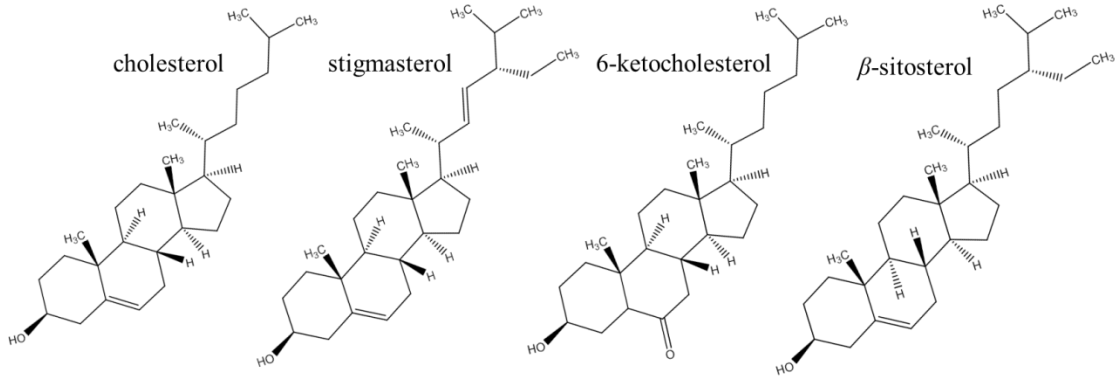

Figure 4-12. Structures of sterols.

Structures of cholesterol, 6-ketocholesterol, *β*-sitosterol, and stigmasterol.These compounds were selected for antimicrobial analysis using FLUOstar growth curves of bacteria grown within their presence. The drawings were adapted from Chemspider using ChemSketch.

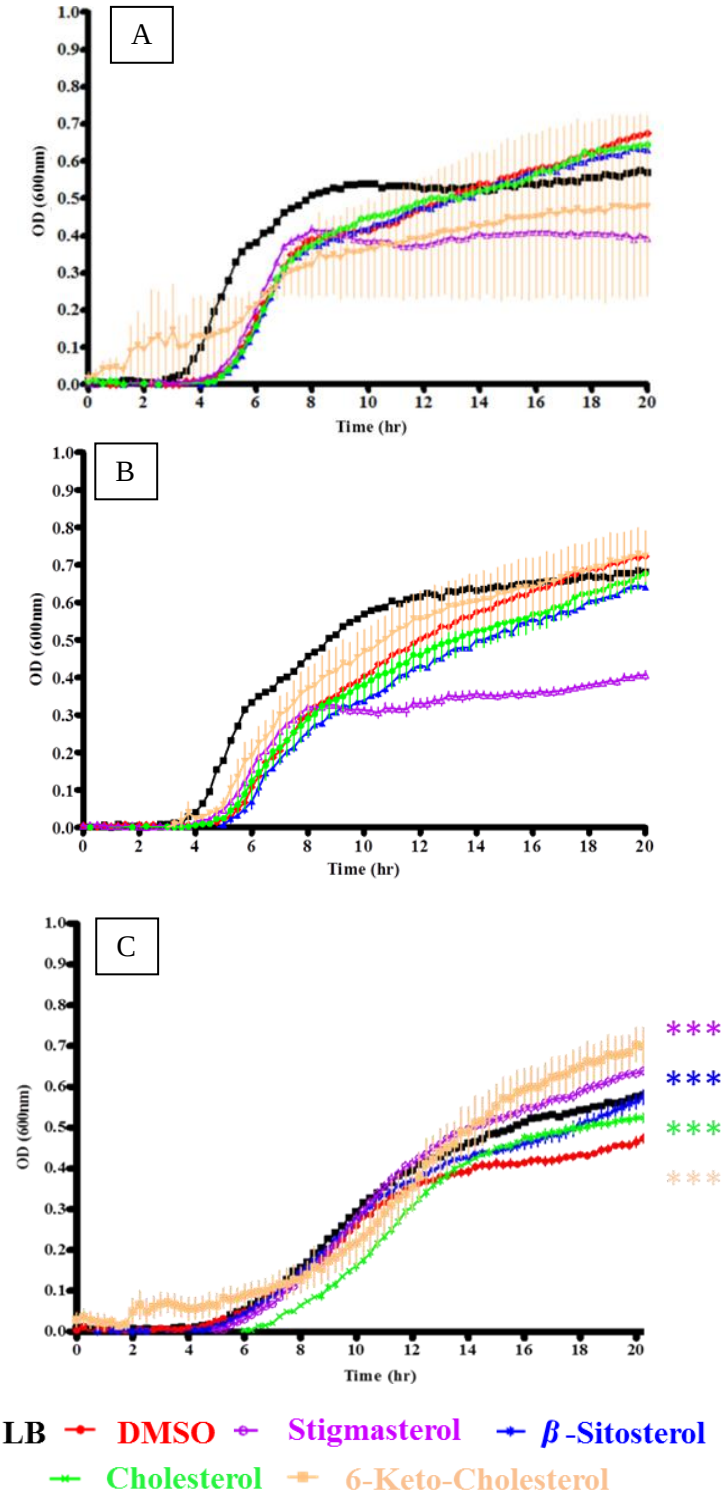

Figure 4-13. FLUOstar growth curves of bacteria grown with pure sterol standards.

Stigmasterol, *β*-sitosterol, cholesterol, and 6-keto-cholesterol (10 µg mL^{-1}), were re-suspended in DMSO into LB cultures for growth curves. A. *E. coli* O157:H7, B. *S.* Typhimurium 10248, and C. *S. aureus* 25923. Error bars represent the standard error, and three biological replicates of the bacteria cultures. The asterisk (***=*P*<0.001) represents the significant difference between the area under the growth curves of bacteria grown in the presence of the extract compared to the DMSO control, using one-way ANOVA Dunnetts multiple comparisons test (95% CI).

4.3 Discussion

4.3.1 Herb species

Several herb extracts were found to be antimicrobial against the tested bacteria (Table 4-4). Pomegranate fruit peel was inhibitory to *S. aureus* 25923 from the water, methanol and ethanol solvent extractions. Previous studies have shown mixed results from that of pomegranate (*Punica granatum*) where ethanol extracts were found to be inhibitory against *Pseudomonas aeruginosa* and *Bacillus subtilis* (inhibition zone ≥7 mm), but no activity against *S. aureus*, or *Salmonella enterica* serotype Choleraesuis (Nascimento et al., 2000). Although these results do not compare with the inhibitory results observed against *S. aureus* as part of this research alternative research by Braga et al., (2005) shows that methanol pomegranate extracts do have antimicrobial activity against *S. aureus* growth and enterotoxin production. The identification of the pomegranate skin as the site of antimicrobial activity is also supported from research performed by McCarrell et al., (2008), where antimicrobial activity from pomegranate rind was found against *S. aureus* and not against the tested Gram-negative bacteria.

Thyme (*Thymus vulgaris*) also had inhibition zones of ≥7 mm against *P. aeruginosa*, and *Candida albicans*. Rosemary (*Rosmarinus officinalis*) had inhibition zones of ≥7mm against *B. subtilis*, and *C. albicans*, and clove (*Syzygium aromaticum*) has activity ranging from 20 to 250 µL against *S. aureus, S. choleraesuis, P. aeruginosa, C. albicans, Klebsiella pneumoniae,* and *Shigella* spp. (Nascimento et al., 2000).

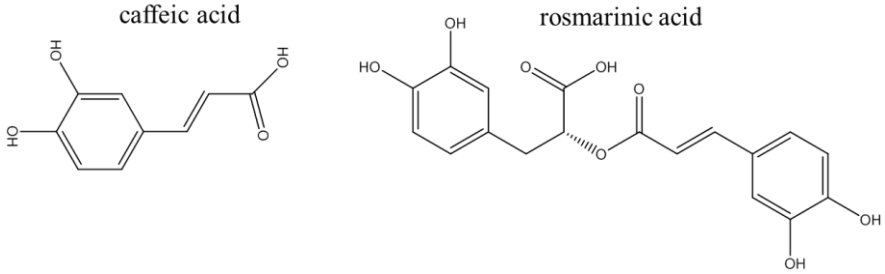

Figure 4-14: Phenolic compounds present within Rosemary.
Caffeic acid and rosmarinic acid structures adapted from Wang et al., (2004) using ChemSketch.

Rosemary extracts were also found to be highly inhibitory as part of this research, and rosemary extracts have been previously highlighted as producers of antimicrobial polyphenols, such as rosmarinic acid, carnosic acid, and caffeic acids, and have been investigated as a food preservative (Lee et al., 2005; Wang et al., 2004).

Aloe vera extracts were antimicrobial towards *S. aureus* 25923, *S.* Typhimurium 10248, and *E. coli* O157:H7. No activity occurred from the water extract, therefore the inhibitory secondary metabolites have medium to high non-polar properties. This data is verified from the research of Arunkumar and Muthuselvam, (2009), where aqueous extracts of *Aloe vera* have no antimicrobial activity against *S. aureus, P. aeruginosa,* or *E. coli*. The major phytocomponents of *Aloe vera* have been identified through GC-MS as terpenes, fatty acids, and sterols, which include phytol, oleic acid, 9,12,15-octadecatrienoic acid methyl ester, and sitosterol (Arunkumar and Muthuselvam, 2009).

Selected herb species have been tested as controls for the efficiency of plant extraction and antimicrobial assay testing. The antimicrobial effects from *Aloe vera*, rosemary and pomegranate skin confirm that the methods used to screen for the antimicrobial potentials of wild tomato relatives are sufficient to yield enough antimicrobial compounds to be effective. It also shows that different solvents are able to extract diverse compounds from the plant material with varied antimicrobial effects, seen in the pomegranate skin which was only antimicrobial from the water, methanol and ethanol solvent extractions suggesting polar compounds are responsible for the antimicrobial effect.

Antimicrobial compounds within herb species are often documented. These are linked to antimicrobial activity through their presence as a major constituent within the plant material. An example of typical methods used to analyse the properties of plant extracts can be found in the research from Arunkumar and Muthuselvam, (2009), where aqueous, methanol, and acetone extracts of *Aloe vera* L. were investigated for antimicrobial activity using disc diffusion assays, and GC-MS analysis was used to identify the main bioactive phytochemical compounds. The complex mixtures of crude plant extracts, makes the identification of individual compounds challenging. Further antimicrobial tests can be performed using a purified fraction of such compounds; the purification can be time consuming and involve large concentrations of crude extract to yield a far reduced concentrated final sample. The literature also lacks the elucidation of the specific antimicrobial mode of action of many of these natural compounds. Metabolomic analysis could be a useful approach to analyse natural antimicrobial compound effects on the bacteria metabolomics, and to elucidate the mode of action of these compounds.

Overall, there seems to be a correlation between the types of antimicrobial compounds extracted from either the fruit or leaf material, where the fruit was shown to be antimicrobial from polar water extracts, and the leaf material was shown to be antimicrobial from more non-polar solvents ranging from methanol to hexane. This suggests that the water extractions of the fruit of the wild tomato relatives as described in the previous chapter (Chapter 3 Figure 3-6 and Figure 3-7) were sufficient to investigate the potential antimicrobial activity.

4.3.2 Solanaceae Species

The antimicrobial activities of the Solanaceae include a range of chemical classes, such as alkaloids, phenolic acids, terpenoids, and sugar esters. *Capsicum* spp., such as green finger chilies and red Scotch bonnet, were inhibitory from all solvent extractions, compared to the green sweet pepper which was not. This confirms the well documented antimicrobial effects of capsaicin (Cowan, 1999), which is present in high quantities of the hot finger chillies and Scotch bonnet, and the sweet pepper contains no capsaicin. The capsaicinoids are alkaloids synthesised through the cinnamic acid pathway and are responsible for the chili peppers hot flavour, these compounds can be found in various concentrations in the form of capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin (I and II), homodihydrocapsaicin (I and II), *N*-vanillyl octanamide, *N*-vanillyl nonanamide, and *N*-vanillyl decanamide (Contreras-Padilla and Yahia, 1998). The fact that the antimicrobial effects can be observed from all eight of the solvent extractions shows that capsaicin would be a very useful compound to use for antimicrobial comparisons with the *S. pennellii* antimicrobial compounds which are also widely dissolvable in the eight solvents.

Further analysis of other Solanaceous plant species using antimicrobial chloroform extracts could highlight the importance of the Solanaceae family as a rich source of unidentified antimicrobial compounds. Sugar esters are present in a range of Solanaceae species, such as the fruits of Cape gooseberry (*Physalis peruviana*) which contain the fatty acid disaccharides 3-*O*-*β*-D-glucopyranosyl-(1-6)-*β*-D-glucopyranoside of ethyl 3-hydroxyoctanoate and the diastereomeric 3-*O*-*α*-L-arabinopyranosyl(1-6)-*β*-D-

glucopyranosides of (3R) and (3S)-butyl 3-hydroxybutanoate (Mayorga et al., 2002). The fruit of Cape gooseberry (*P. peruviana*) also contains glycosidically bound hydroxyl acids, some of which include 3-hydroxybutanoate, 2-methyl propanoic acid, and decanoic acid (Mayorga et al., 2001). This shows that while the leaf material is an important source of sugar esters, fruit could be equally important organs to consider for antimicrobial analysis. The glandular trichome exudates of *S. pyracantum* showed that glucose esters such as 3,4-di-*O*- and 2,3,4-*tri*-*O*-acylated glucose esters were a major proportion of the non-volatile compounds present in leaf material extracted in chloroform (King et al., 1988).

Antimicrobial activity has been observed from the chloroform extracts of the wild tomato relatives (Chapter 3, Figure 3-1), and the initial screening of other Solanaceous species such as the chloroform extract of Apple of Peru, which had antimicrobial activity against *S. aureus* 25923 (Table 4-3). This leads to the hypothesis that phytochemicals with non-polar properties are widely antimicrobial from the Solanaceae family. This activity could be from sugar esters which have amphiphilic properties, and are produced in high concentrations from *S. pennellii* which was antimicrobial from the wide range of solvent extracts. Other phytochemicals, such terpenes, terpenoids, sterols, and fatty acids are candidates for the antimicrobial activity due to their non-polar solubility.

There was also some antimicrobial potential seen from *N. rustica* against *E. coli* O157:H7 from the non-polar extracts (Table 4-3). The trichomes from the tobacco species are known to produce diterpenes in the form of cembratrienols (CBTols), such as α- and β-2,7,11-cebratriene-4-ol which composes the sticky exudate on the tobacco leaf surface (Guo and Wagner, 1995). Tobacco species which produce high levels of CBTols also exhibit enhanced aphid resistance (Wang et al., 2004), and CBTols *cis*-abienol and sugar esters are found to be toxic to tobacco aphids (Severson et al., 1994).

4.3.3 Chemical standards

The FLUOstar growth curves were initially performed using LB broth. The results, however, showed no inhibition from any of the phenolic acids tested, therefore MOPs x

10 minimal media was used to create conditions where the bacteria are not supplied with the optimal additional nutrients to support growth. When the bacteria are under limiting conditions it is easier to detect when a compound is having a negative effect on bacterial growth, because the bacteria are unlikely to overcome the stress. The bacteria are also less likely to resist compound inhibition from bacterial mutations due to a low cell density. LB was used consistently to grow *S. aureus* 25923 because the bacteria would not grow in x 10 MOPs minimal media.

Phenolic, terpenoid, glyoalkaloid and sterol compounds were tested against *E. coli* and *Salmonella*. These compounds were all specifically chosen because they are produced by species of the wild tomato relatives, and several have known insecticidal, toxic, or antimicrobial properties (Chapter 1 Table 1-1 and Table 1-2). There was no antimicrobial activity from the terpenoid methyl-ketones 2-tridecanone, or 2-undecanone, which are known to be produced by certain species of *S. habrochaites* (Fridman et al., 2005; Schmiller et al., 2010). The terpenoid *β*-caryophyllene is a terpenoid which is produced in the essential oil of many plant species, and Duarte et al., (2007) found that *trans*-caryophyllene had an MIC of >1000 µg mL^{-1} against two serotypes of *E. coli* (ETEC 5041-1 and EPEC 0031-2). The terpenoid *α*-pinene was the only compound to show negative effects upon the Gram-negative bacteria in the form of an increased lag phase, whilst the other tested terpenoids generally enhanced bacteria growth. Although there was no activity against the Gram-negative bacteria, including *E. coli* O157:H7, *S.* Typhimurium 10248, or *S.* Enteritidis S1400 as part of this research, *trans*-caryophyllene was shown to be antimicrobial against *S. aureus* 25923. *S. aureus* was also shown to be susceptible to all the tested terpenoids, with the highest inhibition from *α*-pinene (Figure 4-11).

The phenolic acids chlorogenic acid and caffeic acid were significantly inhibitory to *S. aureus* 25923, *E. coli* O157:H7 and *S.* Enteritidis S1400 (Figure 4-5). The phenolic acids were the only class of compounds to show consistent antimicrobial activity against both Gram-positive and Gram-negative bacteria. Although in several cases the phenolic acids increased the growth of the tested bacteria, with ferulic and vanillic acid having a longer growth rate for *S.* Enteritidis S1400, and ferulic acid and sinapic acid also showed a slightly delayed stationary phase in *E. coli* 25923.

There have been previous studies investigating the breakdown of ferulic acid by bacteria as part of complex lignin degradation. The bacteria studied were *Pseudomonas fluorescens* AN103, and it was found to utilize ferulic acid, caffeic acid, vanillin, vanillic acid, and protocatechuic acid (Narbad and Gasson, 1998). It is possible that these compounds which confer an increase in growth as part of this study are likely to be utilised for bacterial metabolism, in their initial form or as a broken down product of the original compound, as vanillic acid, and protocatechuic acid have been proposed as intermediated in the degradation of ferulic acid (Narbad and Gasson, 1998). The antimicrobial potential of phenolic acids used as preservatives against *E. coli* within meat storage has been investigated and the results showed that synergistic effects can be found between combinations of gallic caffeic, rutin and quercetin primarily at 4^{o}C and concentrations of 100 to 200 µg mL^{-1} (Vaquero et al., 2010).

The phenolic acids had mixed effects on the bacteria growth, with vanilic acid increasing growth and chlorogenic acid decreasing growth for the Gram-negative bacteria (Figure 4-5). The glycoalkaloids had a consistent antimicrobial effect upon the Gram-negative bacteria, particularly *α*-solanine (Figure 4-7 and Figure 4-8). Studies involving mice which were prophylactically treated with glycoalkaloids from a water-alcohol extract of sprouts from potato (*S. tuberosum*), were subsequently more resistant to a lethal challenge with *S.* Typhimurium (Gubarev et al., 1998). The potato sprout extract is known to contain the glycoalkaloids solanine, chaconine, and demissin, which have toxic properties to animals in high doses (30 mg kg^{-1}). It is believed that these compounds stimulate the host's innate immunity, allowing better protection against challenge with pathogenic bacteria such as *S.* Typhimurium (Gubarev et al., 1998).

Further analysis of this phenomenon reveals that the glycoalkaloids, such as solanine are the compounds which confer this protective effect against *S.* Typhimurium in mice, not the aglycones such as solanidine or diosgenin (Gubarev et al., 1998). Therefore, the sugar containing compounds such as glycoalkaloids have been previously shown to provide a protective effect against the invasion of *S.* Typhimurium. Within this study we found that *α*-solanine was antimicrobial against *E. coli* and *Salmonella* species (Figure 4-7 and Figure 4-8), suggesting that the protective effects seen within the mice study could have been caused by directly bactericidal effects rather than or additional to any effects upon the hosts innate immunity. The glycoalkaloids showed no inhibition

against *S. aureus* 25923, which shows there could be a specific mode of action of this compound, which prevents its affect upon Gram-positive bacteria.

The sterols showed the least amount of antimicrobial activity across all the bacteria species tested (Figure 4-13). The only slight activity was seen from stigmasterol against *S.* Typhimurium 10248. The compounds 6-keto-cholesterol and stigmasterol showed an increase in growth in *S. aureus* 25923. This suggests that free sterols have no antimicrobial effect on either Gram-positive or Gram-negative bacteria at the selected concentration. This compares with current research where rather than acting as antimicrobial agents, sterols have been found to prevent the activities of antimicrobial peptides upon membranes (Verly et al., 2008). However, there have been studies to suggest that modified sterols can have antimicrobial activities against micro-organisms, such as tosylhydrazone cholesterol derivatives which have inhibitory activities against *C. albicans* at a concentration of 1.5 μg mL^{-1} (Loncle et al., 2004). The reduced growth of *S.* Typhimurium 10248 in the presence of stigmasterol suggests that stigmasterol may have some moderate antimicrobial activity. This is supported by stigmasterol derivatives which were found to have moderate antimicrobial activity against bacteria, which included 6-α-hydroxystigmast-4-en-3-one, and stigmast-5-ene-3β, 7α,20ζ-triol (Zhao et al., 2005).

The analysis of the antimicrobial properties of several compounds which have been discussed in the literature as having antibacterial, antifungal and insecticidal properties (Cowan, 1999) have been useful to confirm that compounds widely present in tomato species can be antimicrobial. This initial analysis of known compounds has shown that certain phenolic acids, glycoalkaloids, and terpenoids such as chlorogenic acid, *α*-solanine and *α*-pinene have an antimicrobial activity against the *S.* Enteritidis S1400, *S.* Typhimurium 10248, *E. coli* O157:H7, *E. coli* 25922, and *S. aureus* 25923. There is also increased growth in the presence of ferulic acid and 2-undecanone, particularly for *Salmonella* sp. and this highlights the issue that certain metabolites can be used by the bacteria as carbon sources and nutrients. When a plant extract is collected it will contain a variety of metabolites which could be both beneficial and harmful to the bacteria and the antimicrobial properties of the metabolites in a plant extract could be masked by the beneficial metabolites. There will be a need to separate secondary metabolites from each other within an extract through collecting fractions using different solvents.

The pure standards tested for antimicrobial activity within this chapter show that secondary metabolites found within plants from different classes can have individual effects on bacteria growth, and therefore, there may be synergistic or opposing effects from a mixture of these compounds within a crude plant extract. Plants can be extracted for specific classes of compounds using combinations of polar and non-polar solvents, and through isolating major classes of compounds through targeted extractions; individual compound effects can be assessed. This reduces the interactions between multiple compound types within the samples which can lead to synergistic and conflicting effects.

Through isolating compounds directly from trichomes the interaction of other leaf metabolites can be reduced, and the secondary metabolite pool can be restricted for better identification of potentially antimicrobial compounds. Analysis of isolated fractions of plant extracts using GC-MS identification can determine which classes of compounds are responsible for the discovered antimicrobial activities. Comparisons between these identified compounds and the pure compound antimicrobial results can be made which has the potential to strengthen the hypothesis that specific compounds are responsible for the observed antimicrobial potential.

Chapter 5

Identification of antimicrobial compounds

5.1 Introduction

The wild tomato relative screen described in Chapter 1 identified two main wild tomato accessions possessing antimicrobial activity. Additional Solanaceae antimicrobial analysis demonstrated that *N. rustica* and *S. melongena* have antimicrobial activities. All of these antimicrobial Solanaceae plants have a high trichome load, and therefore it is hypothesised that the antimicrobial secondary metabolites are likely to be produced from the trichomes of these plants.

This chapter describes the identification of antimicrobial compounds from the antimicrobial Solanaceae species, which includes *N. rustica*, *S. melongena, S. pennellii, S. habrochaites,* and *S. lycopersicum* cv. M82 used as a negative plant control. The start of this chapter includes the identification of compounds from the solvent extracts of dried plant material, which looks into the identification of the phenolic and isoprenoid compound classes within the plant material. Thin layer chromatography (TLC) and solid phase extraction (SPE) was used to separate and identify the active fractions for further analysis. SPE was utilised as part of the research within this chapter to separate and concentrate compound classes for a more targeted analysis of extracts. GC-MS analysis of fractions collected through TLC or SPE was used as the primary mode of identification using the NIST database (Chapter 2 sections 2.2.5, 2.2.4, and 2.5.3) and through comparisons with chemical standards.

5.2 Results

5.2.1 Analysis and identification of dried leaf material extracted with chloroform

Thin layer chromatography (TLC) was used to separate the chloroform extracts from wild tomato relatives into bands using silica gel and a solvent system of hexane:chloroform:methanol (5:5:0.7 v/v). The bands were recovered from the silica scrapings through centrifuging in chloroform:methanol (9:1 v/v). These bands were subjected to alliance identification and disk diffusion analysis.

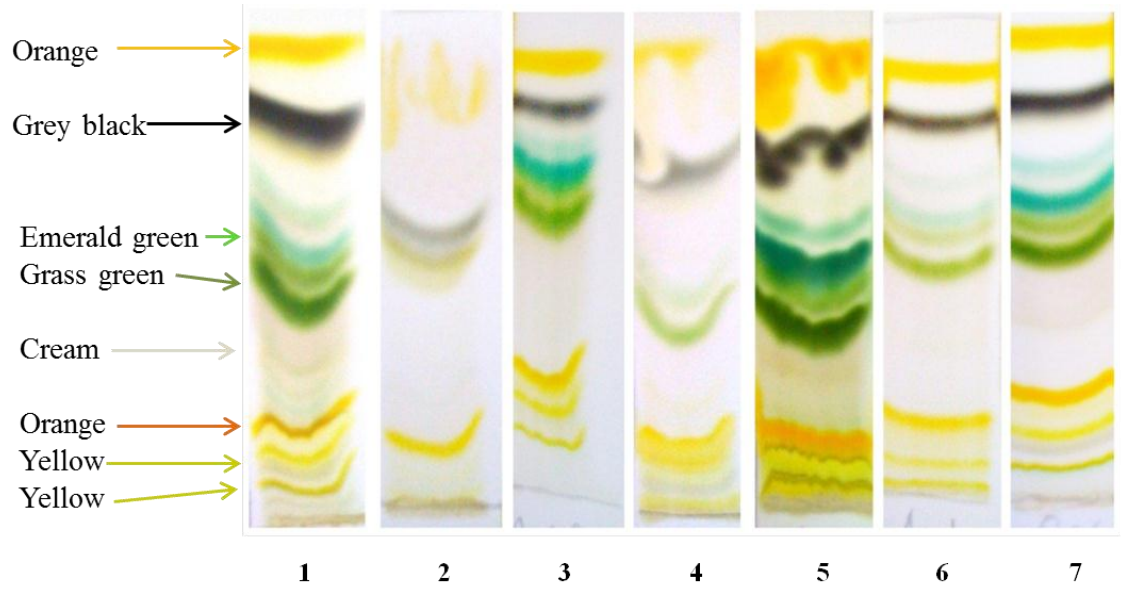

Figure 5-1.TLC plates of Solanaceae leaf extracts.

Bands show the seperation of pigmented compounds from the chloroform extracts of Solanaceae species leaf material on silica gel and a solvent system of hexane:chloroform:methanol (5:5:0.7 v/v), 1. *S. lycopersicum* cv. M8, 2. Rosemary, 3. Apple of Peru, 4. Finger chilli, 5. *S. chmielewskii*, 6. Aubergine, 7. *S. peruvianum*. The bands were taken based on colour and R_f values, and the *S. lycopersicum* cv. M82 TLC strip has been labelled to show the regions which were investigated.

TLC purification of chloroform extracts from *Rosmarinus officinalis* (Rosemary) and Solanaceae species such as *S. lycopersicum* cv. M82, *Nicandra physalodes* (Apple of Peru), *Capsicum annuum* (Finger chillies), *S. peruvianum*, *S. chmielewskii*, and *S. melongena* (Aubergine) were run on an Alliance HPLC system (Table 5-1). The main peaks identified were *β*-carotene, pheophytin, chlorophyll, and lutein from all the tested plant extracts. Although the R_f values of each plant extract varied, the band colours and identifications of the green and black bands as chlorophylls and the yellow and orange bands identified as the carotenoids remained consistent across the plants.

The wild tomato relatives had a higher number of visible coloured bands, and *S. chmielewskii* had the strongest colour intensity from the green bands of chlorophyll. The wild tomato relative bands ran with consistently good separation of coloured compounds with this selected solvent system and silica TLC plates, compared to the Rosemary and Finger chillies which has the poorest stability in band separation (Figure 5-1).

Table 5-1. Identifications compounds on TLC from chloroform extracts of 1 g of dried plant leaf material.

Average R_f values	Band Number/Colour	*S. peruvianum*	*S. melongena* (Aubergine)	*Nicandra physalodes* (Apple of Peru)
0.85	Orange	*β*-carotene	*β*-carotene	*β*-carotene
0.74	Grey black	Pheophytin a	Pheophytin a	Pheophytin a
0.64	Emerald green	Pheophytin a	Chlorophyll b and Lutein	Pheophytin a
0.49	Grass green	Chlorophyll b	Chlorophyll b and Lutein	Chlorophyll b
0.35	Cream	-	Pheophytin a	Pheophytin a and b
0.22	Orange	Lutein	Lutein	Lutein
0.14	Yellow	Unknown carotenoid	Lutein and Luteoxanthin	Unknown carotenoid
0.07	Yellow	Luteoxanthin	Luteoxanthin	Luteoxanthin

Identification based on co-chromatography and similar spectral properties with authentic standards using an Alliance HPLC-PDA, selecting the primary identified peak(s). In the case were no standards were commercially available then reference data was utilised (Britton et al., 2004).

Disc diffusion assays were performed using the TLC bands from the chloroform extracts of dried leaf material. The results were highly variable, and inhibiton was generally relatively low compared to the ciprofloxacin antibiotic control for most of the plant species, with the exception of Rosemary fractions against *S. aureus* 25923. The plant extract TLC bands were compared to the DMSO control zone of inhibtion diameters (two-way ANOVA Dunnetts multiple comparisons test, 95% CI), and the results are represented in Figure 5-2. Rosemary TLC bands with an R_f of 0.5, 0.35 and 0.2 had a significant level of antimicrobial activity similar to the antibiotic control against *S. aureus* 25923 (P<0.001). When all the plants were compared it is difficult to see patterns of bands at R_f values which consistently had inhibitory effects against all the tesed bacteria, such as R_f 0.95 of which *S. peruvianum* was not shown to be antimicrobial, but all the other tested plants were antimicrobial to at least one of the tested bacteria.

E. coli O157:H7 had the highest susceptibility generally against the plant extracts, although the effects of the DMSO should be taken into account, and is likely to be responsible for either synergistically acting with the plant compounds, or directly inhibiting the bacteria. All the plants tested had at least one TLC band extract which was significantly different to the DMSO control for *E. coli* O157:H7, *S.* Typhimurium 10248 and *S. aureus* 25923 (P<0.001).

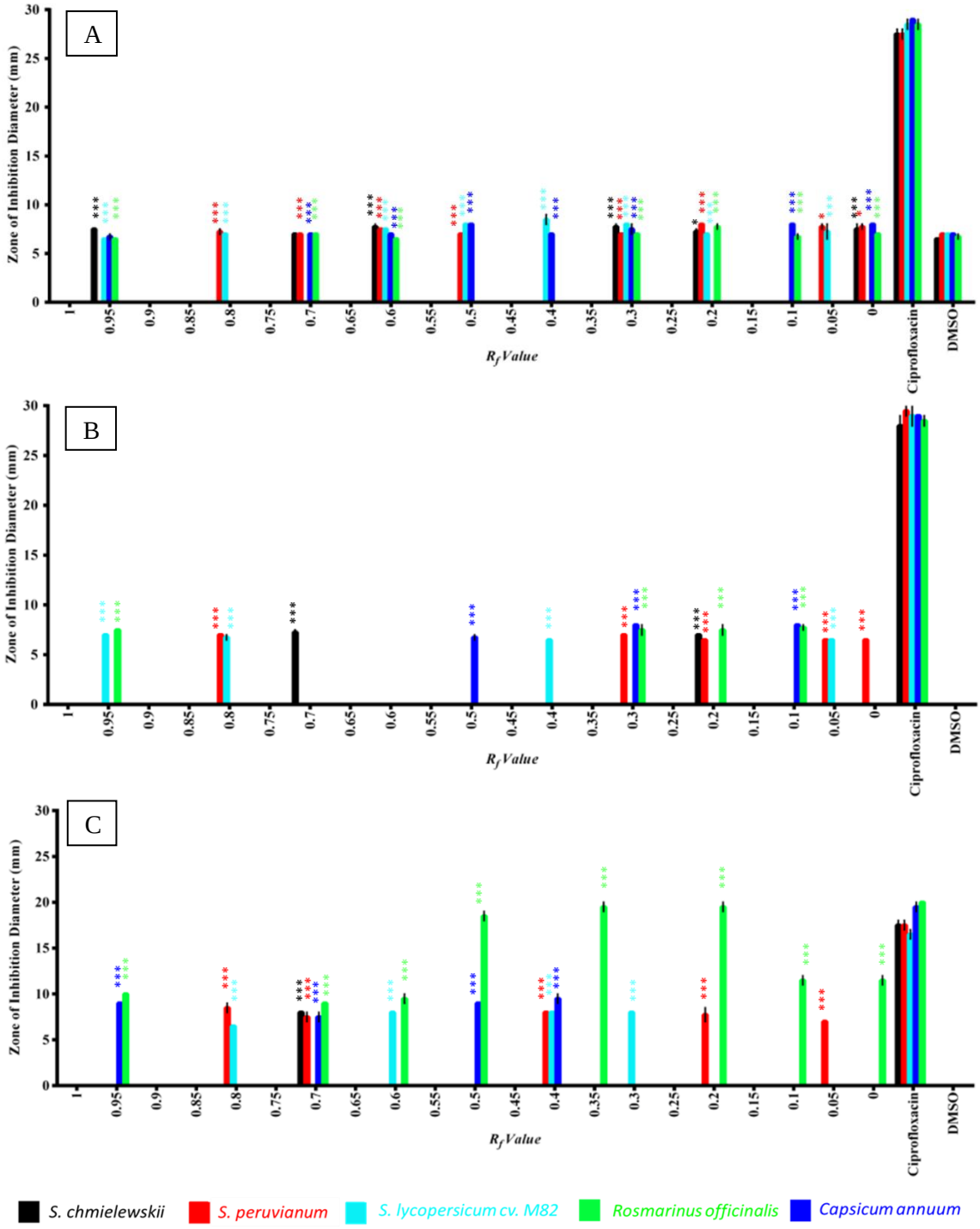

Figure 5-2. Disc diffusion assay for antimicrobial activityof chloroform extracts of dried leaf material.

S. chmielewskii (0.29 mg disc^{-1}), *S. peruvianum* LA2744 (0.58 mg disc^{-1}), *S. lycopersicum* cv. M82 (0.43 mg disc^{-1}), *Capsicum annuum* (Finger chillie) (0.43mg disc^{-1}) and Rosemary (0.7 mg disc^{-1}), A. *E. coli* O157:H7, B. *S.* Typhimurium 10248 C. *S. aureus* 25923. Error bars represent the standard error from two biological replicates and two technical replicates. The asterisk (*=P<0.05 and ***=P<0.001) represents the significant difference between the zone of inhibition diamerter (mm) of bacteria grown in the presence of the extract compared to the DMSO control, using two-way ANOVA Dunnetts multiple comparisons test (95% CI).

5.2.1.1 Identification of compounds using agilent HPLC

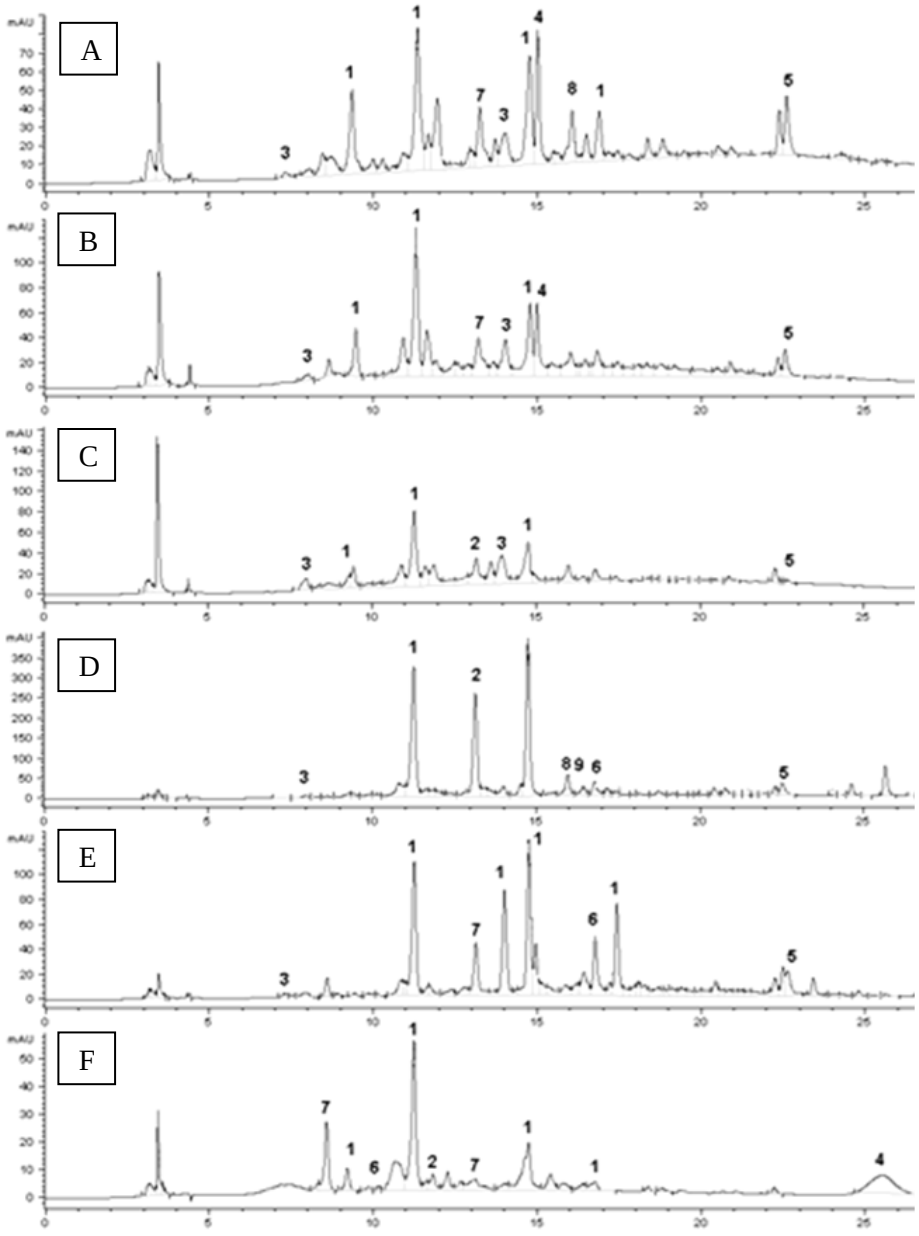

Figure 5-3. Chromatograms from HPLC Agilent analysis of acidified water:methanol (80:20 v/v) extracts of wild tomato relatives.

Plant extract chromatograms; A. *S. lycopersicum* "cerasiforme" LA2675, B. *S. lycopersicum* cv. M82, C. *S. peruvianum* LA2744, D. *S. habrochaites* LA1779, E. *S. pennellii* LA0716, F. *S. habrochaites* LA1918. The identified peaks are numbered; 1. chlorogenic acid, 2. caffeic acid, 3. cinnamic acid, 4. rutin, 5. quercetin, 6. ferulic acid, 7. isoferulic acid, 8. *p*-coumaric acid, 9. sinapic acid. They were detected at 320 nm. The chromatograms are an example of one technical replicate from a pooled sample of three plant replicates.

Various wild tomato species were extracted in methanol:water (80:20 v/v) and acidified using HCL (1% v/v), for analysis on the Agilent HPLC system. Identified peaks are numbered in Figure 5-3, and they were detected at 320 nm, through comparisons with the retention times and spectra of chemical standards. The main peaks were chlorogenic acid, rutin, and caffeic acid. The peaks were similar between all the plant species with most of the peaks running between 8 and 17 min. *S. pennellii* LA0716 which has been shown to have antimicrobial activities was shown to have several derivatives of chlorogenic acid which were not present within the other plant species.

5.2.2 Identification of active fractions from fresh leaf trichomes extracted with chloroform

Extraction of the leaf trichomes through directly soaking the leaves in chloroform was selected as the method for further isolation of potentially antimicrobial secondary metabolites from the plants under investigation. *S. pennellii* trichomes produce high levels of sugar esters. Therefore, *S. pennellii* trichome extracts were separated into two fractions using a mixture of hexane and methanol:water (50:50 v/v) according to the method described by Mudd et al., (1988). The green hexane fraction was hypothesised to contain non-polar fatty acids, and the methanol:water yellow oily residue fraction was thought to be the fraction with the isolated more polar sugar esters.

It was found through testing the extracts in growth curve analysis that for *S.* Typhimurium 10248 the water:methanol (50:50 v/v) ($P<0.001$) and the hexane ($P<0.01$) fractions were significantly different from the DMSO control. *S. aureus* 25923 was also inhibited from both extracts with significantly different AUC compared to the DMSO control ($P<0.001$) and with no linear growth seen from either extract (Figure 5-5). GC-MS analysis of the extracts showed overlapping regions of peaks between 55 to 62 min, with two extra regions within the polar fraction between 45 to 50 min and 65 min and above (Figure 5-4).

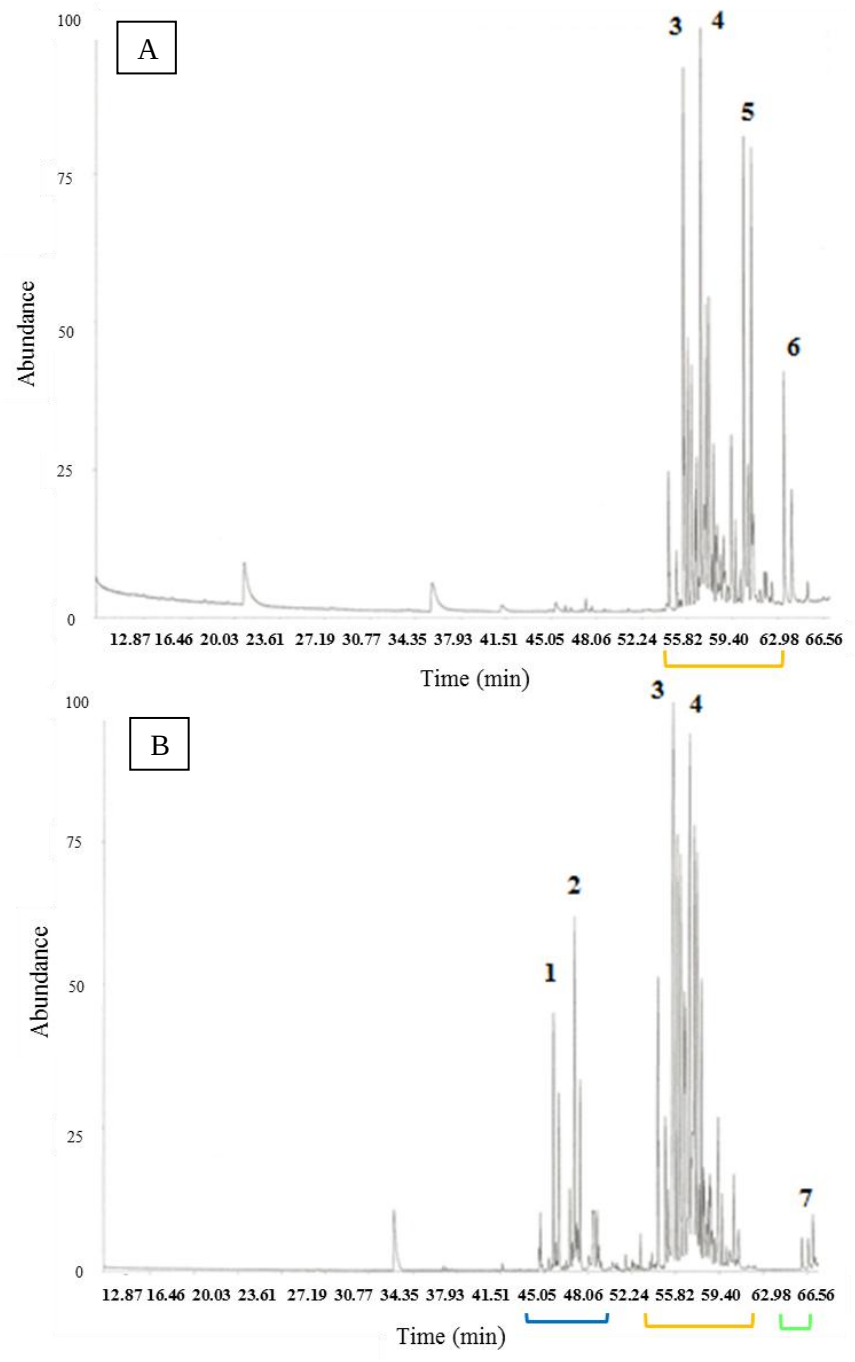

Figure 5-4: GC-MS spectra from *S. pennellii* trichome extracts; A. hexane fraction, and B. water:methanol (50:50 v/v).

Blue (45 to 50 min), orange (55 to 60 min) and green (65> min); 1. 1,3-diisobutyrin, trimethylsilyl at 46.4 min, 2. glycine, N-[(3*α*,5*β*,12*α*)-3,12-dihydroxy-, 3. 1,3-dioxlan-4-propenoic acid at 55.9 min, 4. cholic acid at 56.5 min, 5. hexatriacontane at 61.1 min, 6. tetratriacontane at 63.3 min, and 7. *α*-D-glucopyranoside, 1,3,4,6-tetrakis-*O*- 66.7 min. The GC-MS spectra show the results from an example of one technical replicate of one plant extract separated into two fractions, two further biological replicates were analysed and gave similarly identified compounds.

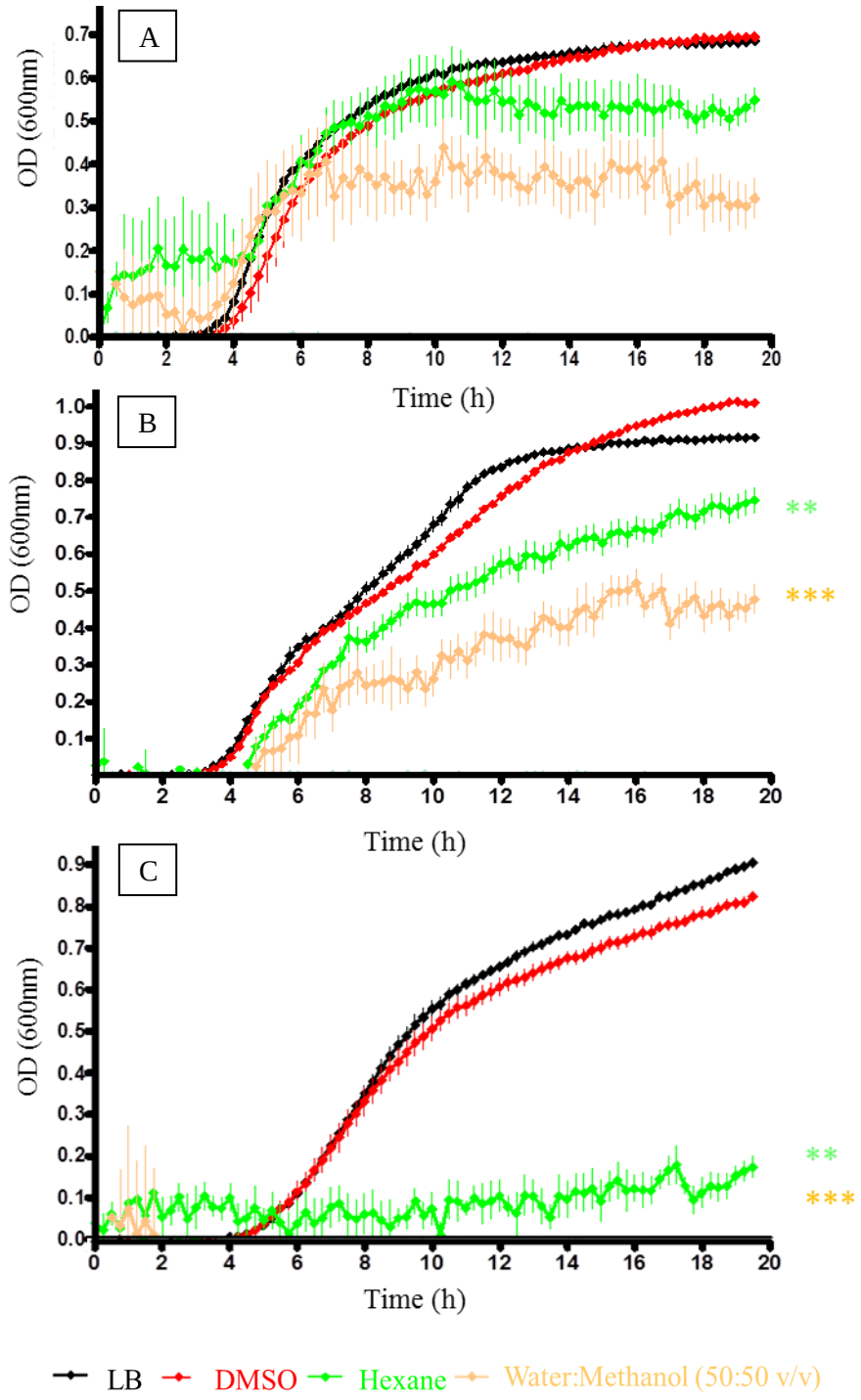

Figure 5-5. FLUOstar growth curves of bacteria grown with *S. pennellii* trichome extract separated into polar and non-polar fractions.

A. *E. coli* O157:H7, B. *S.* Typhimurium, 10248. C. *S. aureus* 25923. *S. pennellii* chloroform trichome extract, sub fractioned with polar methanol:water (50:50 v/v), and non-polar hexane. The extracts were re-suspended in DMSO and tested in LB (5% v/v) to a concentration of 12.5 mg mL^{-1}. Error bars represent the standard error from a pool of three biological replicates, and three technical replicates. The asterisk (*=P<0.05 and ***=P<0.001) represents the significant difference between the zone of inhibition diamerter (mm) of bacteria grown in the presence of the extract compared to the DMSO control, using one-way ANOVA Dunnetts multiple comparisons test (95% CI).

5.2.2.1 Disc diffusion assays of TLC bands from Solanaceae trichomes

The trichome extractions from *S. pennellii* and other plant species were separated and purified using the methods described by Mudd et al., (1988), where aliquots (5 mL) of extracts from fresh leaves (10 g) dipped in chloroform (50 mL) were run by TLC on silica gel plates using a solvent system of hexane:diethyl ether:glacial acetic acid (60:40:2 v/v) to reveal bands separating at R_f 0.15 (glycolipids), R_f 0.3 (flavonoids), R_f 0.5 (terpenoids) and R_f 0.9 (hydrocarbons). The active fractions from disc diffusion assays of the scraped bands were in the hydrocarbon and glycolipid regions of the plates (Mudd et al., (1988).

The trichome TLC bands from *S. pennellii* LA0716, *S. habrochaites* LA1777, *N. rustica*, *S. melongena*, and *S. lycopersicum* cv. M82 were tested for antimicrobial activity against *E. coli* O157:H7, *S.* Typhimurium 10248 and *S. aureus* 25923 using a disc diffusion assay (Figure 5-6). The results of these disc diffusion assays showed a similar pattern to the one seen from the chloroform extracts of dried leaf material (Figure 5-2). The significant antimicrobial activity seen compared to the DMSO control was from *S. pennellii* R_f 0.95 and 0 (P<0.001) against *S. aureus* 25923. Significantly difference from the DMSO control was also observed from R_f 0.35 to 0.15 (P<0.001) against the Gram-negative bacteria. The highest antimicrobial activity observed was from the *S. habrochaites* LA1777 trichome extract TLC band at R_f 0.55 (P<0.001) against *S. aureus* 25923, where the bacteria was inhibited to a level above the antibiotic control, and this inhibition was not seen against the Gram-negative bacteria.

Additional significant antimicrobial activity compared to the DMSO control was found from *N. rustica* TLC bands at R_f 0.45, 0.35, 0.05 and 0 (P<0.001) against *S. aureus* 25923. There was varied antimicrobial activity from *S. melongena* and *S. lycopersicum* cv. M82 with the most significant TLC band extracts between R_f 0.2 and 0 against *S. aureus* 25923 and *S.* Typhimurium 10248.

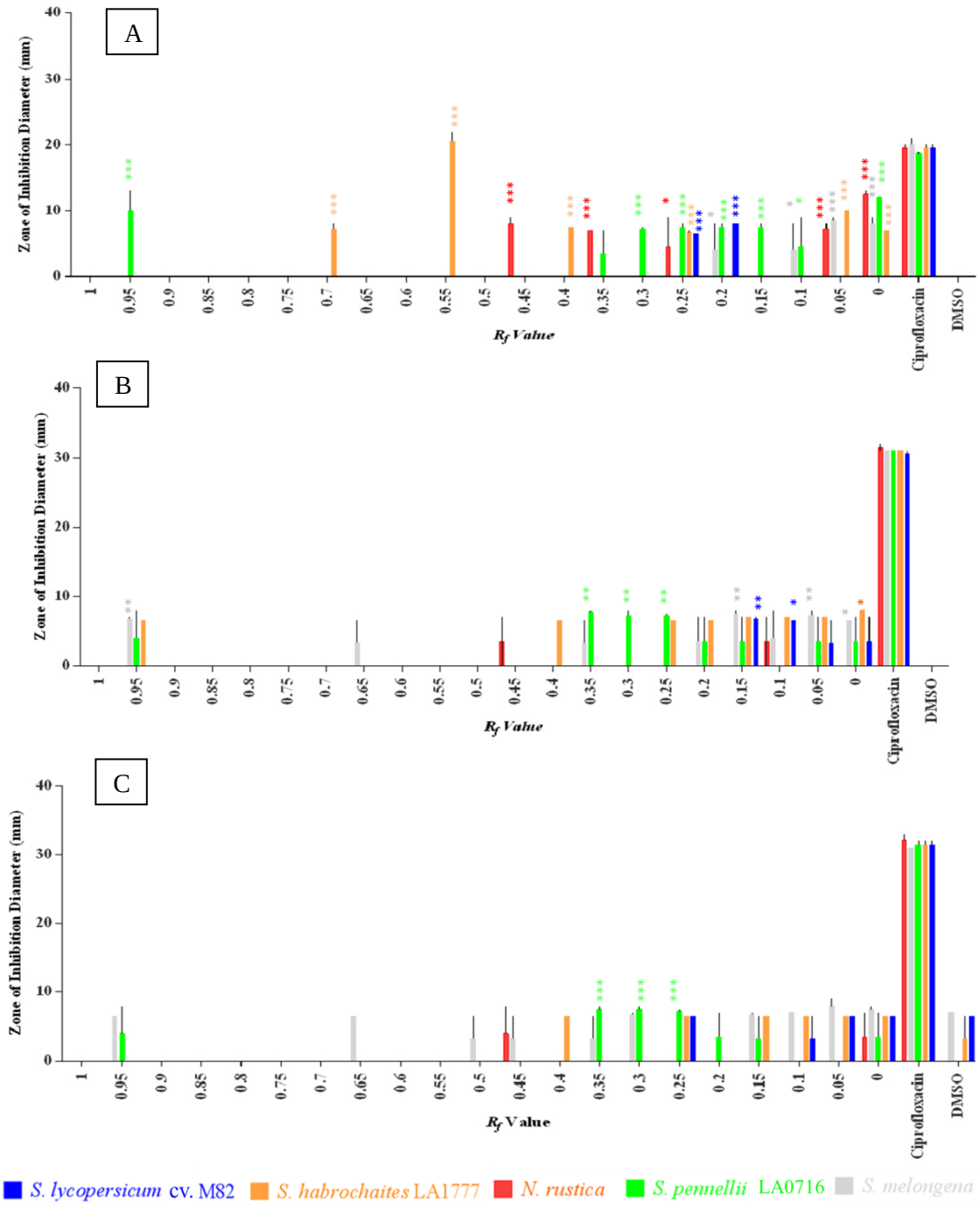

Figure 5-6. TLC bands from trichome chloroform extract.

Extracts were re-suspended in 200 µL DMSO and 10 µL placed on filter paper discs for zones of inhibition diameters in mm, *S. lycopersicum* cv. M82 (blue, average 0.4 mg disc^{-1}), *S. habrochaites* LA1777 (orange, average 0.6 mg disc^{-1}), *N. rustica* (red, average 0.2 mg disc^{-1}), *S. pennellii* LA0716 (green, average 0.6 mg disc^{-1}), and *S. melongena* (grey, average 0.02 mg disc^{-1}). A. *S. aureus* 25923, B. *S.* Typhimurium 10248, and C. *E. coli* O157:H7. Error bars represent the standard error of three biological replicates, and two technical replicates. The asterisk (*=P<0.05, *=P<0.01 and ***=P<0.001) represents the significant difference between the zone of inhibition diamerter (mm) of bacteria grown in the presence of the extract compared to the DMSO control, using two-way ANOVA Dunnetts multiple comparisons test (95% CI).

5.2.3 GC-MS identification of chloroform trichome extracts

Trichome extracts from leaves extracted through dipping the leaves in chloroform were analysed by GC-MS and several components were positively identified through comparisons with chemical standards, although most of the matches were identified using the NIST library database. The compounds with the highest combined match factor and probability were chosen to build a library which was used to label components as putative matches, which provided a good match for the chemical class of the component, rather than a true identified compound, although NIST matches above 90% probability and 800 match factor were generally believed to be correctly identified.

5.2.3.1 Disc diffusion assay of saponified and non-saponified trichome extracts

The inhibition zones from saponified and non-saponified trichome extracts against five bacteria *E. coli* O157:H7, *E. coli* 25922, *S.* Typhimurium 10248, *S.* Enteritidis S1400, and *S. aureus* 25923 showed that generally there were differences between the saponified and non-saponified trichome extracts. It was apparent that the non-saponified extracts of *N. rustica* had a marginal inhibitory effect upon Gram-negative bacteria particularly *E. coli* 25923 and *S.* Typhimurium 10248 ($P<0.05$), where the extract was significantly different from the DMSO control.

The opposite effect was seen from *S. lycopersicum* cv. M82, which largely was not an inhibitory extract, and where the extract was found to be inhibitory such as against *E. coli* O157:H7 and *S.* Enteritidis S1400, it was the saponified extract which had slightly more inhibitory effect. *S. pennellii* trichome extract was the only extract shown to have an inhibitory effect upon the tested Gram-positive bacterium *S. aureus* 25923.

There was also a difference between the saponified and non-saponified extracts, and there was a significant difference compared to the DMSO control for the non-saponified extract ($P<0.001$). *S. pennellii,* however, showed little differences between the saponifed and non-saponifed extracts when tested against the Gram-negative bacteria, with the exception of the non-saponified extract against *S.* Typhimurium 10248 which was significantly different to the DMSO control ($P<0.01$). When comparing the saponified and non-saponified trichome extracts through GC-MS analysis the chromatogram of *S. pennellii* changes the most dramatically (Figure 5-8 G and H). A

more in-depth analysis of these extracts shows that the identified compounds switch from being primarily carbohydrate related compounds in the non-saponified extracts to mainly sterols and miscellaneous compounds (Figure 5-9 A).

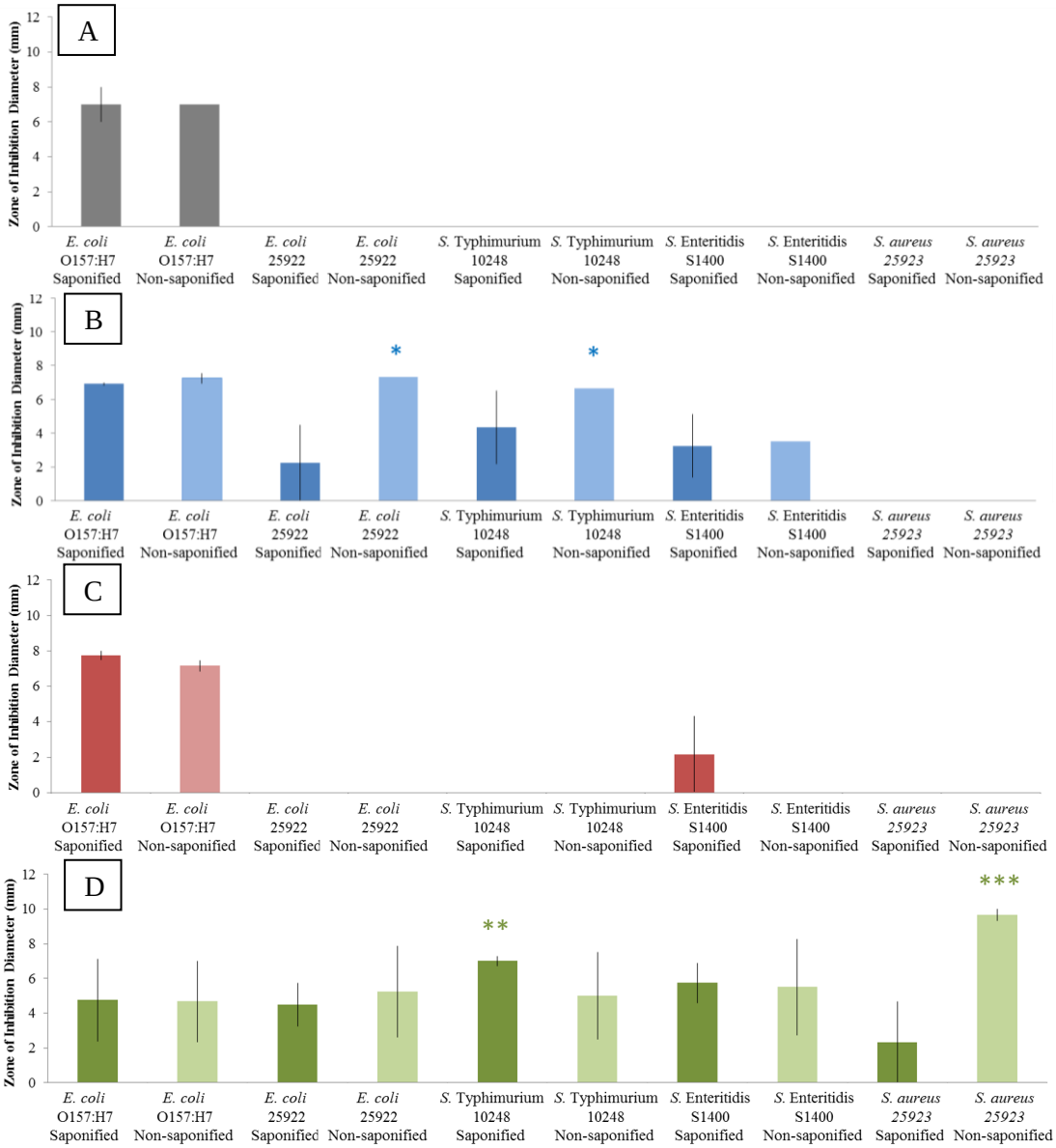

Figure 5-7. Disc diffusion assays of chloroform trichome extracts against bacteria.

Zone of inhibition diameters (mm) of bacteria *E. coli* O157:H7, *E. coli* 25922, *S.* Typhimurium 10248, *S.* Enteritidis S1400, and *S. aureus* 25923. Inhibition in response to trichome extracts saponified and non-saponified, A. DMSO, B. *N. rustica*, C. *S. lycopersicum* cv. M82 and D. *S. pennellii* LA0716. Error bars represent the standard error of three biological plant replicates and two technical replicates tested against each bacterial species. The asterisk (*=P<0.05, **=P<0.01 and ***=P<0.001) represents the significant difference between the zone of inhibition diamerter (mm) of bacteria grown in the presence of the extract compared to the DMSO control, using two-way ANOVA Dunnetts multiple comparisons test (95% CI).

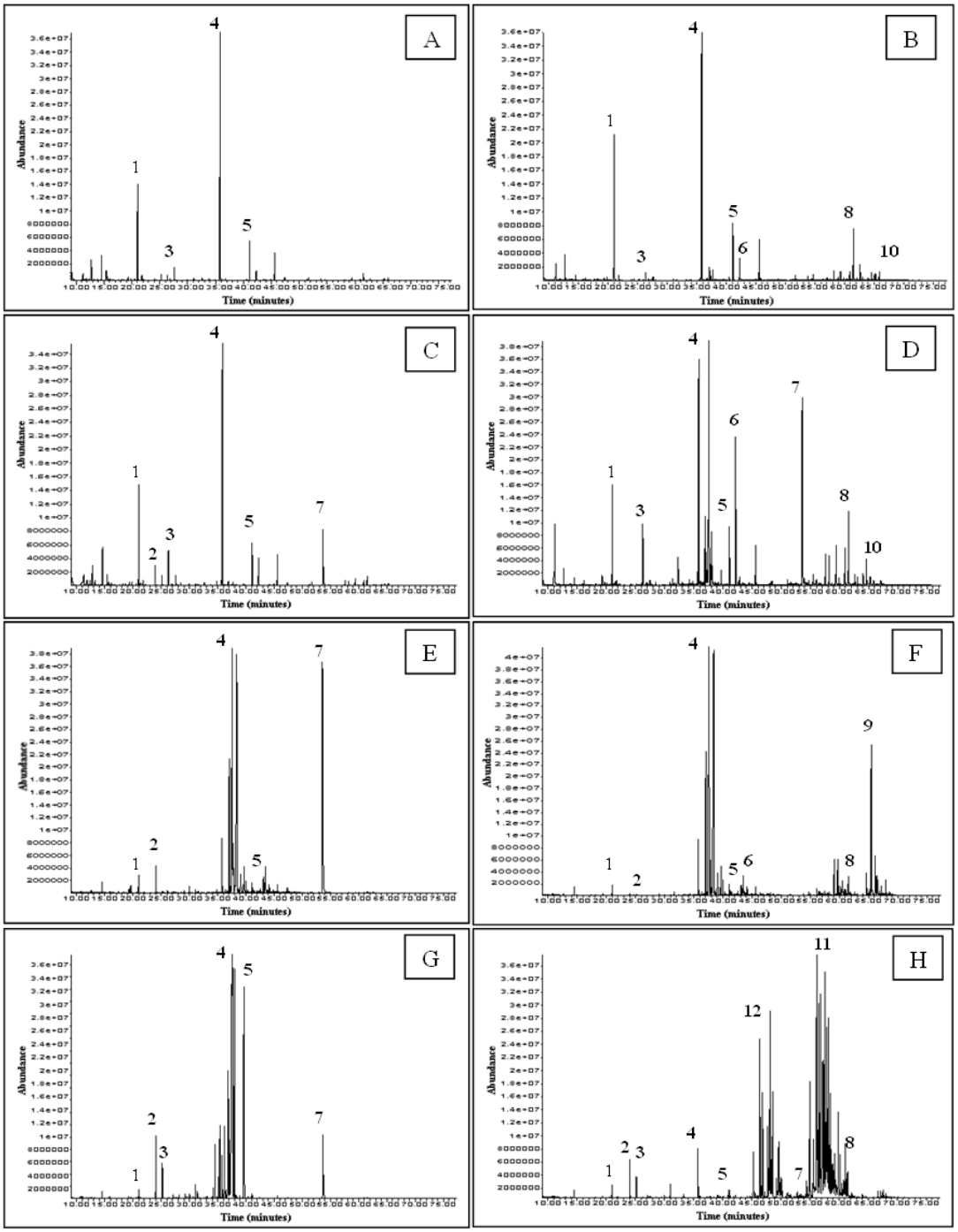

Figure 5-8. GC-MS chromatograms of saponified and non-saponified trichome extracts.

S. lycopersicum c.v.M82 (saponified [A], non-saponified [B]), *N. rustica* (saponified [C], non-saponified [D]), *S. habrochaites* (saponified [E], non-saponified [F]), *S. pennellii* (saponified [G], non-saponified [H]). Consistent peaks are labelled as; 1. Derivatisation contaminant (acetamide 2,2,2-trifluoro-*N*-methyl-*N*-(trimethylsilyl) at 21 min, 2. decanoic acid at 24 min, 3. malic acid at 26.3 min, 4. propanoic acid at 35.8 min, 5. palmitic acid at 41.1 min, 6. inositol, 1,2,3,4,5,6,-hexakis-*O*-(trimethyl) at 42.1 min, 7. sucrose at 55 min, 8. hentriacontane at 61.4 min, 9. pregnane-3,20-dione,17,21-*bis*-(trimethyl silyl)oxy) at 65.4 min, 10. *β*-amyrin at 65.2 min, 11. cholic acid at 57.2 min, and 12. 1,3-diisobutyrin at 46.4 min.

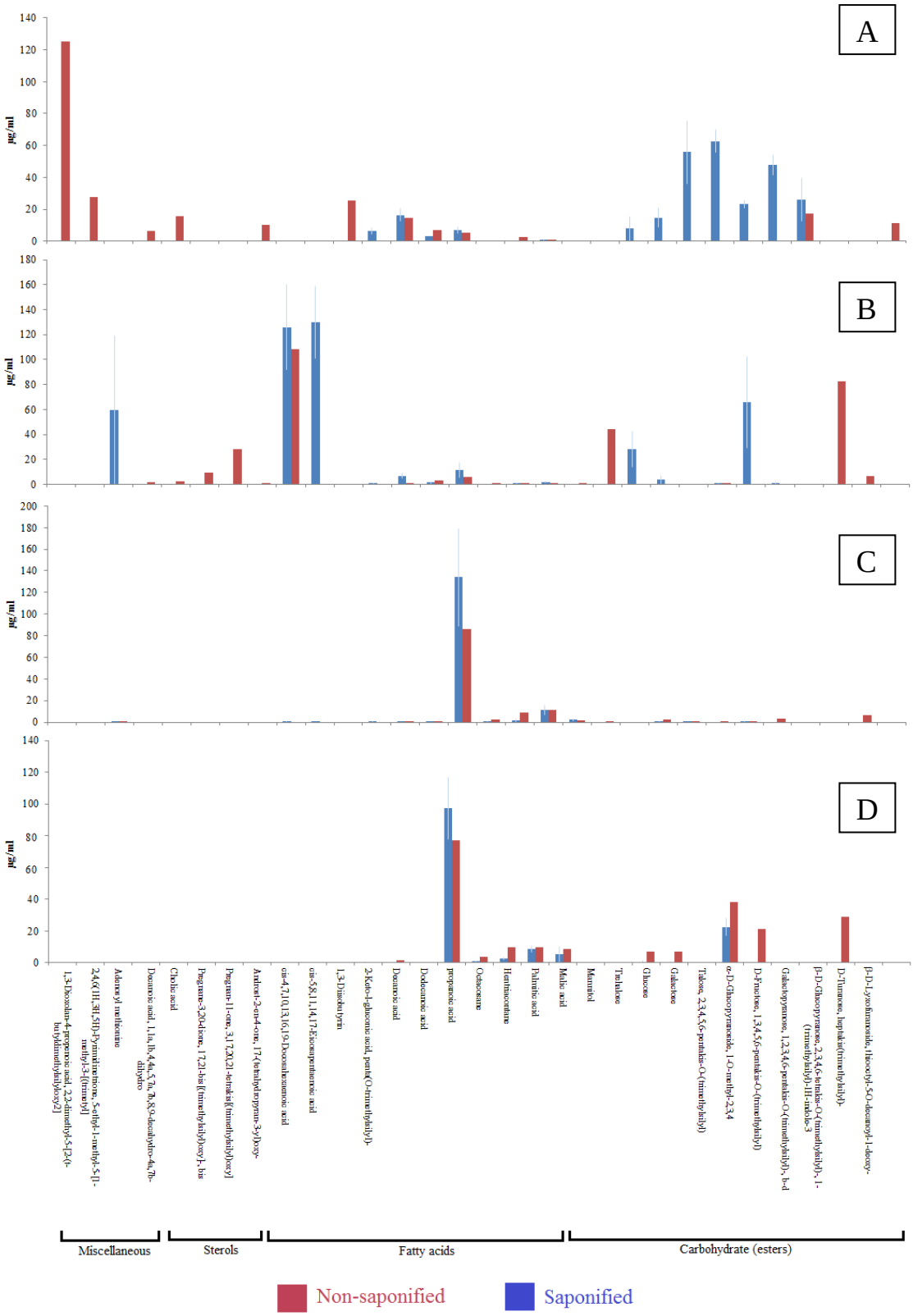

Figure 5-9. GC-MS analysis of extracts of fresh leaves extracted in chloroform.

Comparisons were made between the extract after saponification with NaOH (5% w/v) and acid neutralisation with HLC (5% v/v) (saponified in blue) and non-saponified in red. A. *S. pennellii* LA0716, B. *S. habrochaites* LA1777, C. *S. lycopersicum* cv. M82, and D. *N. rustica*. Error bars represent three biological replicates and three technical replicates, see Appendix 1 for total list of compounds.

The changes in the other plant trichome extracts are less dramatic, such as *S. habrochaites,* which retains all its compound classes but as different derivatives, such as the sugar esters which change from 2,3,4-*O*-*α*-D-glucopyranoside sugar related compounds in the non-saponified extract to *β*-turanose related compounds in the saponified extract. The concentration of adenosyl methionine and fatty acid *cis*-5,8,11,14,17-eicosapentaenoic acid in the *S. habrochaites* was increased in the saponified extract compared to the non-saponified extract, which contained sterols. *S. lycopersicum* cv. M82 and *N. rustica* had a similar profile of components, and both plants primarily produced fatty acids such as propanoic acid, this was consistent in both the saponified and non-saponified samples. *N. rustica* did however produce more carbohydrate related compounds compared to *S. lycopersicum* cv. M82, and these were primarily produced from non-saponified extracts (Figure 5-9 E and D).

5.2.4 GC-MS analysis of TLC bands of trichome extracts

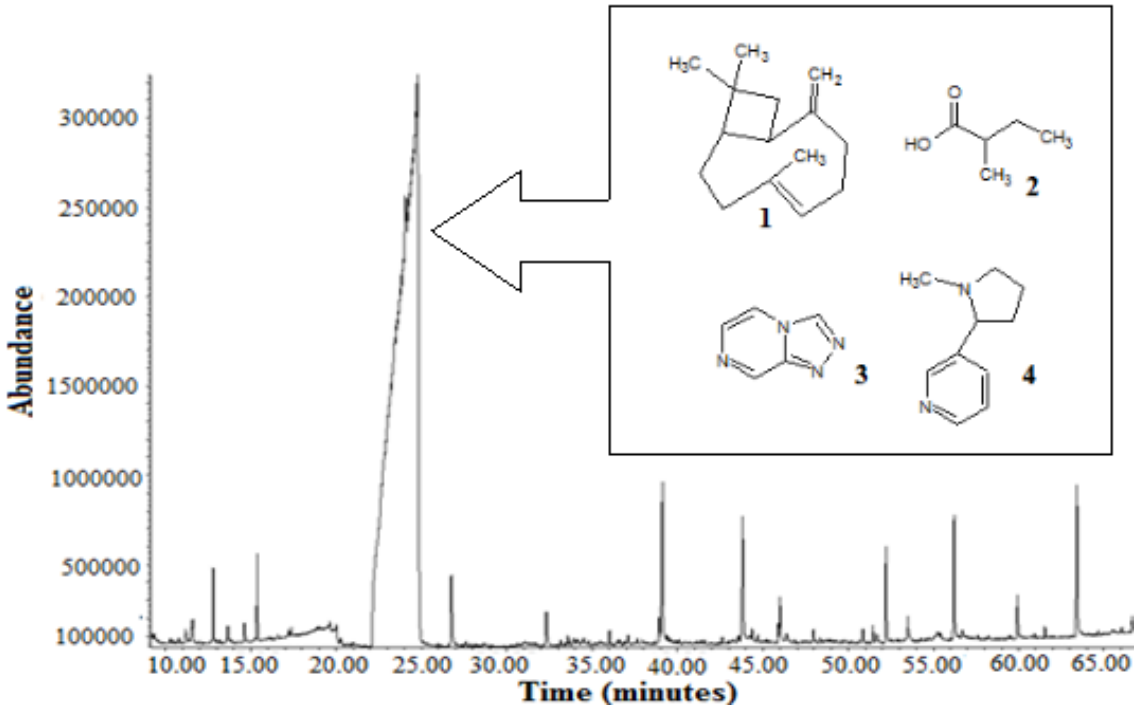

Figure 5-10. *N. rustica* TLC band (R_f 0) GC-MS chromatogram.

Components identified through NIST matches within the large peak between 22 to 25 min; 1. caryophyllene at 24.05 min, 2. 2-methylbutyric acid at 24.09 min, 3. 1,2,4-triazolo[4,3,-a]pyrazine at 24.5 min, and 4. nicotine at 24.7 min. The highest area of the peak was attributed to nicotine. Data is from one technical replicate of a pool of three plant replicates.

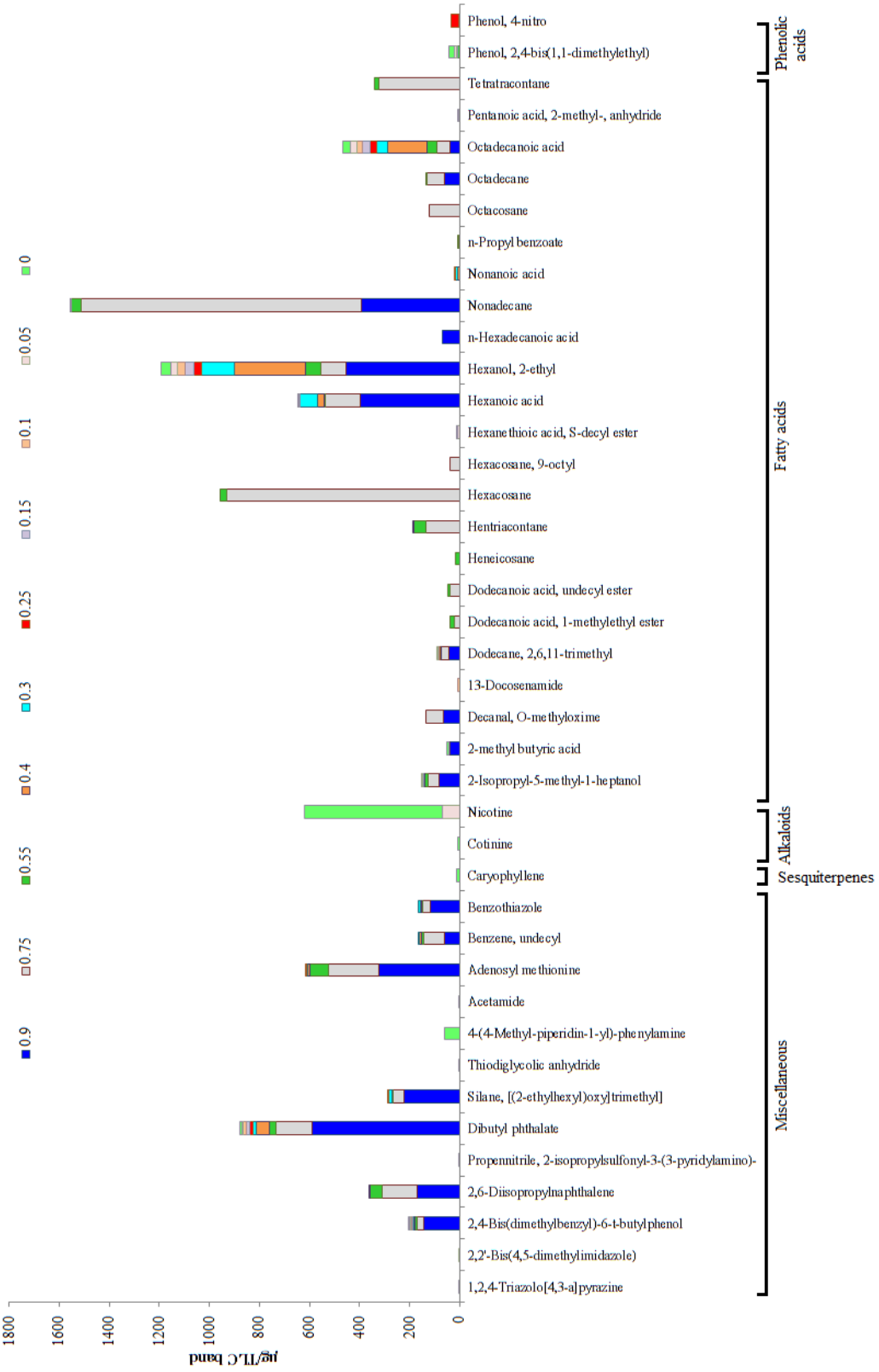

Figure 5-11. TLC and GC-MS analysis of *N. rustica* fresh leaves extracted in chloroform.

Crude trichome extracts were separated into different R_f fractions by TLC on silica gel plates in a solvent system of dichloromethane: hexane, and glacial acetic acid (40:60:2 v/v). The samples represent one technical replicate of three pooled plant samples of 12 separate TLC bands, see Appendix 2 for total list of compounds.

The trichome extracts were separated into bands which have different polarities through using TLC where silica gel plates were used with a solvent system of hexane:diethyl ether:glacial acetic acid (60:40:2 v/v). A trichome extract from 5 mL of dried fresh chloroform leaf extract was chromatographed on several plates (20x20 cm) and the bands were scraped and pooled from plates and from three biological replicates of each plant. Each plant produced an average of 12 bands, which were taken from the line of origin to the solvent front giving a good coverage of the total plate which was taken for GC-MS analysis.

The TLC bands from *N. rustica* shows that the more non-polar bands of R_f 0.9 and 0.75 contain a range of mainly miscellaneous compounds such as dibutyl phthalate and fatty acids such as nonadecane, 2-ethyl hexanol, hexacosane and hexanoic acid. The most antimicrobial extract of *N. rustica* was at R_f 0 (Figure 5-10), and the most abundant compound identified within this fraction was nicotine, along with lower levels of other alkaloids and the sesquiterpene caryophyllene. Unlike the trichome extracts from the other plants, *N. rustica* was not shown to produce any sugar related compounds. This does not compare with the full trichome extract which was shown to produce several types of sugar compounds (Figure 5-9 D).

The fractions from *S. lycopersicum* cv. M82 showed less obvious differences between the identified compounds within its TLC bands, where most of the fractions contained a certain amount of fatty acids, such as palmitic acid, octadecane, heneicosane, and hexadecanoic acid 2,3-*bis*[(trimethylsilyl)oxy]propyl ester (α-glyceryl palmitate). There were several fractions which selectively contained specific compounds different to the rest of the sample, such as R_f 0.3 which contained the most sterols in the form of benzothiazole and β-amyrin. The band at R_f 0.2 contained a mixture of the sterol betulin and the fatty acid *cis*-4,3,10,13,16,19-docosahexaenoic acid, and R_f 0.7 showed high levels of glycerine-1,3-dimyristate, 2-*O* (Figure 5-12).

The TLC bands from *S. habrochaites* LA1777 were also analysed through GC-MS and like the other tomato species *S. lycopersicum* cv. M82 which was analysed there is a consistent fatty acid element within the majority of the fractions which include palmitic acid, octadecanoic acid, *bis*(trimethylsilyl)monostearin, and hexadecanoic acid, 2,3-*bis*[(trimethylsilyl)- oxy]propyl ester (α-glyceryl palmitate).

Figure 5-12. GC-MS analysis of *S. lycopersicum* cv. M82 fresh trichome TLC fractions.

Crude trichome extracts were separated into different R_f fractions by TLC on silica gel plates in a solvent system of dichloromethane: hexane, and glacial acetic acid (40:60:2 v/v). The samples represent one technical replicate of three pooled plant samples of 12 separate TLC bands. Data is from one technical replicate of a pool of three plant replicates, see Appendix 3 for total list of compounds.

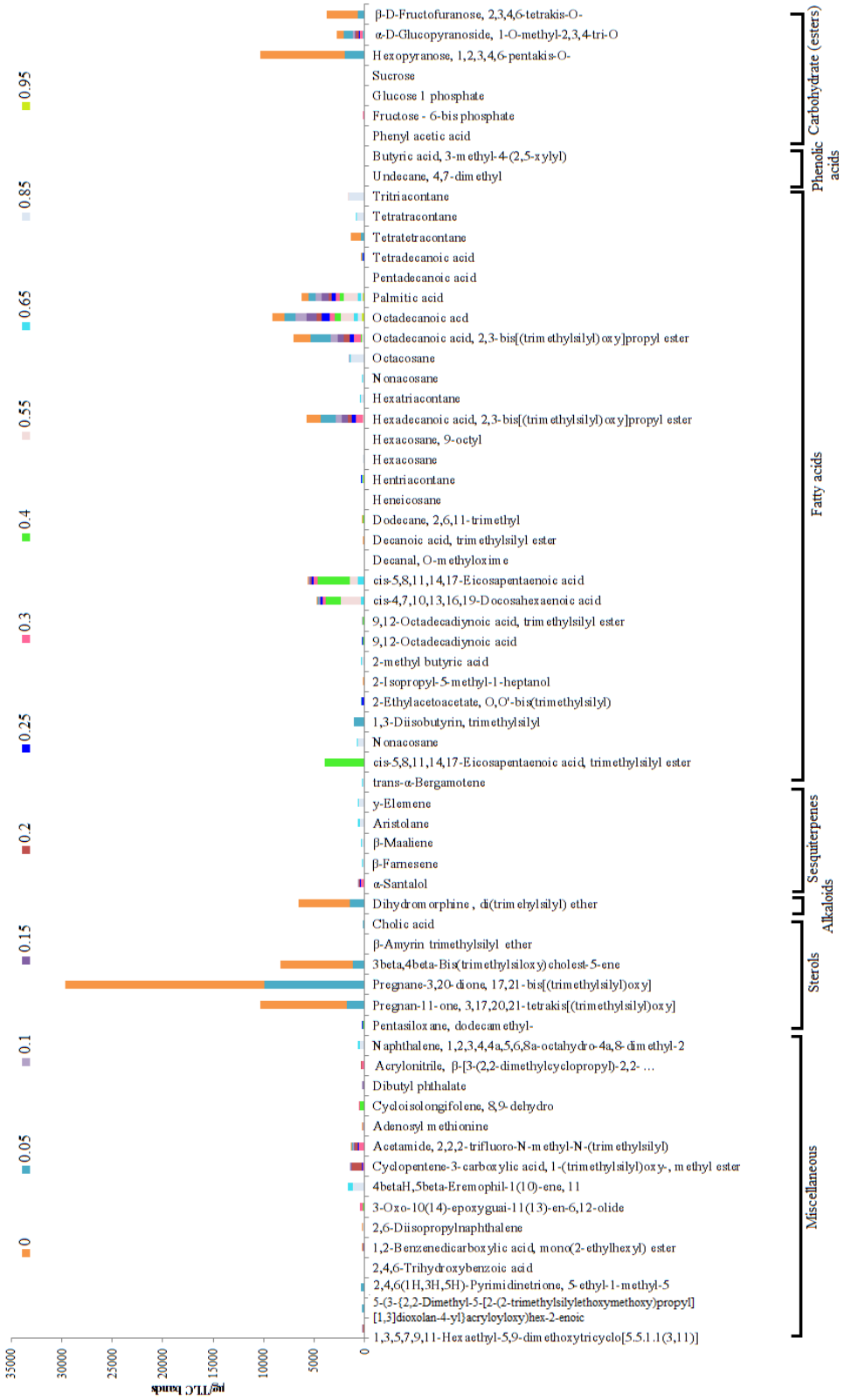

Figure 5-13. GC-MS analysis of *S. habrochaites* LA1777 fresh trichome TLC fractions.

Crude trichome extracts were separated into different R_f fractions by TLC on silica gel plates in a solvent system of dichloromethane: hexane, and glacial acetic acid (40:60:2 v/v). The samples represent one technical replicate of three pooled plant samples of 12 separate TLC bands. Data is from one technical replicate of a pool of three plant replicates, see Appendix 4 for total list of compounds.

The two TLC bands which stand out from the rest of the extracts include R_f 0 which was shown to contain large amounts of sterols and sugar related compounds such as hexopyranose 1,2,3,4,6-pentakis-*O*-, and pregnane-3,20-dione,17,21-*bis*[(trimethylsiloxy)oxy]. The antimicrobial TLC fraction identified in *S. habrochaites* LA1777 was identified as R_f 0.55 (Figure 5-6), and this TLC band was shown to contain some sesquiterpenes but mainly omega 3-fatty acids such *cis*-5,8,11,14,17-eicosapentaenoic acid and *cis*-4,7,10,13,16,19-docosahexaenoic acid (Figure 5-14).

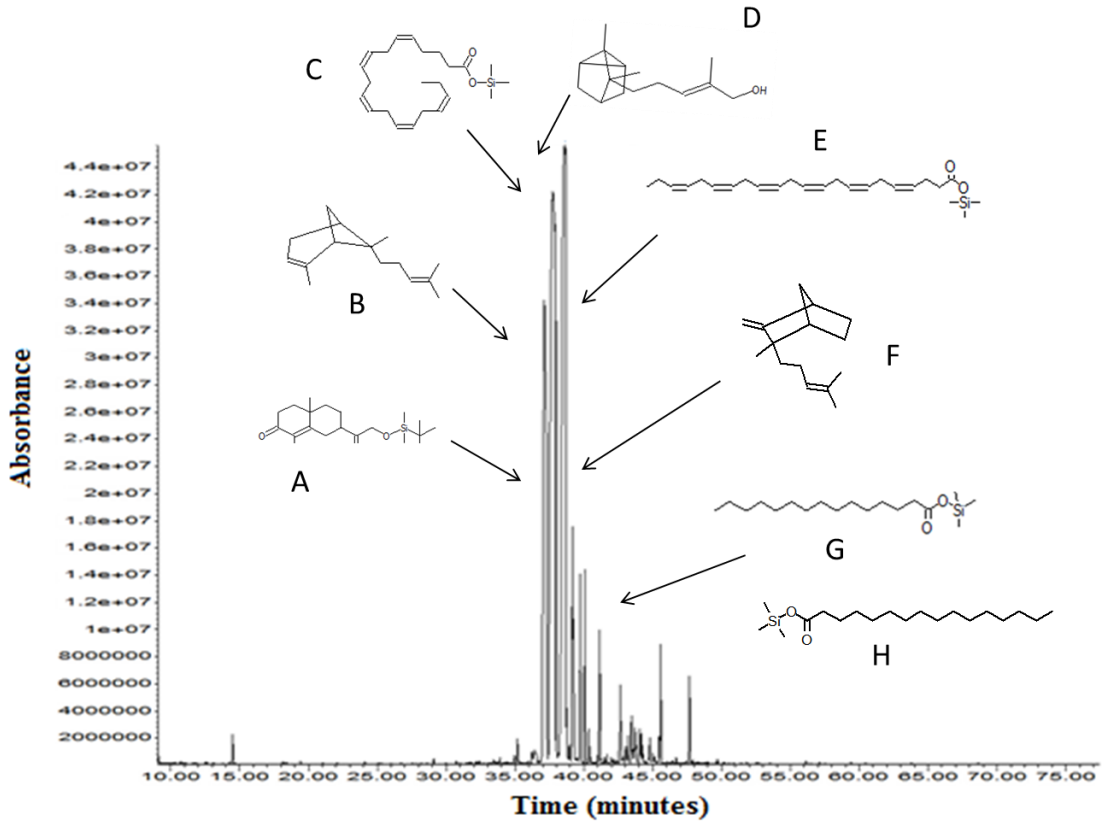

Figure 5-14. *S. habrochaites* LA1777 trichome extract TLC R_f 0.55.

A. bicyclo[4.4.0]dec-1-en-3-one, 2,6-dimethyl-9-[3-(t-butyldimethylsiloxy)prop-1-en-2-yl]- MF. 544 RT. 37.1288 min, B. bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)- MF. 716 RT. 37.2357 min, C. *cis*-5,8,11,14,17-eicosapentaenoic acid, trimethylsilyl ester MF. 628 RT. 37.6808 min, D. *α*-santalol MF. 782 RT. 37.6361min, E. *cis*-4,7,10,13,16,19-docosahexaenoic acid, trimethylsilyl ester MF. 687 RT. 37.9971min, F. bicyclo[2.2.1]heptane, 2-methyl-3-methylene-2-(4-methyl-3-pentenyl)-, (1S-endo)- MF. 814 RT. 38.7629, G. *n*-pentadecanoic acid, trimethylsilyl ester MF. 644 RT. 38.8163 min, and H. hexadecanoic acid MF. 951 RT. 41.1461 min, structures from Chemspider, drawn with Chemsketch (www.acdlabs.com)

5.2.4.1 *S. pennellii* 2D TLC bands trichome extract

A 2D-TLC plate method was used to separate a chloroform extract of *S. pennellii* trichomes. This was performed to move the active fraction at R_f 0 off the line of origin and to further seperate the antimicrobial compounds. The 2D-TLC system included a solvent system of first hexane:diethyl ether:glacial acetic acid (60:40:2 v/v), and then for the second run methanol (100%). The bands ran to an R_f of approximately 0.7 during the second methanol solvent treatment, and this included the antimicrobial fraction at the line of origin which also moved to a second R_f of 0.66.

GC-MS was used to identify the secondary metabolites within the *S. pennellii* trichome extract and showed that 1,3-dioxolan-4-propenoic acid 2,2-dimethyl-5-[2-(I-butyldimthylsilyoxy)propyl] was present in the TLC bands ranging from R_f 0.3 x 0.59 to 0 x 0.66 in concentrations as high 300 µg band^{-1} (Figure 5-15), as well as hexatriacontane. Fatty acids such as hentriacontane, octacosane, oleic acid, and triacontane 1-bromo were present at consistent concentrations within all of the TLC bands. Within the R_f of 0.18 x 0.74 to 0.12 x 0.66 there were compounds identified as *β*-D-lyxofuranoside S-octyl-5-valeroyl, which was the closest accurate NIST match to a sugar ester with links to fatty acids. The TLC fraction believed to contain the active compounds (retention time 65 to 75 min) was R_f 0 x 0.66, and only *α*-D-glucopyranoside was produced at a higher concentration than the other fractions.

Analysis of the MS spectra fragmentation patterns can elucidate the compound structures, by identifying the tallest base peak which is the commonest ion formed, and the molecular ion peak which is the heaviest ion. The components which were selected to investigate were 1,3-dioxolan-4-propenoic acid 2,2-dimethyl-5-[2-(I-butyldimthylsilyoxy)propyl] and *α*-D-glucopyranoside. *S. pennellii* LA0716 ethyl acetate fraction (SPE) at 66.749 min (RI 3568.9) was identified by NIST with a match R of 830 as *α*-D-glucopyranoside, 1,3,4,6-tetrakis-*O*-(trimethylsilyl)-*β*-D-furanosyl 2,3,4,6-tetrakis-*O*-(trimethylsilyl). However, after looking at the fragmentation pattern it is likely that the compound was a glycol-cholesterol compound such as cholesterol *β*-D-glycoside. This is due to the initial identification as a glucose molecule, and the m/z of the ion peaks show fragments which have the same mass as sterols and a hexose sugar (Figure 5-16).

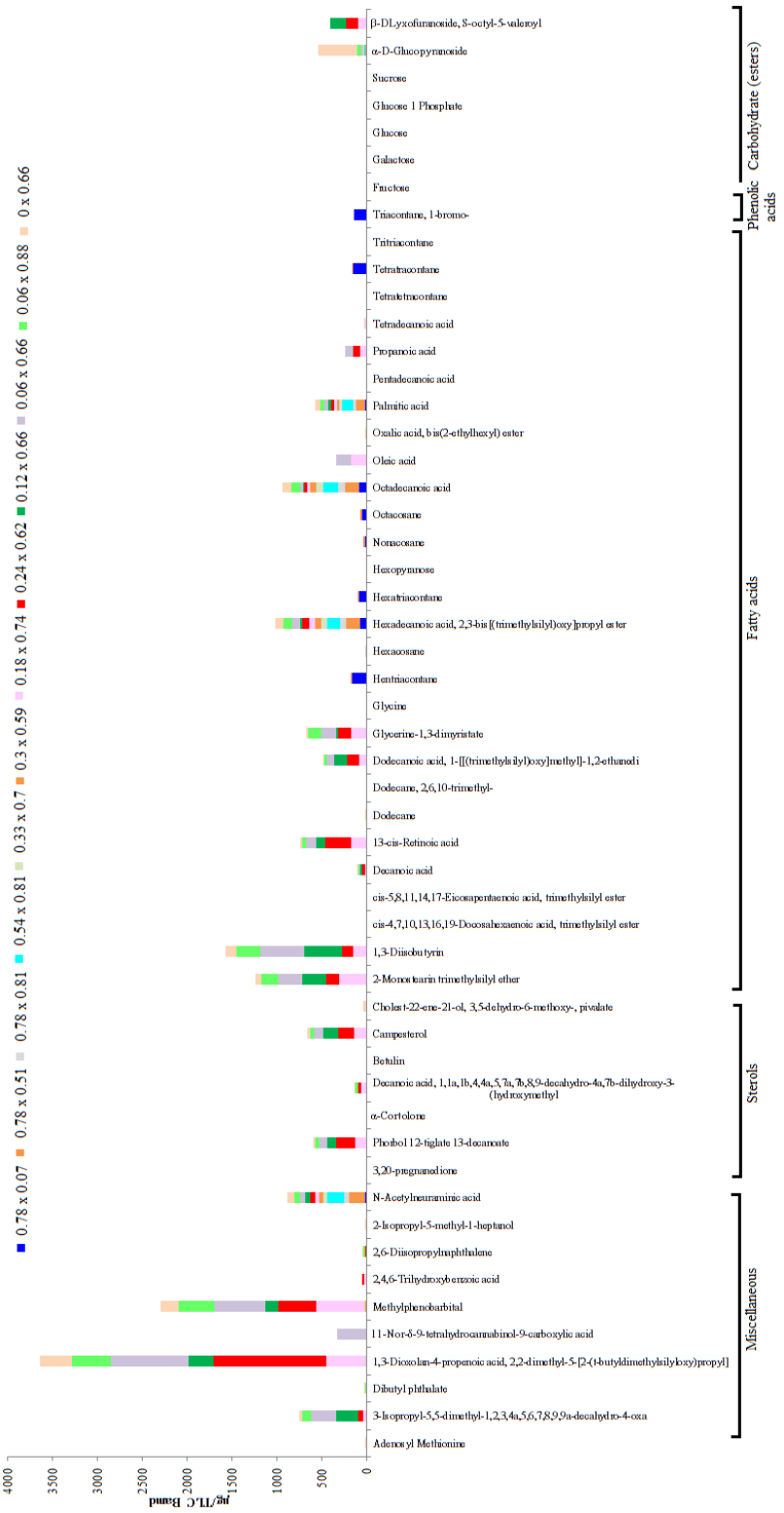

Figure 5-15. GC-MS analysis of *S. pennellii* LA0716 fresh trichome 2D-TLC fractions.

Crude trichome extracts were separated into different R_f fractions by 2D TLC in a solvent system of dichloromethane: hexane, and glacial acetic acid (40:60:2 v/v), followed by methanol. R_f values are represented as the distance travelled by the first dichloromethane: hexane, and glacial acetic acid (40:60:2 v/v) run x the methanol R_f value. The samples represent one technical replicate of three pooled plant samples of 12 separate TLC bands, see Appendix 5 for total list of compounds.

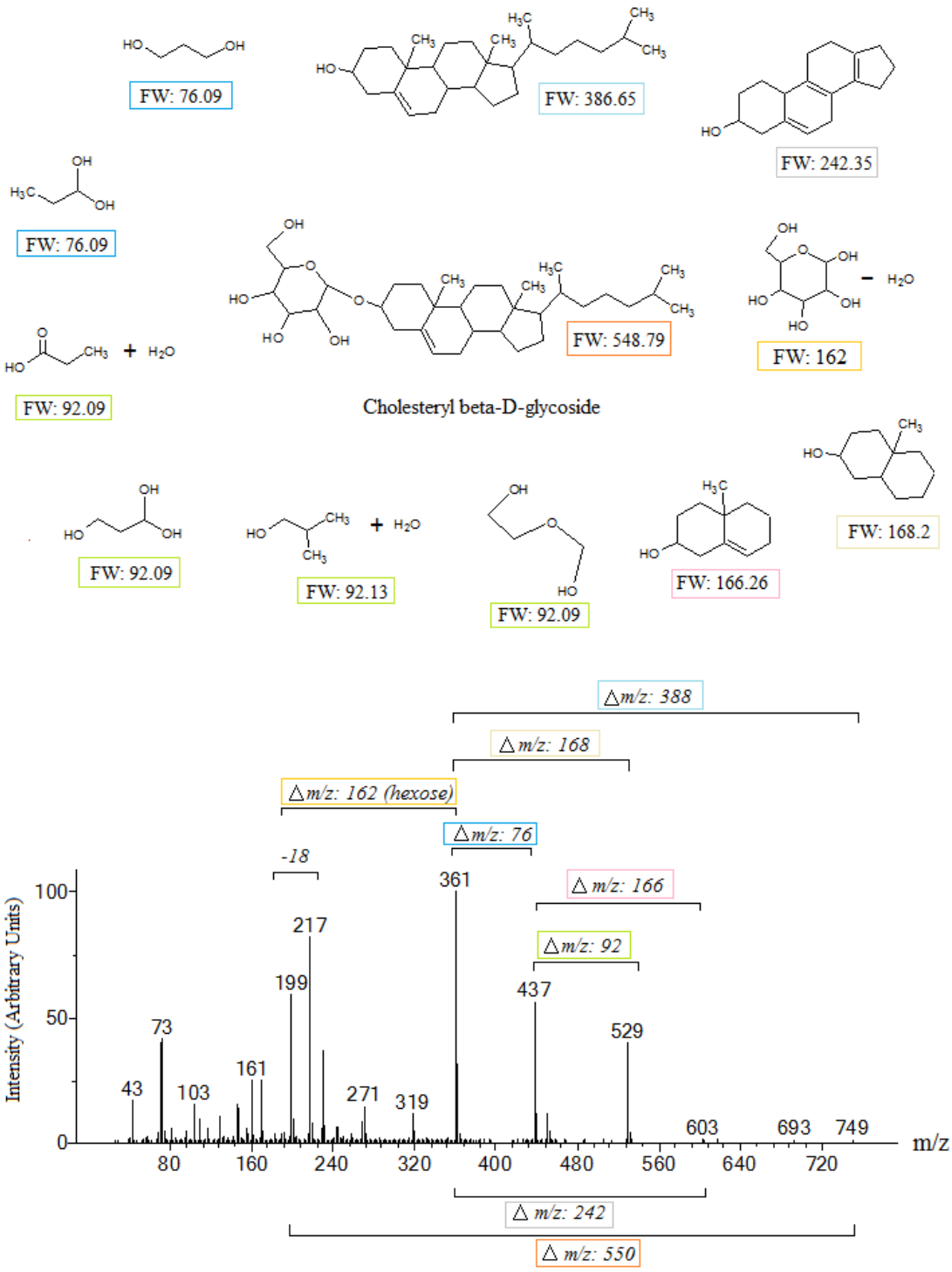

Figure 5-16. The typical MS spectra of the active peaks within the retention time range of 65 to 75 min of *S. pennellii* LA0716 ethyl acetate fraction.

The selected spectra shows the pattern of the peak from *S. pennellii* LA0716 ethyl acetate fraction (SPE) at 66.749 min (RI 3568.9) identified by NIST with a match R of 830 as *α*-D-glucopyranoside, 1,3,4,6-tetrakis-*O*-(trimethylsilyl)-*β*-D-furanosyl 2,3,4,6-tetrakis-*O*-(trimethylsilyl). Chemical structures were adapted from Chemspider using ChemSketch.

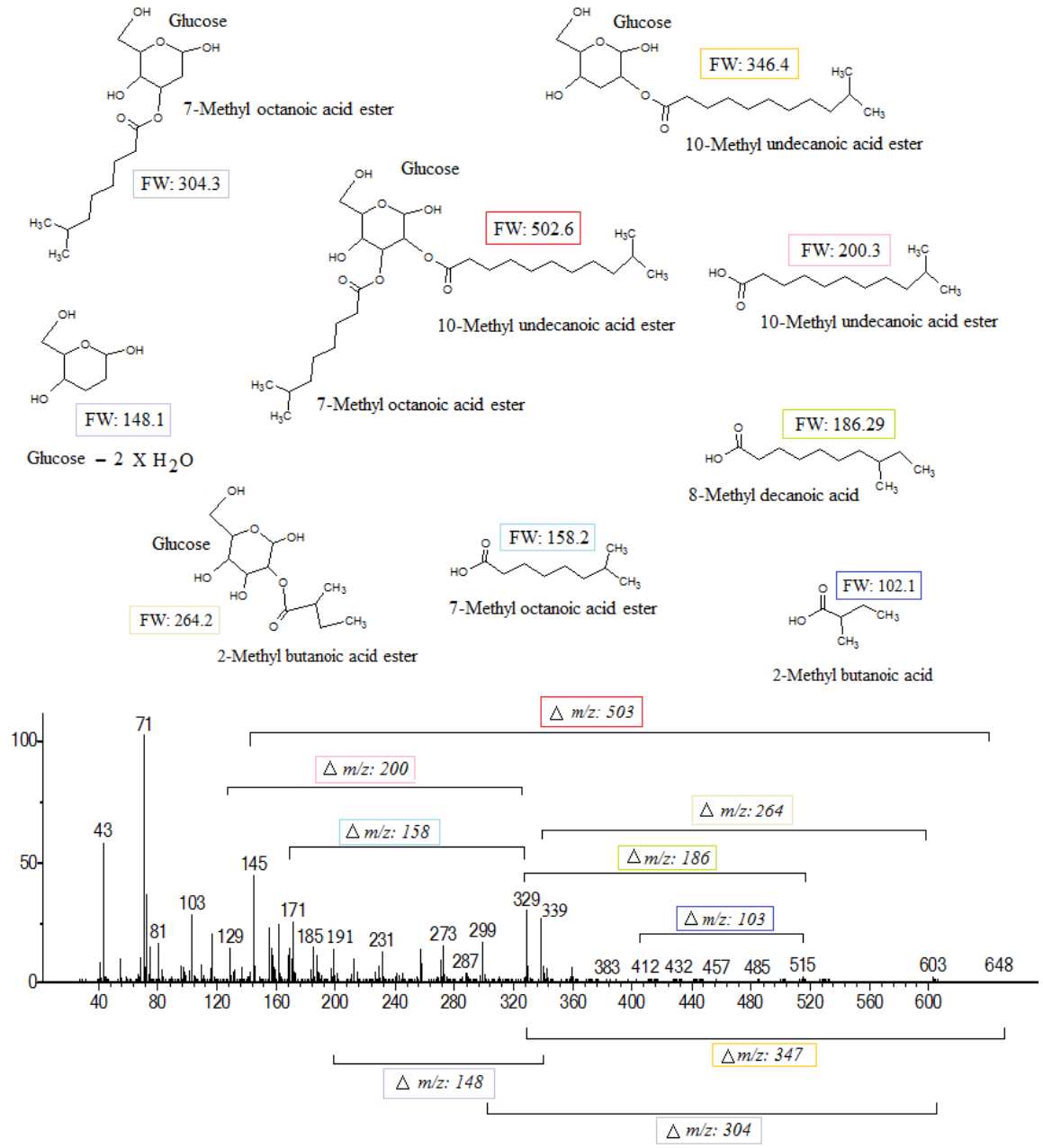

Figure 5-17: Typical MS spectra of the peaks within the retention time range of 55 to 65 min of *S. pennellii* LA0716 2D TLC R_f 0 x 0.66.

The selected spectra shows the pattern of a selected peak from *S. pennellii* LA0716 2D TLC R_f 0 x 0.66 at 56.811 min (RI 3067.8) identified by NIST with a match R of 463 as 1,3-dioxolan-4-propenoic acid, 2,2-dimethyl-5-[2-(t-butyldimethylsilyloxy)propyl]. Chemical structures were adapted from Chemspider using ChemSketch.

These glucose esters match the mass of 1,3-Dioxolan-4-propenoic acid 2,2-dimethyl-5-[2-(I-butyldimthylsilyoxy)propyl] which was present in the majority of the TLC bands. It is not believed to be antimicrobial due to its retention time which was between 55 to 65 min from *S. pennellii* LA0716 2D TLC. Due to its high production comparisons with sugar esters can be made, as high levels of sugar esters are produced by *S. pennellii*. The fragmentation pattern suggests that glucose esters would be a possibility due to the similarities with the masses identified from the ion peaks where fatty acid methyl esters have the same mass, and these are fatty acids known to be produced by *S. pennellii*. The mass of glucose on its own could not be determined from the fragmentation pattern. However, there were several possible matches to the fragmentation of a glucose molecule containing two fatty acids 10-methyl undecanoic acid, and 7-methyl octanoic acid (Figure 5-17).

5.2.5 SPE of chloroform extracts from leaf trichomes of *S. pennellii*

S. pennellii chloroform trichome extract was separated into fractions of different polarity using SPE, the extracts were collected in 1 mL aliquots dried and the compounds were determined using GC-MS. The GC-MS analysis of the collected fractions shows that the more non-polar solvent hexane contained the most of the compound identified through NIST as 1,3-dioxolan-4-propenoic acid, 2,2-dimethyl-5-[2-(t-butyldimethylsilyloxy)propyl], which was also present in the subsequent fractions until ethanol where it was no longer eluted. Hexane also contained fatty acids such as hentriacontane, hexatriacontane, tetratriacontane, and the fat soluble phenol derivative butylated hydroxytoluene.

The hexane:chloroform (50:50 v/v) was the only fraction to contain oleic acid, and the chloroform fraction contained a mixture of compound types including fatty acids such as decanoic acid, *n*-decanoic acid, nonane 5-(2-methylpropyl), the sterol cholest-22-ene-21-ol 3,5-dehydro-6-met, the compound 3,19-epoxyandrosta-5,7-diene, 17-acetoxy-4,4-dimethyl-3-methoxy, and *β*-D-lyxofuranoside, thiooctyl-5-*O*-decan. Chloroform and ethyl acetate (50:50 v/v) fraction contained high levels of 1,3-diisobutyrin, dodecanoic acid, 1-[[(trimethylsilyl)ox, *α*-D-glucopyranoside, 1,3,4,6-tetrakis-*O*-(trimethylsilyl)-*β*-D-fructose and sterols such as cholic acid, glycine *N*-[(3a,5b,12a)-3,12-dihydroxy, and campesterol.

The ethyl acetate fraction was very similar to the fraction which contained both ethyl acetate and ethanol (50:50 v/v) with sterol related and sugar related compounds which were continuing to elute and present in the previous more non-polar fractions. The ethanol fraction contained mainly *β*-D-fructofuranosyl 2,3,4,6-tetrakis-*O*-(trimethylsilyl), and the following methanol and water fractions contained only trace amounts of compounds already observed in the previous fractions (Figure 5-20).

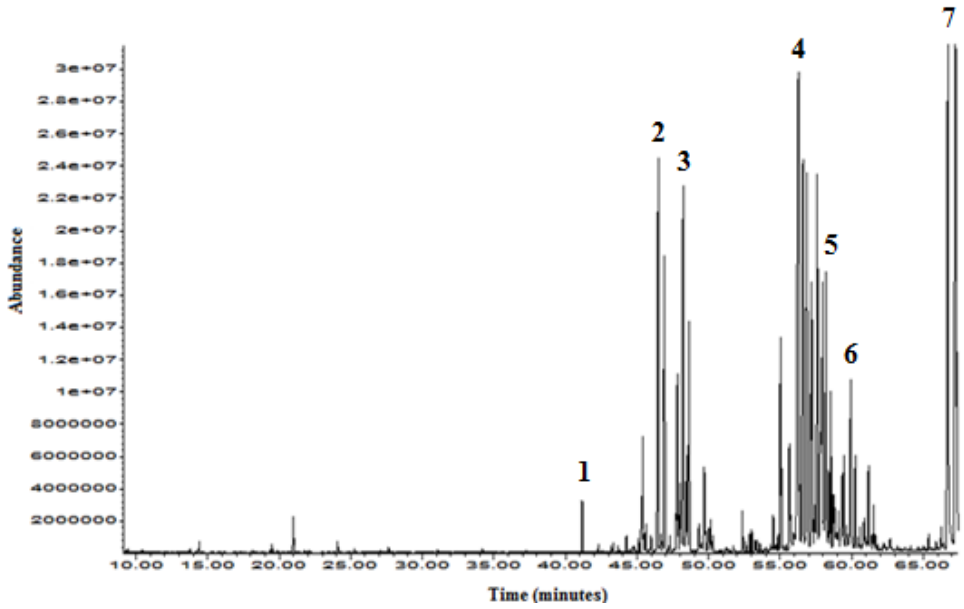

Figure 5-18. *S. pennellii* trichome extract separated using SPE, spectrum shows the ethyl acetate fraction.

Peaks with the highest areas were selected to represent the most abundant compounds within the sample; 1. palmitic acid at 41.1 min, 2. 1,3-diisobutyrin at 46.4 min, 3. glycine, *N*-[(3*α*,5*β*,12*α*)-3,12-dihydroxy- at 48.2 min, 4. 1,3-dioxolan-4-propanoic acid, 2,2-dimethyl-5-[2-(t-butyldimethylsilyl) at 56.5 min, 5. cholic acid at 58.2 min, 6. dodecanoic acid at 60.2 min, and 7. *α*-D-glucopyranoside, 1,3,4,6-tetrakis-*O* at 67.2 min. Data is from one technical replicate of a pool of three plant replicates.

The SPE of *S. pennellii* trichome extract was tested for antimicrobial activity using disc diffusion assay and the results showed that the ethyl acetate (100%) fraction was significantly inhibitory towards *S. aureus* 25923 and was significantly different to the DMSO control ($P<0.001$). This ethyl acetate fraction had a similar level of inhibitory activity towards *S. aureus* 25923 as the ciprofloxacin antibiotic control. The fractions between hexane and ethyl acetate were also significantly different from the DMSO control ($P<0.001$) against *S. aureus* 25923. However, the inhibition against *S.* Typhimurium and *E. coli* O157:H7 was not as high, with only *S.* Typhimurium 10248 significantly different from the DMSO control for fractions between hexane ($P<0.01$) and ethyl acetate: ethanol (50:50 v/v) ($P<0.001$). The level of inhibition reduced

generally from the ethanol fraction onwards, of which these same extracts had no inhibitory effect against *S. aureus*.

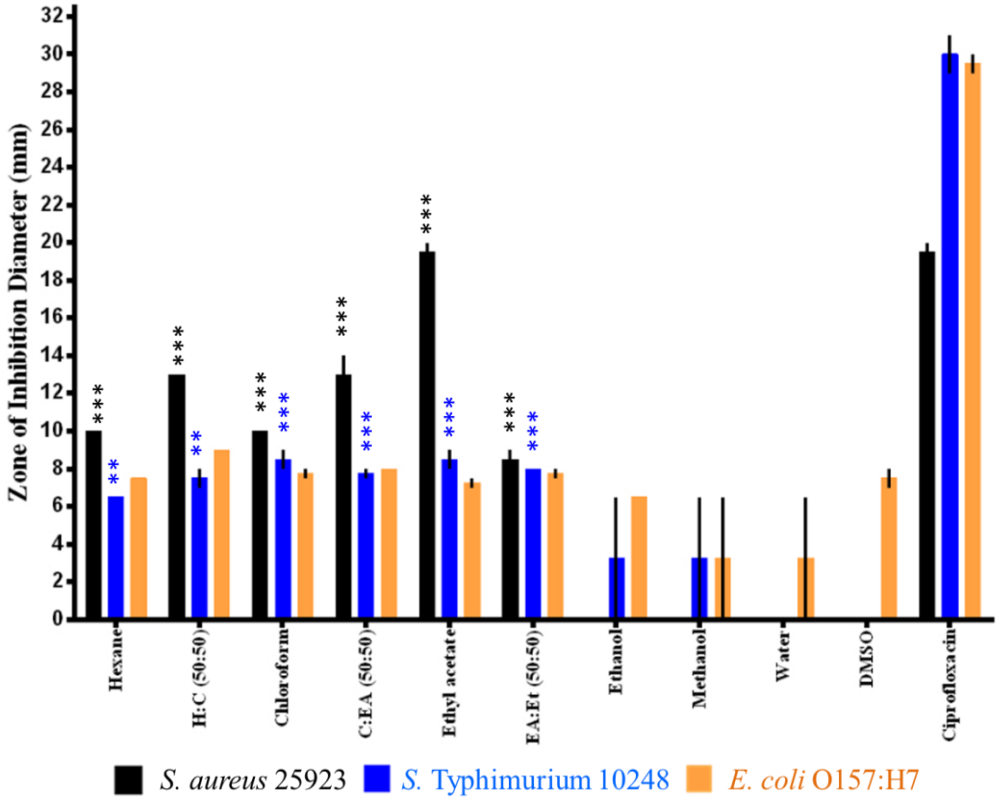

Figure 5-19. Disc diffusion assay of bacteria grown with extracts of SPE fractions from *S. pennellii*. Chloroform extract of trichomes from fresh leaf material were separated into fractions by SPE using solvents increasing in polar properties from hexane to water. The zones of inhibition of *S. aureus* 25923 in black, *S.* Typhimurium 10248 in blue and *E. coli* O157:H7 in orange. Error bars represent standard error of three biological plant replicates and two technical replicates. The asterisk (**=P<0.01 and ***=P<0.001) represents the significant difference between the zone of inhibition diamerter (mm) of bacteria grown in the presence of the extract compared to the DMSO control, using two-way ANOVA Dunnetts multiple comparisons test (95% CI).

The GC-MS spectrum of the SPE extract from *S. pennellii* chloroform leaf extract shows peaks present with retention times above 65 min (Figure 5-18), which compares to the antimicrobial fraction isolated from *S. pennellii* 2D TLC R_f 0 x 0.66, where the antimicrobial compounds are believed to be in the region of 65 to 75 min, due to their absence within the other fractions which had less antimicrobial activity. The compounds within this region are also identified by NIST as glucopyranoside, 1,3,4,6-tetrakis-*O*-(trimethylsilyl)-*β*-D-furanosyl 2,3,4,6-tetrakis-*O*-(trimethylsilyl) or a related sugar, and the fragmentation patterns of these components are similar from both the 2D TLC and the SPE samples suggesting that the compounds are the same.

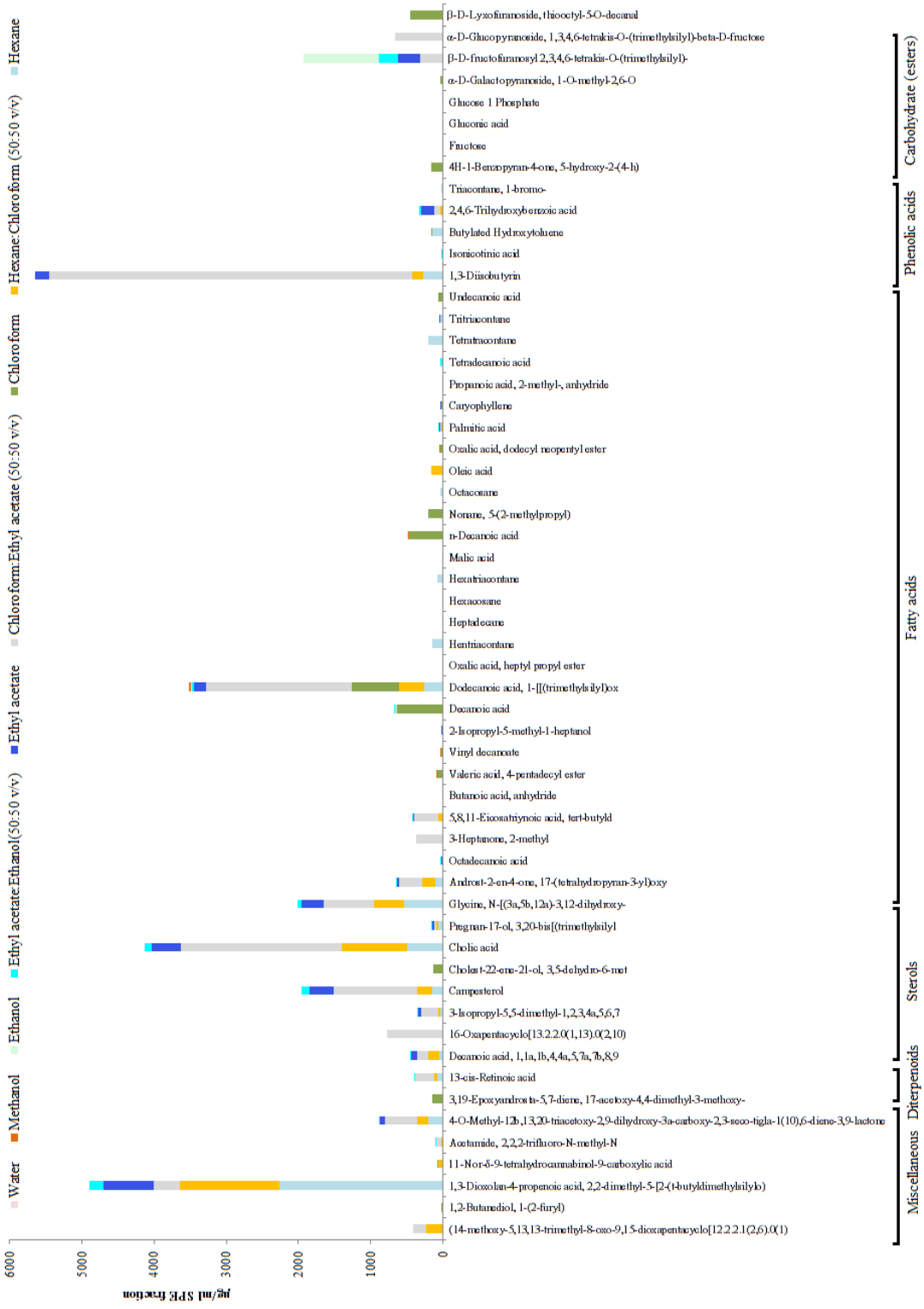

Figure 5-20. GC-MS analysis of *S. pennellii* LA0716 fresh trichome SPE fractions.

S. pennellii LA0716 fresh leaves extracted in chloroform, which was separated into different fractions using SPE with a silica gel column and solvents of increasing polarity from hexane to water. The samples represent one technical replicate of three pooled plant samples, showing 9 different fractions, see Appendix 6 for total list of compounds.

5.2.6 LC-PDA-TOFMS (MAXIS) Identification of antimicrobial fractions

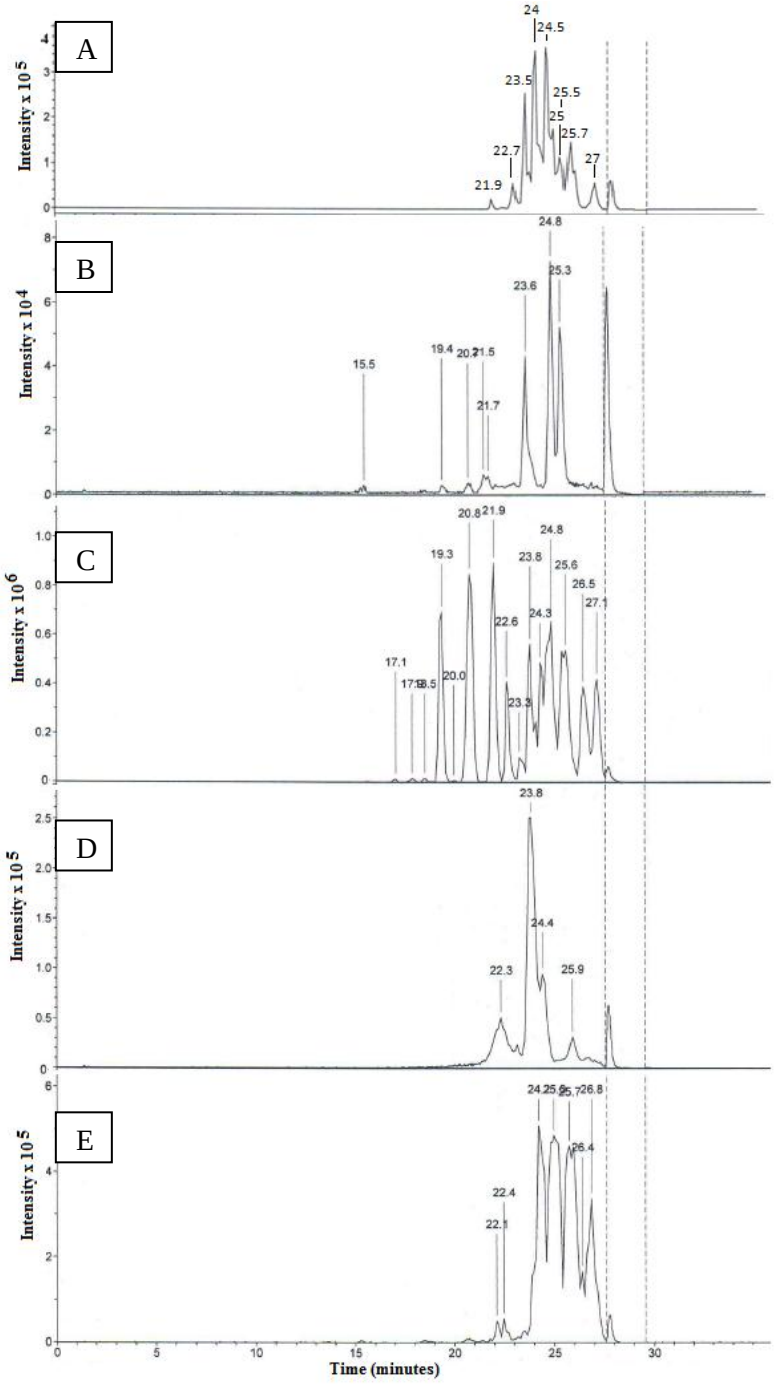

Figure 5-21. LC-MS (MAXIS) chromatograms from antimicrobial fractions of Solanaceae.

Chromatograms from chloroform extracts of trichomes from fresh leaves of Solanaceae species separated into antimicrobial fractions; A. *N. rustica* TLC R_f 0, B. *S. habrochaites* LA1777 TLC R_f 0.55, C. *S. habrochaites* LA1777 TLC R_f 0, D. *S. pennellii* LA0716 TLC R_f 0, E. *S. pennellii* LA0716 SPE ethyl acetate fraction. Calibration was performed using sodium formate calibrant solution which is highlighted in the area between the two dotted lines. The samples (10 µL) were analysed using electrospray ionization, and run on a RP C_{18} 2 µm column using a high resolution Q-TOF mass spectrometer UHR-MAXIS. Data is from one technical replicate of a pool of three plant replicates.

LC-MS was performed using MAXIS analysis of the fractions which showed the most antimicrobial activity was performed. All the compounds eluted near the end of the run showing that all the antimicrobial compounds were non-polar (Figure 5-21). The MAXIS was chosen as an additional platform to identify the compounds within the plant extract, because the samples could be identified using their polarity, and from identification of their accurate masses. Through comparing the fragmentation patterns the likely compounds were identified to specific compound classes with further evidence of their additional structures such as the presence of esters.

5.2.6.1 *N. rustica*

The active fraction from *N. rustica* with an R_f of 0 was analysed using LC-MS (MAXIS), and showed that the compounds were non-polar because they run late on the chromatogram. The peaks clustered together which suggested that they could have been esters with slight changes in the carbon numbers on the fatty acids. This was seen from analysing the three main peaks between 23 to 25 min which had masses decreasing in subsequent values of 14 (CH_2), i.e. 695, 709 and 723 *m/z*.

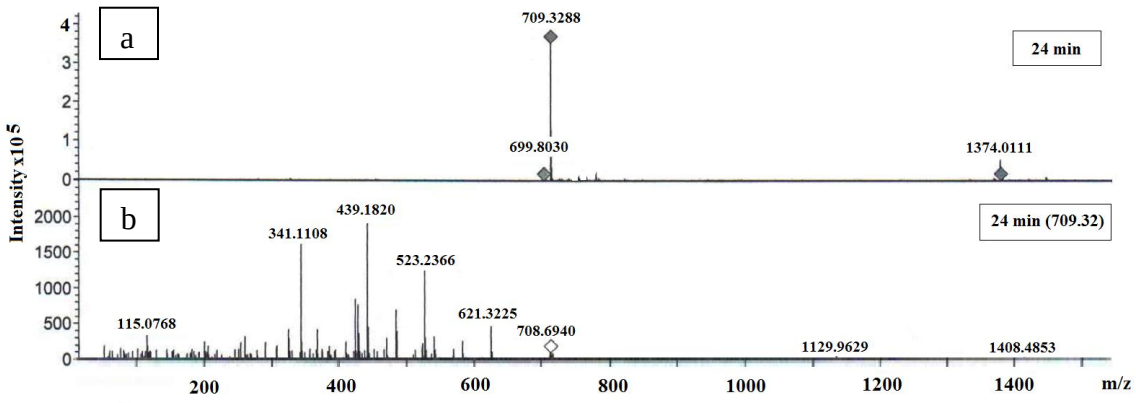

Figure 5-22. *N. rustica* LC-MS (MAXIS) identified ions and fragmentation pattern.

The antimicrobial TLC fraction at R_f 0 was isolated from *N. rustica*. The fragmentation pattern for the ion with an *m/z* of 709, with a retention time of 24 min, was selected as an example of the fragmentation patterns observed from this plant extract. The figure shows the *m/z* of the unfragmented precursor ion (a), and then the fragmentation pattern of this ion underneath (b), (negative ion mode [M-H-]). Data is from one technical replicate of a pool of three plant replicates.

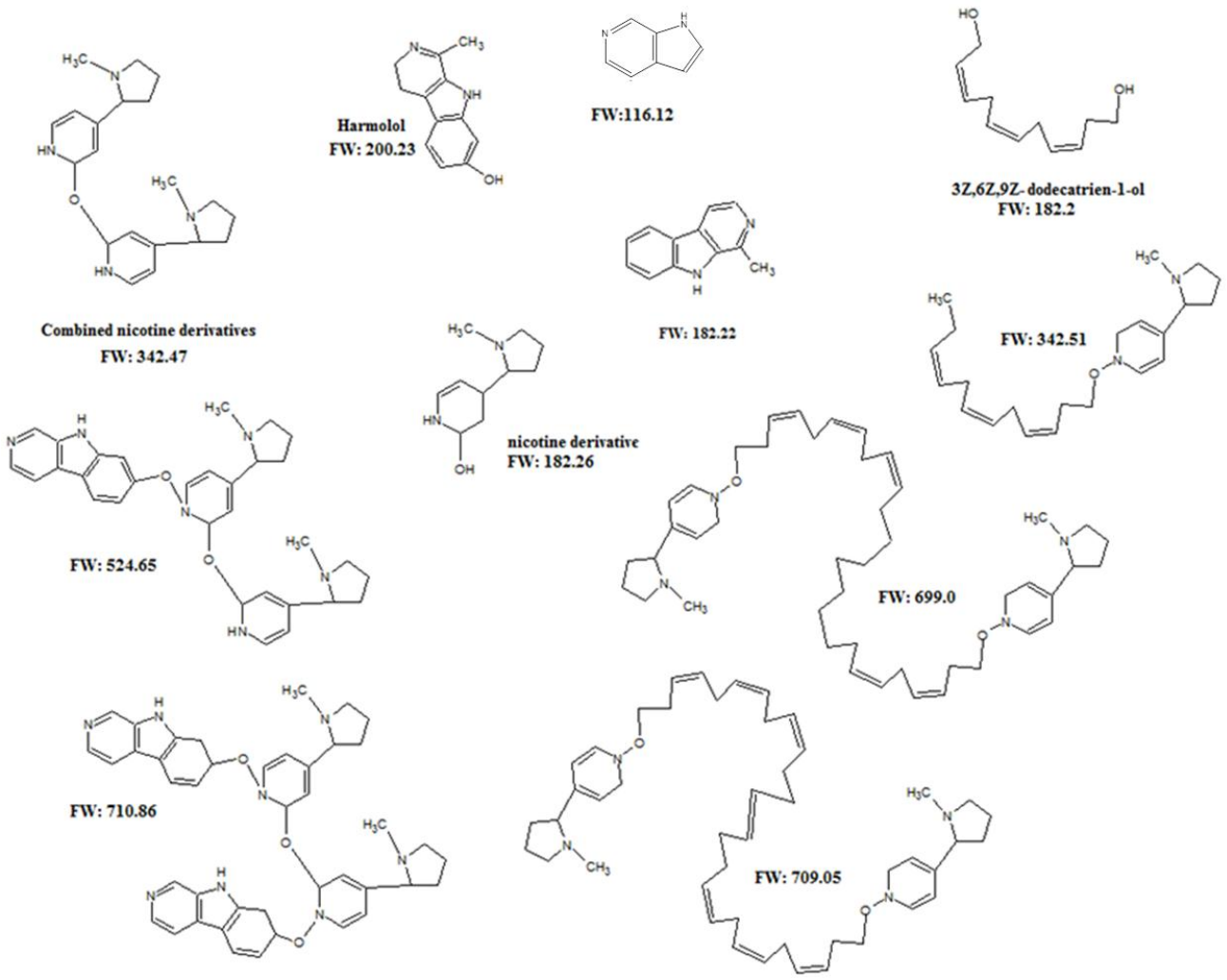

Figure 5-23. Chemical structures representing the putative identification of antimicrobial secondary metabolites from *N. rustica* TLC R_f 0.

Structures were determined through analysis of the fragmentation patterns after LC-MS (MAXIS), structures were re-drawn from Chemspider using ChemSketch

The main ion was found to have a mass of 341.1 Da (342.1 due to [M-H-]), which is the same mass as two nicotine derivatives bound by their hydroxyl groups. The fragmentation pattern of the peak at 24 min also showed multiple ions suggesting that the compound easily fragments during analysis which also showed a similar pattern to the fragmentation of ester compounds. There were also ions with higher masses such as 1409 and 1129 *m/z*. These are likely to be dimers of the original compound. The mass differences between several ions are 182, 98, and 87, where 182 *m/z* could represent the ring structure of a *β*-carboline with an additional CH_3, and 98, and 87 *m/z* could represent fatty acid chains. Additional fatty acid side chains could include 3Z,6Z,9Z-dodecatrien-1-ol, which has a mass of 182.2 Da, and when combined with nicotine the mass matches the ion with 341 *m/z*, and dimers with various chain lengths also match to the original precursor ions.

5.2.6.2 *S. habrochaites*

The antimicrobial TLC band R_f 0.55 was subjected to further analysis using LC-MS (MAXIS). The chromatograms showed that the compounds were non-polar due to their late retention time on a C_{18} column. The antimicrobial fraction had three main peaks at 23.6, 24.8 and 25.3 min. The peaks at 24.8 and 25.3 min were identified as either *α-cis*-santalenoic acid or *α-cis*-bergamotenoic acid due to investigations into their fragmentation patterns which show that the most abundant peak is 233.15 (234.15 due to negative ion mode [M-H-]), which matches the molecular mass of both these compounds.

An additional peak at 23.6 contained the same sesquiterpene carboxylic acids. However, there was an additional ion peak within the fragmentation pattern which suggested that this related to glycosylated versions of *α-cis*-santalenoic acid or *α-cis*-bergamotenoic acid. This was identified from the ion of 821.38 which was identified as having a strong match (100%, -16mDa) to the chemical structure $C_{38}H_{61}O_{19}$, where Chemspider was used to match this chemical structure and mass to 1-*O*-{13-[(2-*O*-hexopyranosylhexopyranosyl)oxy]-16-hydroxy-18-oxokauran-18-yl}hexopyranose.

This seemed unlikely although when the sugars and carboxyl group are removed from the structure of this compound, it has a molecular weight of 234.37 Da. This is the same as *α-cis*-santalenoic acid and *α-cis*-bergamotenoic acid, and therefore it is more likely that this structure is instead a sesquiterpene carboxylic acid attached to multiple sugars, such as seen in the glycoalkaloids.

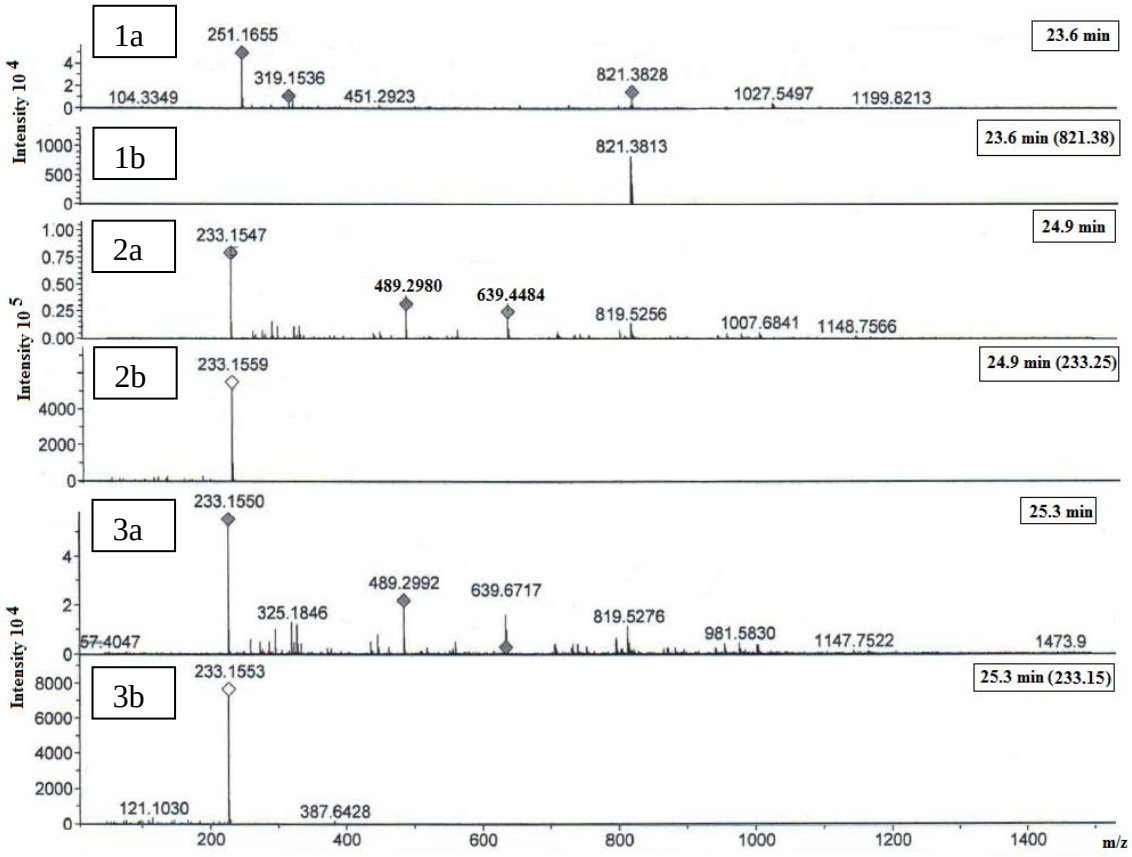

Figure 5-24. *S. habrochaites* LC-MS (MAXIS) identified ions and fragmentation patterns.

The antimicrobial TLC fraction at R_f 0.55 was isolated from *S. habrochaites* LA1777. The fragmentation patterns for three main peaks with retention times of (1) 23.6, (2) 24.8, (3) 24.9 min, were selected as examples of the common fragmentation patterns observed from this plant extract fraction. The figure shows the *m/z* of the unfragmented precursor ion (a), and then the fragmentation pattern of this ion underneath (b), (negative ion mode [M-H-]). Data is from one technical replicate of a pool of three plant replicates.

The exact mass of 822 Da however was not met through adding three sugar molecules to *α-cis*-bergamotenoic acid and could only be reached through adding an additional fatty acid side chain to one of the sugars, such a fatty acid needs to add an extra 87 *m/z* and 2,4-dihydroxy butanoic acid was selected as an example. Another compound which

runs at a similar time to the glycosylated compound has the same mass as *α-cis*-santalenoic acid and *α-cis*-bergamotenoic acid with an additional H_2O, which adds to the probability that the larger molecules are glycosylated sesquiterpene carboxylic acids.

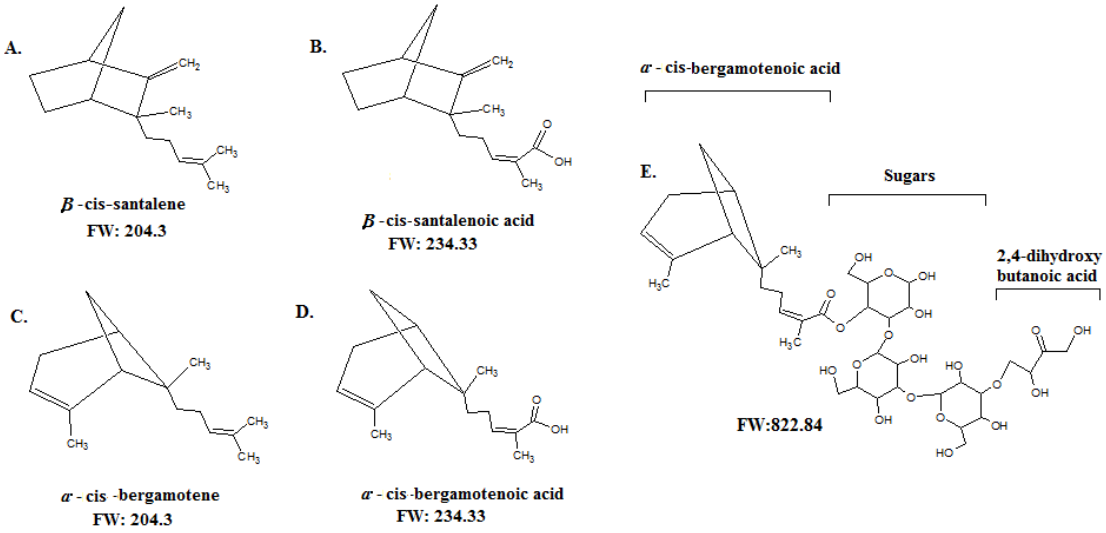

Figure 5-25. Chemical structures representing the putative identification of antimicrobial secondary metabolites from *S. habrochaites* LA1777 TLC R_f 0.55.

Structures are possible matches to the fragmentation patterns of *S. habrochaites* analysis by LC-MS (MAXIS), A. *β-cis*-santalene, B.*β-cis*-santalenoic acid, C. *α-cis*-bergamotene, D. *α-cis*-bergamotenoic acid, E. *α-cis*-bergamotenoic acid attached to a sugar and 2,4-dihydroxy butanoic acid, structures were re-drawn from Chemspider using ChemSketch.

5.2.6.3 *S. pennellii*

The antimicrobial SPE ethyl acetate fraction was subjected to further analysis using LC-MS (MAXIS). The chromatograms showed that the compounds were non-polar, and the antimicrobial fraction had four main peaks at 24.2, 24.8, 25.7, and 26.9 min. These ions within these peaks showed similar fragmentation patterns which suggested esters were present due to a high background of smaller fragments.

The fragmentation ions suggested that the compounds could match to sterol fatty acid esters, such as stigmasterol fatty acid esters or *β*-amyrin fatty acid esters (Figure 5-27). This was due to the mass of stigmasterol matching the mass of 411 Da (412 due to negative ion mode [M-H-]) which ran at 24.8 min. Stigmasterol minus water (-18 Da) could represent the mass of 393 Da, and *β*-amyrin matches to the mass of 425 Da. The combination of sterols and fatty acids which reach similar masses to the ones in the

fragmentation patterns is vast, and other combinations of sterols and fatty acids are likely. Unsaturated fatty acid chains combined with stigmasterol and *β*-amyrin were used as examples where they shared a similar mass to the precursor ions, differences in the masses by 1 *m/z* could be due to additional hydrogen within the fatty acid chain.

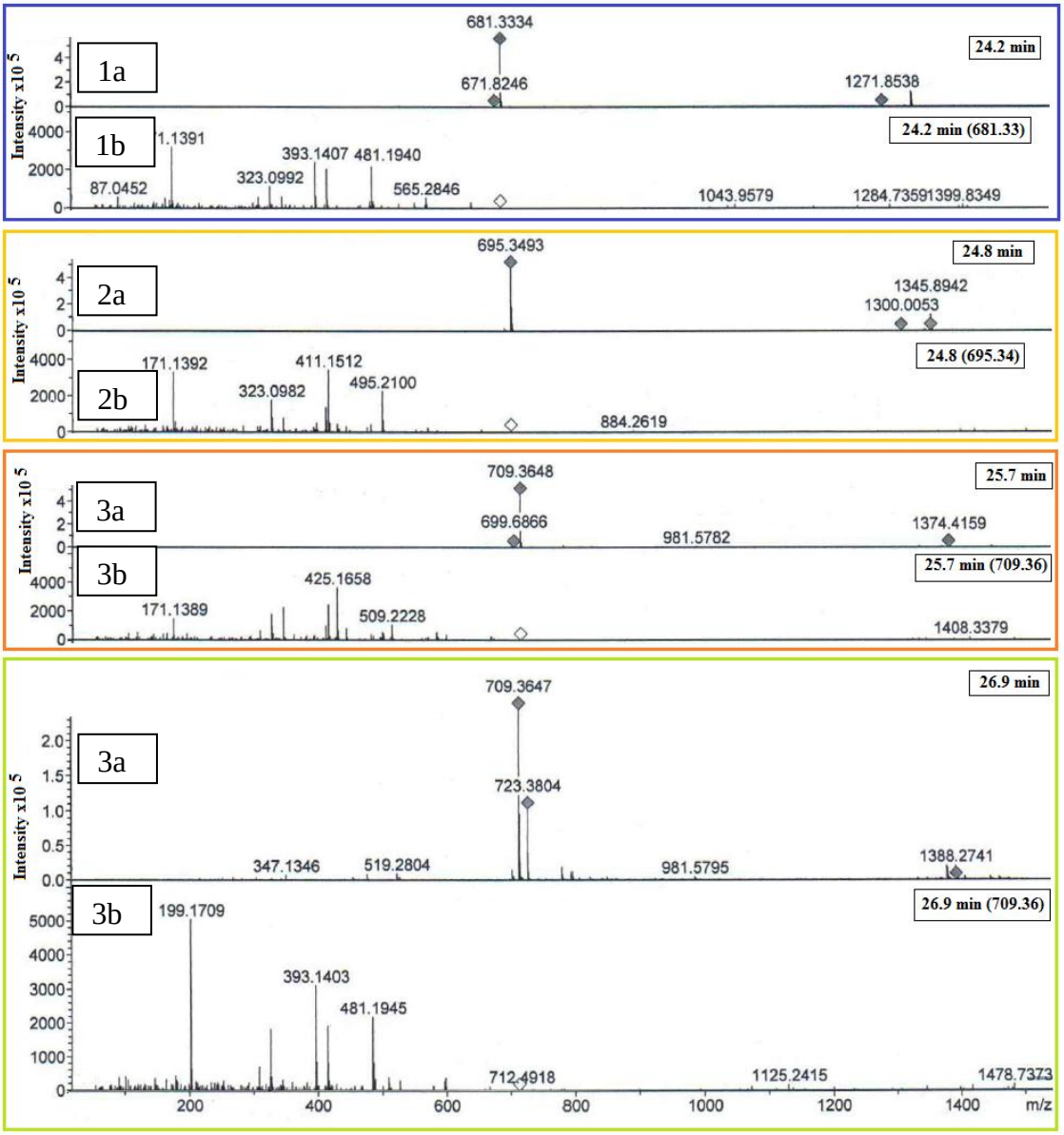

Figure 5-26. *S. pennellii* LC-MS (MAXIS) identified ions and fragmentation patterns.

The antimicrobial SPE ethyl acetate fraction was isolated from *S. pennellii* LA0716. The fragmentation patterns for three main peaks with retention times of (1) 24.2 min, (2) 24.8 min, (3) 25.7 min, (4) 26.9 min, and were selected as examples of the common fragmentation patterns observed from this plant extract fraction. The figure shows the *m/z* of the unfragmented precursor ion (a), and then the fragmentation pattern of this ion underneath (b), (negative ion mode [M-H-]). Data is from one technical replicate of a pool of three plant replicates.

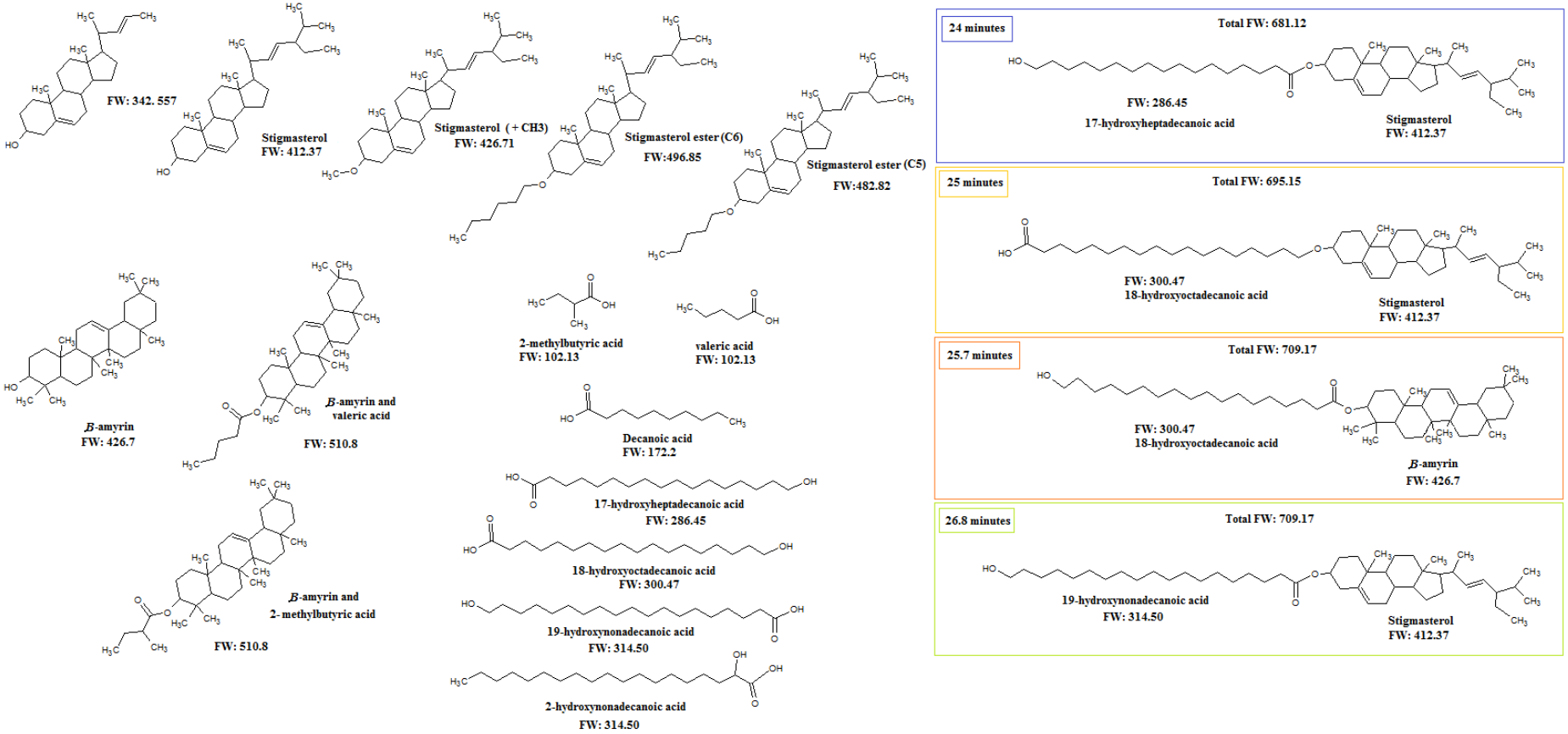

Figure 5-27. Chemical structures representing the putative identification of antimicrobial secondary metabolites from *S. pennellii* SPE ethyl acetate fraction.

Structures are possible matches to the fragmentation patterns of *S. pennellii* analysis by LC-MS (MAXIS), structures were re-drawn from Chemspider using ChemSketch.

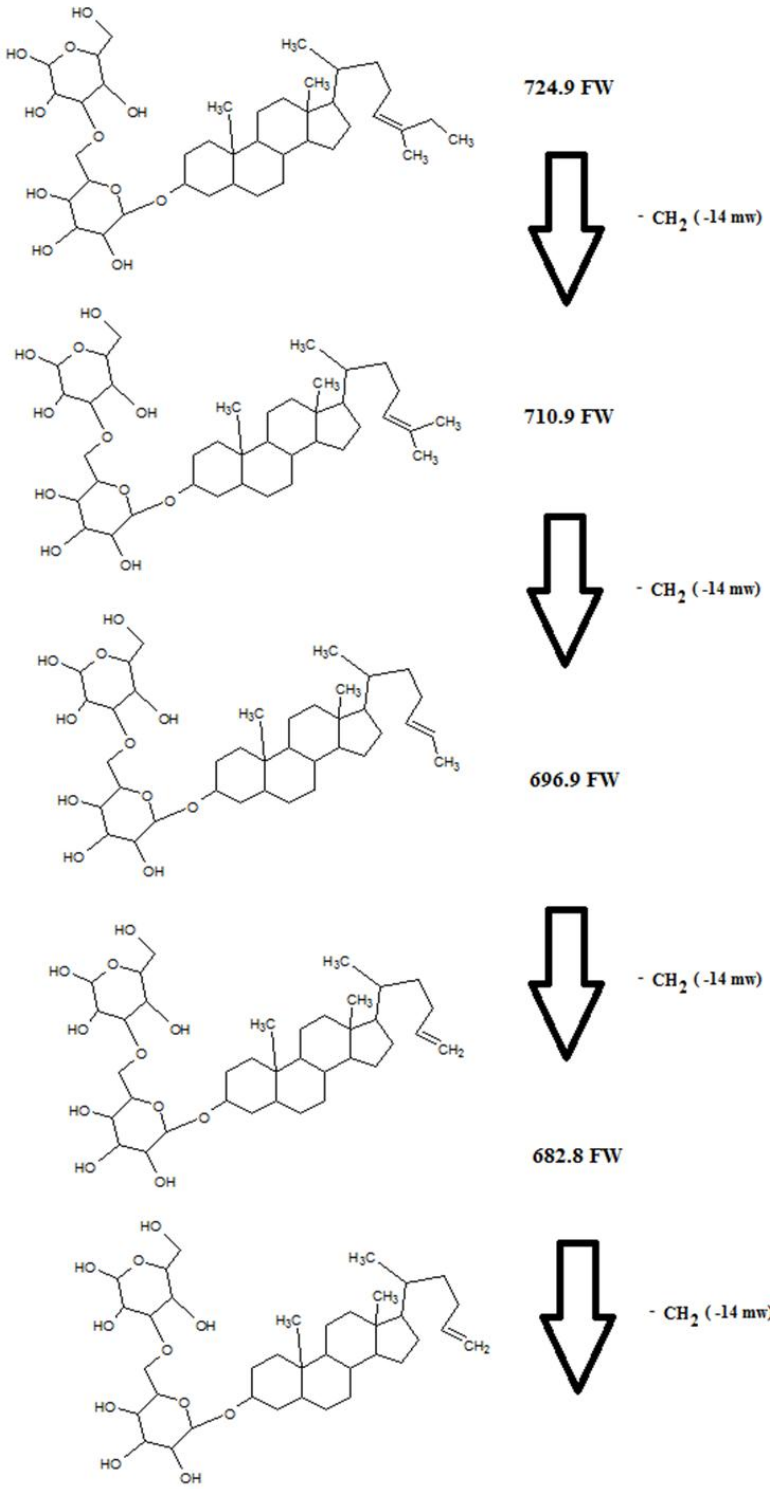

Figure 5-28. Steroidal saponin glycoside structures representing the putative identification of antimicrobial secondary metabolites from *S. pennellii* SPE ethyl acetate fraction.

Structures are possible matches to the fragmentation patterns of *S. pennellii* analysis by LC-MS (MAXIS), structures were re-drawn from Chemspider using ChemSketch.

5.3 Discussion

5.3.1 Agilent HPLC identification of leaf compounds

Identification of compounds using an Agilent HPLC showed that the detection method was either not applicable for glyco-alkaloids or that the extraction method was not optimal. This conclusion was drawn because only phenolic acids were detected on the system and glyco-alkaloids known to be produced by Solanaceae leaves such as α-tomatine were not identified (Figure 5-3). The phenolic acids were compared between the leaf samples from the wild tomato relatives and the results show that the phenolic acids present within the plants are reasonably consistent between the species with the main differences being between the derivatives of the phenolic acids. The two *S. habrochaites* species LA1981 and LA1779 had very different spectra which indicate how different types and concentrations of secondary metabolites can be produced by close genetic relatives, highlighting the need for comprehensive analysis when searching for useful compounds produced by plant species.

5.3.2 TLC and antimicrobial analysis of chloroform trichome leaf extracts

The TLC concentration and purification of chloroform extracts involved extracting 100 mg of dry plant material in chloroform at room temperature for 16 h, separating the extract through TLC and ZIA analysis of the chosen bands (Figure 5-1 and Table 5-1). Whilst this selected antimicrobial method was successful, an additional method often used in bacterial susceptibility testing is bioautography methods, where a TLC plate is either embedded into agar or the TLC overlaid on to the agar surface and compounds are deposited in the same positions as the TLC through diffusion (Choma and Grzelak, 2010). Bioautography was a method which was considered as part of this research. Preliminary results using the technique highlighted some logistical difficulties with using category II bacteria safely and constrictions on the TLC size and therefore separation of bands. Instead the method of scraping the TLC bands directly to yield an extract to be tested in disc diffusion assays was performed with successful results.

The main compounds detected were carotenoids, chlorophylls and pheophytin. The plant with the highest significant antimicrobial activity was rosemary (*Rosmarinus officinalis*) TLC bands with an R_f of 0.5, 0.35 and 0.2 against *S. aureus* 25923. Overall the bands from the beginning and the end of the TLC plate did show a pattern of antimicrobial activity when all the bacteria and all plant species are considered as a whole. An example of a band with consistent activity was from an average R_f value 0.74 which was identified by alliance HPLC as pheophytin, and R_f value 0.07 which was identified as a lutein derivative (Figure 5-2). Pheophytin is known to be a powerful sensitizer for oxidation (Fuse et al., 1997) and carotenoids have antioxidant properties, but these compounds are not typically antimicrobial. This suggests that another compound may be responsible for the antimicrobial effects observed, and due to the amphiphilic nature of sugar esters, they could be an explanation for the wide range and variability in the antimicrobial activities of the chloroform TLC bands.

A hypothesis was proposed that sugar esters could be the antimicrobial compounds responsible for antimicrobial activity from *S. pennellii* and possibly *S. habrochaites* due to the high trichome load from both plant species, and the production of sugar esters from *S. pennellii* trichomes. Further extraction of sugar esters from the trichomes of fresh whole plant material was performed with the intention of reducing the contamination of plant extracts with chlorophylls. Through focusing on extracting the trichome compounds from fresh leaf material in chloroform leaf dips, where sugar esters are known to be produced by *S. pennellii* and other Solanaceae species. This was investigated to confirm or reject the theory that sugar esters are responsible for the antimicrobial activity.

5.3.3 Antimicrobial activity of *S. pennellii* trichome extract

The trichomes of plants are known to produce protective secondary metabolites against herbivore and insect pests. Several methods of trichome specific extraction were investigated, which included the manual removal of trichomes before extraction as described by Yerger et al., (1992), and leaf dips in solvents. The leaf dip method using chloroform as the appropriate solvent was used in the final tests for antimicrobial activity.

The MIC of *S. pennellii* LA0716 and *S. habrochaites* LA1777 from fresh leaf trichome extracts were determined (Figure 3-14 and Figure 3-15), and it was found that through concentration of trichome extracts an enhanced bacterial inhibition could be observed compared to extracting from the whole dried leaf material. In order to determine whether sugar esters were responsible for the antimicrobial activity observed against *S. aureus* 25923. *S. pennellii* trichome extracts were separated into polar water:methanol (50:50 v/v) and a non-polar (hexane) fractions and then subjected to growth curve inhibition analysis of selected bacterial species (Figure 5-5). The results showed that the polar fraction was more inhibitory against *E. coli* O157:H7, *S.* Typhimurium 10248 and *S. aureus* 25923. The differences in antimicrobial activity against the bacteria species were only slightly different, suggesting that either both extracts contain different antimicrobial compounds with different levels of activity, or that both extracts contain the same antimicrobial compounds at different concentrations to each other.

Chloroform extraction of sucrose esters from the glandular trichomes of wild *S. lycopersicum* species has been performed on fresh leaf material extracted in chloroform (King and Calhoun, 1988; King et al, 1990). The residue can be spotted onto TLC silica gel and run with a Chloroform:Methanol (9:1 v/v) solvent system, where the sucrose esters should run to between R_f 0.3 to 0.6 (King and Calhoun, 1988; King et al.,. 1990). Primulin (Malvidine-3-galactoside chloride) was used as a non-destructive dye to visualise the bands at 340 nm, where lipids are bright yellow on a clear background.

The GC-MS analysis of both polar and non-polar *S. pennellii* trichome extract showed that there are three clusters of peaks within the extract, and when extracted in polar water:methanol (50:50 v/v) all three clusters are present 45 to 50, 55 to 60 and 65$\leq$ min, and within the hexane fraction only one cluster is present which has more peaks within it between 55 and 60 min, and two extra peaks within the retention range of 64 min (Figure 5-4). Through visually comparing the retention times of the peaks it could be deduced that the active compounds are the ones present within both samples as both samples demonstrated antimicrobial activity such as the peaks within the retention time of 55 to 60 min. However, this hypothesis does not explain why the polar sample was more antimicrobial, and therefore further analysis of the trichome extracts were investigated using TLC to further separate the trichome extracts. Although these peaks were not identified with standards their high concentration suggested they are

compounds already known to be produced by the plant. *S. pennellii* is known to produce sugar esters in concentrations of up to 40% of their total dry leaf weight and it is likely that most of these peaks are sugar esters, and this is further verified due to the amphiphilic nature of sugar esters which can easily dissolve in both polar and non-polar solvents.

TLC separation of trichome extracts from fresh leaf material which was tested for antimicrobial activity in disc diffusion assays and the results showed antimicrobial activity from *S. pennellii* R_f 0.95 and 0 against *S. aureus* 25923, with a reduced activity against *E. coli* O157:H7 and *S.* Typhimurium 10248 from R_f 0.35 to 0.25. The highest antimicrobial activity observed was from the *S. habrochaites* LA1777 trichome extract TLC band at R_f 0.55 against *S. aureus* 25923, this shows that the antimicrobial compounds present within the two antimicrobial plant species are different classes (Figure 5-6).

5.3.4 Antimicrobial analysis of saponified and non-saponified trichome extracts

The sugar esters and glyco-alkaloids, glyco-sterols and glycosides are specific non-sugar compounds (aglycones), which are attached to a sugar molecule (glycone) via a glycosidic bond, a reaction which liberates water, binding the two compounds with an oxygen molecule. The hypothesis that sugar esters and other glucose bound molecules could be responsible for the antimicrobial activity within *S. pennellii* and other Solanaceae plants was investigated by breaking down the compounds within the samples prior to antimicrobial and GC-MS analysis. Saponification using NaOH and acid hydrolysis with HCL was used to ensure all glycosidic bonds were broken, and then these samples were compared to extracts which were non-saponified. The main results showed that saponification does have an effect on the antimicrobial activity of *S. pennellii* against *S. aureus* 25923 but not against the Gram-negative bacteria (Figure 5-7). This suggests that the compounds which have glycosidic bonds are more antimicrobial, and because this effect is only seen against Gram-positive bacteria it seems that the glucose molecule portion of the compound has an activity specific to the differences in the morphology of Gram-positive bacteria, such as the cell wall.

GC-MS analysis was performed to identify if the spectrum of the plant extracts had changed between the saponified and non-saponified samples (Figure 5-8 and Figure 5-9). The results showed that there were large differences between the samples, and all peaks with a retention time above 55 min were absent in the saponified trichome extracts. This confirms that the peaks which have glycosidic bonds run after 55 min and that it is within these peaks that the antimicrobial activity against *S. aureus* from *S. pennellii* can be identified.

5.3.5 Compounds from the plant trichomes identified by GC-MS

Comparisons were made between the compounds identified within the TLC fractions of different plant species. This identified specific classes of compounds responsible for a underlying level of antimicrobial activity within the Solanaceae species. Whilst glycoside compounds could have been responsible for the antimicrobial against *S. aureus*, additional effects are likely within the crude extract such as activity from fatty acids which are produced as defensive compounds by many plant trichomes. Further separation of the trichome extract through TLC has highlighted a low level of activity against all the tested bacterial species from bands with an R_f of 0.95 which was the most non-polar fraction which ran with the solvent front, and bands between R_f 0.35 and 0 (Figure 5-6). Most of the TLC bands were shown to contain a consistent level of fatty acids and these compounds such as palmitic acid were compounds likely to be responsible for the low level of inhibition observed broadly from the TLC bands across all the tested plant species.

Among the compounds identified within all the extracts of the plant trichomes palmitic acid was the most abundant from *S. lycopersicon* cv. M82, and *N. rustica* (Figure 5-11 and Figure 5-12). Fatty acids also make up a large proportion of *S. pennellii* trichomes, although this is usually in the form of esters attached to glucose molecules via glycosidic bonds. Certain fatty acids have already been discovered to have antimicrobial activities, such as fatty acids from milk in the form of capric acid, myristic acid, linoleic and linolenic acid, which are known to inhibit bacteria such as *S. aureus* in concentrations ranging from 100 to 25µg mL^{-1} (Kelsey et al., 2006).

Medium chain fatty acids such as capric, caprylic and caproic acids have also been found to synergistically reduce the expression of invasion genes in *Salmonella*, and reduced the bacteria colonisation numbers in poultry (Immerseel et al., 2004). Medium chain fatty acids reduce virulence of *Salmonella* at non-bacteriostatic concentrations (2 mM), by decreasing the expression of *hilA* a salmonella pathogenicity island I regulator which is required for invasion (Immerseel et al., 2004). Short chain fatty acids such as formic, propionic and butyric acids are currently used by farmers as a feed additive to reduce the levels of pathogens such as *Salmonella* (Immerseel et al., 2004).

Whilst there were similarities in the types of compounds identified within the plant samples such as the fatty acid decanoic acid, and the sterol cholic acid, consistently similar compounds were not identified from the antimicrobial TLC bands. The three antimicrobial TLC bands, R_f 0.55 from *S. habrochaites*, R_f 0 from *N. rustica*, and R_f 0 from *S. pennellii* all suggested different compound classes as the compounds responsible for the antimicrobial activity within the species. The active compound classes identified within the three plants included alkaloids from *N. rustica*, glyco-sterols in *S. pennellii* and fatty acids and sesquiterpenes from *S. habrochaites*.

5.3.6 Antimicrobial fractions of *N. rustica*

The antimicrobial fraction of *N. rustica* had the same antimicrobial TLC R_f value as *S. pennellii* with an R_f of 0, however the most abundant compounds in *N. rustica* were not the same as the compounds found in *S. pennellii* (Figure 5-6). It was found that the antimicrobial activity of *N. rustica* was likely caused by the high concentrations of the alkaloid nicotine. The *N. rustica* tobacco variety produces high quantities of nicotine and the NIST match was high at 949 (Figure 5-10). The isolation of nicotine through extraction of the leaf trichome is supported from a previous study where nicotine was found to be produced within both trichomes and the mesophyll of *Nicotiana attenuate* Torr. Ex Wats. (Roda et al., 2003). The nicotine compounds ran between 20 and 25 min on the GC-MS, and the separation of these compounds was poor therefore the identification of these compounds were difficult.

Sterols were not found within the extracts of *N. rustica* trichomes, although free sterols have been found within other tobacco species such as *N. tabacum*, with the major sterols including sitosterol, stigmasterol, campesterol, and cholesterol in free, ester, and acylated glycosides (Grunwald, 1975). The composition of sterols found to be produced within tobacco (*N. tabacum* L) and tomato (*S. lycopersicum esculentum* Mill) is very similar, however the concentration of sterols is double to amount found within tomato shoots and roots with the majority of the sterols present within the leaves (Amber et al., 1974).

Carbohydrate related compounds were present in other Solanaceae species tested such as *S. pennellii*. However, none were found to be present within the trichome extracts of *N. rustica*. The presence of sugar esters has been previously found within tobacco species such as *N. glutinosa*, primarily in the form of sucrose esters such as (2,3,4-*tri*-*O*-acyl)-*α*-D-glucopyranosyl)-(3-*O*-acyl-*β*-D-fructofuranoside, and 2,3,4-*tri*-*O*-acyl)-*α*-D-glucopyranosyl-*β*-D-fructofuranoside, with the major fatty acid esters acetic, propionic, 2-methylbutyric, 4-methylpentanoic, 4-methylhexanoic, 5-methylhexanoic, and octanoic acids (Matsuzaki et al., 1988).

There has been some previous research into the antimicrobial properties of nicotine, such as research by Zaidi and Gul (2005) who found that nicotine isolated from *Nicotiana tabacum* was active at 20 µL mL^{-1} against the Gram-negative bacteria *E. coli*, *P. aeroginosa* and the Gram-positive bacteria *Enterococcus faecalis*. When tested against *S. aureus* and *Salmonella* Typhi, Zaidi and Gul (2005) found that nicotine was not inhibitory against these species. These results do not compare with the tobacco extracts high in nicotine which are inhibitory to *S. aureus* 25923 with an average inhibition zone of 12 mm but showed no inhibition against *E. coli* and almost no inhibition against *S.* Typhimurium.

These differences could be related to the concentration of the compounds tested against *S. aureus*. It is possible that other compounds within the crude sample are interfering with the effects or an alternative strain of *E. coli* may have been used within their studies, supported by the varied antimicrobial effects which were discovered between the two *E. coli* strains O157:H7 and 25922 used within this study, such as differences between inhibition levels of saponified and non-saponified *N. rustica* extracts (Figure 5-

7). *N. rustica* analysis of R_f 0 using LC-MS (MAXIS) showed that the antimicrobial compounds are non-polar esters, and the fragmentation patterns suggested that the structures could be mixtures of nicotine and β-carboline fatty acid esters (Figure 5-23). *N. rustica* is known to produce high concentrations of nicotine (Wilbert, 1975), which compares with these findings.

The hypothesis that the antimicrobial compound in *N. rustica* are compounds which include nicotine relies on nicotine being produced by *N. rustica* in a more reactive form such as 6-hydroxy nicotine, as nicotine by itself is poorly reactive (Ohnishi et al., 1982). The addition of a sugar molecule within the antimicrobial samples was not considered likely as this would increase the size of the molecule during the derivatisation process. The *N. rustica* antimicrobial molecules have a retention time between 22 to 25 min on the GC-MS (Figure 5-10), compared to the sugar esters within *S. pennellii* which ran much later due to the TMS groups which attach to the O-H groups on the sugar molecules (Figure 5-18).

The fragmentation patterns and the ions increased by 14 *m/z* within the three main chromatogram peaks suggested that the compounds were fatty acid esters (Figure 5-21A and Figure 5-22 B). The fatty acid 3Z,6Z,9Z-dodecatrien-1-ol was chosen as an example which gave the compounds the same mass as the ion 341 (342 due to negative ion mode [M-H-]) when attached to nicotine, and dimers of this compound also match the masses of the unfragmented precursor ions 699 and 709 *m/z*. Alternative structures could be mixtures of nicotine and β-carboline alkaloids such as harmane, although these structures do not match the fragmentation pattern as accurately (Figure 5-23).

5.3.7 Antimicrobial fractions of *S. habrochaites* LA1777

The antimicrobial TLC fraction of *S. habrochaites* was different from the *S. pennellii*, and *N. rustica* fraction, with an antimicrobial band of compounds at R_f 0.55. The identified compounds were identified as omega 3 fatty acids and sesquiterpenes, and largest concentration was *cis*-5,8,11,14,17-eicosapentaenoic acid (Figure 5-14). These compounds were separate from the other fatty acids which were present in similar concentrations across several bands, and were only found in smaller concentrations within the R_f values of 0.4 and 0.65.

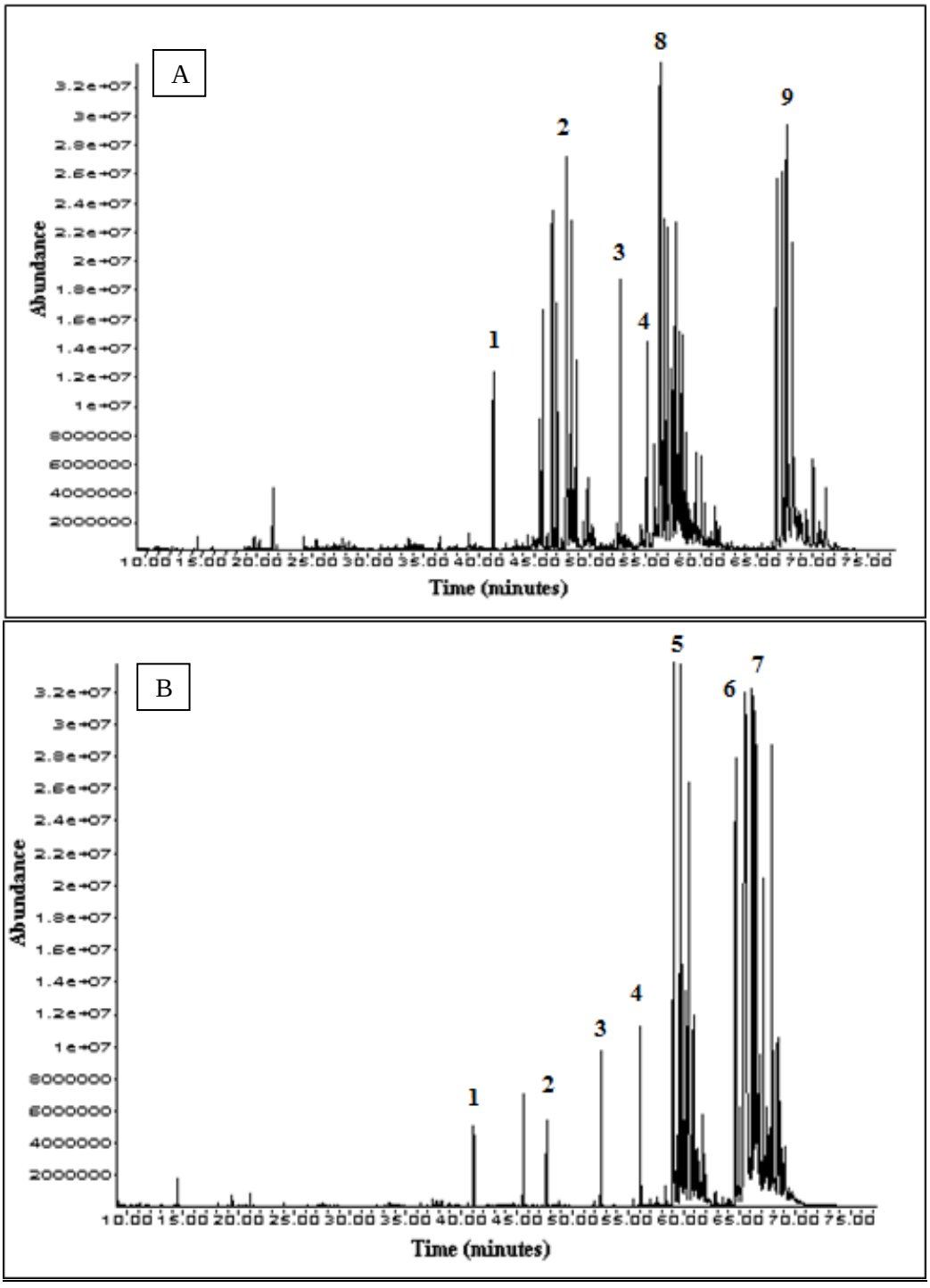

Figure 5-29A. *S. pennellii* TLC band with an R_f of 0, and B. *S. habrochaites* TLC R_f 0.

Components with the highest areas were identified using NIST as; 1. palmitic acid at 41.1 min, 2. nonodecanoic acid at 47.6 min, 3. sucrose at 53.8 min, 4. *bis*(trimethylsily)monostearin at 56.1 min, 5. hexopyranose, 1,2,3,4,6-pentakis-*O*-(trimethylsilyl) at 59.1 min, 6. pregnane-3,20-dione, 17,21-*bis*[(trimethylsilyl)oxy]- at 65.4 min and 7. 3*β*,4*β*-*bis*(trimethylsiloxy)cholest-5-ene at 68.4 min, 8. 1,3-dioxolan-4-propenoic acid, 2,2-dimethyl-5-[2-(t-butyldimethylsilyl)oxy] at 56.5 min, and 9. *α*-D-glucopyranoside, 1,3,4,6-tetrakis-*O*-(trimethylsilyl)-*β*-D-fructose at 67.5 min. Data is from one technical replicate of a pool of three plant replicates.

The NIST matches to these compounds was not strong and alternative matches were linked to sesquiterpenes such as *trans*-*α*-bergamotene, *β*-santanol, and *α*-*cis*-santalol. These alternative compounds have structures similar to class II sesquiterpenes known to be present within *S. habrochaites* LA1777 (Besser et al., 2009). It is more likely that these compounds are sesquiterpenes rather than omega 3 fatty acids, due to this previous research into terpenoids from *S. habrochaites* LA1777 trichomes where the class II sesquiterpenes carboxylic acid derivatives are produced at 93% of total terpenoids the highest being *α*-santalenoic acid.

LC-MS analysis of the active R_f 0.55 fraction also confirmed that these compounds are sesquiterpenes. This analysis identified the *α*-*cis*-santalenoic acid, and *α*-*cis*-bergamotenoic acid to be the main two compounds within the extract, and with additional glycosylated versions of these two compounds also present. Both peaks show the exact mass of both *α*-*cis*-santalenoic acid and *α*-*cis*-bergamotenoic acid which have the same molecular mass but different conformations (Figure 5-24 2b, 3b and Figure 5-25). However, the peak at 24.8 min is most likely to be *α*-*cis*-santalenoic acid because it has the highest intensity and is known to be produced in the highest concentrations within *S. habrochaites* LA1777. This leads to the conclusion that the antimicrobial compounds within *S. habrochaites* LA1777 are sesquiterpene carboxylic acids, and that this activity may be attributed to either these free compounds or the glycosylated versions, or from both types.

Whilst the main active compounds from *S. habrochaites* LA1777 were from R_f 0.55, the TLC fraction R_f 0 also had the same retention time as the active peaks identified within *S. pennellii* at R_f 0, within the retention region of 65 to 70 min (Figure 5-29). GC-MS identification of these peaks showed, only some of the peaks were glucose related compounds and the largest compound class identified at R_f 0 for *S. habrochaites* were sterols such as pregnane-3,20-dione, 17,21-*bis*[(trimethylsilyl)oxy] (Figure 5-29). This suggests that although the compounds have the same retention times, they are not the same.

Further investigations into comparisons between the *S. habrochaites* TLC R_f 0 and *S. pennellii* TLC R_f 0, using LC-MS (MAXIS), show that both fractions contained non-polar compounds with similar fragmentation patterns. This suggested that the

compounds have very similar properties and structures. However, the differences between the extracts give *S. pennellii* antimicrobial activity which was not present in the equivalent fraction of *S. habrochaites*. The similar ions in both plant extracts were 681, 695, and 723 *m/z* and both samples exhibit closely related fragmented ions, such as 495, 481, and 425 *m/z*. *S. pennellii* has several ions which were not present in *S. habrochaites*, which included 709, 699, and 671 *m/z*. Due to the larger mass and higher retention time the ions 709 and 699 could be the antimicrobial compounds in *S. pennellii*, as the antimicrobial compounds ran between 65 and 70 min on the GC-MS chromatogram.

5.3.8 Antimicrobial fractions of *S. pennellii* LA0716

The antimicrobial fraction from *S. pennellii* was identified by two separate methods using TLC and SPE to isolate fractions of a trichome extract and then testing these extracts for antimicrobial activity in disc diffusion assays. The active TLC fraction was R_f 0 from trichome extracts of *S. pennellii*, and the active SPE fraction was identified as the ethyl acetate fraction (Figure 5-19). Analysis of the compounds within these fractions was performed using GC-MS identification, and the active fractions all contain increased concentrations of peaks within the retention region of 65 to 75 min.

The other fractions generally showed consistent levels of the peaks in two clusters between 45 and 65 min. These clusters are believed to be the sugar esters known to be produced in high concentrations by *S. pennellii*. Analysis of the fragmentation patterns was performed of the most abundant peaks within this cluster between 55 to 65 min, specifically 56.811 min (RI 3067.8) was identified through NIST as dioxolan-4-propenoic acid, 2,2-dimethyl-5-[2-(t-butyldimethylsilyloxy)propyl] from *S. pennellii* 2D TLC R_f 0 x 0.66 (Figure 5-15). The fragmentation pattern suggests that these compounds could be sugar esters due to several of the fragment masses corresponding to the molecular weight of known fatty acid methyl esters which combine with glucose in a variety of combinations to give rise to different glucose esters (Figure 5-17).

Analysis of glucose esters using GC-MS has been performed in previous research, and the gas chromatograms of this study are similar to the gas chromatograms of total sugar

esters found within fractions of sucrose esters from tobacco leaf (Severson et al., 1985). Severson et al., (1985) also found that partial hydrolysis of the sucrose esters found within tobacco gave rise to glucose esters which ran together as a group with a lower run time compared to the sucrose esters. This could suggest that the clusters of peaks within *S. pennellii* and *S. habrochaites* could correspond to different types of sugar esters such as glucose and sucrose esters, with the sucrose esters with the highest run time due to a having higher molecular mass. An alternaive theory is that the compounds are similar to steroidal saponin glycosides, where the difference by -14 mw for each identified ions could represent changes fragmentation from the hydrocarbon tail of the steroid (Figure 5-28).

The production of sugar esters within the Solanaceae family is a common occurrence, such as the analysis of non-tuberous *Solanum* species by King et al., 1988 where *S. aethiopsicum, S. corolinense, S. lactum mill, S. maritinum, S. nigrum var chloracaepum, S. pyracantum, S. sturtianum, S. vignoi,* and *S. villorian* were found to produce 2,3,4-*tri*-*O*-acylated sucrose esters, which contrasts with the 2,3,4-*tri*-*O*-acylated glucose esters produced by *S. pennellii*.

Whilst it is hypothesised that the peaks within the region of 65 to 70 min contain the antimicrobial compounds, conflicting evidence should be taken into account such as there were similar compounds in similar concentrations found in within the *S. habrochaites* non-antimicrobial fraction R_f 0. This suggests that either these compounds are not antimicrobial or they are not exactly the same compounds between the plant species. The sugar esters are widely used within industries such as food and detergents due to their biodegradable, non-toxic and amphiphilic properties. Their antimicrobial potentials have been highlighted within research where compounds such as 6-*O*-lauroylsucrose, 6-*O*-lauroylmaltose, and 6-*O*-lauroylmaltotriose were found to be antimicrobial against *Streptococcus sobrinus* at concentrations of 100 µg mL^{-1} (Devulapalle et al., 2004).

The NIST identification of these potentially active compounds was to sugar disaccharides with TMS esters from the derivatisation process, such as *α*-D-glucopyranoside-1,3,4,6-tetrakis-*O*-(trimethylsilyl)-*β*-D-furanosyl-2,3,4,6-tetrakis-*O*-(trimethylsilyl). The identification of sugars through GC-MS analysis is notoriously

difficult due to the similar fragmentation patterns, and the NIST matches from the sugar compounds are poorly identified. Further analysis of the fragmentation patterns suggests that the compounds could be glycosides, and possibly glyco-sterols, such as the compound hypothesised as cholesteryl *β*-D-glycoside (Figure 5-16). An alternative theory is that the active compounds are within these fractions but have not been detected by the selected GC-MS detection method.

If the compounds are glycosylated sterols the hypothesis could be that the sugar portion of the molecule gives the compound additional polar properties which may influence the way the compound affects the bacteria. This polarity could be increasing the uptake of the compound into the bacteria or affecting the bacterial cell membrane. Sterols are important components of the cell membranes of mammals (cholesterol), fungi (ergosterol), plants (stigmasterol), and primitive bacteria (hopanoids) (Dufourc, 2008).

Sterols are generally viewed as membrane reinforcers, due to their influence on ordering the membranes, but they also have a wider role in maintaining the membrane as a temperature stable structure which is fluid enough for cellular processes (Dufourc, 2008). The structure of sterols with their typically 4 to 5 fused ring structures seems to be the key property of a molecule to give an increased order to cell membranes, and slight variations in this structure in the form of branched chains can provide increased stability over a wider temperature range, such as used in plant sterols (Dufourc, 2008).

The *S. pennellii* SPE ethyl acetate fraction was analysed using LC-MS (MAXIS) and hypothesised structures based on the fragmentation patterns suggest that these compounds could be sterol fatty acid esters, such as stigmasterol attached to an 19-hydroxynonadecanoic acid, and *β*-amyrin attached to 18-hydroxyoctadecanoic acid both 709 *m/z* (Figure 5-27). Due to the presence of this compound in the *S. pennellii* sample but not the *S. habrochaites* non-antimicrobial fraction, this is the likely mass of the antimicrobial compounds.

Cholesteryl palmitate has been investigated for its influence on the dynamics of dipalmitoylphosphatidycholine (DPPC) membrane system, and it was found that the ring system of cholesterol is markedly tilted away from the direction of the more typical *β*-cholesterol which appears in line with the normal membrane axis (Dufourc, 2008).

This tilt is due to the pull of the palmitoyl chain as the fatty acid chain is attracted to the membranes hydrophobic interior, and a "horseshoe" confirmation is formed. This suggests that cholesteryl palmitate does not have an optimal conformation required for the van der Waals interactions typically seen from cholesterol membrane packing (Dufourc, 2008). It is possible that this tilt could also be caused through cholesterol being glycosylated to a glucose molecule, and then its subsequent uptake into the bacterial membrane would lead to cell leakage and death.

The antimicrobial compounds from *S. pennellii* are likely to be glycosides, and glucose esters have been identified as potential antimicrobial compounds. There is evidence to support synergistic effects between glucose esters and other compounds, these effects can be seen from examples such as interactions between chitosan and alkyl *β*-D-glucopyranosides (Liu et al., 2004). It was shown that chitosan has a broad spectrum antimicrobial activity, but no antifungal activity towards *C. albicans*, the acyl glucose molecules were only able to inhibit *S. aureus* and the fungi *C. albicans.* However, a mixture of chitosan and acyl glucose was able to inhibit all the tested bacteria and fungi with a lower MIC compared to the chitosan alone, with eight times lower MIC against *S. aureus* (Liu et al., 2004).

The overall results highlighted within this chapter include isolation of antimicrobial fractions from *S. pennellii*, *S. habrochaites*, and *N. rustica*, and the putative identification of the most abundant compounds within these fractions. Due to the shortage of available chemical standards, and the high quantities of plant material which would be required to isolate enough pure compounds from the crude extracts for more accurate chemical identifications, compound classes and structures were identified instead through the use of several analytical machines. The antimicrobial fractions from the identified antimicrobial Solanaceae share similarities in the properties, such as being mainly non-polar and containing fatty acid esters, and all three plant species were mainly antimicrobial towards *S. aureus* 25923. The next chapter investigates how the crude trichome extracts of *S. pennellii* affect the metabolism and physical properties of *E. coli* O157:H7, *S.* Typhimurium 10248 and *S. aureus* 25923, and provides a hypothesis for the likely mode of action of the antimicrobial compounds, and how this is linked to the predicated structures of the compounds.

Chapter 6

Investigating the antimicrobial mode of action of Solanaceae trichome extracts

6.1 Introduction

This chapter describes studies on the potential mode of action of the crude extracts of *S. pennellii* on the bacterial pathogens *S.* Typhimurium 10248, *E. coli* O157:H7, and *S. aureus* 25923. The approach employed was to use metabolite profiling to assess the changes in steady state metabolite levels, following the addition of *S. pennellii* plant extracts to *S. aureus* broth cultures. Determination of metabolic phenotypes potentially demonstrates those biochemical processes affected by the bioactives in the extract and how the bacterium adapts to their presence. It is important to visually determine the structural effects of the extract upon the bacteria. This was achieved through the use of negative staining EM (using a transmission electron microscope). This enabled analysis of the structural effects of the extract upon the bacteria cells, and facilitated visual comparison between the Gram-negative and Gram-positive bacteria.

The use of cold methanol at a low temperature for quenching has been identified as a method which is able to permeabilise the bacteria cells for metabolomic analysis, with less cell leakage compared to other methods such as chloroform/methanol, and hot ethanol/methanol (Maharjan and Ferenci, 2003). Leakage of metabolites into the quenching solution is a serious problem in bacterial metabolomics, as the quenching solution must be able to stop the bacteria metabolism without cell lysis or leakage. In practice it is often a trade-off between the effectiveness of a quenching solution, where it has been found that experiments with less cell leakage have increased inconsistent results (Link et al., 2008). Through analysis of the supernatant these lost metabolites can be recovered and whilst the supernatant is mainly quenching solution it also contains the culture which is useful element to investigate, as this can highlight changes in the metabolites the bacteria uptake for growth, or it could highlight lost metabolites into the culture from cell damage.

The *Salmonella* cell extracts contained a wide selection of non-polar and polar metabolites, and only a few amino acids and some phosphate was lost to the supernatant using the methanol:NaCl solution (60:40 v/v, NaCl 0.9% in water w/v) (see Materials and Methods) showing an effective quenching method has been chosen, with a reduced amount of leakage into the quenching solution. The MICs of compounds were

performed in minimal media and LB for comparison so that the correct concentration could be used. The compounds used in the metabolomic study needed to show an effect on the bacteria without the inhibition of total growth, so a compound concentration lower than the MIC at a sub-inhibitory concentration was used in the cultures.

After identifying the most effective metabolite quenching and extraction methods the metabolomic analysis of *S. aureus* 25923 grown in the presence of *S. pennellii* trichome extracts was performed, with six biological replicates. The samples were run on the GC-MS (see Materials and Methods) in batches of 6 replicates, and the control LB samples were divided and run alongside these batches instead of a single batch, in order to normalise the results.

The challenge of extracting useful scientific conclusions from large data sets is a key aspect of omics research and there are several methods which can be employed to ensure reproducibility, and a better understanding of these results. The process of metabolic analysis goes through several stages, 1; the biological experiment, 2; raw data for data pre-processing, 3; clean data for data pre-treatment, 4; the data is fit for analysis and 5; a list of important metabolites can be produced (Van den Berg et al., 2006). The use of PCA is important for understanding patterns within large data sets. However, caution should be employed due to the possibility of over simplification or the loss of relevant information within the residual variation as part of the PCA data processing. This is why several graphical techniques have been used to visualise the final processed data.

Accurate metabolomic analysis of bacterial cultures can be difficult due to many factors influencing the results, such as the speed of culture quenching, pellet collection, and consistency within the extraction, and derivatisation sample processing. Other influences can lead to anomalous results such as contamination of cultures, the starting number of bacterial cells, and inaccurate data processing.

6.2 Results

6.2.1 Physiological effects of *S. pennellii* extracts upon bacteria, using negative stain EM

S. aureus 25923 had a lower sub-inhibitory concentration tested due to the higher antimicrobial activity of *S. pennellii* trichome extract towards this species (Chapter 3 Figure 3-14). *S. aureus* was grown in the presence of 0.78 mg mL^{-1} of crude trichome extract disolved in DMSO, and there were few differences between the LB, DMSO solvent control and the bacteria grown in the presence of the plant extract (A and B). However, the bacteria grown with *S. pennellii* extract, did show a increase in cell leakage, as the cells showed signs of the breakdown of their cell walls. *S. aureus* 25923 readily absorbed the KPT stain, the dark staining of the cocci was consistent between the test samples and the controls. The cell leakage from the cells was seen predominantly from the samples exposed to the *S. pennelii* extract (Figure 6-1), and is characterised by the contents of the cell being expelled into the surounding environment (Figure 6-1 C).

When *E. coli* O157:H7 was grown in the presence of *S. pennellii* extract there were fewer bacteria, with a poor morphology (Figure 6-2 C), they had lost flagella compared to the LB control. There was also flagella and fimbriae loss in the bacteria grown with only DMSO solvent (Figure 6-2 B). This was consitent with the higher antimicrobial affects that DMSO had upon *E. coli* compared with *S. aureus* and *S.* Typhimurium species. There was also a higher KPT stain uptake and visibly damaged cells with pitted and rough appearance to their cell walls (Figure 6-2 C).

S. Typhimurium 10248 showed a strong difference in the number of flagella at a 25 mg mL^{-1} concetration of *S. pennellii* trichome extract, where almost no flagella or fimbriae are seen attatched to the bacteria and are reduced within the background (Figure 6-3 C), compared to the controls which show many flagella and fimbriae present (Figure 6-3 A and B).

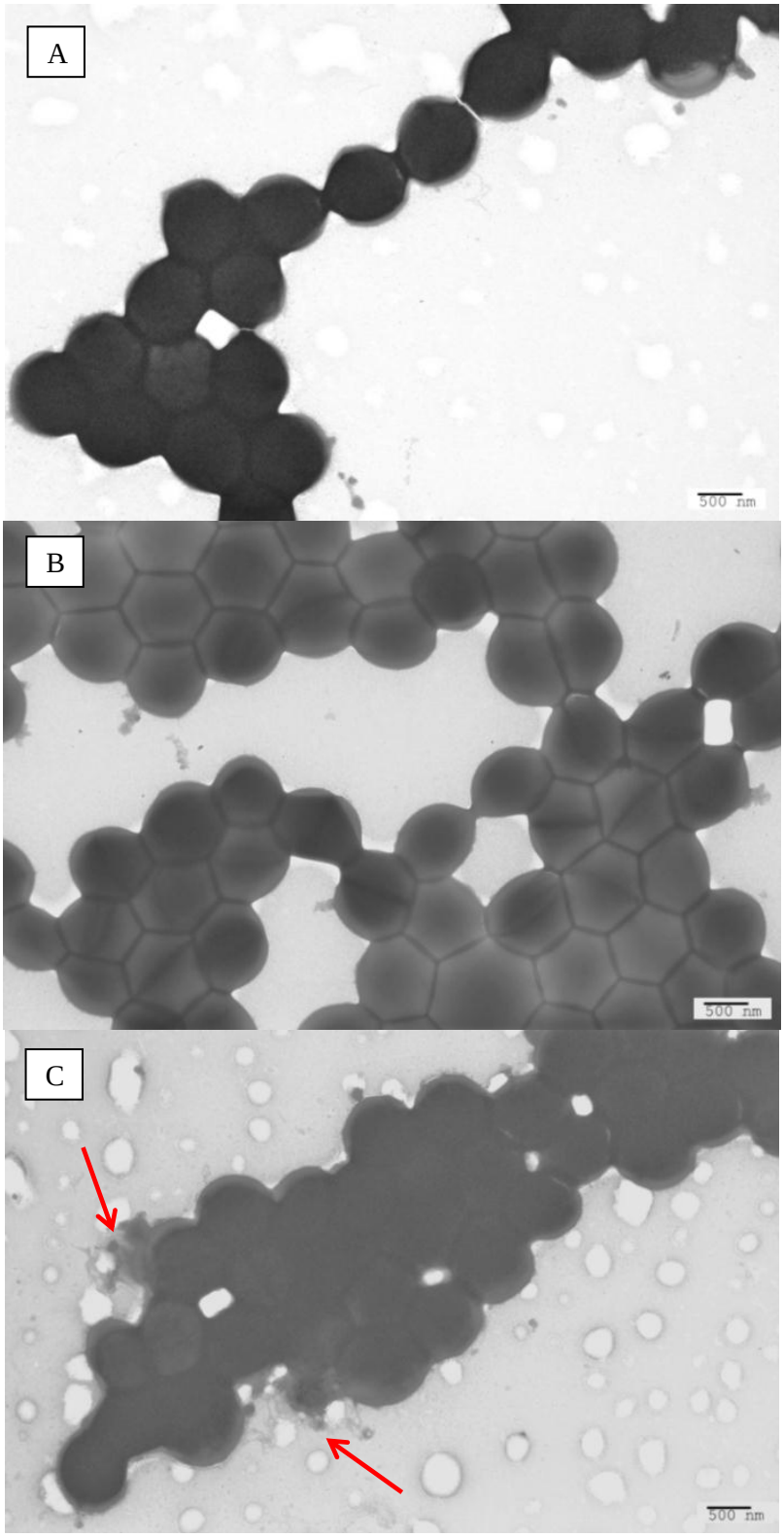

Figure 6-1. *S. aureus* 25923 negatively stained EM of bacterial cultures grown in the presence of *S. pennellii* extract and controls.

Bacteria were recovered from 10 mL cultures containing, A. LB, B. LB and DMSO, C. LB, and *S. pennellii* trichome extract re-suspended in DMSO (0.7 mg mL^{-1}). Red arrows in C show cell damage and leakage of cellular contents. The images were taken using a transmission electron microscope. Images were selected from two biological and two technical replicates, (500 nm, direct mag: 4800 x).

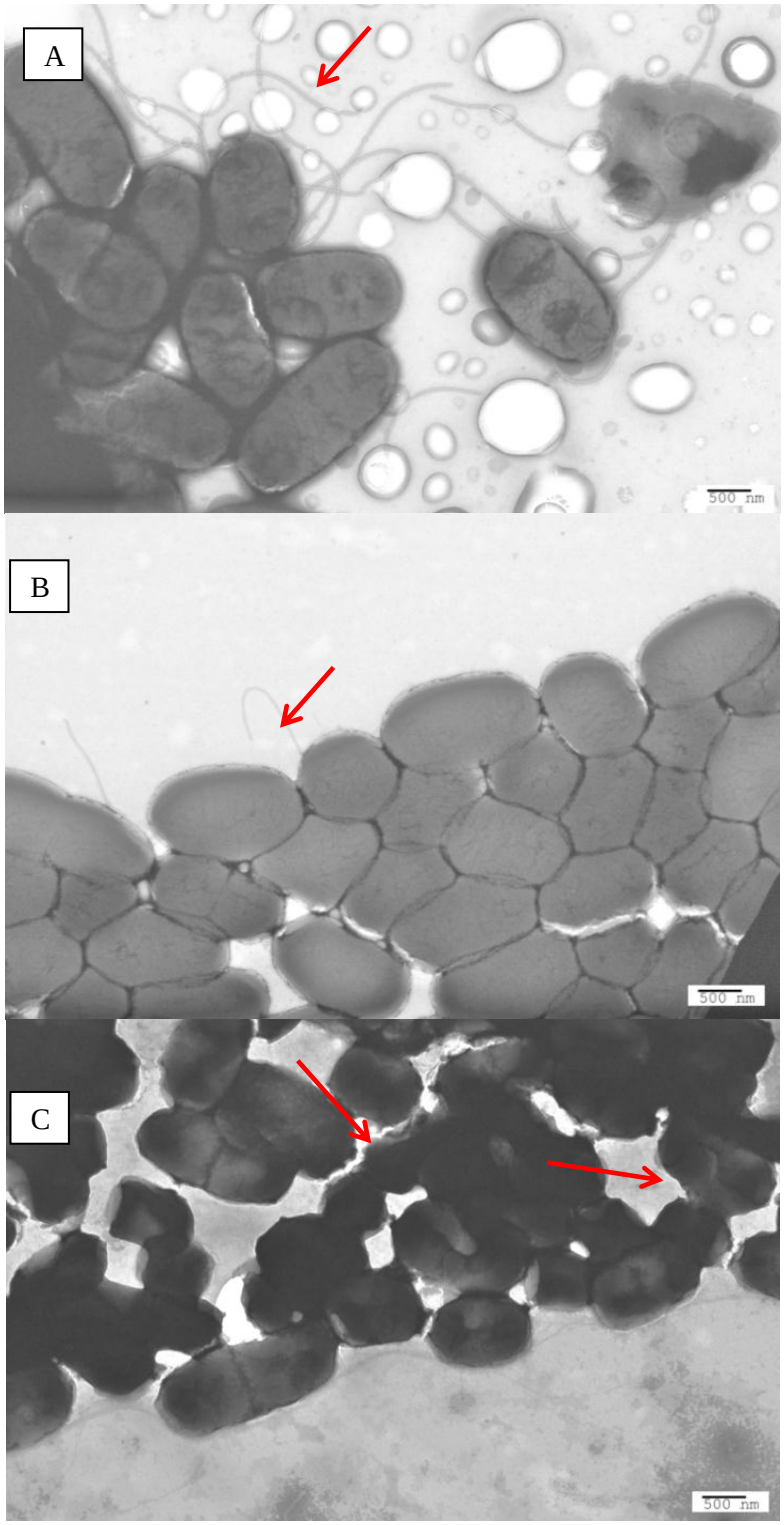

Figure 6-2. *E. coli* O157:H7 negatively stained EM of bacterial cultures grown in the presence of *S. pennellii* extract and controls.

Bacteria were recovered from 10 mL cultures containing, A. LB, B. LB and DMSO, C. LB, and *S. pennellii* trichome extract re-suspended in DMSO (25 mg mL^{-1}). Red arrows in A show many flagella, compared to B where only two flagella are observed. Arrows in C show the rough appearance to the cell wall and increased stain uptake. The images were taken using a transmission electron microscope. Images were selected from two biological and two technical replicates, (500 nm, direct mag: 4800 x).

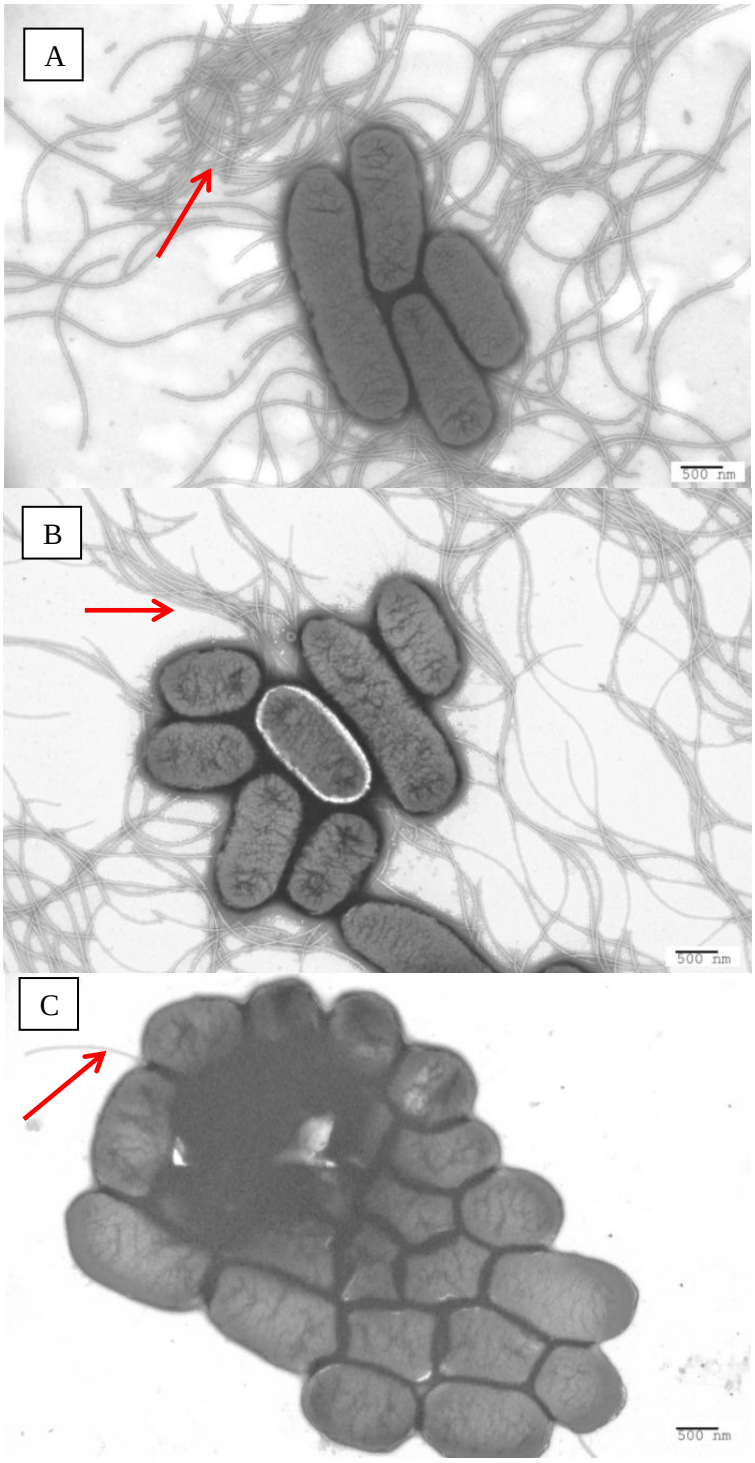

Figure 6-3. *S.* Typhimurium 10248 negative stain EM of bacterial cultures grown in the presence of *S. pennellii* extract and controls.

Bacteria were recovered from 10 mL cultures containing, A. LB, B. LB and DMSO, C. LB, and *S. pennellii* trichome extract re-suspended in DMSO (25 mg mL^{-1}). Red arrows in A and B show many flagella, compared to where only two flagella are observed in C. The images were taken using a transmission electron microscope. Images were selected from two biological and two technical replicates, (500 nm, direct mag: 4800 x).

The types of physiological damage caused by the *S. pennellii* trichome extracts at sub-inhibitory concentrations are shown by the images in Figure 6-4 where A and B show *S.* Typhimurium 10248 which have no leakage of cell contents but show a general loss of flagella and fimbriae, *E. coli* was more affected by the plant extract than *S.* Typhimurium shown in C and B, where additional to flagella loss the cell membrane appears rough. The effects against *S. aureus* were seen in cell rupture, which shows what appears to be releases of cell content see Figure 6-4 E and F.

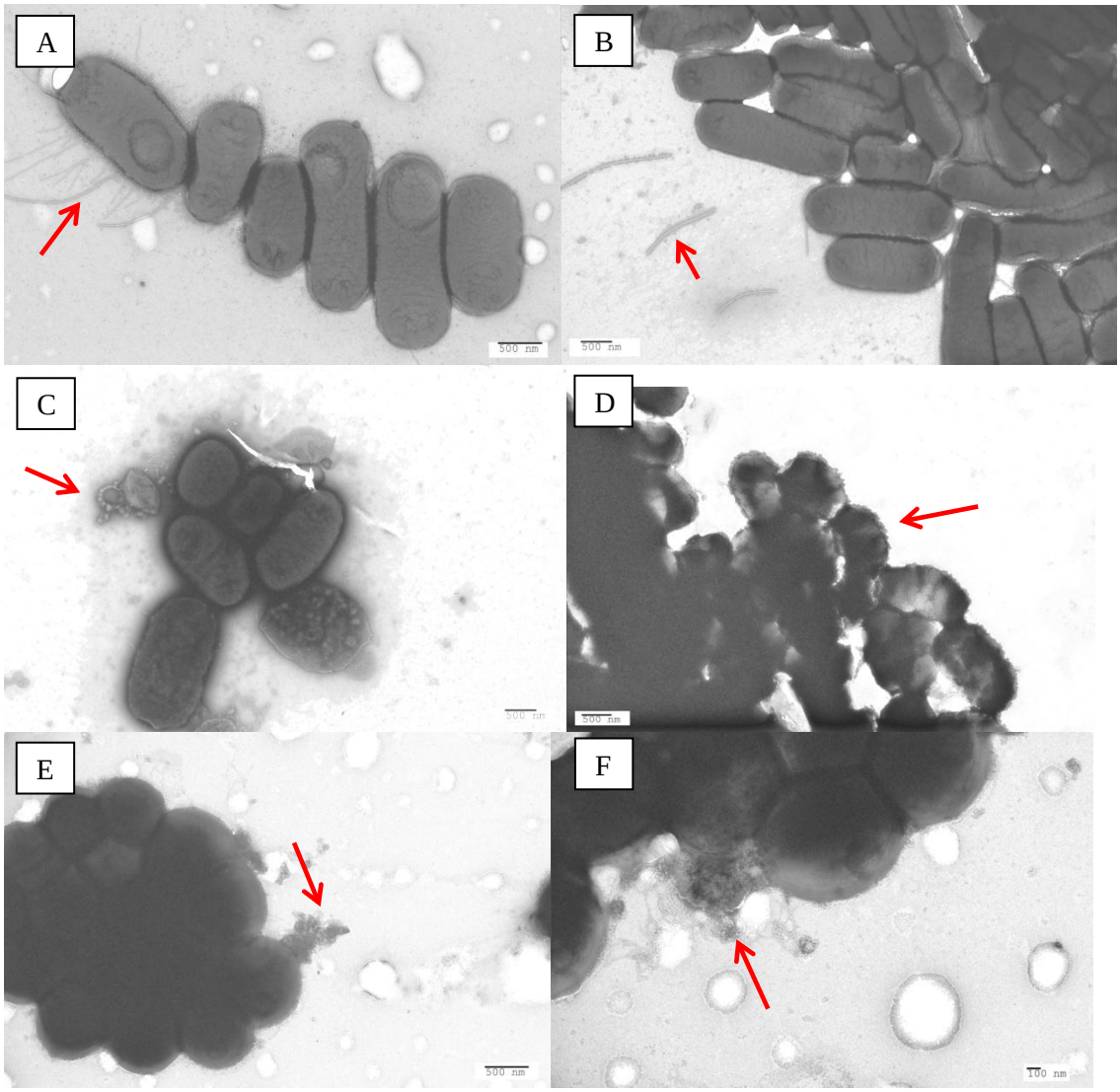

Figure 6-4. Bacteria grown in the presence of *S. pennellii* trichome extracts.

A. *S.* Typhimurium 10248, scale bar 500 nm, mag x 6800, B. *S.* Typhimurium 10248, scale bar 500 nm mag x 4800, C. *E. coli* O157:H7, scale bar 500 nm, mag x 4800, D. *E. coli* O157:H7, scale bar 500 nm mag x4800, E. *S. aureus* 25923, scale bar 500 nm mag x 6800, F., *S. aureus* 25923, scale bar 100 nm mag x 11000x. All images show bacteria grown in the presence of *S. pennellii* trichome extract. Images were selected from two biological and two technical replicates. Red arrows in A and B show the loss of flagella in *S.* Typhimurium 10248, C and D show *E. coli* O157:H7 cell wall damage, E and F show *S. aureus* 25923 cell lysis and release of cell contents.

The use of a sub-inhibitory concentration of *S. pennellii* trichome extract tested against the bacteria was intended to allow them to grow with the expectation that some physiological effects of the extract could still be seen. The CFU mL^{-1} of the bacteria species showed that all the bacteria grew to around 1 x 10^9, with the most differences in growth for *S.* Typhimurium. However, no fold changes in growth were seen from any of the bacteria compared to the LB and solvent controls.

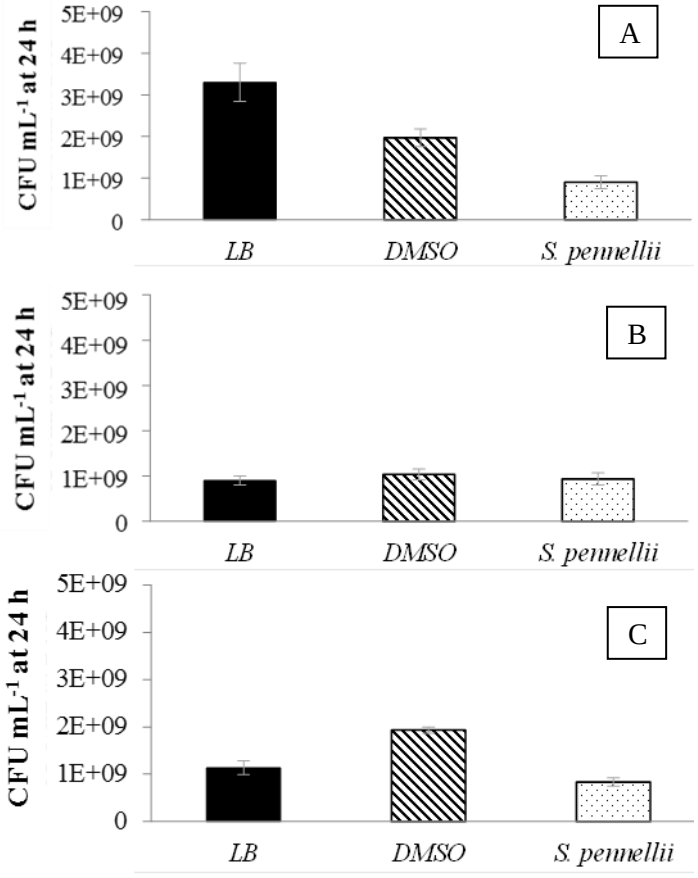

Figure 6-5. Bacterial growth measured in CFU mL^{-1} of the samples grown in the presence of *S. pennellii* extract and controls.

The colony counts from the cultures were measured after 24 h of growth, A. *S.* Typhimurium 10348, B. *E. coli* O157:H7, C. *S. aureus* 25923. Error bars represent two technical replicates and two biological replicates.

6.2.2 Motility assays

Motility assays were performed by culturing the test bacteria in LB, LB and solvent (DMSO), or LB and *S. pennellii* trichome extract in two concentrations (25 mg mL^{-1} and 50 mg mL^{-1}). The results from motility assays demonstrated that the majority of the bacteria showed no difference between the diameter of the migration zones within semi-solid agar from the different cultures, although some visual differences in the

morphology of the migrations could be seen (Figure 6-7). *S. aureus* 25923 showed slight changes in colony morphology, but no migration zones were observed for any of the test cultures from either of the concentrations of *S. pennellii*.

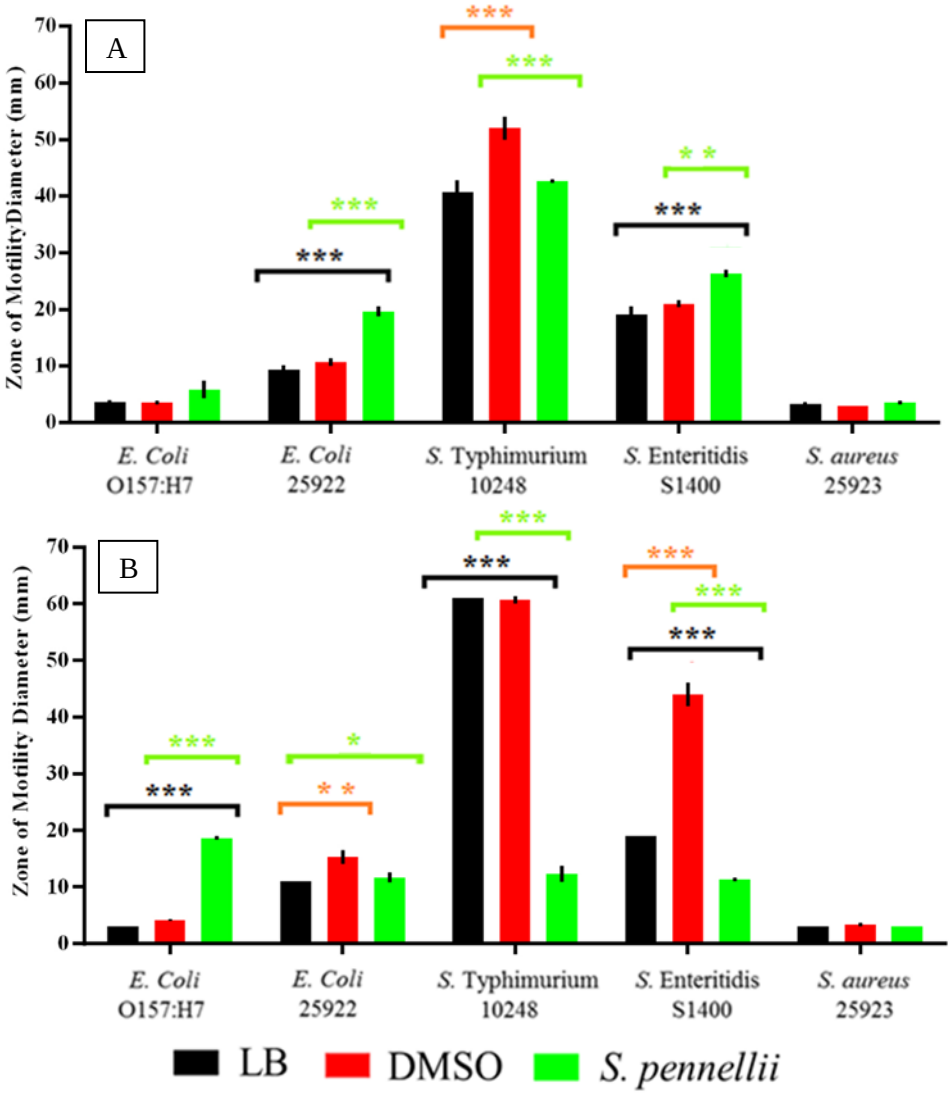

Figure 6-6. Motility zone diameter (mm), for bacteria *E. coli* O157:H7, *E. coli* 25922, *S.* Typhimurium 10248, *S.* Enteritidis S1400, and *S. aureus* 25923.

The black bars represent bacteria grown in only LB, the red bars show the DMSO control, and the green bars represent the bacteria grown within the *S. pennellii* trichome extract. A. Concentration of *S. pennellii* extract dissolved in DMSO and added to LB 25 mg mL^{-1} (0.78 mg mL^{-1} for *S. aureus* 25923), B. concentration of *S. pennellii* extract dissolved in DMSO and added to LB 50 mg mL^{-1} (1.56 mg mL^{-1} for *S. aureus* 25923). Error bars represent three biological replicates of bacteria, and plant extract was pooled from three technical replicates. Significance was determined through two-way ANOVA tukey's multiple comparisons test, * = $P<0.05$, ** = $P<0.01$ and *** = $P>0.001$. The black parentheses compare *S. pennellii* to LB, the red parentheses compare LB to DMSO, and the green parentheses compare *S. pennellii* to DMSO.

S. aureus cultures in the presence of the two concentrations of *S. pennellii* trichomes extract, showed some variations in morphology and one out of three of the biological replicates at both concentrations of *S. pennellii* trichome extract had migration away from the colony centre. The morphology of these bacterial colonies showed lines of bacterial migration away from where the bacteria were originally spotted. The morphology occurred twice as a singular diverging branch away from the colony and once more from one of the replicates at concentration of 25 mg mL^{-1} where the smaller branches left the colony consistently around the whole circumference (Figure 6-8).

The concentration of *S. pennellii* trichome extract at 50 mg mL^{-1} was high enough to show no increased motility in the cultures compared to the controls (Figure 6-7 B), with the exception of *E. coli* O157:H7 which showed increased motility at 50 mg mL^{-1} ($P<0.001$ compared to DMSO and LB) (Figure 6-6 B). *E. coli* 25922 also showed significant difference between the *S. pennellii* extract and the LB control ($P<0.05$). However, both the tested *Salmonella* species had lower zones of motility compared to controls ($P<0.001$) (Figure 6-6 B).

The *S. pennellii* trichome concentration of 25 mg mL^{-1} was shown to have positive effect upon motility, where two of the bacteria species increased their motility compared to the controls; *E. coli* 25922 ($P<0.001$) and *S.* Enteritidis S1400 ($P<0.001$ against LB, and $P<0.01$ against DMSO) (Figure 6-6 A). The two bacteria species tested as part of the EM experiments *E. coli* O157:H7 and *S.* Typhimurium 10248 which had shown flagella loss in the presence of *S. pennellii* extract, did not significantly increase their motility compared to the LB and DMSO controls (Figure 6-6 A).

Although the motility results shows no flagella loss or flagella recovery due to the motility of the bacteria within the semi-solid agar plates, the plant extracts do have morphological effects upon the migration patterns which represent the swarming behaviour of the cells (Figure 6-8). *S.* Typhimurium 10248 had a similar morphology from all the tested cultures, and *E. coli* O157:H7 had no swarming motility and grew as a colony on the surface except for one replicate which had a small motility zone of 9 mm. The lack of motility was also observed from the LB and DMSO controls for *E. coli* O157:H7.

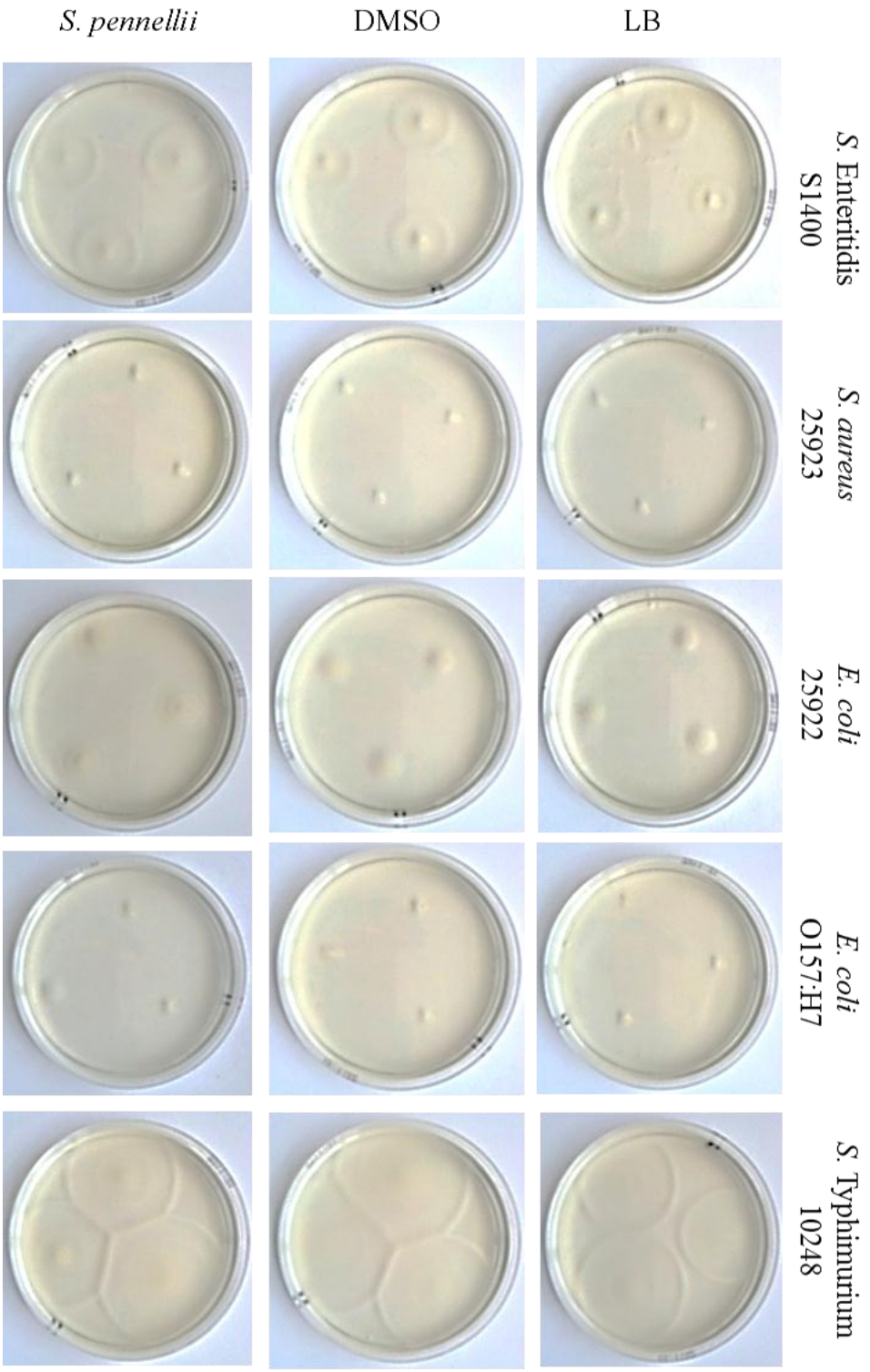

Figure 6-7. Motility assay showing the agar plates and the zones of bacterial migration.

S. Enteritidis S1400, *S. aureus* 25923, *E. coli* 25922, *E. coli* O157:H7, and *S.* Typhimurium 10248. Plates show three technical replicates of 10 µL from bacterial cultures inoculated with LB, DMSO, or *S. pennellii* extract dissolved in DMSO at a concentration of 50 mg mL^{-1} (0.78 mg mL^{-1} for *S. aureus* 25923).

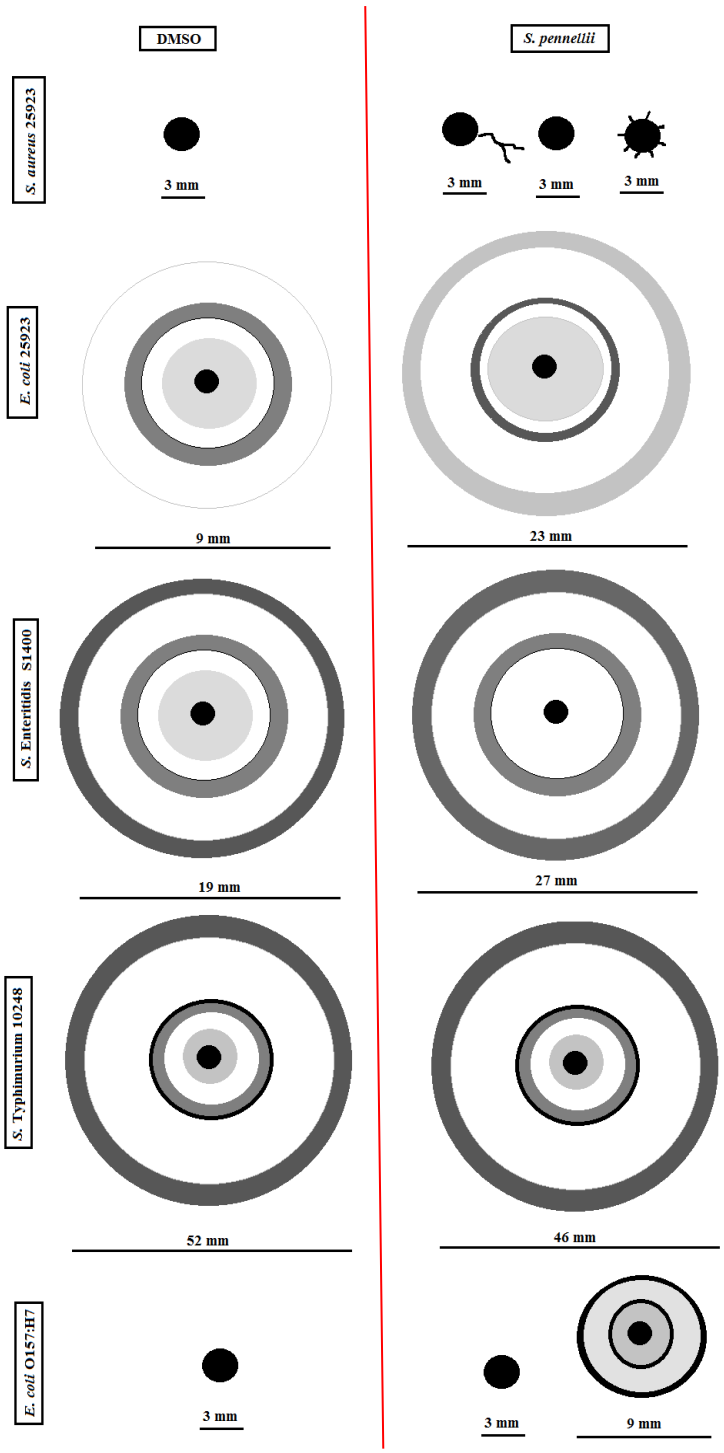

Figure 6-8. Migration morphologies drawn from observation of bacteria after 16 h of growth.
Patterns show the differences between DMSO (left side) and *S. pennellii* trichome extract (right side), showing the morphologies of *S. aureus* 25923, *E. coli* 25922, *S.* Typhimurium 10248, *S.* Enteritidis S1400, and *E. coli* O157:H7 grown in either DMSO or a concentration of 25 mg mL^{-1} of *S. pennellii* trichome extract (0.78 mg mL^{-1} for *S. aureus* 25923). The diameters of the motility zone is stated under each image.

6.3 Metabolomics

Metabolomic analysis was performed for *S. aureus* 25923 cultured in the presence of *S. pennellii* trichome extract (0.78 mg mL^{-1}), which was added to the media after 6 h of normal growth. This experimental design allowed the bacterial samples to be taken at 6 h (mid-exponential phase) and compared to two additional samples taken post challenge 30 min later at 6.30 h, and 18 h later at 24 h. The samples were quantified using an internal standard d4-succinate (1 mg mL^{-1}) for polar samples (Figure 6-9 A 5 and C 5), and d27-myristate (1 mg mL^{-1}) for non-polar samples (Figure 6-9 B 13). The data was normalised to the weight of the dry pellet from each bacterial sample. Culture aliquots were immediately quenched in cold (-20^{o}C) methanol and water containing NaCL 0.9% w/v (60:40 v/v) and then centrifuged. The pellet and sample of the supernatant was evaporated under vacuum, and then extracted for polar and non-polar metabolites (see Methods section for details).

There were visually no dramatic differences seen through comparing the GC-MS chromatograms of the *S. aureus* samples cultured under the different conditions (Figure 6-9 and Figure 6-10). All the samples produced an abundance of components, and the extracts were subjected Principal Component Analysis (PCA) to reduce the data and view clustering of bacterial treatments under different conditions. Analysis of the variations between the bacteria treatments at the different times of mid exponential (6 h), 30 min post treatment (6.30 h), and 18 h post treatment (24 h) showed that there were differences between the samples. PCA of the total data showed that there was separate clustering for the different treatments and for the different times bacteria were sampled.

6.3.1 GC-MS chromatogram analysis

The first stage of the analysis was to analyse the GC-MS chromatograms and to make comparisons between the bacteria cultured under typical conditions within LB only cultures. The pellet was extracted using a method which separates the polar and non-polar metabolites, and the supernatant was also analysed separately. The chromatograms showed clear differences in their profiles between the three conditions, with a good separation of the peaks (Figure 6-9). When visual comparisons were made between the

bacteria grown in LB and the bacteria grown in *S. pennellii* after 24 h (Figure 6-9 B and Figure 6-10 B) it is clear that the non-polar fraction of the pellet shows a cluster of peaks between 45 to 50 min, which is only present within the bacteria cultures containing *S. pennellii* trichome extract.

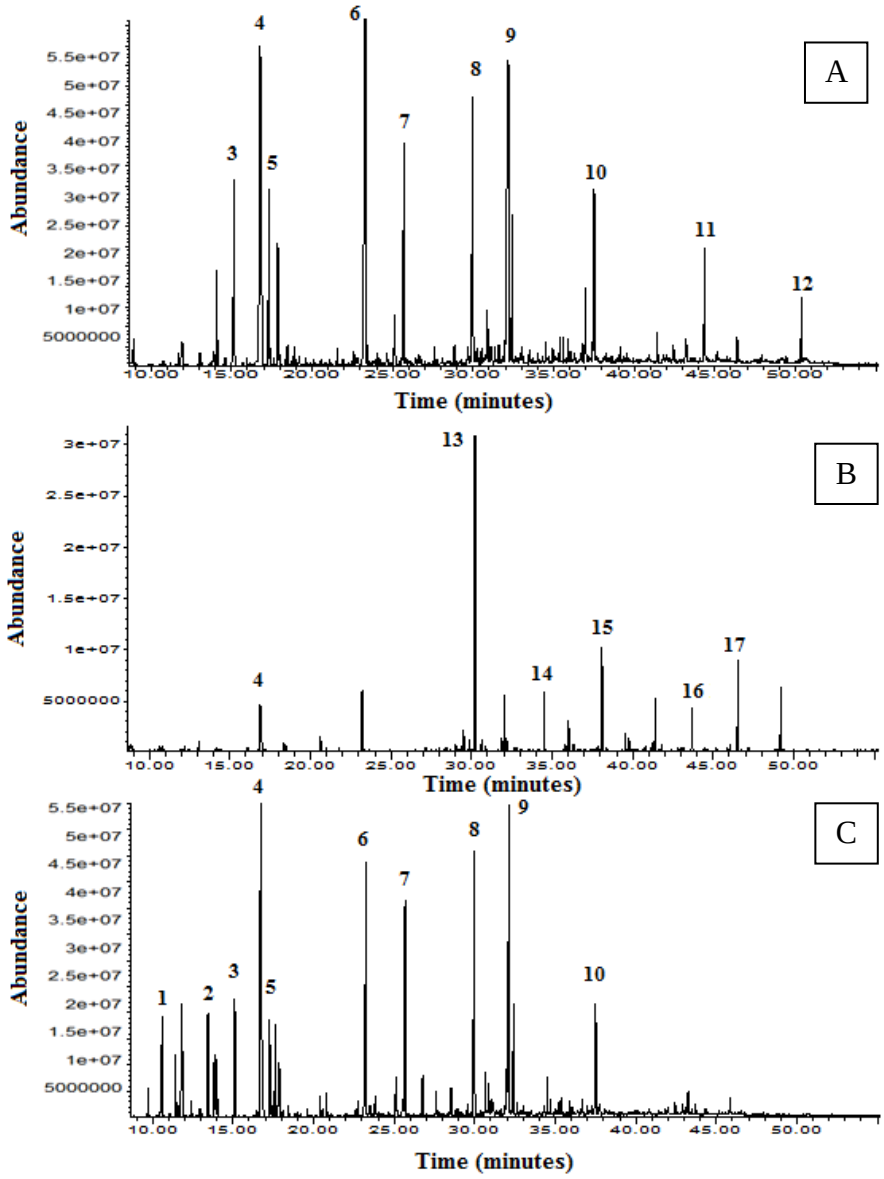

Figure 6-9. GC-MS chromatograms showing the LB culture of *S. aureus* after 24 h.

A.polar extract of bacterial pellet, B. non-polar extract of bacterial pellet, C. supernatant (quenching solution containing media). 1. lactic acid RT. 10.5 min, 2. norleucine RT. 13.4 min, 3. valine RT. 15.1 min, 4. phosphate-RT. 16.7 min, 5. d4-succinate-RT. 17.8 min, 6. pyroglutamic acid-RT. 23.2 min, 7. phenylalanine-RT.25.7 min, 8. ornithine-RT. 29.9 min, 9. lysine-RT. 32.1 min, 10. tryptophan-RT. 37.5 min, 11. adenosine-RT. 44.3 min, 12. disaccharide-RT. 50 min, 13. d27-myristate-RT. 30.2 min, 14. C16:0-RT. 34.5 min, 15. C18:0-RT. 38 min, 16. Glycero-2-ic15:0-RT. 41.2, 17. Glycerol-1-C16:0-RT. 43.6 min, and 18. Glycerol-1-C18-RT. 46.5 min.

These compounds are a contamination from the plant extract which was present in the pellet after centrifugation. These peaks were excluded from the metabolomic analysis because all the bacterial samples were compared to a library of standards and high NIST matched components which did not include these components. The total metabolite list including the average concentrations for each condition at each time is listed in Appendix 7.

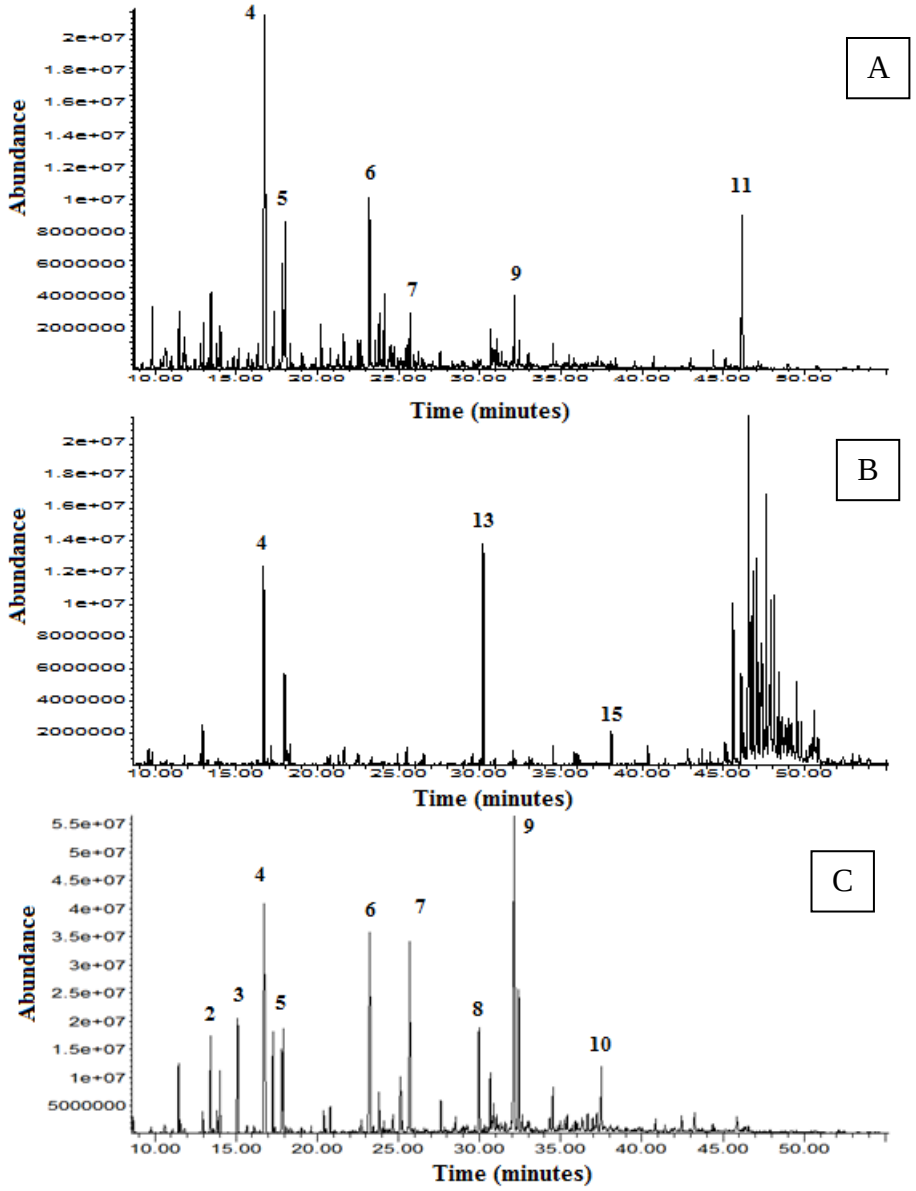

Figure 6-10. GC-MS chromatograms showing the *S. pennellii* challenged culture of *S. aureus* after 24 h.

A. polar extract of bacterial pellet, B. non-polar extract of bacterial pellet, C. supernatant (quenching solution containing media). 2. norleucine RT. 13.4 min, 3. valine RT. 15.1 min, 4. phosphate-RT. 16.7, 5 min. d4-succinate-RT. 17.8 min, 6. pyroglutamic acid-RT. 23.2 min, 7. phenylalanine-RT.25.7 min, 8. ornithine-RT. 29.9 min, 9. lysine-RT. 32.1 min, 10. tryptophan-RT. 37.5 min, 11. adenosine-RT. 44.3 min, 13. d27-myristate-RT. 30.2 min, 15. C18:0-RT. 38 min.

Metabolomic comparisons performed by identifying the most abundant compounds and aligning them in order of concentration can show clear differences between the metabolites produced by *S. aureus* grown under the different conditions. Cultures were all found to contain a spectrum of major DNA related, carbohydrate, fatty acids, amino acids, and several unknown compounds (Figure 6-11). The major fatty acids included (C_9), (C_{11}), (C_{13}), myristic (C_{14}), (C_{15}), palmitic (C_{16}), (C_{17}), stearic (C_{18}), (C_{19}), arachidic (eicosanoic acid) (C_{20}), (iC_{15}), (iC_{16}), (iC_{17}), (aiC_{15}), (aiC_{17}).

When the metabolomic results were presented in ascending order according to most abundant metabolites within the cultures of *S. pennellii* at the different time points, metabolites that had been significantly reduced or increased were observed. The metabolites present in cultures taken after 6 h should all have shown a similar metabolomic profile (Figure 6-11 A), as all the bacteria were subjected to growth in LB only, with no additional solvent or extracts added. The pattern shown after 6 h (mid-exponential) gives a guide of the accuracy of the experiment, because all the cultures only contain LB. The results showed that the method was accurate because all the cultures showed a similar metabolomic trend with only minor fluctuations in the detection of specific compounds relative to each culture, although the concentrations were slightly higher in the pre-*S. pennellii* culture (Figure 6-11 A).

Culture comparisons between the post-30 min of *S. pennellii* extract and the DMSO control, with no change to the LB control (Figure 6-11 B) showed that after a short exposure to the extract and the solvent that there were no differences between the compounds found within the samples. The main increases seen from *S. pennellii* compares with the levels present before the addition of the *S. pennellii* therefore can be excluded. The main differences from the DMSO control, was an increase of guanine and adenine.

The cultures which were incubated the longest after 24 h, which was 18 h post-challenge with *S. pennellii* and DMSO were compared to the LB control, and major differences were observed from the metabolomic profiles (Figure 6-11 C). The cultures containing *S. pennellii* were high in nicotinic acid, adenosine, aspartic acid, fatty acid aiC_{17}:0, *β*-alanine, galactose, norleucine, and serine, which were also absent in the DMSO and LB controls. The LB control showed no additional increases in any of the

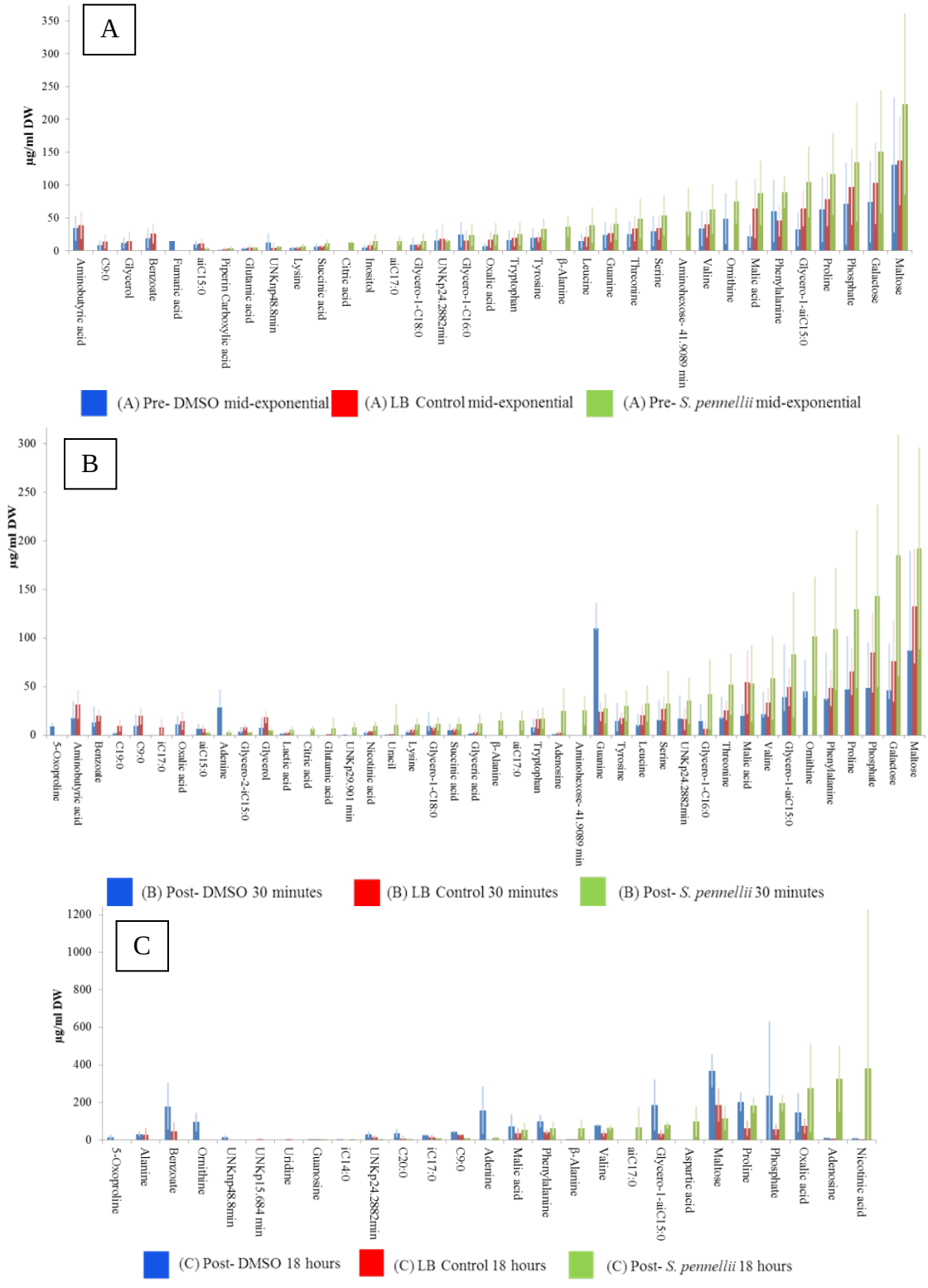

Figure 6-11. The most abundant metabolites identified within the *S. aureus* cultures.

The results show GC-MS identificantion of metabolites from pellets of *S. aureus* 25923; A. samples before addition of solvent or plant extract, B. samples 30 min post challenge with plant extract or solvent, C. samples 18 h post challenge with plant extract or solvent (24 h total culture growth). The blue bars are solvent controls, red bars are LB controls, and the green bars are *S. pennellii* extract (0.78 mg mL^{-1}). Error bars represent the standard error from six biological replicates and one technical replicate each.

compounds, and the DMSO cultures had increases in compounds alternative to *S. pennellii* such as benzoate, ornithine, adenine, glycerol-aiC_{15}:0, and maltose. Whilst the increases in certain compounds were observed from comparing the average compound concentrations, the statistically significant decreases in some compounds were better observed using statistical *t*-tests of the differences between selected cultures.

6.3.2 PCA analysis of *S. aureus* metabolite profiles

Further in depth analysis of the data was performed using metabolomic profile clustering (PCA), from the data of approximately 100 identified components. Comparisons were made between bacteria samples cultured with *S. pennellii*, DMSO and LB only, with the total data separated into two groups of bacteria pellet or supernatant. The data was aligned and processed for *t*-test statistical analysis, using the probability limits of $P<0.05$. Data was interpreted as both a scores plot for interpreting relations among the samples (Figure 6-12), and as a loading plot which interprets the relations among the variables (Figure 6-13). When considering the scores plot samples which are closely clustered together are similar and when interpreting the loading plot variables which are close have a positive correlation.

The PCA scores plot of the bacterial pellets and supernatants compared all the time points and all the culture conditions, which separated into different clusters depending on whether the pellet or the supernatant was analysed (Figure 6-12 and Figure 6-14). When comparing the PCA data for the supernatant samples, clustering was found from the different culture conditions into two main regions (Figure 6-12). The first region contained the LB controls at all the time points (A, B and C) which clustered tightly within one region of the PCA, this region also contained the pre-DMSO mid-exponential (A), and pre-*S. pennellii* mid-exponential (A) cultures.

The second region of clusters contains cultures from the time points 30 min and 18 h post challenge with DMSO and *S. pennellii* trichome extract (B and C). The samples from post-DMSO 30 min (B) has a large circle due to two regions of clustering within the group, where two cultures are similar to the LB control, and two samples cluster to the far right of the PCA. The *S. pennellii* cultures cluster together with the DMSO samples of the same time, which formed two groups within the cluster at 30 min post-challenge (B) and 18 h post-challenge (C) (Figure 6-12).

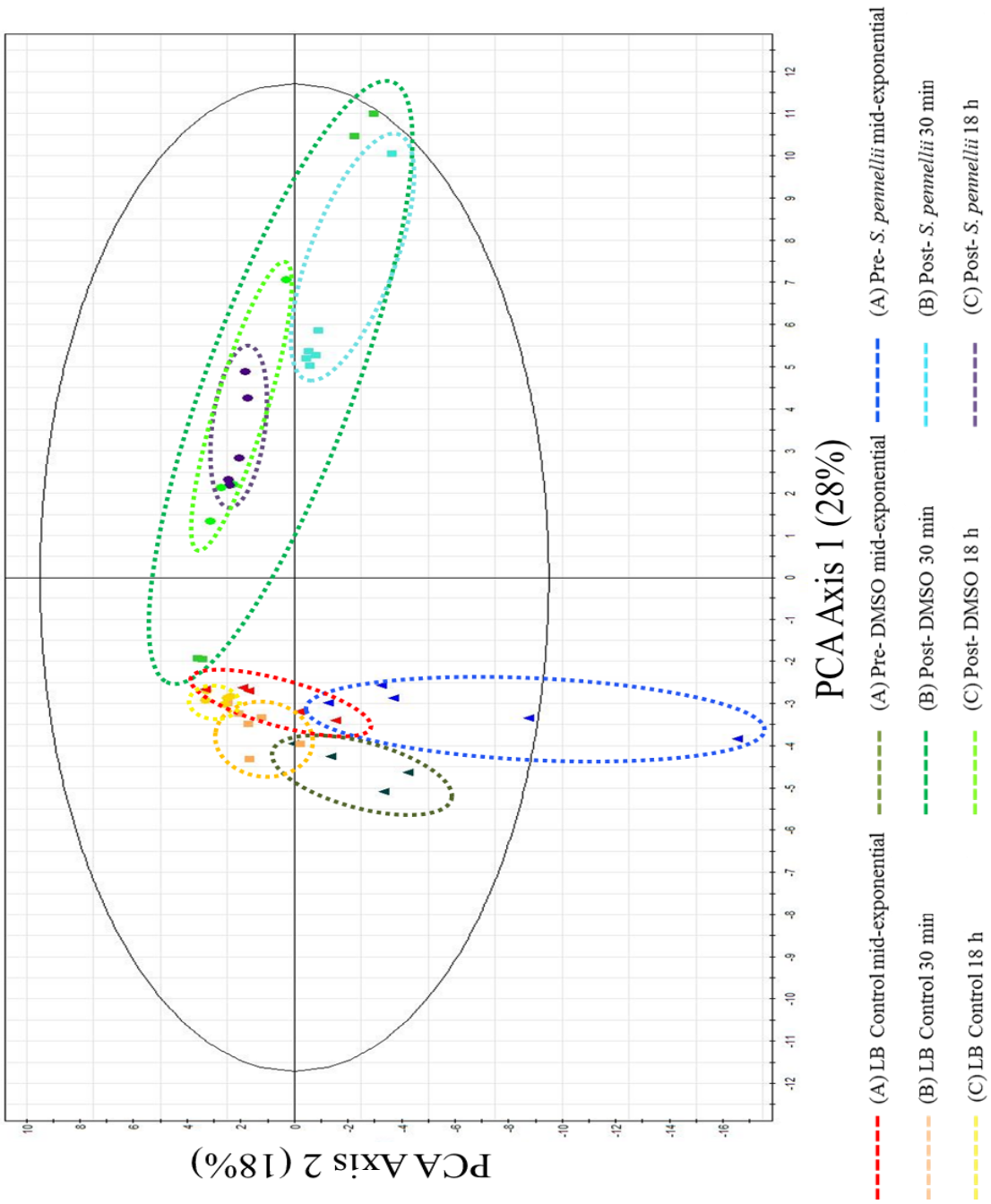

Figure 6-12. PCA of supernatant metabolites of *S. aureus*

Data obtained through growing the bacteria in only LB, LB containing DMSO or LB containing *S. pennellii* trichome extract (0.78 mg mL^{-1}). Data is represented in a score scatter plot, which projects the data onto subspace, showing cluster results from individual test bacterial cultures. Each cluster represents one technical replicate each for six biological replicates. Each culture was measured at; A. 6 h pre-challenge, B. 6.5 h (30 min post-challenge), and C. 24 h (18 h post-challenge). Data obtained using SIMCA-P software. The first component explains 28% of the variation and the second component 18%.

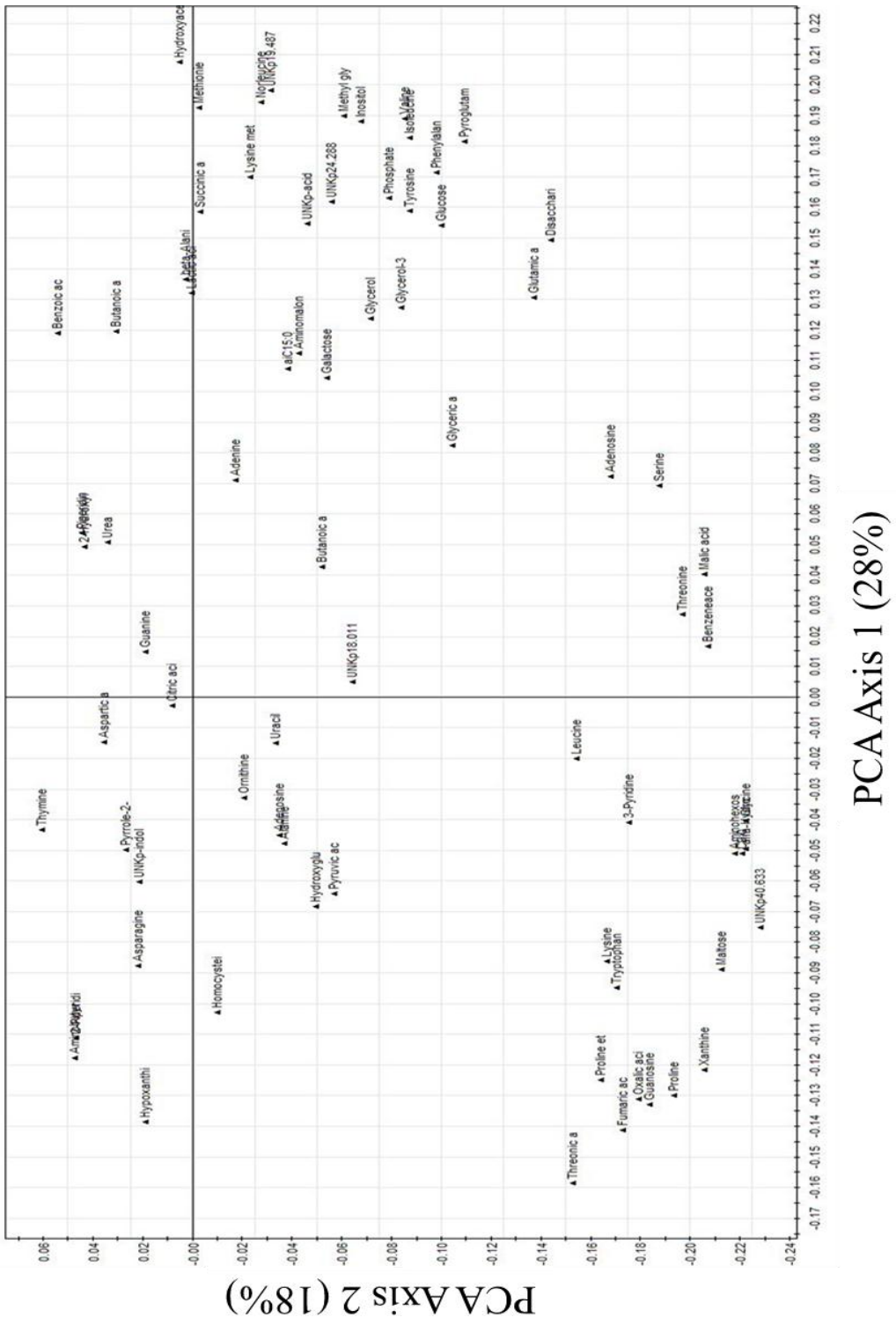

Figure 6-13. PCA of supernatant metabolites of *S. aureus*

Data obtained through growing the bacteria in only LB, LB containing DMSO or LB containing *S. pennellii* trichome extract (0.78 mg mL^{-1}). Data is represented in a loadings plot which plots the relationship between the original variable and the subspace dimetion, showing cluster results from individual test bacterial cultures. Each cluster represents one technical replicate each for six biological replicates. Each culture was measured at; A. 6 h pre-challenge, B. 6.5 h (30 min post-challenge), and C. 24 h (18 h post-challenge). Data is represents components contributing to separation; data obtained using SIMCA-P software. The first component explains 28% of the variation and the second component 18%.

The loading plot reveals useful information about the variables which were influential upon the observations seen within the clustering samples, as the further away a variable lies from the origin the greater the impact the variable has upon the model. The position of the variable also suggested whether the variables had been increased or decreased within samples. An overlay of the metabolites and the clusters highlighted uracil, ornithine, adenosine, hydroxyglutamatic at the centre of the cluster of the LB controls (Figure 6-13). This was subsequently close to 0 on the PCA plot, showing that these compounds did not have a strong effect upon the outcome of the analysis with average properties, and *t*-tests confirmed that the differences between samples were largely not significant.

Within the supernatant samples some of the main variables which were responsible for the clustering of the LB controls are the higher production of thymine, hypoxanthine, piperidine carboxylic acid, and aminobutyric acid, and a negative production of glutamic acid and disaccharides. The main variables involved in separating the DMSO and *S. pennellii* post 18 h (C) were the increase in production of variables benzoic acid and butanoic acid, with the decrease in variables such as threonic acid, fumaric acid, proline and xanthine. Some examples of variables which helped to separate the DMSO and *S. pennellii* post 30 min (B) were an increase in hydroxyacetic acid (glycolic acid), methionine, and norleucine, with a decrease in malic acid, phenylacetic acid, and threonine (Figure 6-13).

The PCA clustering from the bacterial pellet samples showed a similar pattern to the supernatant samples, with two main clusters forming on the left and right of the PCA (Figure 6-14). The LB controls at time points mid-exponential 6 h (A) and LB control 30 min post challenge (B) cluster to the left along with the same time points for DMSO and *S. pennellii*. The cluster on the right of the bacterial pellet PCA was all the samples after 18 h of growth, including the LB control, *S. pennellii* and DMSO. The LB control formed a loose cluster which covered most of the bottom right of the PCA, and the DMSO and *S. pennellii* (C) both clustered next to each other on the top right of the PCA.

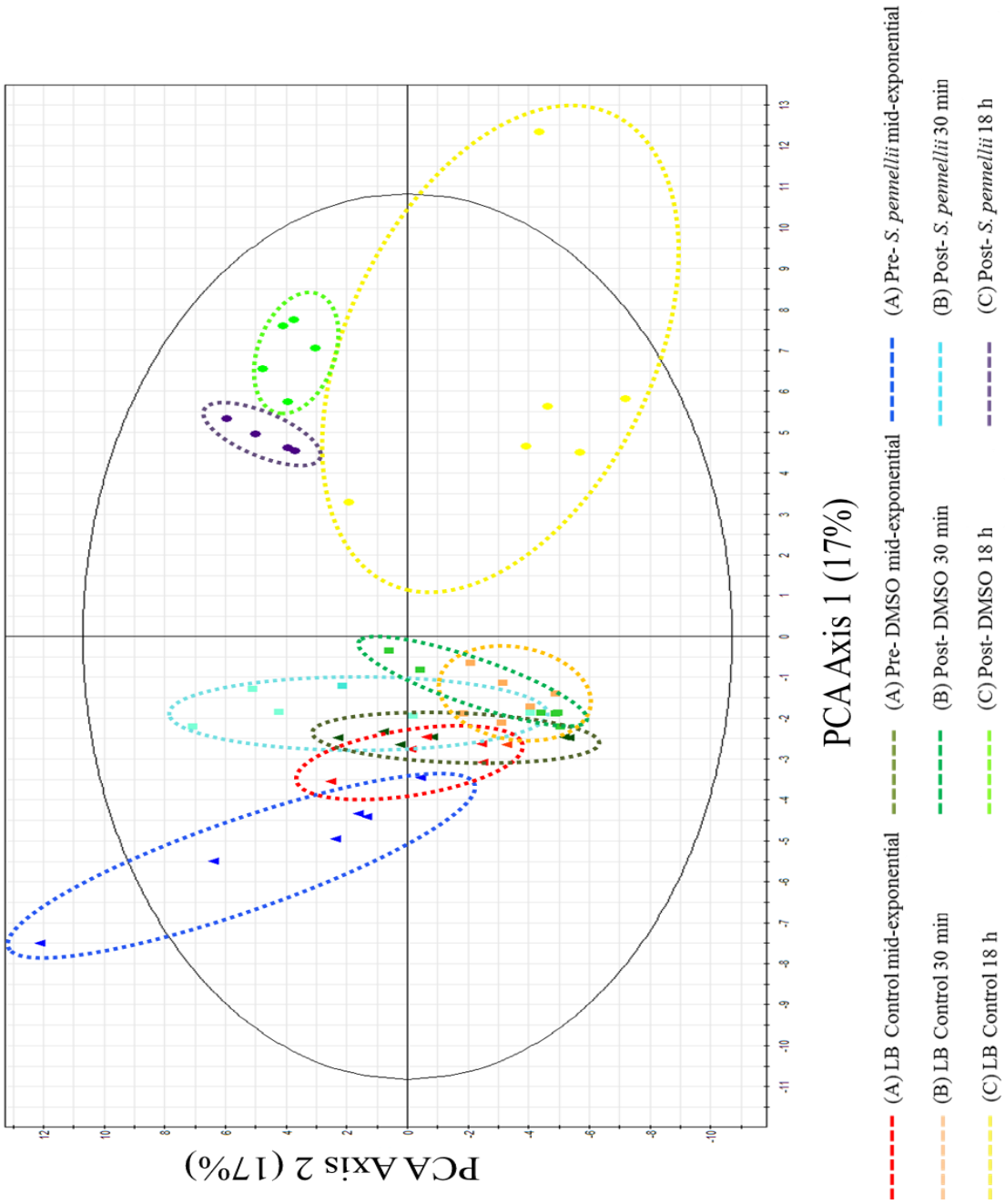

Figure 6-14. PCA of bacterial pellet metabolites of *S. aureus*

PCA of *S. aureus* metabolite data obtained through growing the bacteria in only LB, LB containing DMSO or LB containing *S. pennellii* trichome extract (0.78 mg mL^{-1}). Data is represented in a score scatter plot, which projects the data onto subspace, showing cluster results from individual test bacterial cultures. Each cluster represents one technical replicate each for six biological replicates. Each culture was measured at; A. 6 h pre-challenge, B. 6.5 h (30 min post-challenge), and C. 24 h (18 h post-challenge). Data obtained using SIMCA-P software. The first component explains 17% of the variation and the second component 17%.

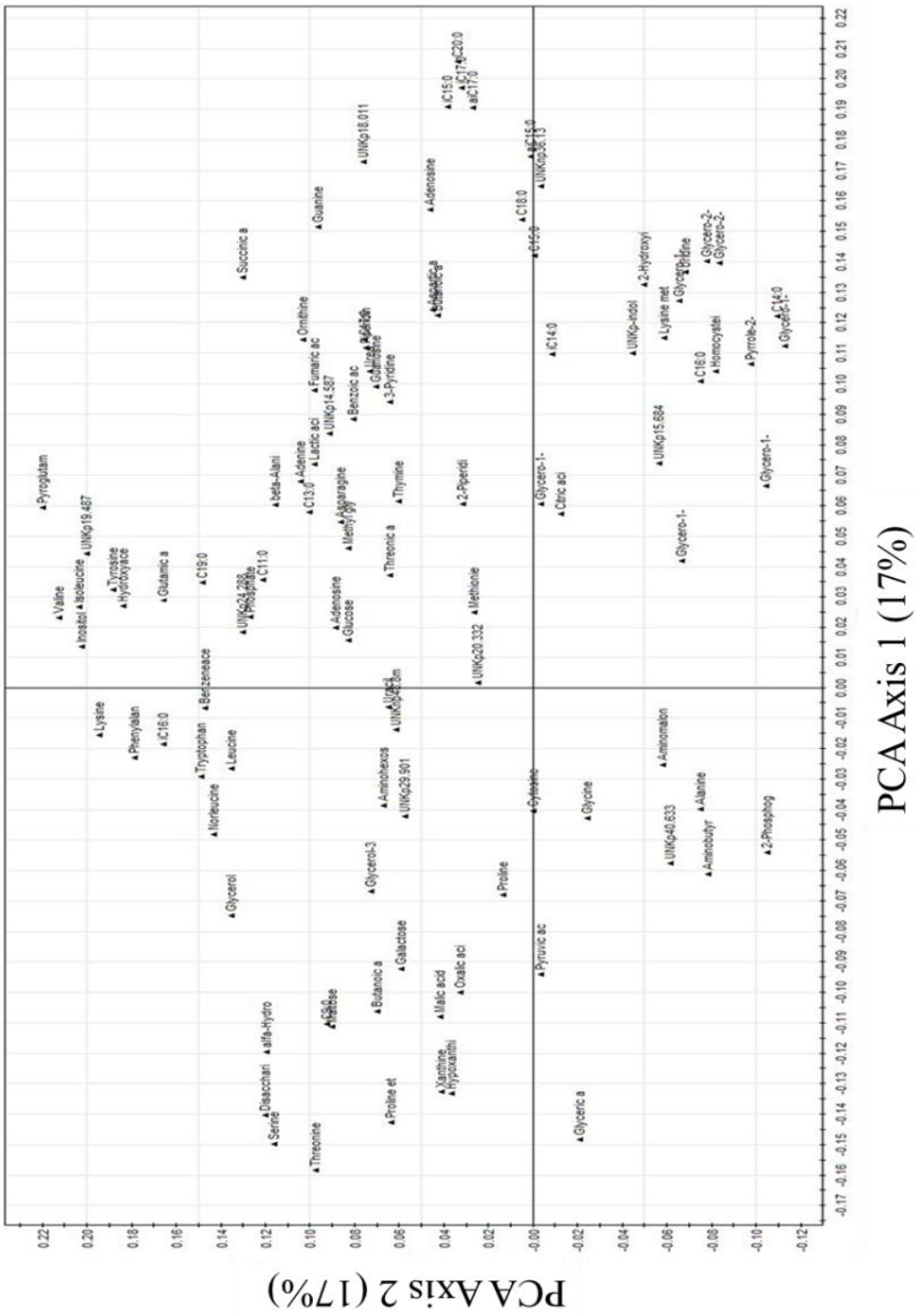

Figure 6-15. PCA of bacterial pellet metabolites of *S. aureus*

PCA of *S. aureus* metabolite data obtained through growing the bacteria in only LB, LB containing DMSO or LB containing *S. pennellii* trichome extract (0.78 mg mL^{-1}). Data is represented in a loading plot which plots the relationship between the original variable and the subspace dimetion, showing cluster results from individual test bacterial cultures. Each cluster represents one technical replicate each for six biological replicates. Each culture was measured at; A. 6 h pre-challenge, B. 6.5 h (30 min post-challenge), and C. 24 h (18 h post-challenge). Data is representes components contributing to separation; data obtained using SIMCA-P software. The first component explains 17% of the variation and the second component 17%.

The results of the loading plot for the bacterial pellet PCA show certain metabolite variables which contribute highly to the clustering of certain types of cultures (Figure 6-14). The LB controls, *S. pennellii* and DMSO (A and B) cluster together, and the variables responsible are an increase in glyceric acid, threonine and serine, with a decrease in fatty acids including iC_{15}, iC_{17}, aiC_{17}, and C_{20}. The cluster of LB after 24 h of normal bacterial growth (C) was shown to be influenced by the increased variables including the fatty acids glycerol-2-C_{18}, glycerol-2-iC_{15}, and C_{14}, and a decrease in the variables iC_{16}, and phenyl alanine. The clustering cultures of post *S. pennellii* 18 h and post DMSO 18 h (C) did cluster apart, however they had similar variables exerting influence over their positions, such as an increase in pyroglutamic acid (5-oxoproline), isoleucine and valine. There was also a decrease in fatty acids such as iC_{15}, iC_{17}, aiC_{17} and C_{20}.

6.3.3 Pathway maps (*t*-test statistical comparisons) of *S. aureus* metabolite profiles

Comparisons were made between the supernatant and bacterial pellet from the cultures of post-*S. pennellii* 18 h (C) and post-DMSO 18 h (C). The *S. pennellii* cultures were compared to the DMSO cultures for two sample *t*-test two-tail statistical analysis (significance at $P<0.05$). These were used to create pathway maps using the Royal Holloway software BioSynLab 1.3 ® (http://www.rhul.ac.uk), showing where compounds have increased or decreased through determining the odds ratios for test and control metabolite data. The supernatant results showed several increases in specific compounds compared to the pellet, where the compounds adenosine, norleucine, lysine, and butanoic acid were significantly increased in the supernatant and decreased in the pellet (Figure 6-16 and Figure 6-17).

There were decreases in the supernatant such as phenyl acetic acid which had increased in the pellet, and increases in alanine occurred in the pellet and supernatant. Other supernatant metabolite decreases were the same as the pellet such as urea and piperidine carboxylic acid. The supernatant had increases in sugars such as an unknown disaccharide and glucose, and these were not significantly increased or decreased within the pellet. There were also amino acids which were increased in either the pellet or supernatant only; such as glutamic acid which was only increased in the supernatant,

adenine and glycine which was only increased in the pellet and aminobutyric acid which only decreased in the supernatant.

The fatty acids were not changed due to a lack of detection within the supernatant, although within the pellet there were clear differences between the increased and decreased fatty acid metabolites of *S. pennellii* compared to DMSO after 24 h. Most of the fatty acids either increase or decrease, and a pattern of decrease in even carbon numbered fatty acids C_{16}, C_{18}, C_{20}, glycerol-1-C_{18}, with an increase in odd numbered carbon fatty acids C_{11}, C_{15}, and C_{19} was observed. This pattern did not include the odd carbon numbered fatty acids C_{17}, iC_{17}, aiC_{17}, iC_{15} or aiC_{15} which also all showed a decrease in the bacterial pellet of *S. aureus* in the presence of *S. pennellii* after 24 h (Figure 6-16 and Figure 6-17).

Table 6-1.The metabolite odds ratios for the supernatant (spent quenching solution) obtained from cultures of *S. aureus* comparing the different growth conditions.

Supernatant	**LB (C) vs DMSO (C)**				***S. pennellii* (B) vs DMSO (B)**				***S. pennellii* (C) vs DMSO (C)**			
Metabolites	**AV***		**StDev**	***t*-test**	**AV***		**StDev**	***t*-test**	**AV***		**StDev**	***t*-test**
Nicotinic acid	10.00	±	0.00	0.02	0.00	±	0.00	1.00	0.00	±	0.00	0.02
Adenosine	0.00	±	0.00	1.00	10.00	±	0.00	0.17	10.00	±	0.00	0.00
Aminobutyric acid	0.61	±	0.03	0.23	0.00	±	0.00	1.00	0.00	±	0.00	0.00
Phenylacetic acid	2.90	±	1.42	0.07	0.00	±	0.00	0.14	0.00	±	0.00	0.03
β-Alanine	0.00	±	0.00	0.01	0.00	±	0.00	0.16	10.00	±	0.00	0.00
Butanoic acid-3 4-diOH	0.00	±	0.00	0.03	0.00	±	0.00	0.12	10.00	±	0.00	0.00
UNKP-disaccharide-44.2984 min	0.00	±	0.00	0.10	0.00	±	0.00	0.15	10.00	±	0.00	0.02
Glucose	0.00	±	0.00	0.04	61.17	±	38.88	0.01	10.00	±	0.00	0.01
Glutamic acid	1.45	±	0.79	0.42	1.34	±	0.90	0.62	2.13	±	0.65	0.03
Leucine	3.25	±	1.70	0.07	1.50	±	0.26	0.40	0.14	±	0.12	0.04
Lysine	0.00	±	0.00	1.00	0.39	±	0.24	0.36	10.00	±	0.00	0.00
Norleucine	0.00	±	0.00	1.00	0.72	±	0.46	0.64	10.00	±	0.00	0.01
Piperidine carboxylic acid	10.00	±	0.00	0.01	0.67	±	0.26	0.59	0.00	±	0.00	0.01
UNKp18.011 min	3.75	±	1.17	0.01	0.00	±	0.00	1.00	0.00	±	0.00	0.01
Urea	10.00	±	0.00	0.04	0.00	±	0.00	1.00	0.00	±	0.00	0.04

The metabolites with quantification based on comparisons with standards of known concentrations, comparing samples by time and condition, as average ± standard error, and *t*-test (two-tailed, unpaired unequal variances), DMSO represented the control in all examples. Data represents the differences in µg mL^{-1} of DW between the supernatant. AV*= Average ratio (test/control). For the full list see Appendix 9.

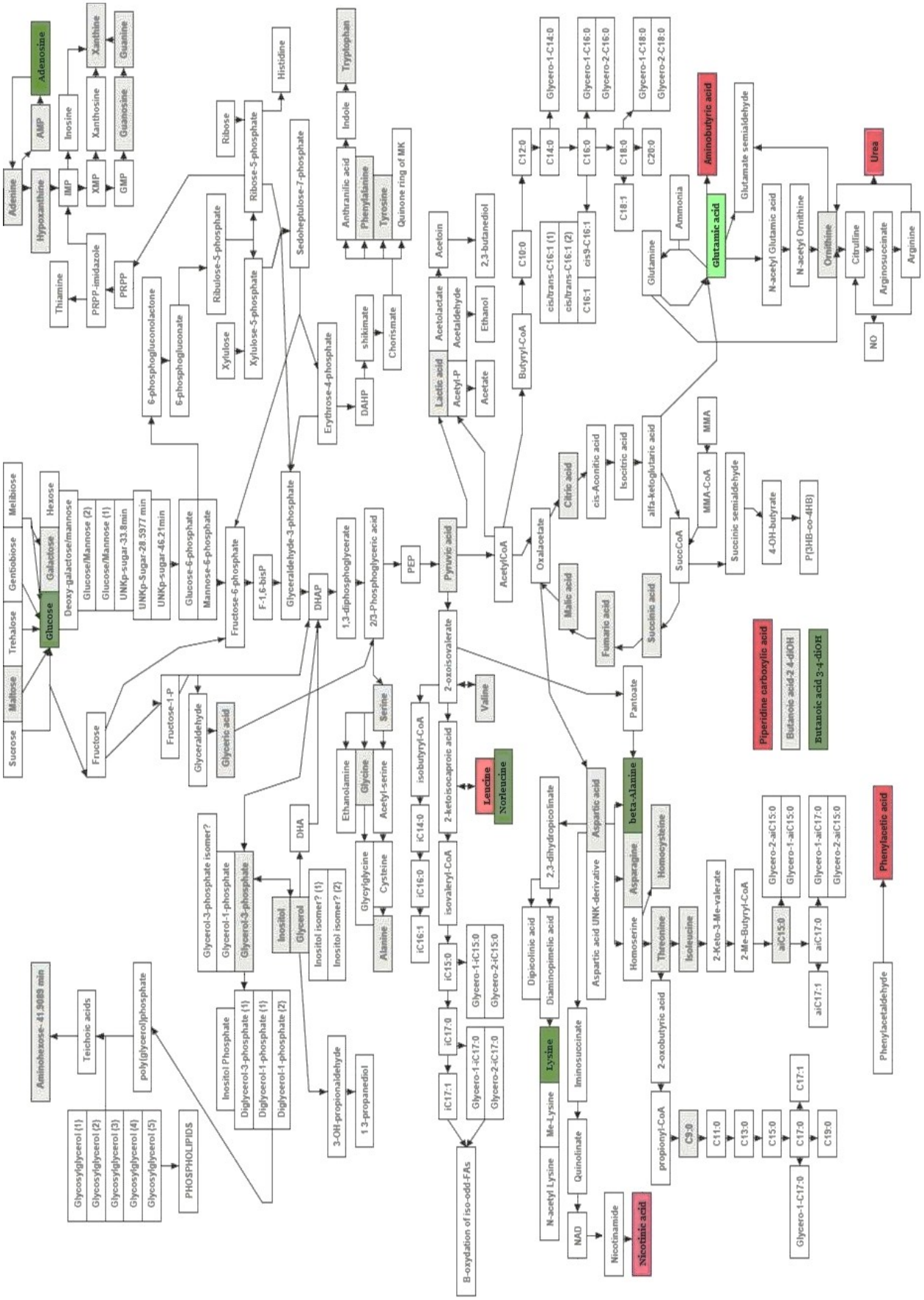

Figure 6-16. Gram-positive metabolic pathway map showing metabolites identified using GC-MS

Data shows differences between the average *S. aureus* 25923 supernatant after 18 h post challenge with *S. pennellii* compared to samples 18 h post challenge with DMSO. Red shows a significant decrease and green shows a significant increase compared to DMSO. The data was positioned using BioSynLab 1.3©. Statistical analysis was *t*-test (2 tailed, unpaired unequal variances).

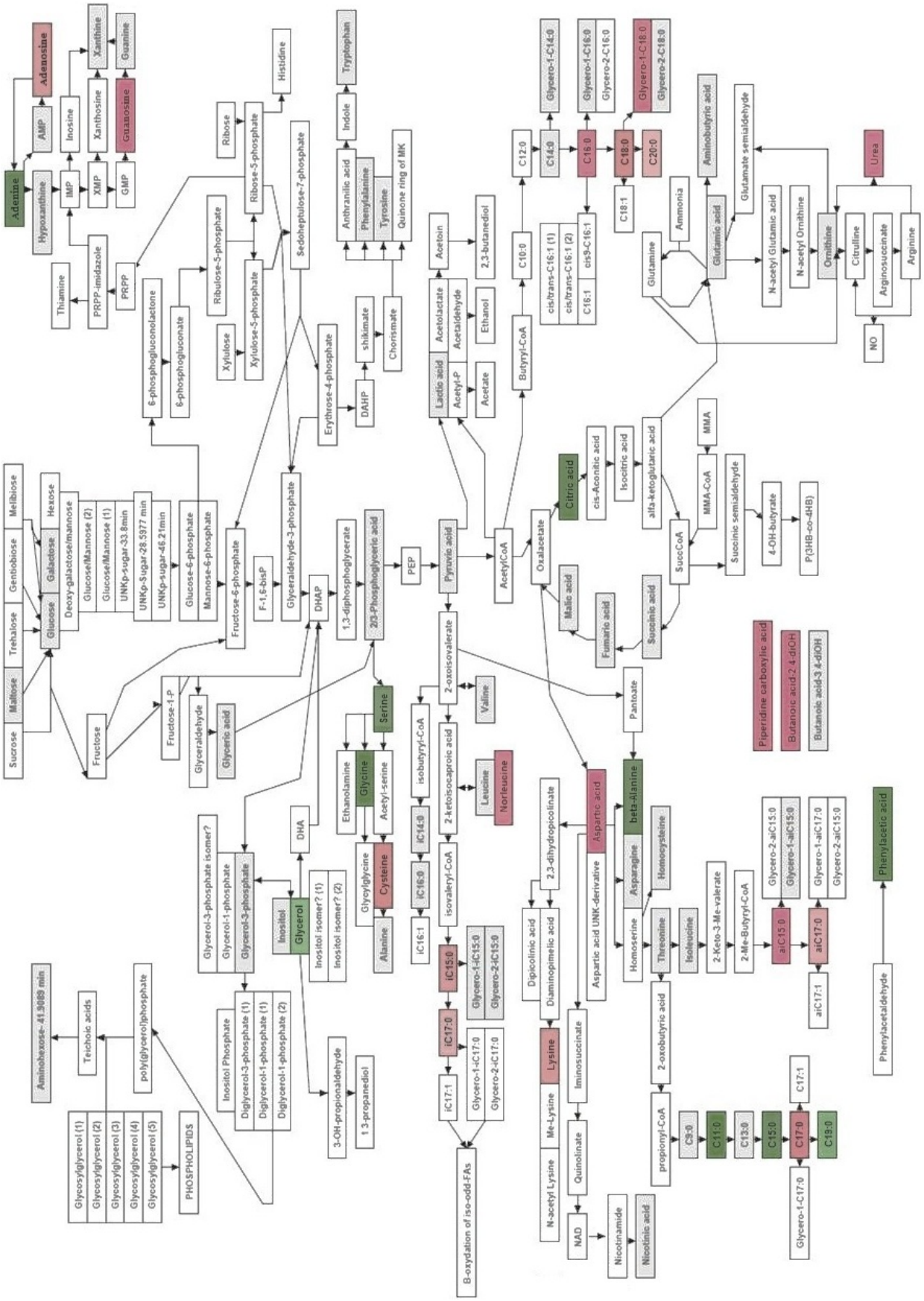

Figure 6-17 Gram-positive metabolic pathway map showing metabolites identified using GC-MS

The data shows differences between the average *S. aureus* 25923 pellet sample after 18 h post challenge with *S. pennellii* compared to samples 18 h post challenge with DMSO. Red shows a significant decrease and green shows a significant increase compared to DMSO. The data was positioned using BioSynLab 1.3©. Statistical analysis was *t*-test (two-tailed, unpaired unequal variances).

Table 6-2. The metabolite odds ratios for the bacterial pellets obtained from cultures of *S. aureus* comparing the different growth conditions.

	LB (C) vs DMSO (C)				*S. pennellii* (C) vs DMSO (C)				*S. pennellii* (B) vs DMSO (B)			
Metabolites	**AV***		**StDev**	***t*-test**	**AV***		**StDev**	***t*-test**	**AV***		**StDev**	***t*-test**
Adenine	1.63	±	0.37	0.04	29.59	±	15.93	0.02	21.40	±	19.72	0.05
Adenosine	1.54	±	0.85	0.37	0.31	±	0.13	0.05	0.40	±	0.24	0.12
aiC15:0	1.12	±	0.51	0.85	0.00	±	0.00	0.01	0.00	±	0.00	0.00
aiC17:0	1.04	±	0.13	0.91	0.43	±	0.29	0.01	0.00	±	0.00	1.00
Aspartic acid	3.88	±	2.68	0.07	0.00	±	0.00	0.03	0.00	±	0.00	0.11
Phenylacetic acid	1.04	±	0.29	0.90	26.19	±	20.14	0.05	10.00	±	0.00	0.01
β-Alanine	0.00	±	0.00	1.00	10.00	±	0.00	0.00	10.00	±	0.00	0.00
Butanoic acid-2 4-diOH	4.63	±	2.36	0.02	0.00	±	0.00	0.01	0.00	±	0.00	1.00
C11:0	0.00	±	0.00	1.00	10.00	±	0.00	0.02	10.00	±	0.00	0.00
C15:0	0.00	±	0.00	0.00	10.00	±	0.00	0.00	0.00	±	0.00	0.00
C16:0	0.45	±	0.22	0.18	0.00	±	0.00	0.01	0.00	±	0.00	0.03
C17:0	4.80	±	2.98	0.05	0.14	±	0.13	0.03	0.00	±	0.00	1.00
C18:0	1.60	±	0.28	0.01	0.19	±	0.13	0.00	0.00	±	0.00	0.12
C19:0	1.32	±	0.38	0.51	5.53	±	1.94	0.01	10.00	±	0.00	0.00
C20:0	1.19	±	0.42	0.55	0.55	±	0.18	0.04	0.00	±	0.00	0.11
Citric acid	0.00	±	0.00	0.05	10.00	±	0.00	0.02	0.00	±	0.00	0.08
Cytosine	0.00	±	0.00	1.00	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Glycerol	1.44	±	1.26	0.60	4.95	±	3.22	0.05	2.83	±	2.42	0.14
Glycero-1-C18:0	0.56	±	0.23	0.18	0.00	±	0.00	0.01	0.69	±	0.17	0.61
Glycine	0.00	±	0.00	0.02	10.00	±	0.00	0.04	0.25	±	0.14	0.48
Guanosine	1.42	±	0.67	0.37	0.00	±	0.00	0.01	1.94	±	1.10	0.13
iC15:0	1.81	±	0.42	0.08	0.31	±	0.09	0.00	0.00	±	0.00	1.00
iC17:0	0.97	±	0.18	0.94	0.44	±	0.14	0.00	0.00	±	0.00	1.00
Lysine	10.00	±	0.00	0.00	0.31	±	0.18	0.00	2.20	±	1.19	0.11
Norleucine	10.00	±	0.00	0.01	0.00	±	0.00	0.01	2.26	±	1.37	0.08
Piperidine carboxylic acid	10.00	±	0.00	0.00	0.00	±	0.00	0.00	0.00	±	0.00	1.00
Serine	0.00	±	0.00	0.05	10.00	±	0.00	0.00	2.13	±	2.18	0.31
UNKnp36.13min	0.00	±	0.00	0.02	10.00	±	0.00	0.00	0.00	±	0.00	1.00
UNKnp48.8min	10.00	±	0.00	0.02	0.00	±	0.00	0.02	0.71	±	0.19	0.66
UNKp14.587 min	0.00	±	0.00	0.02	10.00	±	0.00	0.01	10.00	±	0.00	0.01
UNKp24.2882 min	0.00	±	0.00	1.00	10.00	±	0.00	0.01	10.48	±	7.38	0.03
Urea	10.00	±	0.00	0.00	0.00	±	0.00	0.00	0.00	±	0.00	1.00

The metabolites with quantification based on comparisons with standards of known concentrations, comparing samples by time and condition, as average ± standard error, and *t*-test (2 tailed, unpaired unequal variances), DMSO represented the control in all examples. Data represents the differences in µg mL^{-1} of DW between the bacterial pellets. AV*= Average ratio (test/control). For the full list see Appendix 8.

6.4 Discussion

6.4.1 EM of *S. aureus* and *S. pennellii* extract

Negative stain EM (using a TEM microscope) involves heavy ions interacting with the electron beam to produce phase contrast. A small drop of sample is placed onto a carbon grid for a few min, blotted and then stained for a few seconds, then blotted dry for viewing. The benefits of using negative stain EM is that it produces high contrast due to the stain absorbing much higher amounts than the surrounding medium, and the samples are easy to prepare. The drawbacks of negative stain EM are that the resolution is limited to approximately 20Å, and also artefacts can be common from the staining process, with particles distorted by the staining process.

Bacteria were cultured in the presence of *S. pennellii* trichome extract, at a concentration of 25 mg mL^{-1} for the Gram-negative bacteria *E. coli* O157:H7 and *S.* Typhimurium 10248, and 0.78 mg mL^{-1} of the extract against *S. aureus* 25923. These different concentrations were chosen to have sub-inhibitory effects which allow for bacterial growth similar to the numbers within the LB control, and yet the concentration is high enough to have some physiological effects upon the bacteria. The CFU mL^{-1} of the bacteria show that bacterial growth was largely similar to the LB and DMSO controls, showing that at these concentrations the bacterial growth has been unaffected (Figure 6-5). With the cells able to replicate as usual, the effects of the extracts were investigated with the intention of identifying any morphological changes in the bacteria, which may lead to an indication of the mode of action for the inhibitory effects seen towards bacterial growth at similar and higher concentrations of the extracts.

S. Typhimurium 10248 and *E. coli* O157:H7 were shown to lose their flagella in the presence of *S. pennellii* trichome extract at a sub-inhibitory concentration of 25 mg mL^{-1} (Figure 6-2 C and Figure 6-3 C). The flagella were not seen to be detatched from the bacteria cells, although some detatched flagella were present within the samples, it was reduced as a whole, suggesting that the production of flagella has been halted rather than the extract causing the flagella to detach from the cell membrane. *Salmonella*

flagella loss has been known to occur due to stresses endured by the bacterium which leads to the loss of flagella to a motile form, in order to conserve valuable energy. It is known that *S.* Typhimurium loses its flagella in response to heat stress when grown at 44^{o}C, but flagella and bacteria recover when subsequently incubated at 37^{o}C after a lag phase of one mean bacterium generation time (Kerridge, 1960). There are other chemicals which have been known to induce flagella loss, or to maintain flagella loss after mechanical removal such as 2:4-dinitrophenol (0.001M), and ultraviolet (UV) treatment which reduces bacteria levels to 99.99% and also prevents the regeneration of functional flagella (Kerridge, 1960).

Salmonella and *E. coli* spp. are typically dimorphic, with the ability to become hyperflagellated swarmer cells, normal flagellated cells or non-motile cells, and they can do this in response to their environment, such as stresses or chemical signals from each other through chemoreceptors (Harshey, and Matsuyama 1994). The motility results showed differences in motility between bacteria subjected to two concentrations of the *S. pennellii* extract and the swarming patterns seen on semi solid agar are typical of the morphologies seen previously from *E. coli* and *S.* Typhimurium (Harshey and Matsuyama, 1994).

Swarming occurs within liquid media for *E. coli* and *Salmonella* where two types of swimming occurs, the smooth counter clockwise flagella rotation and the clockwise rotation which produces a tumbling effect (Harshey and Matsuyama, 1994). It is the ability to switch between these flagella rotations which allows bacteria to migrate towards beneficial or away from harmful environments (Yamaguchi et al., 1986; Jones et al., 1992).

It is known that different media and temperature produces different swarming effects upon bacteria, and it has been shown that when a *S.* Typhimurium bacterial culture was first grown at 37^{o}C and then grown at 44^{o}C, the bacteria stop producing new flagella with flagella numbers decreasing from 8 to 2 per cell as the diving cells share its remaining flagella with its daughter cells (Quadling and Stocker, 1962).

E. coli O157:H7 lost fimbriae and flagella in the presence of both the *S. pennellii* trichome extract and the DMSO control. This flagella loss in response to DMSO could

be explained as a similar mechanism which was elicited by the plant extract, where the bacteria were stressed and therefore conserved energy through becoming non-motile. This is because it was seen throughout the study that *E. coli* was susceptible to DMSO and growth was reduced more than that seen by the other bacteria such as *S.* Typhimurium 10248 and *S. aureus* 25923. The visual cell damage was observed in the form of a pitted and rough *E. coli* cell membrane suggested that the *S. pennellii* extract was affecting the outer cell membrane of the bacteria cells.

S. aureus 25923 was shown to be the most susceptible to the *S. pennellii* trichome extract, and the EM results showed that even at a much lower sub-inhibitory concentration of 0.78 mg mL^{-1}, compared to the 25 mg mL^{-1} of the extract used against the Gram-negative bacteria, there was some evidence of cell damage (Figure 6-1 C). This cell damage appeared in the form of cell leakage, although whole cell disruption was minimal, and stain uptake and cell morphology was similar to the LB and DMSO controls. Although the use of sub-inhibitory concentration when accessing the effects of the extract upon selected bacteria is a useful method, the results can be misleading and it has often been found that concentrations of antibiotics lower than the MIC can produce dramatic changes in the bacteria, which are different rather than milder than the effects of the MIC (Lorian, 1975). The sub-inhibitory effects of antibiotics tested against *S. aureus* within a previous study showed characteristic lesions in bacteria, and *S. aureus* was found to grow 3 to 5 times larger than the control cells and contain thickened septa when grown in a sub-inhibitory concentration of penicillin (Lorian, 1975).

6.4.2 Motility assay for bacteria cultured in the presence of *S. pennellii* extract

The motility assay results showed that the flagella loss observed from *E. coli* O157:H7 and *S.* Typhimurium 10248 is a reversible effect of the extract upon the bacteria grown in the presence of the extract when grown in liquid culture (Figure 6-7). It is hypothesised that because the bacteria are motile when subsequently cultured on the semi-solid motility agar, the flagella loss is not permanent and has been recovered by the bacteria. The different concentrations of *S. pennellii* extract show that at the concentration of 50 mg mL^{-1} where visually no bacterial growth was observed, the extracts showed reduced colony numbers and that this is likely to be the reason for the

reduced motility seen for the bacteria, *S.* Typhimurium 10248, and *S.* Enteritidis S1400 (Figure 6-6).

The increase in motility from *E. coli* O157:H7, grown with the 50 mg mL^{-1} of *S. pennellii* trichome extract, did not follow the same trend as the other Gram-negative bacteria, where the opposite effect was observed and motility was decreased (Figure 6-6). This effect could have been a response to growth in the presence of the plant extract, where the bacteria moved away from the colony centre due to residual concentrations of the plant extract from the original plated bacterial culture. An alternative explanation links to the hormesis effects which were seen often in the growth curve assays, where the bacteria have a stimulated effect in response to a low exposure to a toxin, such as the *S. pennellii* extract.

S. aureus 25923 also appeared to move away from the central bacterial colony, and was shown to have an inconsistent morphology to its colony, which remained at approximately 3 mm in diameter under all conditions (Figure 6-8). The lack of motility across the motility agar was expected from *S. aureus* and it was tested primarily as a control bacterium which does not produce flagella. However, the colony morphology showed migration of the bacteria upon the surface of the agar as projections away from the central colony, this morphology was seen from half of the spotted bacterial cultures. Given that *S. aureus* is not a flagellated bacterium the effects observed could be due to some form of contamination, especially as these morphologies were not seen consistently from all the spotted colonies. Although it is possible that the morphologies observed were linked to biofilm production, as *S. aureus* is able to produce biofilms through attachment to a surface, proliferation and aggregation within an ECM (extracellular matrix), and detachment (Periasamy et al., 2011). It is also likely that when the bacteria were spotted onto the plate in liquid culture it may have spread out slightly creating the effect.

The concentration of *S. pennellii* extract at 25 mg mL^{-1} is the same as was used as a sub-inhibitory concentration for the EM experiment, and therefore it can be used to directly compare the flagella loss seen in the EM results and the motility observed in the semi-solid agar plates. The motility results do not compare with the flagella loss observed in the EM experiments. This may be explained by a recovery of the flagella once the

bacteria have been sub-cultured into a new environment where the residual concentration of the original extract within the culture are diluted through diffusion across the agar.

There was also an unexpected result where the *E. coli* O157:H7 was not motile, and the bacteria remained as a colony on the agar surface, all except one replicate which had a small zone of 9 mm diameter (Figure 6-8). This was unexpected as *E. coli* O157:H7 was shown to produce flagella within the EM images. It is possible that although *E. coli* O157:H7 produces flagella, the conditions required for swarming were not optimal, and migration was not initiated. Tarr (1995) has highlighted the difficulty in eliciting motility from *E. coli* O157:H7 even though the bacteria are motile and produce the H7 antigen.

6.4.3 Metabolomic analysis of *S. aureus* in the presence of *S. pennellii* extract

The transcription factor (stationary phase sigma factor) σ^s (RpoS) is a major regulator under many stress conditions controlling more than 140 genes involved in metabolism (Jozefczuk et al., 2010). Bacteria respond to stress through decreasing their central carbon metabolism and down regulating genes involved in cell growth in order to conserve energy and to adjust to the new environment (Jozefczuk et al., 2010). *E. coli* under stressed conditions decreases metabolites involved in glycolysis, the pentose phosphate pathway, and the TCA cycle such as a decrease in glucose-6-phosphate, glyceric acid-3-phosphate, pyruvic acid, succinic acid, erythrose-4-phosphate and ribose-5-phosphate under heat stress (Jozefczuk et al., 2010). Some metabolites such as methionine can decrease after heat and oxidative stress, which is due to the sensitivity of methionine to oxidation (Jozefczuk et al., 2010).

The metabolic profile of *E. coli* has been investigated by Jozefczuk et al., (2010), and they were able to identify 188 metabolites. The metabolite response is more specific when compared with the transcriptomic level of response, which is more general (Jozefczuk et al., 2010). The growth phase is highly influential on metabolomic profiles, because bacteria generally respond to stress through halting growth. Therefore the first hour of application of the inhibitory compound is an important time to sample the culture for metabolomic analysis (Jozefczuk et al., 2010). The largest numbers of

metabolite changes in *E. coli* were seen after 10 min of stress induction (Jozefczuk et al., 2010). This research into *E. coli* metabolite changes was taken into account during these experiments, and two time points were taken for analysis which were 30 min post challenge with *S. pennellii* trichome extract, and a second sample taken after 24 h of bacterial growth (18 h post challenge). Comparisons were made between samples taken at 6 h before challenge with the plant extract, and this allowed comparisons to be made between the short and long term effects of the extract upon the bacteria.

The comparison of *S. aureus* cultures at mid-exponential (6 h), which was before the addition of DMSO or *S. pennellii* should have shown similar metabolomic profiles (Figure 6-11 A). The results showed that the profiles of the cultures after 6 h were similar in terms of metabolomic identifications. The cultures before the addition of the plant extract showed slightly higher metabolite concentrations. The increased concentrations were constant for most of the metabolites which suggests the increases were probably a result of normalising the data to the dry weight of the bacterial pellet. This also showed that there was variability with the experiment and that not all the changes in metabolite profiles were accurate representations of real time effects. It is better to view the results as indications towards metabolomic patterns which should be verified with further studies.

6.4.3.1 Changes in metabolic profiles of the culture supernatants

PCA of the supernatants from the spent media during the quenching step of the bacterial collection was performed, and clustering was found as two distinct groups (Figure 6-12). The controls clustered on left side of the PCA and the cultures containing DMSO and *S. pennellii* plant extract clustered on the right side of the PCA. The clusters were close except for a few outliers, which were probably caused by general variations within the separately grown biological replicates of the bacterial populations. The circle drawn around the culture of post-DMSO 30 min was the largest circle, linking the controls with the test samples. It was likely that the reason for two samples clustering with the LB controls was due to the short exposure time to the DMSO. The effects between the different clustering behaviours seen within this group suggested that the DMSO effects were strong against the bacteria evident though the clustering seen after 18 h of

incubation with DMSO and *S. pennellii* which was dissolved in DMSO. However, the effects did not affect the metabolomic profiles for the two samples which were only grown with the extracts for 30 min, showing that additional time was needed for the DMSO to exert an effect in these samples.

The *S. pennellii* cultures also clustered within the same region as the DMSO cultures. This suggested that the effects of the plant extracts were equal to the effects of the DMSO. Therefore, it must be concluded that because the *S. pennellii* extracts were dissolved in DMSO that the majority of the influence upon clustering was a result of the solvent effects upon the bacterial metabolomic changes. This was further highlighted through the clustering of the cultures on the right, where cultures containing *S. pennellii* co-clustered with cultures containing DMSO of the same time points at either 30 min (B) or 18 h (C) (Figure 6-12).

The score plot was used to identify the metabolite variables which had the highest impact upon the separation of the component samples. Within the supernatant samples of LB controls there were increases in metabolites involved in nucleic acid synthesis such as the nucleotides, thymine, and the purine derivative hypoxanthine. These increases were likely to be linked to the bacteria reproducing successfully within the media, and a negative production of glutamic acid and disaccharides is likely from an increased uptake of sugars and amino acids from the healthy bacteria. The involvement of metabolites within specific metabolic pathways, and the bacterial metabolic network was investigated using the BIOCYC cellular overview tool where *S. aureus* ATCC 51811 was selected as an example species used compare with the metabolomic profile of *S. aureus* 25923.

The variables involved in the separation of clusters of DMSO and *S. pennellii* post 30 min (B) included the increase in glycolic acid which is produced from the hydrolysis of 2-phosphoglycolate (BIOCYC), and increased amino acids in the form of methionine and norleucine. The clusters were also separated by a decrease in malic acid (malate) which is consumed in gluconeogenesis and the TCA cycle (Forsythe et al., 1997), the decrease in amino acid threonine which is consumed in glycine and isoleucine biosynthesis or tRNA charging (Forsythe et al., 1997), and phenylacetic acid which is

likely to be produced from DMSO interactions with the media or bacteria as phenylacetic acid is not typically produced by *S. aureus*.

The main variables involved in separating the DMSO and *S. pennellii* post 18 h (C) treatments were increased carboxylic acids such as benzoic acid which is a break down product of hippurate where glycine is also produced (BIOCYC), and butanoic acid which is produced through phospholipase fatty acid degradation or triacyglycerol degradation (Forsythe et al., 1997). There was also a decrease in metabolites such as threonic acid (threonine), and fumaric acid was also decreased and is typically used as part of mixed acid fermentation and anaerobic respiration (Forsythe et al., 1997). This was along with the decrease in proline also involved in tRNA charging (BIOCYC) and a decrease in xanthine used in the cell for purine nucleotide degradation and the production of XMP (Forsythe et al., 1997).

6.4.3.2 Changes in metabolic profiles of the bacterial pellets

The PCA clusters of the bacterial pellets have clusters on both the left and right, which was similar to the pattern of clustering for the supernatant PCA (Figure 6-12 and Figure 6-14). However, there were differences between the pellet samples and supernatant samples clustering patterns, such as the pellet from the cultures of post-*S. pennellii* 18 h (C) which cluster into a close but separate cluster from the DMSO. There were also only cultures after the 24 h of growth on the right side of the PCA, with little differences between the cluster positions of cultures post-30 min of DMSO and *S. pennellii* compared to the LB controls.

The scatter plot of metabolite variables showed which metabolites had the most effects upon the clustering samples. The LB controls, *S. pennellii* and DMSO (A and B) clustered together (Figure 6-12), and the metabolites responsible were (Figure 6-13); an increase in glyceric acid which is a three carbon sugar acid, increased amino acid threonine which is consumed by biosynthesis of glycine and isoleucine, or used to charge tRNA (Forsythe et al., 1997), and an increase in the amino acid serine which is involved in tRNA charging. There was also a decrease in fatty acids including iC_{15}, iC_{17}, aiC_{17}, and C_{20}, and this decrease could have been due to the lower bacterial levels at mid-exponential phase which account for most of the fatty acids within the samples

due to the high levels of fatty acids within their membranes, because the GC-MS data was normalised to the DW of the bacterial pellets.

The cluster of LB after 24 h of normal bacterial growth (C) was shown to be influenced by the increased metabolites including the fatty acids glycerol-2-C_{18}, glycerol-2i-C_{15}, and C_{14}, and a decrease in the metabolites iC_{16}, and the amino acid phenyl alanine which is consumed through tRNA charging (Forsythe et al., 1997). The positive influence of the fatty acids within the 24 h LB control shows that fatty acids did increase within the bacterial pellet, presumably from cycles of bacterial cell growth and cell death. Previous gas chromatography experiments have investigated the fatty acid methyl ester profiles of different Gram-positive and Gram-negative bacterial cells, and the research completed by Whittaker et al., 2005 found a very similar fatty acid pattern to our study in *S. aureus* where the major fatty acids included iC_{13}, C_{14}, iC_{14}, C_{15}, iC_{15}, aiC_{15}, C_{16}, iC_{16}, C_{17}, iC_{17}, aiC_{17}, C_{18}, iC_{18}, C_{19}, iC_{19}, aiC_{19}, and C_{20}.

The cultures of post *S. pennellii* 18 h and post DMSO 18 h (C) did cluster apart, with similar variables exerting influence over their positions with an increase in pyroglutamic acid (5-oxoproline), which is produced from the hydrolysis of 5-oxoprolyl-peptide (Forsythe et al., 1997), increased amino acid isoleucine which is involved in tRNA charging (Forsythe et al., 1997) and an increased amino acid valine which is involved in alanine biosynthesis, and tRNA charging (Forsythe et al., 1997).

There was also a decrease in fatty acids such as iC_{15}, iC_{17}, aiC_{17} and C_{20}, however, in the LB controls after 24 h the fatty acids were increased. This could have been due to less growth within these cultures, or damage to the cells which may have lost the fatty acids into the supernatant. If the fatty acids were lost to the supernatant, then these increases of fatty acids may not have been detected due to the non-polar nature of these compounds and the rather polar quenching procedure primarily methanol and water. When the supernatant and pellet were compared the amount of fatty acids detected within the supernatant were reduced compared to the bacterial pellet samples.

6.4.3.3 Comparisons between *S. aureus* supernatant and pellet 18 h post challenge with *S. pennellii* trichome extract

The main result from the PCA was that the most difference observed between the samples was after 24 h of bacterial growth (18 h post-challenge with DMSO and *S. pennellii*, or LB control) (Figure 6-12 and Figure 6-14). Using this knowledge the DMSO and *S. pennellii* at 24 h were used for direct comparison, as although they clustered within the same region of the PCA there were still two separate tightly packed clusters for the post-*S. pennellii* 18 h (C) treatments and the post-DMSO 18 h (C) treatments for the bacterial pellet samples. Therefore, although the supernatant clusters together on the PCA for the post-*S. pennellii* 18 h (C) and the post-DMSO 18 h (C) treatments, the differences between the supernatant from *S. pennellii* (C) and DMSO (C) were considered so that the results could be compared to the pellet. Although the supernatant was a mixture of original culture and the metabolites produced by the bacteria, through comparing cultures of *S. pennellii* to DMSO at the same time point, all the differences can be said to represent the effects of the plant sample only.

When the post-*S. pennellii* 18 h (C) treatment was compared to the post-DMSO 18 h (C) treatment and the results were displayed in a metabolic pathway map clear differences were observed between the supernatant samples and the pellet samples (Figure 6-16 and Figure 6-17). The supernatant had increases in an unknown disaccharide and glucose, which were not significantly changed within the pellet between DMSO and *S. pennellii* cultures (C). This increase in sugars within the supernatant was most likely a consequence of an increase in sugars from the addition of the *S. pennellii* extract due to the high amount of sugar esters produced by their trichomes.

As part of metabolic analysis of bacteria it was found that some metabolites increased in response to stress such as certain amino acids which could be an indication of protein degradation (Jozefczuk et al., 2010). The amino acids were alternately increased or decreased between the supernatant and pellet, with the pellet and supernatant usually showing opposite effects. The loss of amino acids from the pellet is typically attributed to the breakdown of proteins and peptides, and therefore it can be assumed that there will be a loss of amino acids into the supernatant from damaged cells.

The overall results show a strong link between the increases in metabolites linked to the cell wall structure of *S. aureus*, particularly the short peptide chains bound to a 5 glycine residue which attach to the NAM (*N*-acetylmuramic acid) sub units which form part of the peptidoglycan layers in the outer cell wall of Gram-positive bacteria such as *S. aureus* (Figure 6-18). The protein chain within peptidoglycan consists of three alanines, one lysine, and one glutamic acid. This ratio of increased alanine within the cell wall of *S. aureus* could explain the increases in alanine found within both the supernatant and the bacterial pellet. The other two amino acids glutamic acid (glutamate) and lysine were also increased, however only within the supernatant, and with a significant decrease in lysine also within the pellet.

This suggests that the general increase in these three amino acids which are known to be present in large numbers within the cell wall of *S. aureus* could be a breakdown of the cell wall which is releasing these amino acids in a free state where they are lost to the supernatant, or a reduction in cell wall synthesis. The exception is alanine which is present in high concentrations within both the pellet and supernatant, this could be because there is around three times more alanine within the cell wall than the other amino acids, therefore some many still remain in the pellet due to a concentration effect.

Glycine forms a pentaglycine chain which is attached to lysine as part of the peptide chain within peptidoglycan. This is a more non-polar metabolite, and this could explain its increased concentrations within the pellet sample, and its absence from the supernatant. This increase within the pellet links with the increase in alanine, so it could also be due to a high concentration of this metabolite within the cell (5 glycine metabolites per peptide chain).

Lipids accumulate on internal bacterial surfaces and can be present as globules within bacterial cells, although less free lipids can be found within the Gram-positive bacteria compared to Gram-negative bacteria cell walls. Lipoproteins and lipopolysaccharides are found within the cell walls. The cell membranes contain a higher amount of lipids which can vary from free fatty acids, neutral fats, and phosphatidic acids (O'Leary, 1962). Within the bacterial pellet there was an increase seen from three of the odd numbered carbon chain fatty acids, although all the other fatty acids and fatty acid related compounds were decreased within the pellet. The overall data showed fatty acid

loss in the pellet and no detection in the supernatant, which can be explained if the fatty acids were lost to the supernatant but not detected due to the polar nature of the quenching solution.

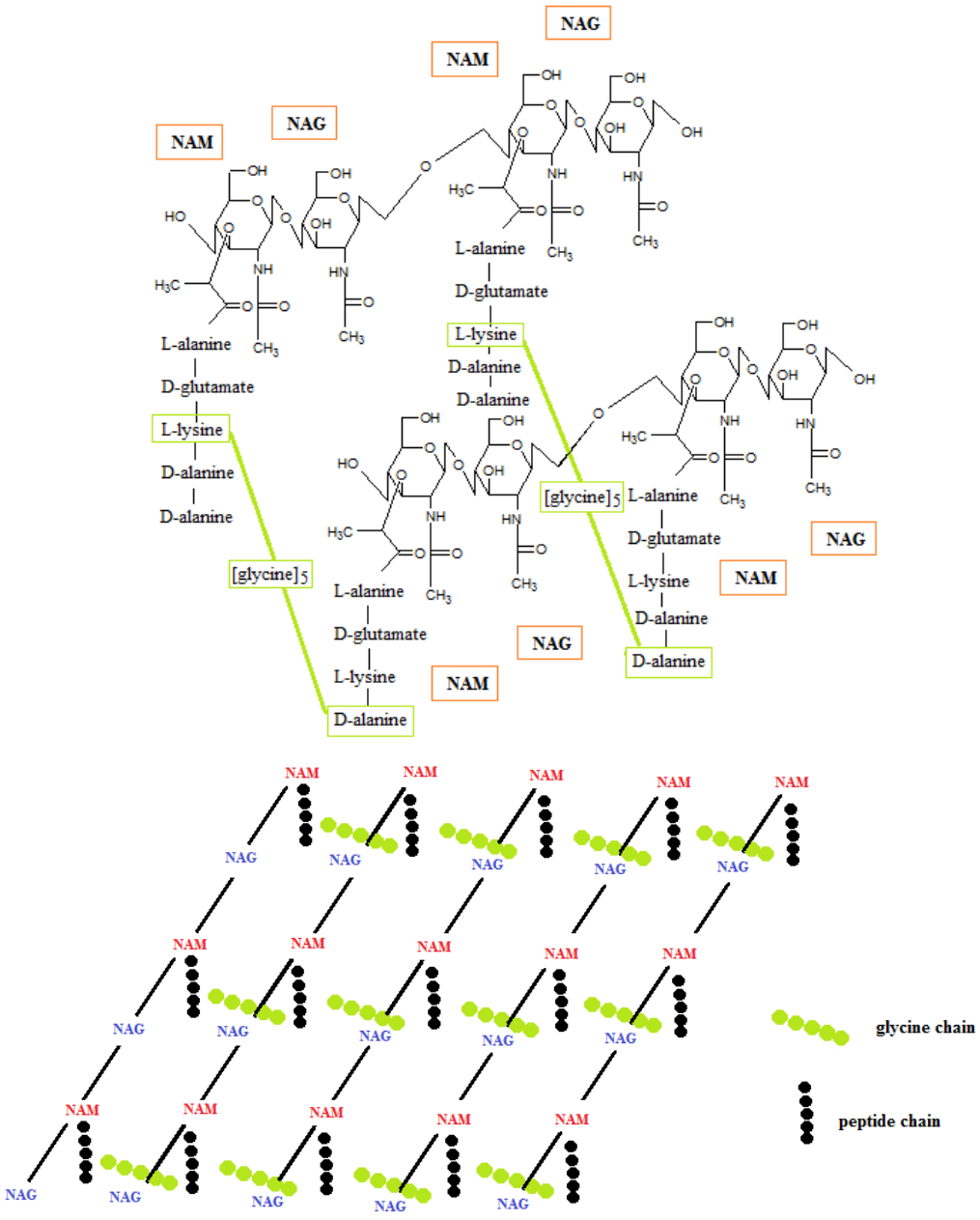

Figure 6-18. Chemical structure of Gram-positive peptidoglycan

Green areas of the diagram represent the 5 glycine chains, and black circles show the 5 amino acid chains. The peptidoglycan structure is typical of *S. aureus* cell wall peptidoglycan. Amino acid chains link via the glycine chains, the backbone of the whole structure includes repeating patterns of linked *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG). Structures adapted from Slack and Snyder (1914) using ChemSketch.

The overall changes specifically associated with the amino acids involved in the cell membrane and the glycine suggests that the cell wall of *S. aureus* is damaged by active compounds within the *S. pennellii* trichome extracts. This in turn makes the bacterial cell membrane vulnerable to damage, which may have included the loss of integrity to the phospholipid bilayer. The *S. pennellii* extracts have a reduced inhibitory effect upon *E. coli* O157:H7 and *S.* Typhimurium 10248 (Chapter 3 Figure 3-14), which could be linked to the double phospholipid bilayer within the Gram-negative cell walls. It suggests that the peptidoglycan layer of *S. aureus* was compromised, which also led to the loss of certain fatty acids. This result can further strengthen the identification of the *S. pennellii* active extracts as compounds with amphiphilic properties allowing them to insert into the cell wall which would cause damage and release of cell wall components.

Chapter 7
General discussion

7.1 General discussion

The studies presented in this thesis have focused on investigating the antimicrobial potential of Solanaceae species. The overall aim was to discover novel secondary metabolites which could be used to inhibit bacterial growth, with a focus on pathogenic bacteria such as *E. coli, Salmonella* and *S. aureus*. The project involved the screening and identification of Solanaceae species with antimicrobial potential, especially wild tomato relatives. Furthermore, the studies compared extracts of these species with the activities of pure compounds. Some of the active secondary metabolites were identified within plant extracts and preliminary studies have provided insights into the mode of actions of the antimicrobial activities.

The selected experimental methods involved comparing the antimicrobial activities of pure compounds, plant species and the sensitivity of bacterial species. It was hypothesised that similarities would emerge which would identify a common class of antimicrobial compounds, produced by Solanaceae. The results obtained in these investigations have shown that there was a high potential for antimicrobial discovery within the Solanaceae species, from a diverse range of compound classes. The hypothesis that Solanaceae plants are related and therefore should produce similar secondary metabolites, with similar antimicrobial potential was found to be largely incorrect. There was diversity within the family such as between species of different sub families and genera, such as the differences between the tobacco species and the wild tomato species, there was also a vast variety between different accessions of the same species. This was evidenced from the antimicrobial activity observed by *S. habrochaites* LA1777 (Chapter 3 Figure 3-1) which was not found in *S. habrochaites* LA1918 (Chapter 3 Figure 3-12), and the variability in the antimicrobial activity of three *S. peruvianum* extracts (LA2777, LA1937 and LA1987) (Chapter 3 Figure 3-13).

There were common correlations in the susceptibility of the bacterial species, where it was found that the Gram-negative bacteria had more resistance to the plant extracts with the highest susceptibility seen from the Gram-positive bacterium *S. aureus*. Although the Gram-negative bacteria were more resistant to the effects of crude Solanaceae plant extracts, they often still had a reduced susceptibility. This showed that some of the

identified extracts have the potential to be used as broad spectrum antimicrobial compounds, and that the restrictions in the observed antimicrobial activity could be due to the difficulty in isolating the pure compounds from the crude plant extract at effective concentrations to be tested against the Gram-negative bacteria.

7.1.1 Comparisons of pure compounds and crude plant extracts

Phenolic acids, glycoalkaloids, and terpenoids are compounds which are produced naturally in plants and are utilised as pest resistance compounds (Cowan, 1999). There has been a vast amount of research into the antimicrobial properties of compounds from these classes. However, the results from current the research are conflicting. This could be due to large variations in the methods used by different researchers, and there is often inhibition from compounds on fungi, insects, and bacteria, but the antimicrobial effects seem to be specific to a number of species rather than having a general wider antifungal or antimicrobial effect.

The growth curves of bacteria cultured in the presence of these compounds demonstrated that certain phenolic acids such as chlorogenic acid, caffeic acid, *p*-coumaric acid have strong activities against both Gram-negative and Gram-positive bacteria (Chapter 4 Figure 4-5). This makes these compounds good candidates for the antimicrobial activity which can be found within Solanaceae species. However, it is unlikely that phenolic compounds are the main source of antimicrobial activity from three main Solanaceae plants investigated as part of this research, due to the typically non-polar nature of the antimicrobial fractions. There was also no inhibition from compounds which have been shown to be antimicrobial in previous research, such as ferulic acid, which was found to be antimicrobial to Gram-positive and Gram-negative bacteria (Wen et al., 2003). This shows that although these compounds can be antimicrobial, there was also a high amount of diversity in the inhibitory range towards different bacterial species for many secondary metabolites. There are large differences within the literature regarding the MICs of plant secondary metabolite compounds, so there may be issues with the stability and volatility of certain compounds under different testing conditions.

The glycoalkaloids such as *α*-solanine were inhibitory in low concentrations of 10 μg mL^{-1} towards the Gram-negative bacteria (Chapter 4 Figure 4-7 and Figure 4-8). This steroidal glycoalkaloid is known also to be toxic to fungi and animal cells, although no antimicrobial activity was found against *S. aureus*. This lack of activity against *S. aureus* suggests that glycoalkaloids are not likely to be responsible for the antimicrobial activity observed within the active Solanaceae plant extracts. The high activity of *α*-solanine against the Gram-negative bacteria suggests that it would be a good candidate for further research into uses as an antibacterial agent. However, the practical uses of *α*-solanine as a drug or food preservative are limited, due to its toxic properties (Dalvi and Bowie, 1983). The sterols also had no effect upon *S. aureus*, where stigmasterol had a reduced activity against *S.* Typhimurium 10248 (Chapter 3 Figure 4-13). This suggests that free sterols are not likely to be the antimicrobial compounds responsible for antimicrobial activity against *E. coli*, *Salmonella* and *S. aureus* from the tested Solanaceae plants.

All the tested terpenoids, such as *α*-pinene and 2-undecanone, had a strong antimicrobial activity against *S. aureus* only (Chapter 4 Figure 4-10). This is interesting because the Solanaceae extracts are generally more inhibitory towards *S. aureus* rather than the Gram-negative bacteria. This could indicate that the antimicrobial secondary metabolites could be similar to non-polar terpenoids, due to the equivalent levels of inhibition. The terpenoids, which have high activity against *S. aureus* are a large class of chemicals, such as caryophyllene, which is a sesquiterpene, and 2-undecanone, a methyl ketone. Gram-positive bacteria such as *S. aureus* have been previously found to be more sensitive to the activity of plant essential oils than Gram-positive bacteria, such as *E. coli* and *Salmonella*, with bacteriostatic concentrations ranging from 0.02 to 0.075% (v/v) (Smith-Palmer et al., 1998).

The use of essential oils as food preservatives has become increasingly investigated, due to consumers' interest in the use of more natural ingredients and preservatives (Smith-Palmer et al., 1998). The susceptibility of Gram-positive bacteria to the essential oils is believed to be a result of the differences in the bacterial outer membrane; the Gram-negative bacterial surface has strong hydrophobicity with low oil permeability (Smith-Palmer et al., 1998). This increased susceptibility of *S. aureus* cell wall to these essential oils has been replicated through the sensitivity seen from *S. aureus* as part of

this research. Metabolomic analysis of *S. aureus* grown in the presence of *S. pennellii* trichome extracts, suggests that the extract affects the metabolites involved in the structure of the Gram-positive cell wall. This provides the theory that the antimicrobial compounds within *S. pennellii* are likely to contain fatty acid esters, and it strengthens the identification of antimicrobial sesquiterpenes in *S. habrochaites*.

The application of these essential oils into food often requires an increased concentration than required during laboratory testing using media; concentrations above 1% are generally unacceptable to the consumer. However, lower concentrations of oils are often sufficient to have bacteriostatic effects, which would be enough to prevent infection (Smith-Palmer et al., 1998). Temperature also affects the antimicrobial properties of some essential oils. Antimicrobial tests at 4^{o}C are less effective than at 37^{o}C for oils such as bay and nutmeg. This is hypothesised to be due to permeability changes in the bacterial membrane, or the release of volatiles at the higher temperature (Smith-Palmer et al., 1998). The antimicrobial activity of *S. pennellii* is not likely to be caused by volatile secondary metabolites, due to the stability of samples during storage, and the consistent inhibitory activity against *S. aureus* at 37^{o}C on plates and within liquid media. These stable properties of the antimicrobial secondary metabolites from plant extracts make them good candidates to be used as food preservatives, and antibacterial products. Glycosides of fatty acids are already routinely used within food, cosmetic and within the pharmaceutical industries (Dembitsky, 2004).

7.1.2 Extraction methods and efficiency of antimicrobial tests

The secondary metabolites within a plant need to be extracted for analysis. This is difficult if they are easily degraded, or difficult to extract from the plant material due to the selection of an extracting solvent which has an opposite chemical polarity to the plant compounds. Polar compounds such as phenolic acids are best extracted in water and methanol, and non-polar solvents can be extracted in solvents such as chloroform, ethyl acetate and hexane. The solvents extract a mixture of compounds based on their solubility within the solvent. The solvent is often too antimicrobial to be used within antibacterial tests; therefore dried extracts are usually re-suspended in a less inhibitory solvent such as DMSO or water. The re-suspending solvents such as DMSO still have

an antimicrobial effect at concentrations above 3% (v/v); this restricts the discovery of antimicrobial compounds which are antimicrobial at a higher concentration threshold. There was a high amount of investigation into the optimal extraction methods to use for extracting the most antimicrobial secondary metabolites from Solanaceae species. However, due to the vast diversity in the types of secondary metabolites produced by the species within this family it was decided that an initial screen using eight solvents increasing in polarity from hexane to water would provide a good indication of where to focus further investigations (Chapter 3 Figure 3-1). This method was used for the wild tomato relatives, and whilst it did not clearly identify the most appropriate solvent to use due to antimicrobial activity from a range of solvents, it did help to identify two wild tomato plants *S. pennellii* and *S. habrochaites*, which showed antimicrobial activity from all solvent extracts, except *S. habrochaites* extracted in water.

The extraction from the trichome hairs was carried out because the two antimicrobial wild tomato relatives had a high trichome load compared to the other tomato species. The trichome hairs which are found widely on many plant species contain antimicrobial compounds to help the plant resist plant pathogens (Fridman et al., 2005; Besser et al., 2009). The collection of trichomes can provide a rich source of concentrated compounds for analysis, and when the trichomes of wild tomato relatives were extracted the non-polar extracts had to be diluted to low concentrations so they would dissolve within the minimal media and LB for growth curve measurements. The water extracts and polar pure phenolic acids could be added in higher concentration to a culture due to the polar properties of the media, and this made these samples the easiest to test for antimicrobial activity and higher concentrations could be tested (Chapter 4 Figure 4-5). The highly non-polar compounds such as the terpenoids were dissolved in acetone and only made up 3% (v/v) of the total growth media (Chapter 4 Figure 4-10 and 4-11), which limits the compound concentrations bringing limitations for antimicrobial testing of these compound classes.

7.1.3 Antimicrobial trichomes extracts of *S. pennellii*

S. pennellii is widely known to produce high levels of sugar esters which have been highlighted as potential antimicrobial agents against both Gram-positive and Gram-

negative bacteria as part of this study. Sugar esters have been previously noted as possible antimicrobial compounds, and have been suggested as good natural food preservatives (Habulin et al., 2008). Sugar fatty acid esters are already widely used as natural surfactants in personal care products and medical supplies, and have antimicrobial activities, such as the inhibition of Gram-positive bacteria such as *B. cereus* by the sugar fatty acid ester sucrose monolaurate (Habulin et al., 2008). The *S. pennellii* sugar esters are candidates likely to be responsible for some of the antimicrobial activity observed within the plant extracts. Antimicrobial activity of sugar esters is likely to be produced through increasing the solubility of their fatty acid side chains within polar environments, and the antimicrobial activity could be a result of the fatty acids.

It is widely accepted that both saturated and unsaturated fatty acids can have a negative effect upon the growth of bacteria, with Gram-positive bacteria more susceptible than Gram-negative bacteria (O'Leary, 1962). The cis-isomers are more inhibitory than the trans configurations, and whilst unsaturated long chain fatty acids stimulate growth, this positive effect decreases with increasing unsaturation. The mode of action of the inhibitory effects of fatty acids has been linked to their effects upon the cell membrane permeability and loss of internal cellular components. An alternative hypothesis is that the fatty acids prevent the use of specific metabolites as part of normal cell metabolism (O'Leary, 1962). Although fatty acids can be inhibitory towards bacteria, they also act as building blocks for essential cellular constituents, primarily in the form of acyl components of phospholipids (Kaneda, 1991).

The fatty acid methyl esters which are produced in high amounts due to their conversion into sugar esters are likely to be the compounds responsible for most of the antimicrobial activity observed against the bacteria. Medium chain fatty acids such as caprylate were found to inhibit *Salmonella* and coliforms within the caecum of pigs, and has been proposed as a potential feed additive, although there are some concerns over the absorption of medium chain fatty acids by the digestive tract, which could be resolved through encapsulating the oils within beads (Messens et al., 2010). Whilst it is believed fatty acids are involved in the activity due to the non-polar nature of the compounds, and the knowledge that *S. aureus* is susceptible to hydrocarbon compounds, the exact inhibitory structure is unknown. Some suggestions could be

glycosylated esters bound to an additional group such as a sterol, or sterol esters, or glucose esters.

The idea that the most antimicrobial activity observed within the *S. pennellii* extracts is not due to free fatty acids is substantiated by the active fractions retention times on the GC-MS (Chapter 5 Figure 5-28 A) which show free fatty acids running in both the antimicrobial and non-antimicrobial fractions, with most of the fatty acids in a cluster between 45 and 50 min. This suggests that if they were the compounds responsible for the antimicrobial activity, then both fractions would have been antimicrobial. The retention time of 55 to 65 min shows the compounds believed to be the sugar esters. A good indication of these compounds being esters is within the shape of the cluster, which resembles a bell shape, typical of compounds which contain esters, which fragment with the GC-MS to give a range of very closely related peaks (Chapter 5 Figure 5-28 A). This effect can also be seen within the third cluster, at 65 to 75 min, although the intensity is reduced, suggesting these compounds are also esters but with fewer possible ways to fragment.

If glyco-sterol esters were present within this 65 to 75 min cluster, it could explain the higher carbon number of these compounds, and the GC-MS fragmentation of the cluster which suggests esters within the compounds. Free sterols, such as cholic acid, campesterol, and betulin, were also found within the trichome samples, showing that sterols are produced by *S. pennellii*, and therefore the production of a modified sterol attached to a glucose and or fatty acid residue becomes more likely. Whilst there was no antimicrobial activity of pure sterol compounds against *S. aureus*, free sterols such as cholesterol are known to incorporate into membranes and can affect their fluidity, suggesting that sterols do have the potential to disrupt membranes (Duperon et al., 1984).

The LC-MS (MAXIS) identification was performed on the *S. pennellii* trichome extract separated into an active SPE ethyl acetate fraction, and the results show that the compounds are mainly non-polar. Through analysis of the fragmentation patterns it was hypothesised that stigmasterol and *β*-amyrin esters were identified as the most abundant compounds within the extract, although the exact sterol and fatty acid esters need to be confirmed with pure standards. The presence of sterol esters identified through LC-MS

within the active *S. pennellii* SPE ethyl acetate fraction suggests that sterol esters could be responsible for the antimicrobial activity observed. It is also interesting to note that free stigmasterol was not found to be inhibitory towards *S. aureus*, and was slightly inhibitory against the Gram-negative bacteria (Chapter 5 Figure 5-19). This could explain the different activities against the Gram-negative and Gram-positive bacteria observed from the *S. pennellii* extract, where the sterol portion of the compound could be inhibitory against the Gram-negative bacteria, and the fatty acid portion of the compound could be causing the antimicrobial activity against *S. aureus*.

Sterols are produced by plants and are involved in the structure of plant membranes the same way as sterols within animals, and bacteria (Grunwald, 1970). There is evidence to suggest that glycosylated sterols and sterol esters would not affect membrane fluidity in studies where cholesteryl glucoside and cholesteryl palmitate had no protective or destructive effects against Barley root samples subjected to leakage conditions using 5% ethanol, and measuring the leakage of electrolytes. Cholesterol was found to reduce leakage at low concentrations of 1 to 10 µM, and increased leakage at 100 µM, the other free sterols, (campesterol, stigmasterol and *β*-sitosterol) are bulkier molecules. This leads reduced penetration of the phospholipid layers, and therefore had less effect on the stability of the membrane (Grunwald, 1971). It is also common for steryl glycosides to be produced as a way to store and transport sterols (Duperon et al., 1984).

These results compare with the free sterols tested as part of this research. However, the suggestion that glyco-sterols and sterol esters would not penetrate the cell wall may not directly compare with Gram-bacteria. *S. aureus* has a different cell wall structure to barley root cells, and the peptidoglycan layer may be affected differently to the plasma membrane. The *S. pennellii* extracts also had a reduced activity against the Gram-negative bacteria, which have a cell wall mainly composed of two plasma membranes with a far reduced peptidoglycan layer. This could indicate that these extracts are more effective at disrupting the thicker peptidoglycan layer of *S. aureus* than the plasma membranes of *E. coli* and *Salmonella*. Although *S. aureus* also has a plasma membrane, the disruption to its protective outer peptidoglycan layer may leave the bacteria more vulnerable to membrane attacks from other sources.

The identification of sterols within prokaryotes is debatable, and although sterols are generally not produced or present in prokaryotes, several studies have identified them, such as 4-methylsterols from methylotrophic bacteria (Volkman, 2003; Poralla, 1982). Sterols are rarely produced by bacteria, and this means that bacterial membranes are formed without sterols when grown in a sterol free environment. However, the mycoplasma bacteria are different and although they are also unable to synthesise sterols, they can uptake cholesterol for growth from the environment, where it is incorporated into their cell membranes (Razin, 1975). It has been previously discovered that bacteria which have an outer cell wall such as Gram-positive bacteria, can incorporate cholesterol into the cytoplasmic membrane, but at much lower levels than the cholesterol requiring mycoplasma bacteria (Razin, 1975). The preferential incorporation into the cytoplasmic membrane rather the outer membrane is believed to be due to twice as much phospholipid molecules, which make up the cytoplasmic membrane, allowing a greater attachment to the cholesterol molecules and a higher viscosity of the lipid domain of the outer membrane (Razin, 1975).

It is interesting to note that Gram-positive bacteria such as *Bacillus megaterium* incorporate more cholesterol than Gram-negative bacteria with a cholesterol content of 23.2 µg mg^{-1} of cell protein, compared to *E. coli* which had a cholesterol content of 7.3 µg mg^{-1} of cell protein (Razin, 1975). Therefore, the uptake of sterols into the plasma membrane in prokaryotes in not impossible, and should still be considered as a possible way of disrupting the membrane if too much is incorporated, or if the sterol is attached through to a fatty acids or a sugar. There is also evidence to suggest that sterols can be destructive to the cell membrane as in the polyene antibiotics produced by *Streptomyces*, which are antifungal compounds that act through forming complexes with ergosterol and damage the cell membrane causing leakage and cell death (Andreoli and Monahan, 1968). The sterol esters are clearly a likely candidate for the antimicrobial compounds within *S. pennellii* (Chapter 5 Figure 5-27), and there is also the possibility that glycosides of these sterol esters can increase their activity which needs to be further investigated.

7.1.4 Antimicrobial *S. habrochaites* trichome extracts

The compounds identified as antimicrobial from *S. habrochaites* in a TLC fraction, R_f value of 0.55 (Chapter 5 Figure 5-6 A), are associated with terpenoids as the GC-MS analysis highlighted the main compounds to be sesquiterpenes, and fatty acids (Chapter 5 Figure 5-14). The fraction which had the R_f value of 0.55 was highly antimicrobial to *S. aureus* 25923 which was inhibited with an average zone of inhibition diameter of 20 mm, equivalent to the same diameter as the ciprofloxacin control. However, there was reduced antimicrobial activity observed against the tested Gram-negative bacteria suggesting that the sesquiterpenes do not have a broad spectrum antimicrobial activity.

S. habrochaites is known to produce high levels of class II sesquiterpenes, such as α-*cis*-bergamotenoic acid, *α*-santalenoic acid, *α-trans*-bergamotenoic acid, and *β-cis*-bergamotenoic acid (Besser et al., 2009). The GC-MS analysis of the active fraction determined through TLC separation, identified the compounds to either be fatty acids such as 5,8,11,14,17-eicosapentaenoic acid or sesquiterpene like compounds such as *α*-santalol (Chapter 5 Figure 5-14). The NIST identification did not provide a high match to these compounds, and had a low percentage score to the matches. This led to further identification of the active R_f 0.55 band using LC-MS (MAXIS) analysis (Chapter 5 Figure 5-24 and 5-25), which confirmed that these compounds have a very similar mass to *α-cis*-santalenoic acid and *α-cis*-bergamotenoic acid, and that glycosylated versions of these compounds are also produced. This narrowed the identification of the antimicrobial compounds to two very similar compounds, and further investigations are needed to determine whether the activity is from the glycosylated sesquiterpene carboxylic acids or the free sesquiterpene carboxylic acids.

S. habrochaites also produce sterol esters, and possible sterol ester glycosides, which can be seen from the similar GC-MS chromatography of the non-polar TLC fractions between *S. habrochaites* and *S. pennellii* where clusters of compounds run between 55 to 65 and 65 to 75 min in both plant species (Chapter 5 Figure 5-28). Further LC-MS comparisons of these fractions show that the compounds are esters with similar fragmentation patterns. The similar fragmentation patterns suggest that *S. habrochaites* also contains sterol esters. However, a lower level of antimicrobial activity was observed from *S. habrochaites*, compared to *S. pennellii*. This suggests that the

concentration of these compounds may be reduced in *S. habrochaites*, or there are structural differences which cause *S. habrochaites* sterol esters to have reduced antimicrobial activity.

7.1.5 Antimicrobial *N. rustica* trichome extracts

N. rustica produces secretions onto its leaf surface in a similar way to *S. pennellii* from a high number of trichomes, and extracts were shown to have antimicrobial activity, with an antimicrobial fraction isolated from TLC of R_f 0. These compounds were analysed using GC-MS and although they ran to the same position on the TLC plate as the active *S. pennellii* band (Chapter 5 Figure 5-6 A), they eluted at a different time on the GC-MS chromatogram, with the main peaks clustering between 20 to 25 min (Chapter 5 Figure 5-10). The NIST identification of these compounds included nicotine and 1,2,4-triazolo[4,3,-a]pyrazine. These identifications are likely due to the high concentrations of nicotine and other pyridine alkaloids produced by *Nicotiana* spp. (Chintapakorn and Hamill, 2003).

This antimicrobial fraction was also analysed using LC-MS (MAXIS) and showed that the compounds within the extract are a mixture of esters, which would explain why the separation of the compounds is poor in the GC-MS and the LC-MS, due to compounds with similar mass running together (Chapter 5 Figure 5-21 and Figure 5-22). Whilst nicotine was identified as the main compound by GC-MS, analysis of the fragmentation pattern suggests more complex molecules are formed, such as a mixture of glycosylated nicotine and β-carboline derivatives. The high production of these alkaloids by *N. rustica* suggests that they are the active compounds within this fraction. Further investigations into the identification of these compounds using pure standards would support this hypothesis, and identify which of the compounds within the mixture are antimicrobial, such as the free nicotine, β-carbolines, or fatty acid esters of the free compounds which may also additionally bind to each other.

7.1.6 Mode of action of *S. pennellii*: metabolomics and EM results

The EM work showed that the loss of flagella observed from *E. coli* O157:H7 and S. Typhimurium 12048 in response to plant extracts needs further investigation (Chapter 6 Figure 6-2 and Figure 6-3). Flagella loss has been found previously in *E. coli* O157:H7 producing non-motile cells when the bacteria were cultured in the presence of 1.0 mM of carvacrol (Burt et al., 2007). However, Burt et al., (2007) also found the *E. coli* O157:H7 cells to have a greater surface smoothness when cultured in carvacrol, which is the opposite observed from *E. coli* O157:H7 grown in the presence of *S. pennellii* trichome extract, where rough and pitted cells were observed.

It suggests that, whilst the *S. pennellii* extract does not have a highly antimicrobial effect upon the Gram-negative bacteria tested, the effects upon the flagella could still have the potential to be important in reducing the invasiveness of the pathogens. Research shows that flagella are not always required for effective invasion of a host species for *Salmonella*, such as the *fliC* mutant of *S.* Enteritidis, which was shown to have a similar invasive effect as a wild type control after 20-day old chicks were challenged with an oral suspension, and that a *flh*D mutant was shown to have an increased invasiveness compared to the wild type (Parker and Guard-Petter, 2001). A similar response has also been shown with *S.* Typhimurium where flagellated non-motile and non-flagellated mutants were shown to have no significant difference in invasiveness in BALBc/Mice compared to a wild type control (Lockman and Curtiss, 1990). It seems that whilst flagella are important for movement towards host cells, the absence of flagella does not reduce the ability of *Salmonella* to initiate infection through entering the Peyer's patches (Jones et al., 1992).

Although flagella may not be necessary for invasion by the bacteria, reducing the amount of flagella produced by the bacteria could have other advantages and disadvantages such as reducing the inflammatory response of the host's immune system to the presence of the bacteria species. It is widely known that when *Salmonella* interact with epithelial cells within the gut, an epithelial pro-inflammatory response is triggered, primarily by the flagellin component of flagella, and this host response can lead to acute inflammatory diarrhea (Zeng et al., 2003). Whilst the host response is part of the disease, it is a necessary step within the host immunity response to eliminating the

infection. Therefore, whilst a bacterium which contains no flagella has challenges due to being non-motile and decreased adhesion to host cells using flagella (H antigen) (Lockman and Curtiss, 1990), it may enhance its survival within a host through evading the hosts immune system.

The loss of flagella could be due to bacterial stress in response to the *S. pennellii* extract, research by Burt et al., (2007), found that the heat shock protein 60 (HSP60) was increased in *E. coli* O157:H7 when cultured in the presence of essential oil containing carvacrol. The loss of flagella is a stress response designed to conserve energy, as flagellin in *E. coli* O157:H7, can make up to 8% of the total cell protein (Burt et al., 2007). The extract may contain compounds which interact with the fluidity of the bacterial cell membranes, which reduces the attachment of flagella in the membrane. The flagella production may be reduced through compounds in the extract directly inhibiting flagella production. The mechanisms behind the flagella loss need to be further investigated before any assumptions can be made. This research has shown that *S. pennellii* trichome extract can cause flagella loss in *E. coli* O157:H7 and *S.* Typhimurium 10248, but that the effects are reversible. The hypothesis that the mode of action involves disrupting the bacterial cell wall would support the flagella loss seen in Gram-negative bacteria, and the subsequent reversal of flagella loss when bacteria are sub-cultured onto motility agar (Chapter 6 Figure 6-6).

The mode of action has been investigated specifically for *S. pennellii* trichome extracts through metabolomic analysis of *S. aureus* grown in the presence of extracts. The interaction with the bacterial cell membrane from the *S. pennellii* extract was shown to be likely (Chapter 6 Figure 6-16 and Figure 6-17), due to the increased changes to the amino acid lipids, and glycine which are involved in the structure of bacterial cell walls, specifically in the peptidoglycan. *S. aureus* has a larger peptidoglycan layer and therefore is more affected by the extract than the Gram-negative bacteria. The effect of the *S. pennellii* extract upon the bacterial cell wall could occur through several different mechanisms.

There are several classes of antibiotics which specifically target the bacterial cell wall for disruption, which can lead to bactericidal or bacteriostatic conditions. Most of the cell membrane antibiotics act upon the peptidoglycan layer, such as penicillins which

work through inhibiting transpeptidase activity which creates amide peptide crosslinks in peptidoglycan, this inhibition is caused through occupying the transpeptidase enzyme site as a competitive substrate (Walsh, 2000). Vancomycin also acts upon the peptidoglycans through forming a complex with the double alanine end of the peptide chains, this also prevents amide cross links leaving the peptidoglycan layer more susceptible to osmotic cell lysis (Walsh, 2000).

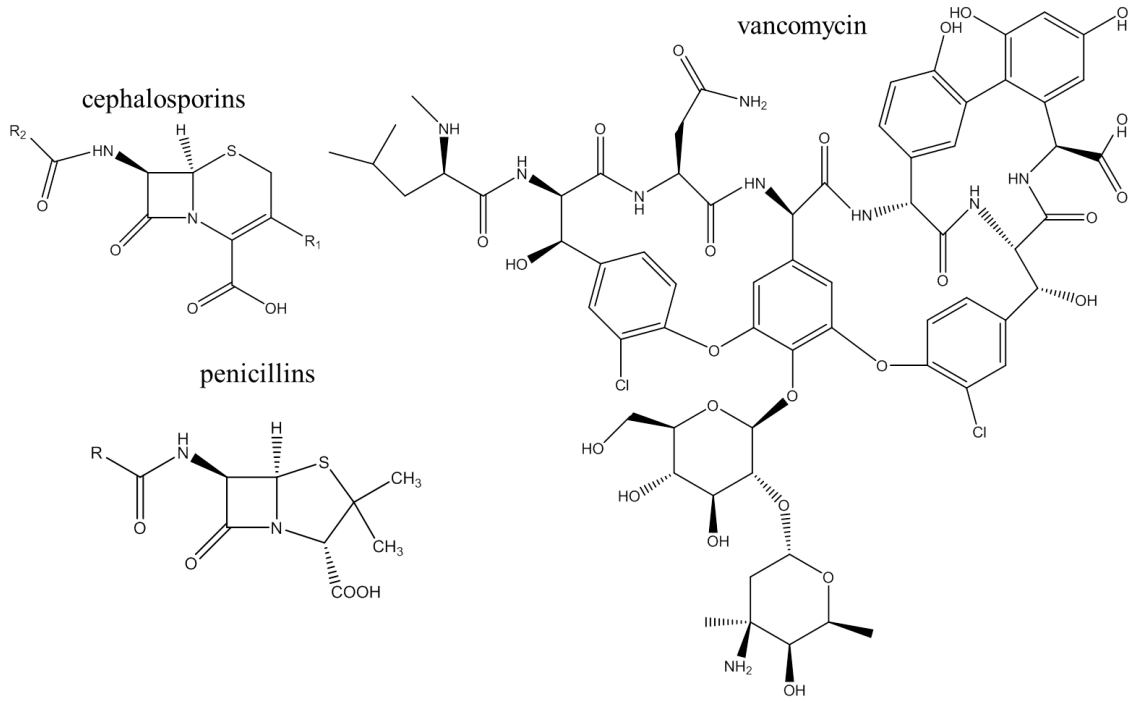

Figure 7-1. Antibiotic structures involved in disruption of cell wall biosynthesis.

Structures represent the antibioitcs compounds; penicillins, cephalosporins, and vancomyin. Structures drawn from Chemspider using ChemSketch.

As part of research into the interaction of sterol esters upon the leakage of barley root cells, it was suggested that bulkier molecules lead to less effective penetration of the phospholipid membrane (Grunwald, 1971). The vancomycin antibiotic works upon the peptidoglycan layer in the cell wall, and its large size reduces cross links and destabilises the peptidoglycan layer. The binding sites within the antibiotic bind to the D-Ala-D-Ala-termini, through 5 hydrogen bonds (Walsh, 2000). This suggests that molecules which contain mixtures of exposed free hydrogen and oxygen may also act in a similar way to vancomycin allowing the compounds to attach to elements within the peptidoglycan layer through hydrogen bonding. The large size of vancomycin increases

its ability to form hydrogen bonds, therefore the large size of the sterol esters in *S. pennellii*, the nicotine-harmolol esters from *N. rustica* and the sesquiterpene carboxylic acid glycosides in *S. habrochaites* could allow these molecules to interact and bind to the peptidoglycan. Their increased sizes due to fatty acid side chains and sugar molecules gives them a bulker properties which suggests they are unable to penetrate the cell plasma membrane, and interactions with the peptidoglycan layer is more likely, such as peptidoglycan interactions with vancomycin.

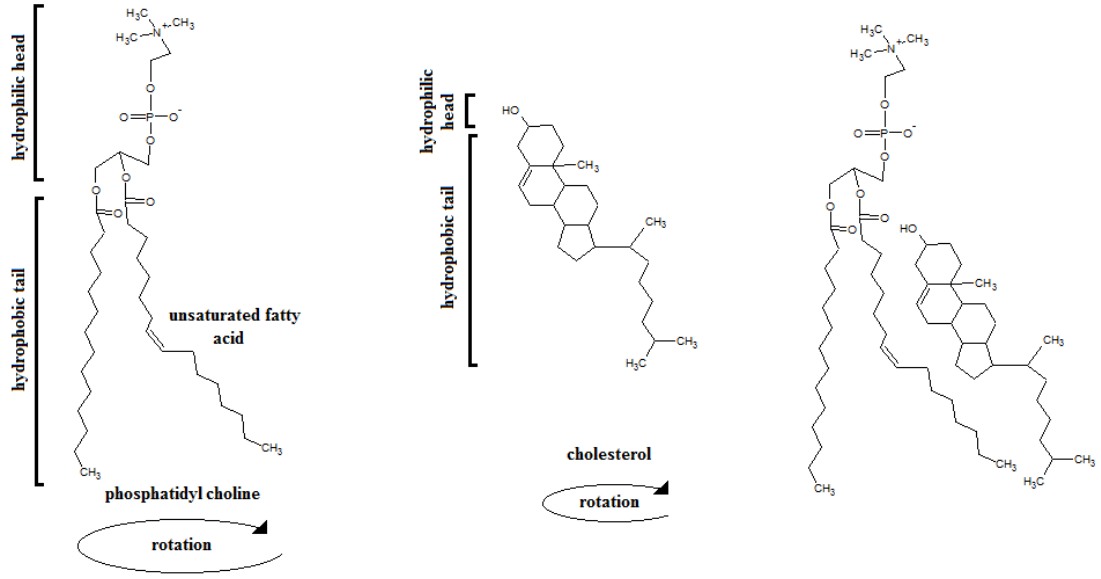

Figure 7-2. Structures of phospholipids cholesterol and phosphatidyl choline.

The diagrams show how the two lipid structures associate with each other within the phospholipid membrane, and their orientation due to the hydrophobic and hydrophilic nature of both compounds. Structures used from Brown (1996), re-drawn using ChemSketch.

Another possibility is that these molecules are able to insert into the plasma membrane and that this is achieved through the amphiphilic properties of glycosylated molecules which also contain fatty acid esters. The sugars give the compound its polar properties, and the fatty acid side chains allow the compound to also be non-polar, which would help with the insertion into the phospholipid bilayer, and could cause disruption to the cell membrane. This hypothesis also suggests that the sterol esters would be more antimicrobial when attached to a sugar molecule. Whilst this is possible, it is unlikely due to the lower levels of inhibition seen in the Gram-negative bacteria, which have cell walls composed of a thin peptidoglycan layer, and two phospholipid layers. This

suggests that the plasma membrane is difficult to penetrate, or that the double layer increases the structural support.

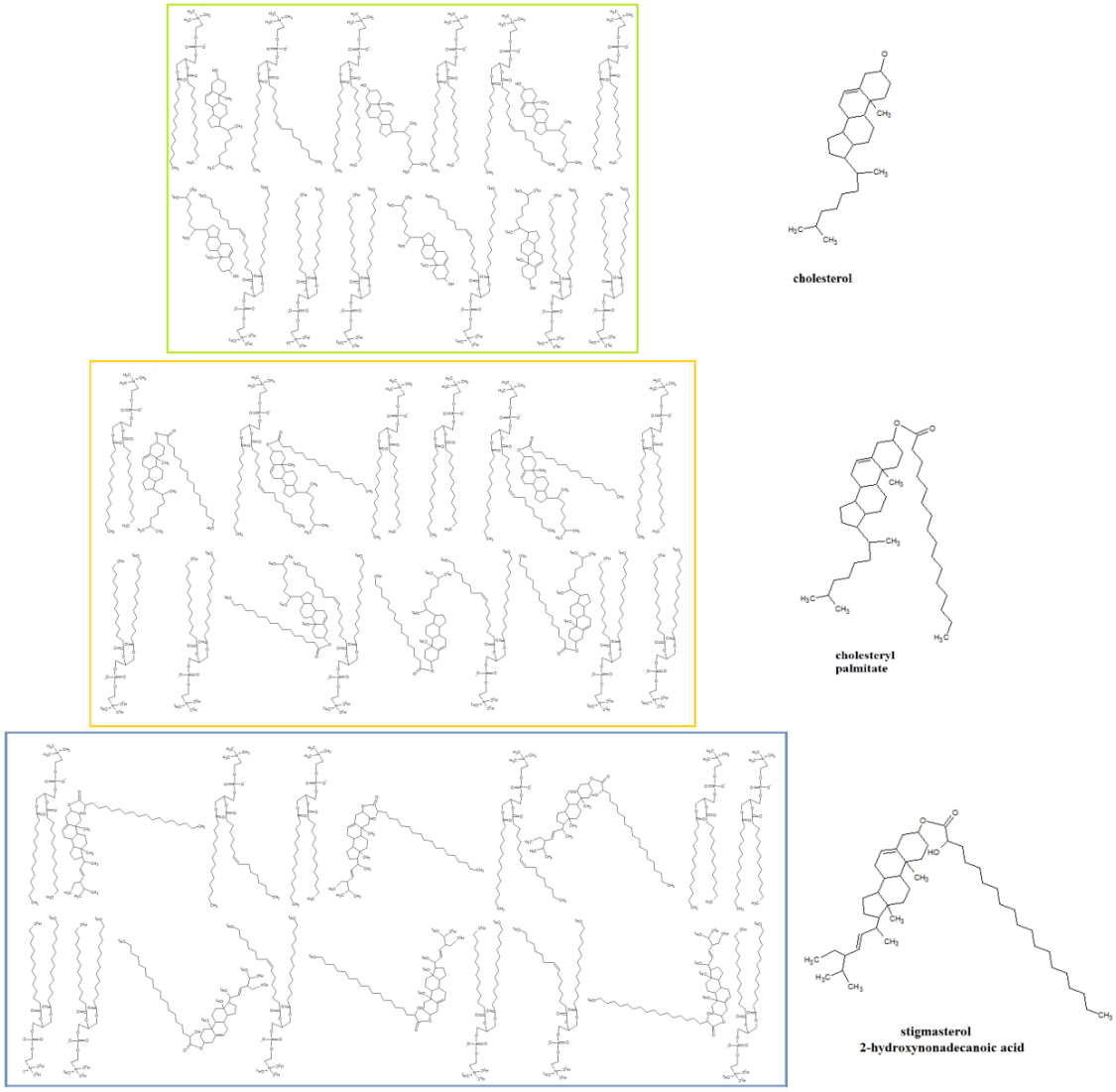

Figure 7-3. Sterol and sterol esters inserted into a phospholipid bilayer.

The structures inside the boxes represent phospholipid bilayers containing phosphatidyl choline (saturated and unsaturated), the green box shows cholesterol inserted into the membrane. The orange box shows the predicted confirmation of cholesterol palmitate inserted into the membrane. The blue box shows the predicted sterol ester produced by *S. pennellii*, stigmasterol-2-hydroxynonadecanoic acid and its predicted confirmation when inserted into the membrane. Structures were drawn from ChemSpider using ChemSketch.

Sterol interaction with membranes has been mostly studied using cholesterol, and its effects upon the membrane dynamics is a well-established ordering-disordering mechanism, where membranes absent in cholesterol are rigid at low temperature and fluid at high temperatures. Cholesterol reduces the rigidity of the membrane at low temperatures, and stabilises the membrane at high temperatures (Dufourc, 2008). Cholesterol is inserted within the bilayer, where its OH group faces the polar head of the phospholipid chains, and the increased presence of cholesterol increases the membrane thickness (Dufourc, 2008) (Figure 7-2). Cholesterol is not static within the membrane, but rotates, which causes the stability within the bilayer, and this rotation is amplified with increased temperature (Dufourc, 2008).

The phospholipids are also in a state of constant moving and rotation (Brown, 1996) (Figure 7-2). Cholesterol esters such as cholesteryl palmitate have a tilted and horse-shoe confirmation due to fatty acid side chain. Therefore, when inserted into the phospholipid membrane, its conformation makes this compound less able to order the membrane, due to diminished van der Waals interactions, which reduces the density of cholesterol packing (Dufourc, 2008) (Figure 7-3).

Is hypothesised that from the analysis of the *S. aureus* metabolomic responses, when cultured in the presence of *S. pennellii* trichome extracts, that compounds within this extract can act upon the bacterial cell wall. However, the modes of action of the other plant extracts (*S. habrochaites* and *N. rustica*) still need to be investigated, and the effects of the extracts upon the Gram-negative bacterial metabolomics should be tested. The antimicrobial mode of action of sesquiterpene carboxylic acids is unknown, but due to the high inhibitory activity against *S. aureus*, and the non-polar properties of the molecules, it is also possible that they affect the stability of the *S. aureus* cell membrane. The antimicrobial mode of action of the predicted alkaloid esters from *N. rustica* could be related to the higher nitrogen content of the molecules, such as the donated hydrogen molecules in the hydrogen bonds on vancomycin come from NH groups, and which would increase the binding capabilities of these molecules to the bacteria.

7.1.7 Summary

This project has identified that, whilst many members of the Solanaceae family are already widely used for food, spices, and medicines, there are more potential uses of Solanaceae plant extracts as antimicrobial agents. Wild tomato relatives *S. pennellii* LA0716, and *S. habrochaites* LA1777 have been found to be antimicrobial from two separate classes of compounds, sterol esters in *S. pennellii* to sesquiterpene carboxylic acids in *S. habrochaites*. The tobacco species *N. rustica* also showed antimicrobial activity, which has been attributed to esters of nicotine and *β*-carboline alkaloids. The similarities between the antimicrobial plant extracts, has been the production of many different ester types of each compound. This creates variety from structurally similar secondary metabolites within the plant, which increases the probability of discovering antimicrobial activity, but also makes the identification of specific antimicrobial compounds challenging.

This research has also highlighted a high level of susceptibility of *S. aureus* towards the antimicrobial extracts, with a decreased susceptibility seen from the Gram-negative bacteria, suggesting that the plant extracts have a mode of activity which is more potent against Gram-positive bacteria. There is also a link between increased antimicrobial activities from Solanaceae plants which have a high trichome load. This is an important finding because high trichome numbers can be visually identified, and can suggest whether a plant is worth investigating for antimicrobial activity. The links between trichomes and the production of protective secondary metabolites are well known in plant resistance to insects and fungi (Bonierbale et al., 1994; Hoeven et al., 2000), and this link can also apply to the potential antimicrobial activity of plants within the Solanaceae family.

The discovery of antimicrobial activity from the trichomes of these three plants has the potential to lead to the use of novel compounds directly from the plants as antimicrobial drugs or preservatives. The use of these compounds in this way would be subject to further tests such as cytotoxicity experiments. Sterol esters would be the most useful antimicrobial compound class, because sterols and fatty acids are already largely consumed from plants as part of our normal diet, and if glucose esters are also responsible for the antimicrobial activity, these compounds would prove to have useful

properties such as a highly amphiphilic nature. The *S. pennellii* sugar esters can also be separated into fractions of bitter and non-bitter tasting compounds, the bitter taste is avoided when at least one of the of the fatty acid side chains on the sugar molecule contains more than 7 carbons (Mudd et al., 1988).

7.2 Future Directions

7.2.1 Metabolic profiling- phenotypic microarray

In addition to the GC-MS analysis of bacteria grown in the presence of plant extracts, the Biolog phenotypic microarrays could be used to determine bacterial metabolic profiles grown with and without the compounds to determine how the bacteria are affected by the compounds. Once an inhibitory compound is identified and purified, it can be tested against a wider range of bacteria, fungi, and insect species to determine if the compound has a more non-specific inhibitory activity.

Phenotypic microarrays (PMs) are used to measure hundreds to thousands of cellular phenotypes simultaneously (Bochner et al., 2001). The Biolog technology involves array plates, with each well containing a tested phenotype. This technology can be used to measure the phenotypes of cells exposed to a certain chemical products, to look for sensitivity, and to identify how these compounds have an effect. Biolog uses a patented redox chemistry which involves cell respiration as a reporter, and positive phenotypes are indicated through the reduction of a tetrazolium dye via the cells active respiration, creating a colour which can be quantified by the OmniLog PM machine through taking images of the colour changes over time (Bochner, 2009). Kinetic graphs can be created from these phenotypic changes, and used to make comparisons between cells analysed under different conditions.

This phenotypic microarray technology could be used to elucidate the effects of compounds on bacteria cells by respiration. It can also be used to discover drug targets which are harmful to infectious bacteria, but not to the plant and animal hosts, and could enable quantification of the cytotoxicity of the compounds within the plant extracts. It also gives a better representation of the effects of compounds over several different physiological states. It can also identify previously unknown target sites and the test can

also be used to identify how compounds might interact with other drugs. The Biolog technology will be a valuable resource to utilise where the compounds of interest can be tested quickly and efficiently against a wide selection of microorganisms.

7.2.2 Further identification of antimicrobial compounds

When testing plant samples initially in bioassays to screen for antimicrobial activities, the compound(s) of interest will be mixed with other non-antimicrobial compounds, and the novel compounds will have to be identified in a background of typical secondary metabolites. The identification of antimicrobial compounds within this research has been largely hypothesis driven, and several analytical platforms have been utilised to investigate which class of compounds are likely to be responsible for the antimicrobial activity observed. Unequivocal identification of purified antimicrobial compounds could not be performed as part of this research for several reasons, such as the limited availability of certain pure compounds to be used as standards in MS.

The purification of a mixture of antimicrobial compounds also requires large concentrations of plant material and a long period of time in order to concentrate enough of the fractions for further analysis, such as further antimicrobial testing and NMR identification. These experiments would be the next stage of identification of the antimicrobial compounds from the plant extracts. The HPLC can also be used to collect fractions during the analysis of a sample, although the concentrations within the fractions would be significantly smaller, they could be amplified through combining and drying multiple samples. This is also a more accurate and a higher separation of compounds achieved than is possible through separating crude plant extracts using SPE cartridges.

Although this further work should be undertaken, the identification of a novel antimicrobial plant fraction is often the limiting stage of many medicinal plants. This can be seen through the widespread use of many different plant essential oils, which contain a variety of compound mixtures. It is largely accepted that the identification of specific antimicrobial compounds within a crude plant extract is a notoriously difficult task. Often within this field of research the most abundant compounds are ascribed

responsible for the antimicrobial activity, such as α-pinene from *R. officinalis* L. essential oil (Santoyo et al., 2005).

7.2.3 Analysis of *S. pennellii* ILs

Whilst some preliminary antimicrobial investigations into the *S. pennellii* ILs were performed as part of this research, only a few selected ILs were tested, and the extraction methods were not initially targeted to the plant trichomes. Due to the antimicrobial activity of *S. pennellii* from the trichome extracts, the *S. pennellii* ILs represent a potential source of secondary metabolite variability, through the introgressed chromosome regions from *S. pennellii* into the domesticated tomato *S. lycopersicum* cv. M82.

Investigating the *S. pennellii* ILs could be important for two reasons. The first is to investigate whether the genetic changes within the plants increase their antimicrobial activity and if this activity is the same as is observed within the wild tomato *S. pennellii*. The second reason is to identify the *pennellii* ILs QTLs which are responsible for the production of these novel antimicrobial compounds. Once the genetic regions linked to the metabolism of these important compounds have been identified, this knowledge could be utilised to amplify these compounds, and such genetic regions could be inserted into other compatible host species.

7.2.4 Trichome proteins and peptides

The main focus of this research was on the secondary metabolites produced as defensive compounds from the leaf trichomes of Solanaceae; however it is also well known that plant trichomes also often produce defensive peptides and proteins (defensins). The Solanaceae species also produce defensive peptides, and many species are known to produce polyphenol oxidase (PPO) which is believed to be responsible for the polymerization of the trichome exudate using O_2, a function which helps to trap insect pests (Yu et al., 1992). The glandular trichomes of several wild tomato species have been investigated for the production of PPO and it was found to be produced by *S. chmielewskii*, *S. cheesmanii*, *S. lycopersicum* cv. VFNT and *S. lycopersicum* cv. Freedom (Yu et al., 1992).

Future work into the investigation of the antimicrobial activities of *S. pennellii* and *S. habrochaites* should include analysing the peptides produced by the trichomes of these species, to determine whether the peptide element of trichome production is responsible as a whole or as part of the antimicrobial activity observed. Whilst it is more likely that the peptides produced by the plant trichomes act as defensive enzymes, which help to create defensive compounds in response to plant stress, the oxidative properties of proteins such as PPO could also be providing some additional antimicrobial effects which should be further investigated.

7.2.5 Concentration of antimicrobial secondary metabolite fractions for further tests against bacteria, fungi and cytotoxic assays

Whilst several of the Solanaceae plant extracts were highly antimicrobial towards the Gram-positive bacteria *S. aureus*, the Gram-negative bacteria were only inhibited at much higher concentrations of the crude trichome extracts. The maximum concentration of the extract in liquid culture was 50 mg mL^{-1} of extract and this was not enough to give a complete inhibition of bacterial growth for *S.* Typhimurium 10248 or *E. coli* O157:H7. The restrictions on this concentration was related to the amount of DMSO solvent which can be used without causing too much inhibition itself upon the tested bacteria, and the general viscosity and dissolvability of the crude extract.

The active fractions from *S. habrochaites* LA1777, *S. pennellii* LA0716, and *N. rustica* have all been identified as part of this research, but further purification and concentration of the crude extracts would be useful in order to determine the accurate MIC of the purified active compounds towards the bacteria. Further investigations into how the extracts affect the bacterial growth should be performed, such as whether the extracts have bactericidal or bacteriostatic effects. Once these compounds have been concentrated from the crude extract it would be appropriate to test the antimicrobial products upon a wider range of bacteria species such as MRSA and *Campylobacter*. The extracts should also be tested against clinically relevant fungi such as *C. albicans*, as many of the compounds within tomato have antifungal properties.

Cytotoxic screening of the antimicrobial secondary metabolites within the identified plant fractions should be performed to determine the safety of the extracts towards

humans and animals. This is important if the extracts are to be developed as phytochemical preservatives or as antibiotic drugs used within animal or human medicine. Such tests would involve treating cells with the plant extract and observing any cell damage which can be highlighted through the use of a dye which can be taken in through damaged cell membranes. Alternatively, normal cell activity can be investigated through monitoring the movement of molecules commonly moved from inside the cell to the surrounding environment, such as lactate dehydrogenase (LDH).

Cytotoxicity screening and in vivo testing would be particularly important for the extracts from *S. habrochaites* and *N. rustica.* This is due to the general toxic properties of alkaloids, which are hypothesised to be produced by *N. rustica*. Also, the sesquiterpene carboxylic acids from *S. habrochaites* would need to be investigated for toxic effects, as sesquiterpene lactones are known to cause allergic reactions and toxic effects towards grazing livestock (Picman, 1986). Some oral toxicity and an animal feeding trial has already been performed for mixtures of 2,3,4-triacylhexoses obtained from *S. pennellii*, where these compounds were found to be non-toxic to laboratory animals, and feeding trials showed that only 33 to 51% of the compounds were absorbed and metabolised (Mudd et al., 1988). These trials concentrated on the sugar esters produced from *S. pennellii*, therefore, the toxicity of sterol ester compounds would also need to be investigated.

7.2.6 Further investigation into flagella inhibition

The EM work identified that *S. pennellii* causes a loss of flagella within *S.* Typhimurium 10248 and *E. coli* O157:H7. The EM results showed drastic changes in the flagella content of bacterial grown in LB compared to within the presence of *S. pennellii* extract particularly against *S.* Typhimurium 10248 (Chapter 6 Figure 6-3). This flagella inhibition was not maintained when sub-cultured onto motility agar and therefore further investigations need to be pursued to determine the cause of this reversible flagella loss.

Further experiments could include using antibodies specific for the flagellin protein which makes up the structure of bacterial flagella, detection using an enzyme-linked immunosorbent assay (ELISA) would show if flagella are present and to what extent

within samples of the bacterial cultures. This would give greater support to the results from the negative EM results, where differences between samples can sometimes be difficult to compare quantitatively. The flagella loss could also be investigated in a variety of ways to determine the reasons behind the flagella loss, once confirmed as a repeatable response to the extract, such as the use of reverse transcriptase polymerase chain reaction (RT-PCR). Through investigating the transcriptional changes such as in the production of flagellin, conclusions could be drawn about whether the flagella loss is imposed upon the bacteria through normal flagella production which is unable to attach to the plasma membrane, or if it is a bacterial response to stress, where flagellin production is reduced to conserve energy.

7.2.7 Investigations into the inhibitory effects of active Solanaceae plant extracts upon biofilms

The motility assay experiment within Chapter 4 showed that some of the tested bacteria had chemotactic movement away from the bacterial plaque, in the form of increased zone of motility, or altered zone morphology. The effects of the plant extracts have currently only been fully tested under liquid broth and solid agar conditions, whilst the motility assay was used, this was primarily to determine whether flagella loss seen within the EM experiments were accurate and maintained. If the extracts were tested against bacteria under a larger variety of environmental and morphological conditions, a better view of their efficiency could be ascertained, such as to determine how effective the extracts would be upon biofilms.

Biofilms act as a protective barrier for the growth of bacteria under stressful conditions, and are known to protect bacteria from several antibiotics through mechanisms such as restricting penetration with an exopolymer matrix, and causing reduced bacterial growth (Lewis, 2001). *Salmonella* can form biofilms in the form of a coordinated formation of colonies with a rdar (red dry and rough) morphology, which consists of aggregating bacteria within a matrix of EPS (exopolysaccharides) and curli fimbriae (White et al., 2010). Metabolic analysis of *Salmonella* cells, which had developed the rdar morphology, were found to produce increased end products of gluconeogenesis, and osmoprotectants such as trehalose, glycine-betaine and glutamate (White et al., 2010).

There is currently a limited understanding about how the biofilm structure develops and how the subsequent detachment for further biofilm proliferation occurs (Periasamy et al., 2012). Biofilms are an important aspect of *S. aureus* infectious life cycle within hospital environments, where *S. aureus* usually forms biofilms within tubes such as catheters and find entry into the body through colonising medical tubing (Beenken et al., 2004). Given that *S. aureus* was the most highly inhibited bacteria as part of this study and that the infectious *S. aureus* species are able to evade antimicrobials through forming biofilms it would be sensible to test these plant extracts upon *S. aureus* biofilms to determine their antimicrobial activity whilst the bacteria exists within its more protective environment. This could increase the potential use of the plant extracts as antiseptic compounds, where they could act as external inhibitors of bacterial growth within environments such as hospitals which require sterile conditions.

7.3 Conclusions

The wild tomato species *S. habrochaites* and *S. pennellii* have antimicrobial activity towards *S. aureus*. This could have considerable implications towards the control of Gram-positive pathogenic bacteria, and therefore further antimicrobial tests should be performed with these plant extracts against bacteria such as MRSA. Identification of chemical classes of secondary metabolites responsible for the antimicrobial activity is an important step towards the identification of the pure chemical structures. The identification of a chemical class can focus the discovery of the antimicrobial compounds through developing targeted extraction procedures.

The negative stain EM allowed bacterial responses to be viewed visually, which is often overlooked within antimicrobial susceptibility testing. However, visual confirmation of cell damage and stress can provide vital information regarding the mode of action of the antimicrobial compounds through analysis of the bacterial response. This was achieved through metabolite analysis of bacteria grown within the presence of the antimicrobial compounds. Additional omics platforms such as transcriptomics could also have been pursued, and would be useful to support the metabolomic results obtained from *S. aureus* grown in the presence of *S. pennellii*. These results, which suggest the *S. pennellii* extract affects the bacterial outer peptidoglycan layer, give strong evidence for membrane disruption as the mode of action of the compounds within the extract. This

could also explain the flagella loss in the Gram-negative bacteria, where increased outer membrane fluidity may be preventing attachment of the flagella.

The discovery of novel antimicrobial compounds is an important area of science due to the continued need for new antibiotics in response to antibiotic resistant strains of bacteria. Whilst most of our current antibiotics are derived from bacterial and fungal sources, the use of plant sources as antimicrobials is rare (Cowan, 1999). The vast diversity in complexity and properties of plant secondary metabolites is a natural resource which has been used in human medicine for thousands of years. The use of plants as antimicrobial agents is less common, which show there is a large resource available to us, which should yield structurally new compounds with no previous influences upon the current resistance mechanisms of our bacterial pathogens. This research which has investigated the antimicrobial potential of several wild tomato relatives shows that antimicrobial activity is a natural and frequent property of many plant species, with three novel species including *N. rustica*, *S. habrochaites*, and *S. pennellii* identified with significant antimicrobial potential.

The development of antimicrobials from plant material has additional benefits, such as the potential to produce these compounds through genetic intervention in species that are more amenable to agricultural production. This could provide a natural antibacterial preservative to the fruit, and the leaves, which may prevent the recent outbreaks of *Salmonella* spp. from contaminated vegetable products.

If the identified antimicrobial compounds from this research are unsuitable to be directly used as antibiotic drugs or preservatives, their structures and mode of actions could still lead to the discovery of novel bacterial target sites to be used for future synthetic drug development. The increasing antimicrobial resistance mechanisms developing within bacterial populations are putting pressure on the currently available antibiotics, which, is coupled with the desires of the general public for the increased use of natural products as substitutes for less trusted synthetic chemicals. This adds an increased importance to the discovery of novel antimicrobial compounds, which is why this research which has identified three chemically different antimicrobial fractions from three separate Solanaceae species are of potential commercial and health-related importance.

Appendices

Appendix 1

Table 1. Saponified and non-saponified trichome extracts.

Analysed by GC-MS and the compounds were identified using Amdis (NIST), a combined list of examples showing the most abundant peaks from the plants p (n-s); *S. pennellii* non-saponified, M82 (s); *S. lycopersicum* cv. M82 saponified, h(n-s); *S. habrochaites* non-saponified, h (s); *S. habrochaites* saponified, n (n-s); *N. rustica* non-saponified, n (s); *N. rustica* saponified, std; compounds verified with a pure chemical standard.

Compounds	Molecular formula	Ref Ion	RT (min)	RI	TLC (R_f)	Amdis Match (R)
1,3-Dioxolan-4-propenoic acid, 2,2-dimethyl-5-[2-(t-butyldimethylsilyloxy2]	$C_{17}H_{32}O_5Si$	329	56.305	3042.7	p (n-s)	581
2,4,6(1H,3H,5H)-Pyrimidinetrione, 5-ethyl-1-methyl-5-[1-methyl-3-[(trimetyl]	$C_{18}H_{36}N_2O_4Si_2$	339	58.7015	3614.7	p (n-s)	454
Adenosyl methionine	$C_{15}H_{23}N_6O_5S$	149	51.477	2687.9	M82 (s)	std
Decanoic acid, 1,1a,1b,4,4a,5,7a,7b,8,9-decahydro-4a,7b-dihydro	$C_{40}H_{64}O_8$	203	46.684	2306.8	p (n-s)	475
Cholic acid	$C_{24}H_{40}O_5$	339	58.281	3591.4	p (n-s)	552
Pregnane-3,20-dione, 17,21-bis[(trimethylsilyl)oxy]-, bis	$C_{29}H_{54}N_2O_4Si_2$	519	65.504	5306.5	h (n-s)	530
Pregnan-11-one, 3,17,20,21-tetrakis[(trimethylsilyl)oxy]	$C_{33}H_{66}O_5Si_4$	417	59.696	3213.2	h (n-s)	550
cis-4,7,10,13,16,19-Docosahexaenoic acid	$C_{25}H_{40}O_2Si$	146	39.806	2000.9	h (s)	705
cis-5,8,11,14,17-Eicosapentaenoic acid	$C_{23}H_{38}O_2Si$	291	37.896	1923.1	h (s)	629
1,3-Diisobutyrin	$C_{14}H_{28}O_5Si$	145	46.527	2298.8	p (n-s)	782
Androst-2-en-4-one, 17-(tetrahydropyran-3-yl)oxy-	$C_{24}H_{36}O_3$	339	58.364	3146.3	p (n-s)	554
2-Keto-l-gluconic acid, penta(*O*-trimethylsilyl)-	$C_{21}H_{50}O_7Si_5$	292	34.558	1793.1	p (n-s)	878
Decanoic acid	$C_{11}H_{22}O_2$	143	20.86.3	1339.4	p (n-s)	960
Dodecanoic acid	$C_{13}H_{26}O_2$	171	27.224	1535.7	p (n-s)	963
propanoic acid	$C_3H_6O_2$	312	35.846	1842.6	p (n-s)	std
Octacosane	$C_{28}H_{58}$	217	54.675	2960	p (n-s)	929
Hentriacontane	$C_{31}H_{64}$	99	61.51	3304.7	p (n-s)	915
Palmitic acid	$C_{16}H_{32}O_2$	313	41.191	2059.5	p (n-s)	std
Malic acid	$C_{13}H_{30}O_5S$	147	26.269	1503.8	p (n-s)	966
Mannitol	$C_6H_{14}O_6$	319	38.04	1929	p (n-s)	std
Trehalose	$C_{12}H_{22}O_{11}$	361	67.275	3595.7	n (n-s)	std
Glucose	$C_6H_{12}O_6$	319	37.572	1910	n (n-s)	std
Galactose	$C_{22}H_{55}NO_6Si_5$	319	37.774	1918.4	n (s)	std
Talose, 2,3,4,5,6-pentakis-*O*-(trimethylsilyl)	$C_{21}H_{52}O_6Si_5$	204	39.622	1993.1	p (n-s)	953
α-D-Glucopyranoside, 1-*O*-methyl-2,3,4	$C_{36}H_{86}O_{11}Si_8$	529	66.627	3567	p (n-s)	722
D-Fructose, 1,3,4,5,6-pentakis-*O*-(trimethylsilyl)	$C_{36}H_{86}O_{11}Si_8$	361	53.589	2881.8	p (n-s)	807
Galactopyranose, 1,2,3,4,6-pentakis-*O*-(trimethylsilyl)-, b-d	$C_{21}H_{52}O_6Si_5$	404	37.502	1907.2	p (n-s)	782
β-D-Glucopyranose, 2,3,4,6-tetrakis-*O*-(trimethylsilyl)-, 1-(trimethylsilyl)-1H-indole-3	$C_{31}H_{59}NO_7Si_5$	547	67.161	3590	h (n-s)	902
D-Turanose, heptakis(trimethylsilyl)-	$C_{33}H_{78}O_{11}Si_7$	318	66.145	3538.7	h (n-s	566
β-D-Lyxofuranoside, thiooctyl-5-*O*-decanoyl-1-deoxy-	$C_{21}H_{42}O_2Si$	329	56.988	3076.8	p (n-s)	570

Appendix 2

Table 2. *N. rustic*a trichome extracts separated by TLC were analysed by GC-MS.

The compounds were identified using Amdis (NIST), a combined list of examples showing the most abundant peaks from all the TLC bands, std; compounds verified with a pure chemical standard.

Compounds	Molecular Formula	Ref Ion	RT (min)	RI	TLC (R_f)	Amdis Match (R)
1,2,4-Triazolo[4,3-a]pyrazine	$C_9H_{11}NOS$	161	24.569	1451	0	437
13-Docosenamide-(Z)	$C_{22}H_{43}NO$	144	24.622	1452.7	0	855
Thiodiglycolic anhydride	$C_4H_4O_3S$	84	24.683	1454.5	0	948
2,3-Dihydro-2,2,4-trimethyl-1,4-benzoxazepine	$C_{11}H_{13}N_3O_2S$	161	24.622	1452.7	0	885
2,4-*bis*(dimethylbenzyl)-6-t-butylphenol	$C_{28}H_{34}O$	149	51.486	2631.1	0.9	647
2,6-Diisopropylnaphthalene	$C_{16}H_{20}$	212	31.447	1679.1	0.9	874
2-Isopropyl-5-methyl-1-heptanol	$C_{11}H_{24}O$	111	20.574	1330.9	0.9	825
2-methyl butyric acid	$C_5H_{10}O_2$	147	24.061	1435.4	0.9	std
2-Propanol, 1-(2-methoxypropoxy)-	C_2H_5NO	84	34.709	1455.3	0	973
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{16}H_{22}O_4$	149	38.93	1892	0.25	965
Benzene, undecyl	$C_{17}H_{28}$	92	34.54	1792.4	0.9	827
Benzothiazole	C_7H_5NS	135	17.615	1248.6	0.9	931
Caryophyllene	$C_{15}H_{24}$	189	24.061	1435.3	0	852
Cotinine	$C_{10}H_{12}N$	176	32.324	1711	0	948
Decanal, *O*-methyloxime	$C_{11}H_{23}NO$	73	17.376	1242.4	0.9	847
Dibutyl phthalate	$C_{16}H_{22}O_4$	149	38.939	1965.5	0.9	955
Dodecane, 2,6,11-trimethyl	$C_{15}H_{32}$	113	33.612	1758.2	0.9	804
Dodecanoic acid, 1-methylethyl ester	$C_{15}H_{30}O_2$	200	30.282	1638.9	0.75	827
Dodecanoic acid, undecyl ester	$C_{23}H_{46}O_2$	201	50.312	2574.9	0.75	897
Heneicosane	$C_{21}H_{44}$	57	58.249	3140.4	0.05	927
Hentriacontane	$C_{31}H_{64}$	155	61.554	3307.1	0.75	912
Hexacosane	$C_{26}H_{54}$	393	59.494	3203.1	0.75	842
Hexacosane, 9-octyl	$C_{34}H_{70}$	365	56.034	3028.8	0.75	820
Hexanethioic acid, S-decyl ester	$C_{13}H_{26}OS$	99	53.519	28.75.5	0	774
Hexanoic acid	$C_6H_{12}O_2$	60	9.288	1037.3	0.9	925
Hexanol, 2-ethyl	$C_8H_{18}O$	57	10.936	1078.3	0.9	878
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	129	39.062	1970.6	0.9	707
Nonacosane	$C_{29}H_{60}$	127	57.619	3108.4	0.75	885
Nonanoic acid	$C_9H_{18}O_2$	129	18.927	1283.8	0.4	878
n-Propyl benzoate	$C_{10}H_{12}O_2$	105	19.102	1288.8	0.75	889
Octacosane	$C_{28}H_{58}$	99	54.719	2962.4	0.75	898
Octadecane	$C_{18}H_{38}$	113	35.101	1813.9	0.9	933
Octadecanoic acid	$C_{18}H_{36}O_2$	284	43.767	2173.8	0.9	621
Pentanoic acid, 2-methyl-, anhydride	$C_9H_{18}O_2$	159	23.123	1406.9	0	765
Phenol, 2,4-bis(1,1-dimethylethyl)	$C_{14}H_{22}O$	191	26.751	1519.9	0.55	835
Phenol, 4-nitro	$C_6H_5NO_3$	139	27.338	1539.3	0.3	895
Nicotine	$C_{10}H_{14}N_2$	84	24.762	1457.2	0	949
Nonadecane	$C_{19}H_{40}$	99	44.713	2216.6	0.9	937
Adenosyl methionine	$C_{15}H_{23}N_6O_5S$	149	51.486	2688.8	0.9	std
2,2'-*bis*(4,5-dimethylimidazole)	$C_{10}H_{14}N_4$	189	52.223	2760.4	0	739
4-(4-Methyl-piperidin-1-yl)-phenylamine	$C_{12}H_{18}N_2$	189	56.209	3027.5	0	741
Silane, [(2-ethylhexyl)oxy]trimethyl	$C_{10}H_{26}OSi_2$	147	12.995	1130.3	0.9	771
Tetratracontane	$C_{44}H_{90}$	394	60.958	3276.9	0.75	903

Appendix 3

Table 3. *S. lycopersicum* cv. M82 trichome extracts separated by TLC and analysed by GC-MS. The compounds were identified using Amdis (NIST), a combined list of examples showing the most abundant peaks from all the TLC bands, std; verified with a chemical standard.

Compounds	Molecular formula	Ref Ion	RT (min)	RI	TLC (R_f)	Amdis Match (R)
1,3-Diisobutyrin, trimethylsilyl	$C_{14}H_{28}O_5Si$	43	46.413	2293.3	0	755
1,3-Dioxolan-4-propenoic acid, 2,2-dimethyl-5-[2-(t-butyldimethylsilyloxy)p	$C_{17}H_{32}O_5Si$	145	60.3146	3703.9	0.45	750
2,4,6(1H,3H,5H)-Pyrimidinetrione, 5-ethyl-1-methyl-5-[1-methyl-3-[(trimethyl]	$C_{18}H_{36}N_2O_4Si_2$	339	58.7015	3614.7	0.9	816
1,2-Benzenedicarboxylic	$C_{16}H_{22}O_4$	149	51.433	2625.7	0.4	940
2,6-Diisopropylnaphthalene	$C_{16}H_{20}$	197	33.027	1736.9	0.9	906
2-Ethylacetoacetate, *O*,*O*'-bis(trimethylsilyl)	$C_{12}H_{26}O_3Si_2$	259	39.587	1991.7	0.1	650
2-Isopropyl-5-methyl-1-heptanol	C11H24O	125	20.576	1331	0.9	848
3-Isopropyl-5,5-dimethyl-1,2,3,4a,5,6,7,8,9,9a-decahydro-4-oxa	$C_{17}H_{26}N_{2O}$	214	45.391	2247.1	0.45	802
5,8-Dihydroxy-4a-methyl-4,4a,4b,5,6,7,8,8a,9,10-decahydro-	$C_{15}H_{22}O_3$	394	43.127	2144.7	0.1	679
9,12-Octadecadiynoic acid	$C_{18}H_{28}O_2$	306	40.025	2010.2	0.7	584
Acetamide, 2,2,2-trifluoro-*N*-methyl-*N*-(trimethylsilyl)	$C_6H_{12}F_3NOSi$	184	21.027	1344.3	0.9	581
Adenosyl methionine	$C_{15}H_{23}N_6O_5S$	149	51.433	2683.9	0.7	std
α-D-Glucopyranoside, 1-*O*-methyl-2,3,4-*tri*-*O*-trimethylsilyl	$C_{16}H_{38}O_6Si_3$	193	59.372	3196.9	0.1	556
Benzothiazole	C_7H_5NS	135	17.551	1247.1	0.4	838
β-Amyrin trimethylsilyl ether	C_7H_5NS	498	65.12	3486.7	0.35	796
Betulin	$C_{30}H_{50}O_2$	498	65.978	3530	0.35	721
cis-4,7,10,13,16,19-Docosahexaenoic acid	$C_{23}H_{38}O_2Si$	291	39.745	1998.2	0.9	676
cis-5,8,11,14,17-Eicosapentaenoic acid	$C_{20}H_{30}O_2$	93	37.476	1906.3	0.9	651
Cycloisolongifolene, 8,9-dehydro	$C_{15}H_{22}$	143	31.658	1687.2	0.3	724
Decanal, *O*-methyloxime	$C_{11}H_{23}NO$	201	17.378	1242.4	0.9	833
Decanoic acid, 1,1a,1b,4,4a,5,7a,7b,8,9-decahydro-4a,7b-dihydro	$C_{40}H_{64}O_8$	214	46.945	2319.4	0.9	800
Decanoic acid, trimethylsilyl ester	$C_{13}H_{28}O_2Si$	229	25.226	1612.7	0.9	806
Dibutyl phthalate	$C_{16}H_{22}O_4$	149	38.913	1964.3	0.35	915
Dodecane, 2,6,11-trimethyl	$C_{15}H_{32}$	85	26.165	1500.5	0.9	887
Glutamine	$C_5H_{10}N_2O_3$	156	26.63	1497.5	0.45	std
Glycerine-1,3-dimyristate, 2-*O*-trimethylsilyl	$C_{34}H_{68}O_5Si$	85	1497.5	1497.5	0.45	507
Glycine	$C_2H_5NO_2$	229	19.563	1264	0.7	std
Heneicosane	$C_{21}H_{44}$	57	58.189	3137.5	0.3	824
Hentriacontane	$C_{36}H_{74}$	57	64.099	3435.4	0.7	583
Hexacosane	$C_{26}H_{54}$	393	59.459	3201.3	0.9	769
Hexacosane, 9-octyl	$C_{34}H_{70}$	365	55.99	3026.4	0.05	628
Hexadecanoic acid, 2,3-*bis*[(trimethylsilyl)oxy]propyl ester	$C_{25}H_{54}O_4Si_2$	371	52.564	2791.7	0.7	915
Hexatriacontane amdis	$C_{31}H_{64}$	99	64.059	3433.6	0.7	858
Nonacosane	$C_{29}H_{60}$	365	57.567	3106.1	0.7	842
Octacosane	$C_{28}H_{58}$		54.717	2962.2	0.7	941
Octadecane	$C_{18}H_{38}$	57	35.087	1813.5	0.9	933
Octadecanoic acid, 2,3-*bis*[(trimethylsilyl)oxy]propyl ester	$C_{27}H_{58}O_4Si_2$	399	56.14	3034	0.45	920
Palmitic acid	$C_{16}H_{32}O_2$	313	41.066	2057.6	0.9	std
Pentadecanoic acid	$C_{18}H_{38}O_2Si$	299	38.755	1958.1	0.35	846
Silane, [(2-ethylhexyl)oxy]trimethyl	$C_{11}H_{26}OSi$	459	13.018	1132.4	0.9	std
Tetradecanoic acid	$C_{17}H_{36}O_2Si$	285	36.291	1860	0.9	917
Tetratetracontane	$C_{44}H_{90}$	449	65.724	3517.6	0.7	855
Tetratracontane	$C_{34}H_{70}$	393	60.966	3277.3	0.7	874
Tritriacontane	$C_{33}H_{68}$	99	61.615	3309.8	0.7	767
α-Linolenic acid	$C_{21}H_{38}O_2Si$	121	41.445	2070.2	0.1	606
Octadecanic acid	$C_{21}H_{44}O_2Si$	341	45.63	2257.9	0.9	959

Appendix 4

Table 4. *S. habrochaites* trichome extracts separated by TLC and analysed by GC-MS.

Compounds were identified using Amdis (NIST), a combined list of examples showing the most abundant peaks from all the TLC bands, std; verified with a chemical standard.

Compounds	Molecular formula	Ref Ion	RT (min)	RI	TLC (R_f)	Amdis Match (R)
1,3,5,7,9,11-Hexaethyl-5,9-dimethoxytricyclo[5.5.1.1(3,11)]	$C_{14}H_{36}O_{10}Si_6$	503	64.147	3438	0.2	783
5-(3-{2,2-Dimethyl-5-[2-(2-trimethylsilylethoxymethoxy)propyl] [1,3]dioxolan-4-yl}acryloyloxy)hex-2-enoic	$C_{28}H_{52}O_8Si_2$	145	56.454	3049.9	0.1	592
2,4,6(1H,3H,5H)-Pyrimidinetrione, 5-ethyl-1-methyl-5	$C_{18}H_{36}N_{2O}4Si_2$	145	48.376	3043.4	0.85	650
2,4,6-Trihydroxybenzoic acid	$C_{19}H_{38}O_5Si_4$	443	61.212	3289.7	0.2	808
1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	$C_{16}H_{22}O_4$	149	51.425	2625.6	0.95	946
2,6-Diisopropylnaphthalene	$C_{15}H_{32}$	99	32.352	1712.1	0.95	852
3-Oxo-10(14)-epoxyguai-11(13)-en-6,12-olide	$C_{15}H_{20}O$	156	42.619	2121.7	0.4	598
4*β*H,5*β*-Eremophil-1(10)-ene, 11	$C_{18}H_{34}OSi$	204	34.487	1790.9	0.85	783
Cyclopentene-3-carboxylic acid, 1-(trimethylsilyl)oxy-	$C_{10}H_{18}O_3Si$	155	43.136	2144.9	0.4	753
Nonacosane	$C_{29}H_{60}$	421	62.771	3368.3	0.85	828
Acetamide, 2,2,2-trifluoro-*N*-methyl-*N*-(trimethylsilyl)	$C_6H_{12}F_3NOSi$	285	20.968	1342.5	0.95	652
Adenosyl methionine	$C_{15}H_{23}N_6O_5S$	149	51.141	2536.6	0.85	std
Cycloisolongifolene, 8,9-dehydro	$C_{15}H_{22}$	202	31.666	1687.2	0.4	774
Dibutyl phthalate	$C_{16}H_{22}O_4$	149	38.904	1964.1	0.95	935
cis-5,8,11,14,17-Eicosapentaenoic acid	$C_{23}H_{38}O_2Si$	291	38.483	1947.1	0.4	701
α-Santalol	$C_{15}H_{24}O$	294	41.48	2071.1	0.25	732
Acrylonitrile, *β*-[3-(2,2-dimethylcyclopropyl)-2,2-dimethylcyclopropyl-	$C_{13}H_{19}N$	318	43.951	2182	0.4	607
Octadecanoic acd	$C_{21}H_{44}O_2Si$	341	45.598	2256.5	0.85	955
Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl	$C_{15}H_{24}$	189	27.557	1546.6	0.85	926
Pregnan-11-one, 3,17,20,21-tetrakis[(trimethylsilyl)oxy]	$C_{33}H_{66}O_5Si_4$	417	59.705	3214	0.5	542
Pregnane-3,20-dione, 17,21-*bis*[(trimethylsilyl)oxy]	$C_{29}H_{54}N_2O_4Si_2$	519	65.523	3507.1	0.4	551
3*β*,4*β*-*bis*(trimethylsiloxy)cholest-5-ene	$C_{33}H_{62}O_2Si_2$	547	67.967	3630.3	0.5	447
β-Amyrin trimethylsilyl ether	$C_{33}H_{58}OSi$	218	65.26	3494.1	0.4	927
Cholic acid	$C_{24}H_{40}O_5$	343	57.164	3085.6	0.1	567
Dihydromorphine , *di*(trimehylsilyl) ether	$C_{23}H_{37}NO_3Si_2$	519	66.408	3552	0.5	536
β-Farnesene	$C_{15}H_{24}$	161	25.226	1471.4	0.85	917
β-Maaliene	$C_{15}H_{24}$	204	27.732	1552.5	0.85	902
Aristolane	$C_{15}H_{26}$	161	27.881	1557.4	0.85	932
y-Elemene	$C_{15}H_{24}$	121	24.368	1444.8	0.85	954
trans-*α*-Bergamotene	$C_{15}H_{24}$	119	23.807	1427.6	0.85	937
1,3-Diisobutyrin, trimethylsilyl	$C_{14}H_{28}O_5Si$	43	45.388	2246.8	0.95	566
2-Ethylacetoacetate, *O,O'*-*bis*(trimethylsilyl)	$C_{12}H_{26}O_3Si_2$	259	39.596	1991.9	0.95	593
2-Isopropyl-5-methyl-1-heptanol	$C_{11}H{=}O$	111	20.6	1331.7	0.95	831
2-methyl butyric acid	$C_5H_{10}O_2$	94	24.008	1433.8	0.85	std
9,12-Octadecadiynoic acid	$C_{21}H_{36}O_2Si$	266	13.091	1132.7	0.65	727
9,12-Octadecadiynoic acid, trimethylsilyl ester	$C_{14}H_{22}O_3Si$	117	29.099	1597.5	0.95	645
cis-4,7,10,13,16,19-Docosahexaenoic acid	$C_{25}H_{40}O_2Si$	119	36.994	1887.2	0.95	611
cis-5,8,11,14,17-Eicosapentaenoic acid	$C_{25}H_{40}O$	291	37.458	1905.4	0.95	627
Decanal, *O*-methyloxime	$C_{11}H_{23}NO$	281	17.3652	1242.1	0.85	820
Decanoic acid, trimethylsilyl ester	$C_{13}H_{28}O_2Si$	229	24.078	1435.9	0.95	944
Dodecane, 2,6,11-trimethyl	$C_{16}H_{34}$	85	26.19	1501.2	0.95	858
Heneicosane	$C_{21}H_{44}$	97	58.189	3137.5	0.4	837
Hentriacontane	$C_{31}H_{64}$	99	61.492	3304	0.4	890
Hexacosane	$C_{36}H_{74}$	393	59.459	3201.4	0.85	758
Hexacosane, 9-octyl	$C_{34}H_{70}$	365	55.99	3026.4	0.85	746
Hexadecanoic acid, 2,3-*bis*[(trimethylsilyl)oxy]propyl ester	$C_{25}H_{54}$	371	52.572	2792.3	0.95	914
Hexatriacontane	$C_{36}H_{74}$	57	64.103	3435.4	0.95	859
Nonacosane	$C_{29}H_{60}$	99	57.584	3107	0.85	883
Octacosane	$C_{28}H_{58}$	57	54.675	2960.3	0.95	924

Octadecanoic acid, 2,3-*bis*[(trimethylsilyl)oxy]propyl ester	$C_{27}H_{58}O_4Si_2$	399	56.104	3032.2	0.85	934
Palmitic acid	$C_{16}H_{32}O_2$	313	41.129	2057	0.95	std
Pentadecanoic acid	$C_{15}H_{30}O_2$	299	39.306	1980.3	0.85	std
Pentasiloxane, dodecamethyl-	$C_{12}H_{36}O_4Si_5$	369	15.632	1132.2	0.2	948
Tetradecanoic acid	$C_{17}H_{36}O_2Si$	285	36.293	1859.9	0.95	940
Tetratetracontane	$C_{44}H_{90}$	57	61.002	3279	0.85	856
Tetratracontane	$C_{44}H_{90}$	449	65.742	3518.3	0.85	764
Tritriacontane	$C_{33}H_{68}$	436	61.702	3314.7	0.85	725
Undecane, 4,7-dimethyl	$C_{13}H_2$	135	39.149	1973.9	0.4	795
Butyric acid, 3-methyl-4-(2,5-xylyl)	$C_{13}H_{18}O$	119	39.167	1974.7	0.4	472
Phenyl acetic acid	$C_8H_8O_2$	193	19.846	1309.4	0.4	std
Fructose -1- 6-bis phosphate	$C_6H_{10}O_{12}P_2$	149	51.149	2537	0.85	std
Glucose 1 phosphate	$C_6H_{13}O_9P$	161	34.345	1781	0.85	std
Hexopyranose, 1,2,3,4,6-pentakis-*O*-	$C_{21}H_{52}O_6Si$	417	59.179	3187.1	0	580
Sucrose	$C_{12}H_{22}O_{11}$	361	53.86	2905.3	0.65	std
α-D-Glucopyranoside, 1-*O*-methyl-2,3,4-*tri*-*O*	$C_{21}H_{52}O_6Si_5$	193	59.372	3197	0.85	532
β-D-Fructofuranose, 2,3,4,6-tetrakis-*O*-	$C_{24}H_{61}O_9PS$	519	67.188	3591.2	0.5	560

Appendix 5

Table 5. *S. pennellii* trichome extracts separated by 2D TLC, bands were analysed by GC-M S

Compounds were identified using Amdis (NIST), a combined list of examples showing the most abundant peaks from all the 2D TLC bands, std; verified with a chemical standard.

Compounds	Molecular formula	Ref Ion	RT (min)	RI	TLC (R_f)	Amdis Match (R)
1,3-Diisobutyrin	$C_{14}H_{28}O_5Si$	145	46.395	2292.5	0.74 x 0.18	780
1,3-Dioxolan-4-propenoic acid, 2,2-dimethyl-5-[2-(t-butyldimethylsilyloxy)propyl]-	$C_{17}H_{32}O_5Si$	329	56.209	3037.3	0.74 x 0.18	463
13-*cis*-Retinoic acid	$C_{23}H_{36}O_2Si$	255	49.751	2520.7	0.74 x 0.18	574
11-Nor-*δ*-9-tetrahydrocannabinol-9-carboxylic acid	$C_{21}H_{28}O_4$	329	56.822	3068.2	0.74 x 0.18	530
2,6-Diisopropylnaphthalene	$C_{16}H_{20}$	197	32.998	1735.8	0.66 x 0	891
2,4,6-Trihydroxybenzoic acid	$C_{19}H_{38}O_5Si_4$	443	61.448	3301.5	0.74 x 0.18	805
Oxalic acid, *bis*(2-ethylhexyl) ester	$C_{18}H3_4O_4$	842	50.82	2625	0.07 x0.78	845
2-Isopropyl-5-methyl-1-heptanol	$C_{17}H_{26}N_2O$	460	45.476	2250.4	0.66 x 0.06	568
Pregnan-11-one, 3,17,20-*tris*[(trimethylsilyl)oxy]-, (3*α*,5*β*,20S)-	$C_{30}H_{58}O_4Si_3$	449	59.731	3214.9	0.07 x0.78	463
3-Isopropyl-5,5-dimethyl-1,2,3,4a,5,6,7,8,9,9a-decahydro-4-oxa	$C_{17}H_{26}N_2O$	214	45.344	2245	0.74 x 0.18	563
Triacontane, 1-bromo-	$C_{30}H_{61}Br$	421	62.684	3364.1	0.07 x0.78	795
α-Cortolone	$C_{21}H_{34}O_5$	519	65.488	3505.3	0.62 x 0.21	576
Adenosyl Methionine	$C_{15}H_{23}N_6O_5S$	149	51.141	2536.6	0.66 x 0	std
α-D-Glucopyranoside, 1,3,4,6-tetrakis-*O*-(trimethylsilyl)-*β*-D-fructofuranosyl 2,3,4,6-tetrakis-*O*-(trimethylsilyl	$C_{36}H_{86}O_{11}Si_8$	543	67.617	3612.8	0.07 x0.78	725
β-D-Lyxofuranoside, S-octyl-5-valeroyl	$C_{18}H_{34}O_5S$	229	59.459	3201.5	0.74 x 0.18	524
Betulin	$C_{30}H_{50}O_2$	498	66.063	3534.4	0.66 x 0	710
Campesterol	$C_{31}H_{56}OSi$	343	60.809	3269.3	0.74 x 0.18	539
Cholest-22-ene-21-ol, 3,5-dehydro-6-methoxy-, pivalate	$C_{33}H_{54}O_3$	145	57.346	3539.7	0.74 x 0.18	409
Decanoic acid, 1,1a,1b,4,4a,5,7a,7b,8,9-decahydro-4a,7b-dihydroxy-3-(hydroxymethyl)-1,1,6,8-tetramethyl	$C_{40}H_{64}O_8$	214	46.886	2316.5	0.74 x 0.18	482
Dibutyl phthalate	$C_{16}H_{22}O_4$	149	38.956	1966.1	0.66 x 0.12	928
cis-4,7,10,13,16,19-Docosahexaenoic acid, trimethylsilyl ester	$C_{25}H_{40}O_2Si$	156	38.229	1936.8	0.74 x 0.18	658
Dodecane, 2, 6, 11 tri methyl	$C_{15}H_{32}$	113	27.54	1546	0.74 x 0.18	878
Dodecanoic acid, 1-[[(trimethylsilyl)oxy]methyl]-1,2-ethanedi	$C_{13}H_{28}O_2S$	229	24.052	1435	0.74 x 0.18	914
Hexopyranose, 1,2,3,4,6-pentakis-*O*-(trimethylsilyl)-	$C_{23}H_{38}O_2Si$	291	37.449	1905.1	0.74 x 0.18	662
Fructose	$C_6H_{12}O_6$	307	37.073	1890.1	0.66 x 0	std
Galactose	$C_6H_{12}O_6$	319	37.66	1913,7	0.66 x 0	std
D-Glucose, 2,3,4,5,6-pentakis-*O*-(trimethylsilyl)-, *O*-methyloxyme, (1E)-	$C_{22}H_{55}NO_6Si_5$	319	37.66	1913.7	0.66 x 0	779
Glucose 1 Phosphate	$C_6H_{13}O_9P$	217	34.295	1783.3	0.66 x 0	std
Glycerine-1,3-dimyristate	$C_{34}H_{68}O_5Si$	339	58.399	3148	0.62 x 0.21	509
Glycine	$C_2H_5NO_2$	174	19.563	1264	0.74 x 0.18	std
Hentriacontane	$C_{31}H_{64}$	111	61.589	3331.2	0.62 x 0.21	std
Hexacosane	$C_{26}H_{54}$	393	59.424	3199.8	0.07 x0.78	721
Hexadecanoic acid, 2,3-*bis*[(trimethylsilyl)oxy]propyl ester	$C_{25}H_{54}O_4Si_2$	371	52.555	2790.9	0.74 x 0.18	909
Hexatriacontane	$C_{36}H_{74}$	421	64.13	3436.9	0.07 x0.78	878
Hexopyranose, 1,2,3,4,6-pentakis-*O*-(trimethylsilyl)-	$C_{21}H_{52}O_6Si_5$	199	59.126	3184.4	0.07 x0.78	358
2,6-Diisopropylnaphthalene	$C_{16}H_{20}$	155	33.007	1736.2	0.07 x0.78	875
Nonacosane	$C_{29}H_{60}$	99	57.584	3106.8	0.07 x0.78	878
Octacosane	$C_{28}H_{58}$	155	54.684	2960.7	0.07 x0.78	936
Octadecanoic acid	$C_{27}H_{58}O_4S$	399	56.112	3032.5	0.74 x 0.18	864
Oleic acid	$C_{21}H_{42}O_2Si$	339	57.304	3092.8	0.66 x 0.12	563
Palmitic acid	$C_{16}H_{32}O_2$	313	41.121	2056.5	0.74 x 0.18	std
Pentadecanoic acid	$C_{15}H_{30}O_2$	299	38,755	1958	0.66 x 0	std
Propanoic acid	$C_3H_6O_2$	312	35.767	1839.6	0.74 x 0.18	std
Sucrose	$C_{12}H_{22}O_{11}$	361	53.42	2658.2	0.74 x 0.18	std

Tetradecanoic acid	$C_{17}H_{36}O_2Si$	285	36.284	1859.7	0.74 x 0.18	890
Tetratetracontane	$C_{44}H_{90}$	449	65.724	3517.3	0.07 x0.78	739
Tetratracontane	$C_{34}H_{70}$	393	61.028	3280.4	0.07 x0.78	855
Tritriacontane	$C_{33}H_{68}$	127	61.632	3311.1	0.07 x0.78	788
Dodecane, 2,6,10-trimethyl-	$C_{15}H_{32}$	127	44.666	2214.1	0.07 x0.78	833
D-Glycero-D-galacto-2-Nonulosonic acid, 5-(acetylamino)-3,5-dideoxy-4,6	$C_{27}H_{61}NO_9Si_5$	175	48.279	2384.4	0.74 x 0.18	507
Decanoic acid, 1,1a,1b,4,4a,5,7a,7b,8,9-decahydro-4a,7b-dihydroxy-3-(hydroxymethyl	$C_{40}H_{64}O_8$	214	54.965	2974.7	0.74 x 0.18	560
2-Monostearin trimethylsilyl ether	$C_{27}H_{58}O_4Si_2$	399	56.315	3042.8	0.07 x0.78	688

Appendix 6

Table 6. *S. pennellii* trichome extracts separated by SPE, fractions were analysed by GC-MS.

The compounds were identified using Amdis (NIST), a combined list of examples showing the most abundant peaks from all the fractions after SPE of the extract. Fractions are represented by- H; hexane, CL; chloroform, H:CL; hexane:chloroform (50:50 v/v), EA; ethyl acetate, and CL:EA; chloroform:ethyl acetate (50:50 v/v).

Compounds	Molecular formula	Ref Ion	RT (min)	RI	TLC(R_f)	Amdis Match (R)
(14-methoxy-5,13,13-trimethyl-8-oxo-9,15-dioxapentacyclo[12.2.2.1(2,6).0(1)	$C_{24}H_{34}O_7$	127	56.892	3071.9	H	553
1,3-Dioxolan-4-propenoic acid, 2,2-dimethyl-5-[2-(t-butyldimethylsilylo)	$C_{17}H_{32}O_5Si$	329	56.296	3041.7	H	557
Oxalic acid, heptyl propyl ester	$C_{12}H_{22}O_4$	371	50.864	2631.7	H	713
Acetamide, 2,2,2-trifluoro-*N*-methyl-*N*	$C_6H_{12}F_3NOSi$	285	20.977	1342.8	H	655
Octadecanoic acid	$C_{21}H_{44}O_2Si$	341	45.633	2258.1	H	943
4-*O*-Methyl-12b,13,20-triacetoxy-2,9-dihydroxy-3a-carboxy-2,3-seco-tigla-1(10),6-diene-3,9-lactone	$C_{27}H_{36}O_{10}$	214	55.035	29.78.3	H	610
3,19-Epoxyandrosta-5,7-diene, 17-acetoxy-4	$C_{24}H_{34}O_4$	300	58.32	3143.9	CL	465
13-*cis*-Retinoic acid	$C_{23}H_{36}O_2Si$	357	49.331	2480.3	H:CL	575
Decanoic acid, 1,1a,1b,4,4a,5,7a,7b,8,9	$C_{50}H_82O_9$	474	46.956	2320	H	458
16-Oxapentacyclo[13.2.2.0(1,13).0(2,10)	$C_{25}H_{38}O_4$	339	58.005	3127.9	H:CL	566
3-Isopropyl-5,5-dimethyl-1,2,3,4a,5,6,7	$C_{17}H_{26}N_{2O}$	214	45.414	2248	H	579
Campesterol	$C_{31}H_{56}OSi$	343	61.212	3289.8	H	575
Cholest-22-ene-21-ol, 3,5-dehydro-6-met	$C_{33}H_{54}O_3$	343	48.647	2413.4	H:CL	560
Cholic acid	$C_{24}H_{40}O_5$	339	57.654	3110.2	H:CL	557
Pregnan-17-ol, 3,20-*bis*[(trimethylsilyl	$C_{27}H_{52}O_3Si_2$	343	48.016	23714	H:CL	533
Glycine, *N*-[(3a,5b,12a)-3,12-dihydroxy-	$C_{26}H_{43}NO_5$	343	48.209	2381.1	H:CL	544
Caryophyllene	$C_{15}H_{24}$	285	24.07	1435.6	EA	934
5,8,11-Eicosatriynoic acid, tert-butyld	$C_{26}H_{42}O_2Si$	339	58.776	3166.8	H:CL	574
Butanoic acid, anhydride	$C_8H_{14}O_3$	329	54.547	3384.8	CL:EA	540
2-Isopropyl-5-methyl-1-heptanol	$C_{11}H_{24}O$	154	20.6	1331.6	H	840
Decanoic acd	$C_{13}H_{28}O_2Si$	229	24.079	1435.9	H	862
Decanoic acid, trimethylsilyl ester	$C_{13}H_{28}O_2Si$	229	24.079	1436	H	789
Dodecanoic acid, 1-[[(trimethylsilyl)ox	$C_{30}H_{60}O_5Si$	367	60.246	3241.4	H	606
Androst-2-en-4-one, 17-(tetrahydropyran-3)-	$C_{24}H_{36}O_3$	339	58.408	3148.2	H	537
Hentriacontane	$C_{31}H_{64}$	99	61.615	3310.1	H	921
Hexacosane	$C_{26}H_{54}$	393	59.522	3204.3	H	638
Hexatriacontane	$C_{36}H_{74}$	421	64.13	3436.9	H	879
Malic acid	$C_{13}H_{30}O_5Si_3$	147	26.404	1489.9	CL:EA	std
Octacosane	$C_{28}H_{58}$	57	54.728	2962.7	H	919
Oleic acid, trimethylsilyl este	$C_{21}H_{42}O_2Si$	343	57.365	3095.7	H:CL	545
Oxalic acid, dodecyl neopentyl ester	$C_{10}H_{18}O_4$	145	59.921	59.9212	CL:EA	452
Palmitic acid	$C_{16}H_{32}O_2$	313	41.173	2058.5	H	std
11-Nor-*δ*-9-tetrahydrocannabinol-9-carboxylic	$C_{21}H_{28}O_4$	329	60.011	3229.3	H:CL	429
Propanoic acid, 2-methyl-, anhydride	$C_{14}H_{20}O_2$	293	26.602	1515	H:CL	858
Tetradecanoic acid, trimethylsilyl ester	$C_{17}H_{36}O_2Si$	285	36.336	1861.7	H:CL	913
Tetratracontane amdis match	$C_{34}H_70$	393	61.037	3280.8	H:CL	854
Tritriacontane, 3-methyl	$C_{33}H_{68}$	155	64.682	3464.6	H:CL	916
Undecanoic acid	$C_{14}H_{30}O_2Si$	229	25.226	1471.3	CL:EA	623
1,3-Diisobutyrin, trimethylsilyl	$C_{14}H_{28}O_5Si$	329	46.471	2296.3	H	774
Isonicotinic acid	$C_6H_5NO_2$	180	18.616	1233.1	CL:EA	std
Butylated Hydroxytoluene	$C_{15}H_{24}O$	293	26.602	1515	H:CL	740
2,4,6-Trihydroxybenzoic acid, tetra-TMS	$C_{19}H_{38}O_5Si_4$	443	61.519	3305.2	CL:EA	808
Triacontane, 1-bromo-	$C_{30}H_{61}Br$	421	62.736	3366.9	H	767
Fructose	$C_6H_{12}O_6$	307	37.274	1898.3	EA	std
Gluconic acid	$C_6H_{12}O_7$	319	37.37	1902.1	EA	std
Glucose 1 Phosphate	$C_6H_{13}O_9P$	217	34.207	1780.2	EA	std
α-D-Galactopyranoside, 1-*O*-methyl-2,6-*O*	$C_{21}H_{52}O_6Si_5$	204	37.499	1907.2	H:CL	782
(trimethylsilyl)-*β*-D-fructofuranosyl 2,3,4,6-tetrakis-*O*-(trimethylsilyl)-	$C_{36}H_{86}O_{11}Si_8$	543	52.844	2816.3	E	820
α-D-Glucopyranoside, 1,3,4,6-tetrakis-*O*-(trimethylsilyl)-*β*-D-fructose	$C_{36}H_{86}O_{11}Si_8$	543	67.249	3594.1	EA	835
β-D-Lyxofuranoside, thiooctyl-5-*O*-decan	$C_{23}H_{44}O_5S$	339	58.566	3156.2	H:CL	576

Appendix 7

Table 7. Total of raw data from *S. aureus* metabolomic analysis of bacteria cultured in the presence of *S. pennellii* 0.78 mg mL^{-1} extract, with LB and solvent controls.

The total metabolite concentrations measured using GCMS and Amdis (NIST) identification, with quantification based on comparisons with standards of known concentrations, represented per hour as average ± standard error. Data represents the µg mL^{-1} of DW the bacterial pellet.

	DMSO	DMSO	DMSO	LB	LB	LB	*S. pennellii*	*S. pennellii*	*S. pennellii*
Compounds	6 h	6.30 h	24 h	6h	6.30 h	24 h	6 h	6.30 h	24 h
2/3 Phosphoglyceric acid	0.59 ± 0.25	2.06 ± 1.03	0.00 ± 0.00	0.73 ± 0.37	2.56 ± 1.25	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2-Hydroxyisocaproic acid	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.53 ± 0.59	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
5-Oxoproline	62.76 ± 49.71	47.13 ± 54.77	202.97 ± 50.76	78.58 ± 41.40	65.56 ± 24.25	61.76 ± 41.00	116.64 ± 62.63	129.74 ± 80.86	184.23 ± 41.71
Adenine	1.27 ± 0.71	1.17 ± 1.14	11.01 ± 2.50	2.12 ± 1.26	2.64 ± 1.98	6.77 ± 2.89	0.00 ± 0.00	25.00 ± 23.03	325.73 ± 175.28
Adenosine	9.51 ± 5.64	6.81 ± 5.24	31.83 ± 17.55	11.16 ± 7.30	6.44 ± 3.66	20.69 ± 19.39	2.98 ± 2.30	2.70 ± 1.63	9.96 ± 4.27
aiC15:0	0.00 ± 0.00	2.65 ± 1.30	31.49 ± 14.33	0.00 ± 0.00	4.35 ± 1.48	28.08 ± 34.53	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
aiC17:0	0.00 ± 0.00	0.00 ± 0.00	34.35 ± 4.32	0.00 ± 0.00	0.00 ± 0.00	32.94 ± 27.10	0.00 ± 0.00	0.00 ± 0.00	14.66 ± 10.11
Alanine	34.40 ± 19.03	17.82 ± 17.31	0.00 ± 0.00	38.81 ± 21.24	31.62 ± 14.79	18.98 ± 19.75	0.00 ± 0.00	0.00 ± 0.00	7.32 ± 6.24
alfa-Hydroxyglutaric acid	0.47 ± 0.19	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	59.39 ± 36.26	25.35 ± 14.71	0.00 ± 0.00
Aminobutyric acid	0.00 ± 0.00	0.60 ± 0.44	0.00 ± 0.00	0.80 ± 0.42	0.80 ± 0.15	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Aminohexose- 41.9089 min	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.85 ± 0.63	2.78 ± 5.20	0.00 ± 0.00
Aminomalonic acid	0.00 ± 0.00	0.99 ± 0.50	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
AMP	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	14.88 ± 6.24	15.15 ± 10.40	67.98 ± 105.17
Asparagine	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	98.60 ± 81.70
Aspartic acid	18.97 ± 15.27	13.08 ± 16.80	179.16 ± 123.71	25.83 ± 14.73	20.16 ± 5.90	46.13 ± 49.09	0.26 ± 0.09	0.00 ± 0.00	0.00 ± 0.00
Benzoate	0.19 ± 0.11	0.61 ± 0.34	1.73 ± 0.28	0.13 ± 0.04	1.51 ± 2.16	0.50 ± 0.61	0.00 ± 0.00	0.00 ± 0.00	6.46 ± 4.74
β-Alanine	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.86 ± 0.31	4.74 ± 1.52
Butanoic acid 2,4-diOH	0.57 ± 0.55	0.00 ± 0.00	3.51 ± 1.78	0.56 ± 0.41	0.57 ± 0.36	0.76 ± 0.42	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Butanoic acid 3,4-diOH	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.42 ± 0.09	0.00 ± 0.00	1.07 ± 0.87	0.00 ± 0.00	0.00 ± 0.00
C11:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	2.76 ± 0.47	4.53 ± 2.77

C13:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.33 ± 0.13	0.00 ± 0.00	1.09 ± 0.25	5.38 ± 4.36
C14:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.66 ± 0.55	3.35 ± 1.47	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C15:0	0.00 ± 0.00	0.22 ± 0.03	0.00 ± 0.00	0.38 ± 0.21	0.00 ± 0.00	1.32 ± 0.49	0.00 ± 0.00	0.00 ± 0.00	1.26 ± 0.19
C16:0	0.00 ± 0.00	2.06 ± 1.71	4.44 ± 2.15	1.46 ± 0.82	9.80 ± 6.15	9.91 ± 7.52	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C17:0	0.00 ± 0.00	0.00 ± 0.00	35.73 ± 22.17	0.00 ± 0.00	0.00 ± 0.00	7.45 ± 14.71	6.37 ± 3.09	0.00 ± 0.00	4.90 ± 4.49
C18:0	7.96 ± 7.76	9.51 ± 12.36	43.27 ± 7.50	14.01 ± 10.73	19.83 ± 8.97	27.09 ± 2.84	0.00 ± 0.00	0.00 ± 0.00	8.39 ± 5.74
C19:0	0.00 ± 0.00	0.00 ± 0.00	4.20 ± 1.23	0.00 ± 0.00	0.00 ± 0.00	3.19 ± 2.93	12.53 ± 0.79	6.62 ± 2.51	23.27 ± 8.17
C20:0	0.17 ± 0.13	0.70 ± 0.88	5.85 ± 2.08	0.63 ± 0.60	2.31 ± 0.81	4.93 ± 2.54	0.00 ± 0.00	0.00 ± 0.00	3.20 ± 1.04
C9:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.53 ± 0.28	0.00 ± 0.00	0.00 ± 0.00
Citric acid	1.86 ± 1.69	0.82 ± 0.91	0.00 ± 0.00	3.62 ± 2.64	3.29 ± 1.88	3.25 ± 2.52	0.00 ± 0.00	0.00 ± 0.00	4.04 ± 2.57
Cysteine	15.65 ± 16.78	16.92 ± 23.97	28.96 ± 15.96	18.23 ± 22.27	16.52 ± 11.34	14.79 ± 8.97	15.53 ± 2.73	35.46 ± 23.80	4.78 ± 2.18
Cytosine	149.24 ± 125.82	0.00 ± 0.00	0.00 ± 0.00	0.33 ± 0.11	0.26 ± 0.11	0.00 ± 0.00	0.29 ± 0.10	0.00 ± 0.00	0.00 ± 0.00
Fumaric acid	0.37 ± 0.30	0.00 ± 0.00	3.90 ± 0.69	0.65 ± 0.45	0.58 ± 0.31	1.67 ± 1.04	0.67 ± 0.36	0.00 ± 0.00	9.87 ± 9.04
Galactose	3.63 ± 1.24	0.00 ± 0.00	0.00 ± 0.00	4.50 ± 6.33	0.91 ± 0.45	0.00 ± 0.00	4.46 ± 2.12	7.05 ± 10.93	0.00 ± 0.00
Glucose	3.22 ± 3.32	1.62 ± 1.83	1.48 ± 0.75	5.27 ± 3.41	3.13 ± 2.23	1.93 ± 1.24	5.86 ± 3.56	12.13 ± 9.58	37.22 ± 70.38
Glutamic acid	32.29 ± 25.47	38.90 ± 54.62	186.58 ± 136.74	64.46 ± 27.45	49.59 ± 19.75	33.67 ± 18.33	104.84 ± 54.13	83.05 ± 64.58	80.19 ± 18.57
Glyceric acid	1.06 ± 0.13	0.00 ± 0.00	0.00 ± 0.00	0.95 ± 0.34	0.78 ± 0.34	0.00 ± 0.00	1.17 ± 0.33	0.00 ± 0.00	0.00 ± 0.00
Glycero-1-aiC15:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.40 ± 0.20	0.34 ± 0.26	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Glycero-1-C14:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.04	0.11 ± 0.03	0.27 ± 0.08	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Glycero-1-C16:0	4.56 ± 4.70	3.56 ± 5.10	5.04 ± 2.04	7.63 ± 5.55	8.13 ± 2.75	6.80 ± 2.36	0.00 ± 0.00	3.17 ± 0.68	6.21 ± 0.93
Glycero-1-C18:0	11.90 ± 8.82	7.63 ± 10.83	11.14 ± 4.53	14.53 ± 14.59	18.53 ± 6.11	19.95 ± 11.81	0.00 ± 0.00	5.25 ± 1.34	0.00 ± 0.00
Glycero-1-iC15:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	9.09 ± 8.83	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Glycero-2-C18:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.51 ± 0.91	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Glycero-2-iC15:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.29 ± 0.56	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Glycerol	24.35 ± 18.92	14.81 ± 17.73	5.56 ± 4.87	15.70 ± 15.43	6.56 ± 7.99	3.86 ± 4.98	24.03 ± 16.68	41.89 ± 35.83	27.54 ± 17.90
Glycerol-3-Phosphate	9.01 ± 9.93	9.46 ± 14.04	3.89 ± 2.48	9.11 ± 3.81	7.56 ± 2.96	5.10 ± 3.33	14.98 ± 11.11	11.70 ± 6.87	9.40 ± 8.22
Glycine	23.80 ± 19.29	109.95 ± 261.72	0.00 ± 0.00	26.40 ± 13.67	24.09 ± 9.60	14.09 ± 8.72	41.14 ± 24.45	27.36 ± 15.72	8.55 ± 6.60

Glycolic acid	0.76	±	0.93	0.37	±	0.44	1.26	±	0.67	0.46	±	0.05	0.40	±	0.10	0.00	±	0.00	0.89	±	0.41	1.04	±	0.58	2.28	±	0.78
Guanine	0.38	±	0.21	0.00	±	0.00	3.75	±	2.12	0.37	±	0.14	0.00	±	0.00	2.31	±	1.61	0.69	±	0.37	0.42	±	0.23	2.06	±	0.76
Guanosine	0.77	±	0.61	0.73	±	0.64	4.21	±	2.00	1.23	±	0.73	1.01	±	0.37	2.98	±	2.09	2.00	±	1.07	1.42	±	0.81	0.00	±	0.00
Homocysteine	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	3.98	±	2.58	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Hypoxanthine	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.13	±	0.06	0.10	±	0.03	0.00	±	0.00	0.17	±	0.08	0.00	±	0.00	0.00	±	0.00
iC14:0	0.00	±	0.00	0.73	±	0.70	1.45	±	1.15	0.81	±	0.50	0.77	±	0.34	1.07	±	0.96	0.00	±	0.00	0.00	±	0.00	0.75	±	0.41
iC15:0	0.00	±	0.00	0.00	±	0.00	25.98	±	6.02	0.89	±	0.81	8.22	±	8.33	14.37	±	10.97	0.00	±	0.00	0.00	±	0.00	7.95	±	2.39
iC16:0	4.88	±	4.51	4.24	±	5.73	12.23	±	5.56	8.35	±	6.51	0.00	±	0.00	3.93	±	3.87	14.66	±	10.08	4.43	±	0.34	10.70	±	2.74
iC17:0	0.00	±	0.00	0.00	±	0.00	7.33	±	1.39	0.00	±	0.00	0.00	±	0.00	7.58	±	6.57	0.00	±	0.00	0.00	±	0.00	3.23	±	1.01
Inositol	2.26	±	1.92	1.49	±	1.54	5.13	±	0.62	3.17	±	1.84	2.71	±	1.46	3.04	±	1.35	4.54	±	2.94	5.62	±	3.65	5.82	±	2.87
Isoleucine	14.88	±	11.85	10.45	±	11.72	44.66	±	10.19	20.81	±	15.31	20.54	±	10.30	23.51	±	18.44	38.59	±	27.30	32.54	±	18.77	45.55	±	12.30
Lactic acid	3.79	±	2.89	2.93	±	3.12	20.54	±	24.28	4.85	±	2.32	5.49	±	2.83	13.37	±	15.00	7.10	±	4.41	11.14	±	6.04	14.80	±	11.51
Leucine	21.67	±	17.19	19.97	±	12.52	72.31	±	66.04	64.30	±	45.80	54.37	±	32.79	35.94	±	26.06	88.04	±	49.21	53.08	±	39.19	54.27	±	37.36
Lysine	130.95	±	102.88	87.31	±	102.90	367.97	±	88.15	137.04	±	68.15	132.80	±	58.82	185.98	±	88.25	223.25	±	137.87	192.08	±	103.82	115.32	±	66.22
Malic acid	1.50	±	1.49	0.00	±	0.00	0.00	±	0.00	2.13	±	1.54	1.78	±	1.10	0.78	±	0.64	3.17	±	2.52	0.00	±	0.00	0.00	±	0.00
Maltose	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	3.18	±	1.58	0.00	±	0.00	0.00	±	0.00
Methionine	0.00	±	0.00	2.44	±	1.87	9.76	±	3.57	0.00	±	0.00	4.17	±	0.92	2.84	±	1.10	0.00	±	0.00	9.63	±	4.67	382.62	±	845.75
Methyl glycerate	0.98	±	0.80	0.68	±	0.68	4.20	±	2.09	1.45	±	0.83	1.23	±	0.64	1.20	±	0.57	2.09	±	1.34	3.08	±	2.08	31.47	±	38.59
Nicotinic acid	0.00	±	0.00	28.35	±	18.53	158.32	±	127.80	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	2.96	±	2.91	11.70	±	6.98
Norleucine	48.74	±	38.79	44.92	±	33.15	95.09	±	50.71	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	74.72	±	33.51	101.61	±	61.33	0.00	±	0.00
Ornithine	6.59	±	5.13	11.27	±	9.09	146.80	±	104.92	17.15	±	12.76	14.75	±	9.52	74.81	±	39.71	24.92	±	17.16	0.00	±	0.00	276.14	±	235.77
Oxalic acid	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.43	±	0.12	0.31	±	0.19	0.29	±	0.09	0.87	±	0.40	0.00	±	0.00	0.00	±	0.00
Phenylacetic acid	0.00	±	0.00	0.00	±	0.00	2.40	±	0.68	0.00	±	0.00	0.00	±	0.00	2.31	±	1.45	36.38	±	15.71	14.93	±	8.99	62.89	±	48.37
Phenylalanine	60.16	±	47.49	37.80	±	47.28	99.66	±	34.69	45.77	±	24.14	48.51	±	18.69	40.70	±	11.81	88.79	±	24.16	109.01	±	62.87	61.71	±	36.74
Phosphate	71.34	±	62.51	48.82	±	47.08	235.06	±	397.06	96.98	±	57.50	85.03	±	41.06	57.10	±	30.89	134.53	±	90.71	143.18	±	93.77	197.49	±	43.51
Piperidin carboxylic acid	0.00	±	0.00	0.00	±	0.00	0.74	±	0.28	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Piperin Carboxylic acid	0.00	±	0.00	8.86	±	4.27	13.74	±	14.38	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	1.40	±	1.12	0.00	±	0.00	0.00	±	0.00

Proline	1.15	±	0.95	0.00	±	0.00	0.00	±	0.00	2.83	±	1.97	1.90	±	1.16	2.69	±	1.11	3.90	±	2.43	0.00	±	0.00	0.00	±	0.00
Pyrrole-2-carboxylic acid	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.18	±	0.05	0.60	±	0.44	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Pyruvic acid	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	1.55	±	1.07	0.37	±	0.12	0.00	±	0.00	0.96	±	0.51	0.00	±	0.00	0.00	±	0.00
Serine	29.67	±	23.41	15.39	±	21.18	0.00	±	0.00	34.59	±	17.99	27.11	±	12.11	0.91	±	0.73	53.84	±	30.80	32.73	±	33.53	21.52	±	6.13
Succinic acid	6.13	±	4.70	5.25	±	6.49	60.05	±	32.36	6.82	±	2.59	5.85	±	1.95	16.11	±	16.48	11.30	±	6.17	11.79	±	6.60	27.50	±	16.73
Threonic acid	0.25	±	0.20	0.37	±	0.19	0.00	±	0.00	0.30	±	0.18	0.29	±	0.11	0.42	±	0.27	0.51	±	0.24	0.00	±	0.00	14.86	±	25.74
Threonine	25.15	±	19.93	18.04	±	21.92	0.00	±	0.00	33.78	±	19.48	25.45	±	10.38	0.66	±	0.44	48.63	±	30.01	52.28	±	31.70	0.00	±	0.00
Thymine	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	1.18	±	0.96	0.00	±	0.00	0.00	±	0.00	7.68	±	9.28
Tryptophan	15.85	±	14.55	8.24	±	8.53	20.73	±	14.94	19.90	±	13.07	16.64	±	9.73	16.09	±	9.12	25.53	±	17.83	17.26	±	10.93	19.77	±	16.11
Tyrosine	19.49	±	14.95	14.61	±	18.83	39.61	±	7.60	20.50	±	7.90	17.61	±	6.07	24.06	±	11.99	33.21	±	16.08	30.24	±	15.83	30.05	±	18.88
UNKnp36.13min	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.58	±	0.33	0.00	±	0.00	0.00	±	0.00	0.42	±	0.15
UNKnp48.8min	12.26	±	15.02	3.31	±	4.99	14.19	±	8.77	4.37	±	7.49	0.00	±	0.00	0.00	±	0.00	6.24	±	1.78	2.35	±	0.63	0.00	±	0.00
UNKp14.587 min	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.10	±	0.06	0.00	±	0.00	0.13	±	0.08	0.00	±	0.00	0.11	±	0.06	0.38	±	0.19
UNKp15.684 min	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.08	±	0.06	4.44	±	5.49	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
UNKp18.011 min	0.00	±	0.00	1.17	±	1.37	11.31	±	5.32	0.69	±	0.52	2.30	±	0.83	9.67	±	8.23	0.13	±	0.08	4.79	±	2.85	12.80	±	9.66
UNKp19.487 min	0.73	±	0.58	0.58	±	0.40	1.98	±	0.87	1.08	±	0.64	0.91	±	0.51	0.95	±	0.48	1.32	±	0.75	2.13	±	1.44	2.82	±	1.03
UNKp24.2882min	0.00	±	0.00	0.77	±	0.49	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	8.10	±	5.71	9.12	±	3.79
UNKp29.901 min	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	4.52	±	1.51	0.00	±	0.00
UNKp40.6336 min	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.53	±	0.23	0.40	±	0.15	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
UNKP-Disaccharide-44.2984 min	74.15	±	61.83	46.02	±	48.54	0.00	±	0.00	103.11	±	62.44	76.08	±	41.73	0.00	±	0.00	150.76	±	93.57	185.01	±	124.41	45.79	±	76.32
UNKp-indole deriv.-36.145 min	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.16	±	0.04	0.34	±	0.09	2.60	±	3.48	0.33	±	0.17	0.00	±	0.00	0.00	±	0.00
Uracil	0.95	±	0.74	0.61	±	0.54	2.71	±	1.33	1.31	±	0.96	1.17	±	0.38	2.11	±	1.39	1.89	±	1.14	10.51	±	21.44	3.78	±	4.47
Urea	0.00	±	0.00	0.00	±	0.00	1.03	±	0.36	0.16	±	0.07	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Uridine	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.14	±	0.04	4.49	±	2.56	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Valine	33.57	±	26.64	21.38	±	23.50	77.05	±	6.89	40.30	±	20.19	33.74	±	15.06	36.72	±	17.24	63.14	±	37.74	58.62	±	43.07	65.39	±	14.02
Xanthine	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.40	±	0.21	0.26	±	0.12	0.00	±	0.00	0.52	±	0.25	0.00	±	0.00	0.00	±	0.00

Appendix 8

Table 8. *S. aureus* metabolomic analysis of bacteria cultured in the presence of *S. pennellii* 0.78 mg mL^{-1} extract showing the average and statistical differences between the bacterial pellet samples. The metabolites with quantification based on comparisons with standards of known concentrations, comparing samples by time and condition, as average ± standard error, and *t*-test (two-tailed, unpaired unequal variances), DMSO represented the control in all examples. Data represents the differences in µg mL^{-1} of DW between the bacterial pellets. AV*= Average ratio (test/control).

	LB C vs DMSO C				***S. pennellii* C vs DMSO C**				***S. pennellii* B vs DMSO B**			
Metabolites	**AV***		**StDev**	***t*-test**	**AV***		**StDev**	***t*-test**	**AV***		**StDev**	***t*-test**
2-Hydroxyisocaproic acid	0.00	±	0.00	0.11	0.00	±	0.00	1.00	0.00	±	0.00	1.00
2/3-Phosphoglyceric acid	0.00	±	0.00	1.00	0.00	±	0.00	1.00	0.00	±	0.00	0.00
Nicotinic acid	10.00	±	0.00	0.05	0.07	±	0.04	0.06	0.10	±	0.10	0.02
Adenine	1.63	±	0.37	0.04	29.59	±	15.93	0.02	21.40	±	19.72	0.05
Adenosine	1.54	±	0.85	0.37	0.31	±	0.13	0.05	0.40	±	0.24	0.12
AMP	0.00	±	0.00	1.00	10.00	±	0.00	0.22	10.00	±	0.00	0.02
aiC15:0	1.12	±	0.51	0.85	0.00	±	0.00	0.01	0.00	±	0.00	0.00
aiC17:0	1.04	±	0.13	0.91	0.43	±	0.29	0.01	0.00	±	0.00	1.00
Alanine	0.00	±	0.00	0.10	10.00	±	0.00	0.06	0.00	±	0.00	0.05
alfa-Hydroxyglutaric acid	0.00	±	0.00	1.00	0.00	±	0.00	1.00	10.00	±	0.00	0.01
Aminobutyric acid	0.00	±	0.00	1.00	0.00	±	0.00	1.00	0.00	±	0.00	0.02
Aminohexose- 41.9089 min	0.00	±	0.00	1.00	0.00	±	0.00	1.00	10.00	±	0.00	0.25
Aminomalonic acid	0.00	±	0.00	1.00	0.00	±	0.00	1.00	0.00	±	0.00	0.00
Asparagine	0.00	±	0.00	1.00	10.00	±	0.00	0.05	0.00	±	0.00	1.00
Aspartic acid	3.88	±	2.68	0.07	0.00	±	0.00	0.03	0.00	±	0.00	0.11
Phenylacetic acid	1.04	±	0.29	0.90	26.19	±	20.14	0.05	10.00	±	0.00	0.01
Benzoate	3.49	±	0.57	0.01	3.74	±	2.74	0.09	0.00	±	0.00	0.01
β-Alanine	0.00	±	0.00	1.00	10.00	±	0.00	0.00	10.00	±	0.00	0.00
Butanoic acid-2 4-diOH	4.63	±	2.36	0.02	0.00	±	0.00	0.01	0.00	±	0.00	1.00
Butanoic acid-3 4-diOH	0.00	±	0.00	1.00	0.00	±	0.00	1.00	0.00	±	0.00	1.00
C9:0	0.00	±	0.00	1.00	0.00	±	0.00	1.00	0.00	±	0.00	1.00
C11:0	0.00	±	0.00	1.00	10.00	±	0.00	0.02	10.00	±	0.00	0.00
C13:0	0.00	±	0.00	0.00	10.00	±	0.00	0.05	10.00	±	0.00	0.00
C14:0	0.00	±	0.00	0.01	0.00	±	0.00	1.00	0.00	±	0.00	1.00
C15:0	0.00	±	0.00	0.00	10.00	±	0.00	0.00	0.00	±	0.00	0.00
C16:0	0.45	±	0.22	0.18	0.00	±	0.00	0.01	0.00	±	0.00	0.03
C17:0	4.80	±	2.98	0.05	0.14	±	0.13	0.03	0.00	±	0.00	1.00
C18:0	1.60	±	0.28	0.01	0.19	±	0.13	0.00	0.00	±	0.00	0.12
C19:0	1.32	±	0.38	0.51	5.53	±	1.94	0.01	10.00	±	0.00	0.00
C20:0	1.19	±	0.42	0.55	0.55	±	0.18	0.04	0.00	±	0.00	0.11
Citric acid	0.00	±	0.00	0.05	10.00	±	0.00	0.02	0.00	±	0.00	0.08
Cytosine	0.00	±	0.00	1.00	0.00	±	0.00	1.00	0.00	±	0.00	1.00
UNKP-disaccharide-44.2984 min	0.00	±	0.00	1.00	10.00	±	0.00	0.25	4.02	±	2.70	0.04
Fumaric acid	2.34	±	0.42	0.01	2.53	±	2.32	0.21	0.00	±	0.00	1.00
Galactose	0.00	±	0.00	1.00	0.00	±	0.00	1.00	10.00	±	0.00	0.17
Glucose	0.77	±	0.39	0.51	25.15	±	47.55	0.32	7.49	±	5.92	0.04
Glutamic acid	5.54	±	4.06	0.07	0.43	±	0.10	0.16	2.14	±	1.66	0.23
Glyceric acid	0.00	±	0.00	1.00	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Glycerol	1.44	±	1.26	0.60	4.95	±	3.22	0.05	2.83	±	2.42	0.14
Glycerol-3-Phosphate	0.76	±	0.49	0.53	2.42	±	2.12	0.21	1.24	±	0.73	0.74
Glycero-1-aiC15:0	0.00	±	0.00	0.05	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Glycero-1-C14:0	0.00	±	0.00	0.00	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Glycero-1-C16:0	0.74	±	0.30	0.24	1.23	±	0.18	0.29	0.89	±	0.19	0.86
Glycero-1-C18:0	0.56	±	0.23	0.18	0.00	±	0.00	0.01	0.69	±	0.17	0.61
Glycero-1-iC15:0	0.00	±	0.00	0.08	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Glycero-2-C18:0	0.00	±	0.00	0.02	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Glycero-2-iC15:0	0.00	±	0.00	0.01	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Glycine	0.00	±	0.00	0.02	10.00	±	0.00	0.04	0.25	±	0.14	0.48
Guanine	1.63	±	0.92	0.26	0.55	±	0.20	0.15	10.00	±	0.00	0.01
Guanosine	1.42	±	0.67	0.37	0.00	±	0.00	0.01	1.94	±	1.10	0.13
Homocysteine	0.00	±	0.00	0.03	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Glycolic acid	10.00	±	0.00	0.01	1.81	±	0.62	0.06	2.81	±	1.58	0.05
Hypoxanthine	0.00	±	0.00	1.00	0.00	±	0.00	1.00	0.00	±	0.00	1.00

iC14:0	1.36	±	1.08	0.58	0.51	±	0.28	0.25	0.00	±	0.00	0.05
iC15:0	1.81	±	0.42	0.08	0.31	±	0.09	0.00	0.00	±	0.00	1.00
iC16:0	3.11	±	1.41	0.03	0.87	±	0.22	0.60	1.04	±	0.08	0.94
iC17:0	0.97	±	0.18	0.94	0.44	±	0.14	0.00	0.00	±	0.00	1.00
Inositol	1.69	±	0.20	0.02	1.14	±	0.56	0.62	3.78	±	2.45	0.04
Isoleucine	1.90	±	0.43	0.06	1.02	±	0.28	0.90	3.11	±	1.80	0.04
Lactic acid	1.54	±	1.82	0.59	0.72	±	0.56	0.65	3.81	±	2.07	0.02
Leucine	2.01	±	1.84	0.30	0.75	±	0.52	0.61	2.66	±	1.96	0.10
Lysine	10.00	±	0.00	0.00	0.31	±	0.18	0.00	2.20	±	1.19	0.11
Malic acid	0.00	±	0.00	0.05	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Maltose	0.00	±	0.00	1.00	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Methionine	3.44	±	1.26	0.01	39.21	±	86.68	0.38	3.95	±	1.91	0.01
Methyl-glycerate	3.51	±	1.75	0.03	7.49	±	9.18	0.19	4.53	±	3.06	0.04
Norleucine	10.00	±	0.00	0.01	0.00	±	0.00	0.01	2.26	±	1.37	0.08
Ornithine	1.96	±	1.40	0.21	1.88	±	1.61	0.31	0.00	±	0.00	0.03
Oxalic acid	0.00	±	0.00	0.00	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Phenylalanine	2.45	±	0.85	0.02	0.62	±	0.37	0.13	2.88	±	1.66	0.05
Phosphate	4.12	±	6.95	0.37	0.84	±	0.19	0.84	2.93	±	1.92	0.06
Piperidine carboxylic acid	10.00	±	0.00	0.00	0.00	±	0.00	0.00	0.00	±	0.00	1.00
Proline	0.00	±	0.00	0.01	0.00	±	0.00	1.00	0.00	±	0.00	1.00
5-Oxoproline	3.29	±	0.82	0.00	0.91	±	0.21	0.54	2.75	±	1.72	0.07
Pyrrole-2-carboxylic acid	0.00	±	0.00	0.04	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Pyruvic acid	0.00	±	0.00	1.00	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Serine	0.00	±	0.00	0.05	10.00	±	0.00	0.00	2.13	±	2.18	0.31
Succinic acid	3.73	±	2.01	0.04	0.46	±	0.28	0.09	2.24	±	1.26	0.11
Threonic acid	0.00	±	0.00	0.03	10.00	±	0.00	0.27	0.00	±	0.00	0.01
Threonine	0.00	±	0.00	0.03	0.00	±	0.00	1.00	2.90	±	1.76	0.06
Thymine	0.00	±	0.00	0.05	10.00	±	0.00	0.14	0.00	±	0.00	1.00
Tryptophan	1.29	±	0.93	0.57	0.20	±	0.14	0.07	10.00	±	0.00	0.01
Tyrosine	1.65	±	0.32	0.05	0.76	±	0.48	0.34	2.07	±	1.08	0.15
UNKnp36.13min	0.00	±	0.00	0.02	10.00	±	0.00	0.00	0.00	±	0.00	1.00
UNKnp48.8min	10.00	±	0.00	0.02	0.00	±	0.00	0.02	0.71	±	0.19	0.66
UNKp14.587 min	0.00	±	0.00	0.02	10.00	±	0.00	0.01	10.00	±	0.00	0.01
UNKp15.684 min	0.00	±	0.00	0.14	0.00	±	0.00	1.00	0.00	±	0.00	1.00
UNKp18.011 min	1.17	±	0.55	0.72	1.13	±	0.85	0.77	4.09	±	2.43	0.03
UNKp19.487 min	2.09	±	0.92	0.06	1.43	±	0.52	0.20	3.70	±	2.50	0.05
Cysteine	1.96	±	1.08	0.13	0.17	±	0.08	0.03	2.10	±	1.41	0.21
UNKp24.2882min	0.00	±	0.00	1.00	10.00	±	0.00	0.01	10.48	±	7.38	0.03
UNKp29.901 min	0.00	±	0.00	1.00	0.00	±	0.00	1.00	10.00	±	0.00	0.00
UNKp40.6336 min	0.00	±	0.00	1.00	0.00	±	0.00	1.00	0.00	±	0.00	1.00
UNKp-indole deriv.-36.145 min	0.00	±	0.00	0.17	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Uracil	1.28	±	0.63	0.51	1.40	±	1.65	0.63	17.34	±	35.37	0.31
Urea	10.00	±	0.00	0.00	0.00	±	0.00	0.00	0.00	±	0.00	1.00
Uridine	0.00	±	0.00	0.02	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Valine	2.10	±	0.19	0.00	0.85	±	0.18	0.15	2.74	±	2.01	0.10
Xanthine	0.00	±	0.00	1.00	0.00	±	0.00	1.00	0.00	±	0.00	1.00

Appendix 9

Table 9. *S. aureus* metabolomic analysis of bacteria cultured in the presence of *S. pennellii* 0.78 mg mL^{-1} extract showing the average and statistical differences between the supernatant (quenching solution) samples.

The metabolites with quantification based on comparisons with standards of known concentrations, comparing samples by time and condition, as average ± standard error, and *t*-test (2 tailed, unpaired unequal variances), DMSO represented the control in all examples. Data represents the differences in µg mL^{-1} of DW between the supernatant (quenching solution). AV*= Average ratio (test/control).

	LB C vs DMSO C				*S. pennellii* B vs DMSO B				***S. pennellii* C vs DMSO C**			
Metabolites	**AV***		**StDev**	***t*-test**	**AV***		**StDev**	***t*-test**	**AV***	+	**StDev**	***t*-test**
2-Hydroxyisocaproic acid	8.47	±	5.68	0.08	0.00	±	0.00	1.00	0.00	±	0.00	0.06
Nicotinic acid	10.00	±	0.00	0.02	0.00	±	0.00	1.00	0.00	±	0.00	0.02
Adenine	2.76	±	1.51	0.10	0.00	±	0.00	0.10	0.31	±	0.07	0.09
Adenosine	0.00	±	0.00	1.00	10.00	±	0.00	0.17	10.00	±	0.00	0.00
AMP	0.00	±	0.00	1.00	10.00	±	0.00	0.00	0.00	±	0.00	1.00
aiC15:0	0.00	±	0.00	1.00	10.00	±	0.00	0.00	0.00	±	0.00	1.00
Alanine	0.00	±	0.00	0.15	10.00	±	0.00	0.02	0.00	±	0.00	1.00
Hydroxyglutaric acid	0.00	±	0.00	1.00	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Aminobutyric acid	0.61	±	0.03	0.23	0.00	±	0.00	1.00	0.00	±	0.00	0.00
Aminohexose-41.9089 min	0.00	±	0.00	1.00	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Aminomalonic acid	0.00	±	0.00	1.00	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Asparagine	0.00	±	0.00	1.00	0.00	±	0.00	0.17	0.00	±	0.00	1.00
Aspartic acid	1.36	±	1.28	0.67	0.00	±	0.00	1.00	0.00	±	0.00	0.12
Phenylacetic acid	2.90	±	1.42	0.07	0.00	±	0.00	0.14	0.00	±	0.00	0.03
Benzoate	20.36	±	6.17	0.01	10.00	±	0.00	0.00	1.49	±	0.44	0.09
β-Alanine	0.00	±	0.00	0.01	0.00	±	0.00	0.16	10.00	±	0.00	0.00
Butanoic acid-2 4-diOH	0.00	±	0.00	1.00	10.00	±	0.00	0.00	0.00	±	0.00	1.00
Butanoic acid-3 4-diOH	0.00	±	0.00	0.03	0.00	±	0.00	0.12	10.00	±	0.00	0.00
C9:0	0.00	±	0.00	1.00	10.00	±	0.00	0.00	0.00	±	0.00	1.00
Citric acid	1.61	±	1.08	0.37	0.00	±	0.00	1.00	0.00	±	0.00	0.06
UNKP-disaccharide-44.2984 min	0.00	±	0.00	0.10	0.00	±	0.00	0.15	10.00	±	0.00	0.02
Fumaric acid	0.00	±	0.00	0.01	1.41	±	0.23	0.49	0.00	±	0.00	1.00
Galactose	10.00	±	0.00	0.00	0.00	±	0.00	1.00	0.74	±	0.36	0.25
Glucose	0.00	±	0.00	0.04	61.17	±	38.88	0.01	10.00	±	0.00	0.01
Glutamic acid	1.45	±	0.79	0.42	1.34	±	0.90	0.62	2.13	±	0.65	0.03
Glyceric acid	0.00	±	0.00	1.00	0.60	±	0.16	0.50	0.00	±	0.00	1.00
Glycerol	10.00	±	0.00	0.03	10.00	±	0.00	0.00	2.26	±	1.01	0.05
Glycerol-3-Phosphate	0.00	±	0.00	1.00	5.98	±	3.00	0.01	0.00	±	0.00	1.00
Glycine	0.00	±	0.00	0.10	0.56	±	0.57	0.52	0.00	±	0.00	1.00
Guanine	2.11	±	1.87	0.32	0.29	±	0.16	0.28	0.00	±	0.00	0.11
Guanosine	0.00	±	0.00	1.00	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Homocysteine	0.00	±	0.00	1.00	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Glycolic acid	10.00	±	0.00	0.01	0.00	±	0.00	1.00	0.81	±	0.16	0.43
Hydroxyglutaric acid	0.00	±	0.00	1.00	1.06	±	0.28	0.92	0.00	±	0.00	1.00
Hypoxanthine	0.00	±	0.00	0.01	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Inositol	4.40	±	2.79	0.09	0.00	±	0.00	1.00	0.88	±	0.22	0.74
Isoleucine	3.18	±	1.55	0.06	1.57	±	0.26	0.33	0.94	±	0.33	0.83
Lactic acid	1.54	±	1.23	0.53	1.18	±	0.21	0.75	1.88	±	1.75	0.36
Leucine	3.25	±	1.70	0.07	1.50	±	0.26	0.40	0.14	±	0.12	0.04
Lysine	0.00	±	0.00	1.00	0.39	±	0.24	0.36	10.00	±	0.00	0.00
Malic acid	0.00	±	0.00	0.01	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Maltose	0.00	±	0.00	0.01	0.57	±	0.20	0.49	0.00	±	0.00	1.00
Methionine	10.00	±	0.00	0.03	2.46	±	0.40	0.04	1.35	±	0.45	0.30
Methyl-glycerate	4.74	±	2.54	0.06	0.00	±	0.00	1.00	0.95	±	0.25	0.88
Norleucine	0.00	±	0.00	1.00	0.72	±	0.46	0.64	10.00	±	0.00	0.01
Ornithine	0.83	±	1.02	0.79	1.37	±	0.32	0.52	1.26	±	0.35	0.71
Oxalic acid	0.00	±	0.00	0.04	1.09	±	0.21	0.87	0.00	±	0.00	1.00

Phenylalanine	2.93	±	1.52	0.08	0.00	±	0.00	1.00	0.66	±	0.37	0.31
Phosphate	5.84	±	4.64	0.13	0.00	±	0.00	1.00	0.74	±	0.29	0.58
Piperidine carboxylic acid	10.00	±	0.00	0.01	0.67	±	0.26	0.59	0.00	±	0.00	0.01
Proline	0.00	±	0.00	0.03	1.39	±	0.37	0.47	0.00	±	0.00	1.00
5-Oxoproline	4.90	±	2.82	0.07	0.00	±	0.00	1.00	0.77	±	0.21	0.50
Pyrrole-2-carboxylic acid	0.00	±	0.00	1.00	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Pyruvic acid	0.00	±	0.00	1.00	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Serine	0.00	±	0.00	0.02	0.94	±	0.17	0.92	0.00	±	0.00	1.00
Succinic acid	6.90	±	2.58	0.02	0.00	±	0.00	1.00	0.44	±	0.08	0.05
Threonic acid	0.00	±	0.00	0.00	0.00	±	0.00	1.00	0.00	±	0.00	1.00
Threonine	0.00	±	0.00	0.06	1.67	±	0.42	0.47	0.00	±	0.00	1.00
Thymine	0.00	±	0.00	0.03	0.92	±	0.26	0.90	0.00	±	0.00	1.00
Tryptophan	1.07	±	0.92	0.90	0.00	±	0.00	1.00	0.33	±	0.09	0.22
Tyrosine	2.43	±	0.59	0.01	7.30	±	1.29	0.00	1.04	±	0.49	0.88
UNKp18.011 min	3.75	±	1.17	0.01	0.00	±	0.00	1.00	0.00	±	0.00	0.01
UNKp19.487 min	4.27	±	2.04	0.05	0.00	±	0.00	1.00	0.99	±	0.29	0.98
UNKp24.2882min	0.00	±	0.00	1.00	10.00	±	0.00	0.00	0.00	±	0.00	1.00
UNKp40.6336 min	0.00	±	0.00	1.00	0.62	±	0.32	0.55	0.00	±	0.00	1.00
UNKp-indole deriv.-36.145 min	0.00	±	0.00	0.17	0.00	±	0.00	1.00	0.00	±	0.00	1.00
UNKp-acid sugar lactone-43.306 min	0.00	±	0.00	1.00	1.34	±	0.25	0.55	0.00	±	0.00	1.00
Uracil	3.35	±	2.27	0.13	0.66	±	0.19	0.59	0.00	±	0.00	0.06
Urea	10.00	±	0.00	0.04	0.00	±	0.00	1.00	0.00	±	0.00	0.04
Valine	3.72	±	1.88	0.06	0.00	±	0.00	1.00	0.86	±	0.22	0.63
Xanthine	0.00	±	0.00	1.00	2.21	±	0.70	0.10	0.00	±	0.00	1.00

Appendix 10 Addresses of suppliers

Agilent Technologies, South Queensferry, West Lothian, EH30 9TG, UK
AHVLA, Woodham Lane, New Haw, Addlestone, Surrey, KT15 3NB
Beckman Coulter, Oakley Court, High Wycombe, *HP1*1 1JU, UK
Bruker Daltonics, Fahrenheitstr, 4D-28359 Bremen, Germany
BMG LABTECH, GmbH, Allmendgruen 8, D-77799 Ortenberg, Germany
Chiltern Seeds Limited, Crowmarsh Battle Barns, 114 Preston Crowmarsh, Wallingford, OX10 6SL, England.
Dionex Softron GmbH, Dornierstrasse 482110 Germering, Germany
Eppendorf, Eppendorf AG, Barkhausenweg 1, 22339 Hamburg, Germany
Extrasynthese, Z.I Lyon Nord, B.P 62, 69726 Genay Cedex, France
Falcon, 2100 Derry Road West, Suite 100, Mississauga, ON L5N 0B3, Canada
Fisher Scientific UK Ltd, Bishop Meadow Road, Leicestershire, LE11 5RG
Gilson, Wilford Industrial Estate, Wilford, Nottingham, NG11 7EP, UK
Hichrom Ltd, 1 The Markham Centre, Station Road, Theale, Berkshire, RG7 4PE, UK
Kitchen Garden Seeds, 23 Tulip Drive PO Box 638 Bantam, Connecticut, USA
Merk Milipore, Building 6, Croxley Green Business Park, Watford, WD18 8YH, UK
Oxoid Limited, Wade Road, Basingstoke, Hampshire, RG24 8PW, UK
Phenomenex, Inc., Queens Avenue, Hurdsfield Ind. Est. , Cheshire,SK10 2BN, UK
Scotts® Levington, Paper Mill Lane, Bramford, Ipswich, Suffolk, IP8 4BZ
Sigma-Aldrich, 3050 Spruce St, St. Louis, MO 63103 USA
SP SCIENTIFIC, The Sovereign Centre, Farthing Road, Ipswich, Suffolk,IP1 5AP,UK
Techne, Bibby Scientific Limited, Beacon Road, Stone, Staffordshire ST15 0SA, UK
Tomato Genetics Resource Centre, Department of Plant Sciences, Mail Stop 3, University of California, One Shields Avenue, Davis, CA 95616, USA
Royal Holloway, Royal Holloway, University of London, Egham Hill, Egham ,TW20 0EX, UK
MKS Instruments UK Ltd - Umetrics UK, Unit 3-4, Cowley Way, Weston Road, Crewe, Cheshire, CW1 6AG, UK
Waters Corporation, 34 Maple Street Milford, MA 01757, USA
YMC Incorporated, 3233 Burnt Mill Drive, Wilmington NC, 28403, New Hanover County

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