The bacterial diversity associated with the gut of *Gonipterus* sp.n.2 fed on two different *Eucalyptus* hosts

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Declaration

I declare that the thesis “The bacterial diversity associated with the gut of *Gonipterus* sp.n.2 fed on two different *Eucalyptus* hosts”, which I hereby submit for the Master of Science degree at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE:  DATE: .1/12/2021……..

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Summary

The *Eucalyptus* snout beetle is an oligophagous invasive insect pest of *Eucalyptus* species. The beetle is attracted to emerging leaves containing high concentrations of essential oils consisting of a complex mixture of mono and sesquiterpenes. In *Eucalyptus*, terpenoids can function as insect repellents through powerful odours and bitter taste. Furthermore, on a molecular scale, terpenoids are involved in breaking down cell membranes and negatively affecting ion transport. Regardless of the elaborate chemical defences of *Eucalyptus* species, the *Eucalyptus* snout beetle is able to tolerate and overcome high concentrations of terpenoids. We, therefore, hypothesize that the microbial communities associated with the gut of *Gonipterus* spp. are involved in secondary metabolite detoxification. *Gonipterus* sp. n. 2 adults were reared on two *Eucalyptus* genotypes with significantly different biochemical profiles. Metabolomic analysis of the leaf and beetle frass profiles indicated that there was a dramatic decrease in terpenoid concentrations from leaf to frass. Moreover, several biotransformation products of eucalyptol, α-pinene and d-limonene were found in the frass, suggesting that *Gonipterus* sp. n. 2 gut microbial communities may facilitate in *Eucalyptus* secondary metabolite detoxification. Following this discovery, a 16s rRNA gene sequencing approach was used to investigate the bacterial diversity of the host plants, gut and frass of *Gonipterus* sp. n. 2. Additionally, to determine if the *Gonipterus* gut microbial communities detoxified *Eucalyptus* secondary metabolites *in vivo*, an attempt was made to disrupt the gut bacterial community through antibiotic treatment. Sequencing results indicated that plant bacterial communities were enriched with Clostridiales and Sphingomonadales whereas gut and frass associated bacterial communities were dominated by Enterobacteriales. Furthermore, a core gut microbial community was detected, with a large percentage of the bacterial families shared between the *Gonipterus* beetle groups despite diet differences. Antibiotic treatment failed to disrupt the gut microbial communities of *Gonipterus* sp. n. 2, as no significant difference was observed between antibiotic and control beetle treatments. Therefore, more research is required to determine the role of *Gonipterus* gut microbial communities in detoxifying *Eucalyptus* secondary metabolites.

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Chapter 1: Literature review

1.1 Introduction

The genus *Gonipterus*, known commonly as the *Eucalyptus* snout beetle, consists of 20 Tasmanian and Australian species belonging to the tribe Gonipterini found in the family Curculionidae (Tooke, 1955). Members of *Gonipterus* are oligophagous herbivores feeding on the genus *Eucalyptus* (Mally, 1924). *Gonipterus* species are highly similar morphologically and biologically but may prefer different *Eucalyptus* hosts (Health *et al.*, 2018). Because of the similarity in their morphology, they were misidentified after their detection outside of their native range (Schröder et al., 2019). The *Eucalyptus* snout beetle was initially found in New Zealand in 1890 where it was described as *Gonipterus scutellatus*. When it arrived in South Africa in 1917 and spread into neighbouring African countries, the beetle was given five other names (Schröder et al., 2019; Tooke, 1955). Eventually a revision of the genus was performed by A.M Lea, placing the *Eucalyptus* snout beetle under a single species name, *G. scutellatus* (Schröder et al., 2019; Tooke, 1955).

Its increasing spread and the lack of an effective biocontrol agent prompted further research on *Gonipterus scutellatus* and its biological control, host specificity and taxonomy (Schröder et al., 2019). After performing a study on the *G. scutellatus* oviposition behaviour on seven naturally co-occurring *Eucalyptus* species, it was suggested that *G. scutellatus* consisted of "a complex of sibling species," due to the large discrepancies between oviposition preferences in different populations (Clarke et al., 1998; Loch, 2008). This hypothesis was further strengthened by Loch (2008) indicating that the lack of success with the biological control agent, *Anaphes nitens*, could be associated with the existence of different species of *Gonipterus*. This information added to calls to revise the taxonomy of Gonipterinae. Analyses of *Gonipterus* male genitalia indicated morphological differences in the adephegal schlerites of the genitalia (Barratt et al., 2018). In the most recent taxonomic investigation of *G. scutellatus*, Mapondera et al. (2012) confirmed the presence of 10 different species in *G. scutellatus sensu lato* using a DNA barcoding approach followed by morphological identification of the adephegal schlerites in the male genitalia. Of the 10 different species identified, eight were found to be cryptic species, five had been described previously and the other five consisted of undescribed species, each now denoted as *Gonipterus* sp. n. 1-5 (Mapondera et al., 2012).

In its natural range in Australia, *Gonipterus* species cause little damage to *Eucalyptus* plantations due to a number of predators and parasitoids which control the beetle population (Schröder et al., 2019). However, outside of this range, in other *Eucalyptus* growing countries, the *G. scutellatus* complex proliferated without constraints (Tooke, 1955). From the Mapondera et al. (2012) study, three species from the *G. scutellatus* complex were identified as invasive. These include *G. platensis*, *G. pulverulentus* and *Gonipterus* sp. n. 2, all causing significant damage to *Eucalyptus* hosts and severe economic losses in the silvicultural industry (Schröder et al., 2019). These three invasive *Gonipterus* species appear as invasive pests across several countries. *G. platensis* was identified in New Zealand, South America, USA, Spain, France, Portugal and Western Australia. On the other hand, *G. pulverulentus* was reported only in Uruguay. *Gonipterus* sp. n. 2 was found to occur in Madagascar, South Africa, France, Italy and Western Australia (Health et al., 2018; Leschen et al., 2014; Schröder et al., 2019).

Eucalyptus susceptibility to *Gonipterus scultellatus sensu lato* was poorly understood and varied between different studies (Clarke et al., 1998; Health et al., 2018; Loch, 2006; Newete et al., 2011; Tooke, 1955). On the one hand, *E. globulus* and *E. viminalis* were thought to be the most susceptible to *Gonipterus scutellatus senso lato* (Loch, 2006; Mally, 1924; Tooke, 1955). In reinterpreted and reapplied data of the identified *G. scutellatus sensu stricto* and their distribution found that additional *Eucalyptus* hosts *E. grandis*, *E. nitens*, *E. longifolia* and *E. propinqua* were also considered highly prone to *Gonipterus* feeding (Schröder et al., 2019). However, determining the *Gonipterus* feeding preference of *Eucalyptus* species is complicated by the differences in experimental parameters between studies (Schröder et al., 2019).

1.2 Behaviour and biology of *Gonipterus* species

Gonipterus species undergo four larval instars and have two egg-laying periods, in spring and summer. Adult females oviposit 8-10 faecal capsules, consisting altogether of approximately 800 eggs, onto tender new leaves (Health et al., 2018) (Fig. 1A). While hatching, larvae eat through the leaf surface underneath the egg capsule and continue to feed on the soft tissue of young leaves. Larvae appear green with black lined sides and black spots surrounded with

a sticky slime layer that aids in the attachment to *Eucalyptus* leaves (Tooke, 1955) (Fig. 1B). Towards the end of the fourth instar, larvae drop to the ground to initiate pupation. Young adults emerge after 3 months and take a further month to become mature adults. Adults are adept flyers, responsible for the spread and migration to new *Eucalyptus* hosts (Health et al., 2018) (Fig. 1C). Adult beetles feed on the margins of mature leaves resulting in scalloped edges. Additionally, whilst holding onto young shoots, adults gnaw into soft bark creating deeply pitted marks along the stem (Mally, 1924; Tooke, 1955).

Figure 1: (A) The egg (B) larvae and (C) adult of *Gonipterus* sp. n. 2 (Schröder, 2021)

1.3 The structure and function of the alimentary canal in Gonipterini

The gut of members belonging to Gonipterini can be divided into a long foregut, short midgut and a lobed hindgut (Fig. 2) (Calder, 1989; Leschen et al., 2014). The differentiated regions of the gut promote various functions of absorption, digestion and excretion (Chapman et al., 2013). Such processes occur through a simple tube, known as the alimentary canal, that runs from the mouth to the anus (Chapman et al., 2013). The hindgut and foregut are made up of an ectodermal layer that is absent in the midgut. Instead, epithelial cells of the midgut secrete an endodermal sheet, consisting of a non-cellular lining known as the peritrophic membrane (Engel and Moran, 2013).

Gonipterini have an elongated foregut (15% of digestive tract) (Fig. 2). Prominent features of the foregut are the well-developed crop and a bulbous proventriculus (Fig. 2) (Calder, 1989). Food enters through the mouth and is transported by the oesophagus, a short thin tube containing several setae and pegs, towards the crop. The crop is an organ that temporarily

holds food before releasing it as manageable portions into the proventriculus (Chapman et al., 2013). With the discharge of food material, the proventriculus performs preliminary mechanical disruption with the use of eight basal plates (Calder, 1989; Leschen et al., 2014).

Food material passes through a pyloric valve, entering the midgut. The shorter midgut of Gonipterini consists of anterior non-papilliose and posterior papillose regions (Calder, 1989). An important feature of the midgut is the long film fundamentally characterised in most insects as the peritrophic membrane. The peritrophic membrane is important in dividing the inner and outer membrane of the midgut into what is called the endo- and ecto-peritrophic space (Chapman et al., 2013; Engel and Moran, 2013). The peritrophic membrane divides food material from the epithelial lining and in doing so protects the epithelial cells from mechanical damage, large toxins and pathogenic microbes as well as compartmentalises mixtures of food and digestive enzymes (Chapman et al., 2013; Franzini et al., 2016).

Following passage through the midgut, food substrate proceeds into the hindgut through a pyloric valve (Chapman et al., 2013). The pyloric valve prevents backwards flow of food material. Once within the pylorus of the hindgut, food material is introduced to two sets of six cryptonephric excretory organs known as the Malpighian tubules (Fig. 2) (Calder, 1989). The Malpighian tubules act as an extension of the anterior hindgut that function to redirect the waste in the pylorus and channel it towards the rectum. Remaining food residue is transported from the pylorus to the rectum by a narrow tube known as the ileum. In the rectum, an enlarged sac, rectal pads absorb water remaining in the food residue before excretion by the anus (Chapman et al., 2013).

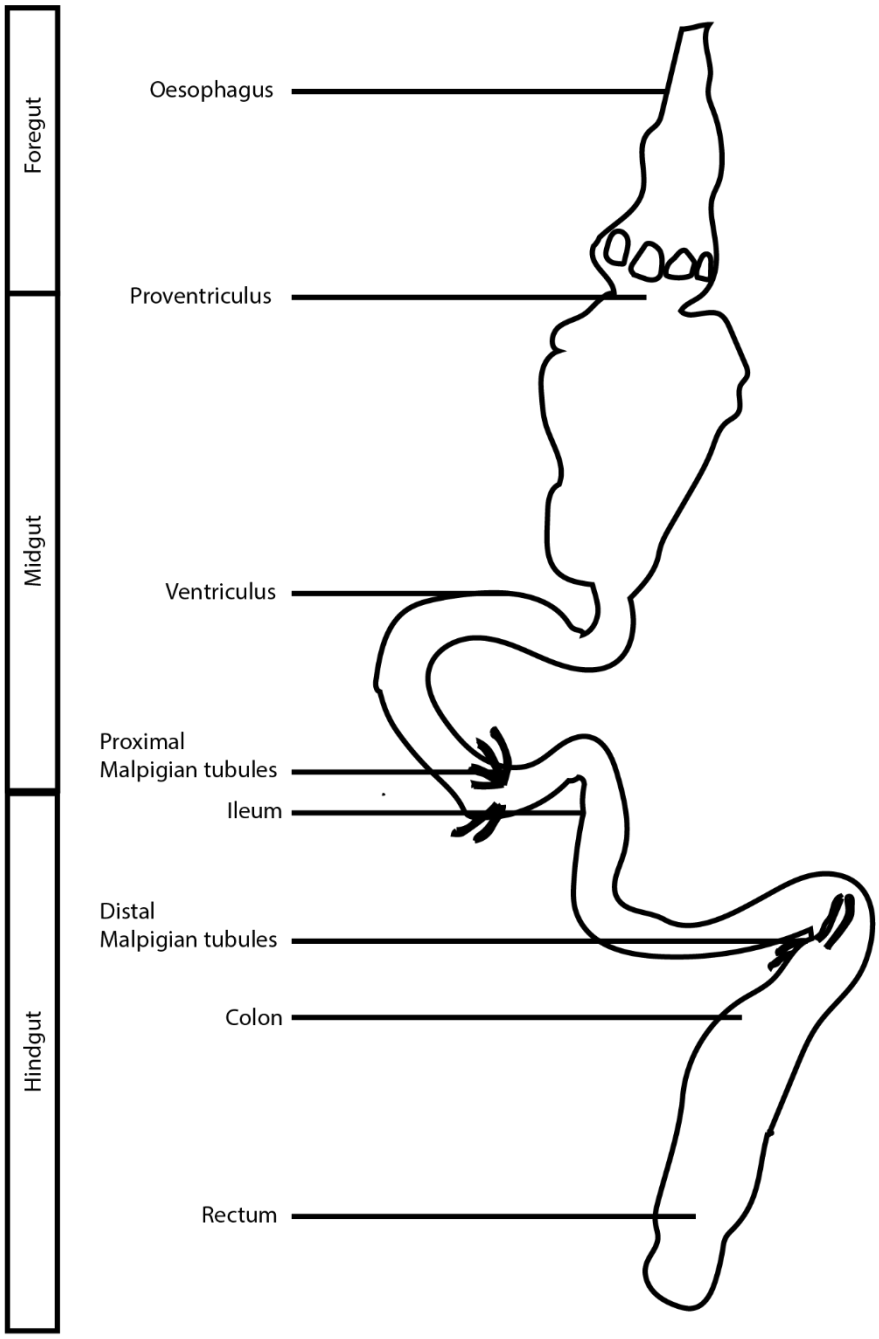

Figure 2: Alimentary canal of adult *Gonipterus* species

1.4 *Eucalyptus* plant secondary metabolites

Plant secondary metabolites often function as chemical defences towards microorganisms and animal and insect herbivores (Nishida, 2014). Secondary metabolites are categorised according to their structure and biosynthesis pathway as phenolics, terpenoids or alkaloids (War et al., 2012). The activity of secondary metabolites limits the host range of insect herbivores by inhibiting plant polymer digestion and nutrient acquisition or directly toxifying the insect host (Boone et al., 2013; Mason et al., 2016).

The *Eucalyptus* Snout beetle's diet is exclusively comprised of *Eucalyptus* foliage containing chemically complex secondary metabolites (Tooke, 1955). A characteristic of *Eucalyptus* foliage is the presence of specialised structures referred to as glandular trichomes (Goodger et al., 2018). Glandular trichomes contain complex mixtures of plant secondary metabolites known as essential oils (Goodger et al., 2018).

Essential oils are volatile mixtures of organic hydrocarbons (Upadhyay, 2010). *Eucalyptus* essential oils are important defence chemicals made up of mixtures of mono- and sesqui-terpenes and phenylpropanoids (Brezáni and Karel, 2013). Essential oils are antimicrobial, antifungal, insecticidal and nematocidal (Batish et al., 2008; Dhakad et al., 2018). The major secondary metabolites of *Eucalyptus* essential oils are 1,8 cineole, *d*-limonene, *p*-cymene, *α*-terpineol and *α*-phellandrene (Brezáni and Karel, 2013). All of these compounds have insecticidal and antimicrobial properties (Batish et al., 2008). However, it is not known how *Eucalyptus* essential oils affect *Gonipterus* species or what kind of detoxification systems they require to detoxify these metabolites.

1.5 Detoxication of Secondary Metabolites by insects

Metabolic detoxification is an essential process in insect herbivores as it shields the host from endogenously produced plant secondary metabolites and xenobiotics (Heidel-Fischer and Vogel, 2015). The enzymes involved in metabolic detoxification transform harmful secondary metabolites into non-toxic compounds that are easily excretable (Panini et al., 2016). Metabolic detoxification occurs through two phases, the first of which involves hydrolysis and oxidation of the substrate, and the second phase operates by conjugating phase one products

(Yu, 2005). The two phases are performed by a series of enzymes known as carboxylesterases, p450 cytochrome monoxygenases, UDP-glucuronyl-transferases and glutathione-S-transferases (Yu, 2005). The mechanisms by which the enzymes operate and detoxify is explored below.

1.6 Detoxification enzymes in insects

1.6.1 Cytochrome p450 Monoxygenases

Cytochrome p450 monoxygenases (CYP450s) are a large superfamily of heme thiolate phase I enzymes (Panini et al., 2016). Insect species encode approximately 100 different CYP450s (Feyereisen, 1999). Because of the diversity of CYP450 monoxygenases, CYP450s can metabolise a broad range of substrates, these include epoxidation, hydroxylation, N-dealkylation and desulfuration (Heckel, 2018). CYP450s oxidise toxic lipophilic secondary metabolites and insecticides into polar metabolites (Panini et al., 2016). For the reaction to occur, the enzyme requires molecular oxygen and two electrons from a cytochrome p450 reductase enzyme. In the reaction, CYP450 catalyse the insertion of a cleaved oxygen resulting in an oxidised product and a molecule of water (Heckel, 2018).

Bark beetles contain CYP450s that detoxify high concentrations of monoterpenes encountered from the resins of their host plants (Cui et al., 2016). The oxidised products are converted into parts of the pheromone system found in the antennal and the gut tissues (Cui et al., 2016). *Ips pini* (Coleoptera: Curculionidae), for instance, can convert myrcene into an aggregation pheromone made up of a mixture of ipsedienol and ipsenol (Blomquist et al., 2021). Additionally, many *Dendroctonus* sp. (Coleoptera: Curculionidae) contain CYP6DE1, a CYP450 that hydroxylates stored *α*-pinene into components of the beetle's aggregation pheromone (Blomquist et al., 2021; Chiu et al., 2019).

1.6.2 Carboxylesterases

Carboxylesterases are another large group of phase I detoxifying enzymes that act on a wide range of plant secondary metabolite classes (Hatfield et al., 2016). Carboxylesterases can attach to ester bonds in plant secondary metabolites and hydrolyse the substrate into an acid

or an alcohol (Ross et al., 2010). This transformation causes an increase in the compound's polarity; therefore, it is more readily excreted by the insect host (Panini et al., 2016).

An important pest of stored grains, *Tribolium castaneum* (Coleoptera:Tenebronidae), qualitatively produces carboxylesterases against the insecticide malathion (Wool et al., 1982). In these specific resistant populations of *T. castaneum*, carboxylesterases catalyse the hydrolysis of malathion into less toxic and more excretable malathion mono- and di-carboxylic acids (Haubruge et al., 2002).

1.6.3 Transferases

Transferases are a group of phase II enzymes that catalyse the conjugation of atoms from a donor substrate to a receiving compound (Panini et al., 2016). Glutathione-S- transferases play a role in catalysing the conjugation of reduced endogenous glutathione, whilst UDP-glucuronyl-transferases conjugate compounds with sugars into a soluble glycosidic product (Bretschneider, 2016; Heidel-Fischer and Vogel, 2015). These reactions convert reactive molecules into more water-soluble excretable compounds (Panini *et al.*, 2016).

Four upregulated glutathione-S-transferases were identified in *Dendroctonus armandi* (Coleoptera: Curculionidae) in the presence of pine terpenes (Dai et al., 2016). Furthermore, in a second study 16 more glutathione-S-transferases were identified from the gene family DaGST (*Dendroctonus armandi* glutathione-S-transferases). These genes were observed to have a higher expression under conditions of monoterpene fumigation (Gao et al., 2020). Thus, further hinting that these GSTs facilitate in negating the deleterious impact of terpenoids on *D. armandi* (Dai et al., 2016; Gao et al., 2020).

In a second example, two overexpressed UDP-glucoronosyl-transferases were identified in a comparison of transcription profiles between imidacloprid non-resistant and resistant populations of the *Leptinotarsa decemlineata* (Coleoptera: Chrysomilidae) (Kaplanoglu et al., 2017). Silencing one of the two UDP-glucuronsyl-transferases reinforced the susceptibility of the previously resistant population of the *L. decemlineata* to the insecticide imidacloprid (Kaplanoglu et al., 2017). This study thus indicated that UDP-glucuronsyl-transferases have a role in detoxifying imidacloprid (Kaplanoglu et al., 2017).

1.7 The cost of detoxification

Plant chemical defences pose a threat to insect herbivores (Nair et al., 2018). Insect herbivores, therefore, require morphological, behavioural, and biochemical adaptations to overcome plant chemical defences (José Gosalbes et al., 2010; Simon et al., 2015). However, the additional metabolic activities required to adapt to plant chemical defence mechanisms represents a trade-off against insect growth and development (Castañeda et al., 2009; Zvereva and Kozlov, 2016). Instead, microbial communities colonising the gut have been shown to have beneficial effects on their insect hosts that outweigh any metabolic costs (Castañeda et al., 2009; Douglas, 2015).

It is well known that many insects require microbial symbionts to supplement essential nutrients to their diet (Anbutsu et al., 2017; Heddi and Nardon, 2005; Kuriwada et al., 2010; Toju et al., 2010). Recently, studies on the roles of insect intestinal microbial communities have expanded to include the detoxification of plant secondary metabolites (Berasategui et al., 2016; Boone et al., 2013; Ceja-Navarro et al., 2015; Zhang et al., 2020). Studies in Curculionid beetles, *Hypothenemus hampei*, *Hylobius abietus*, *Dendroctonus valens* and *Curculio chinensis*, have shown that intestinal microbial communities can detoxify caffeine, monoterpenes, diterpenes, sesquiterpenes and saponins (Berasategui et al., 2016; Boone et al., 2013; Ceja-Navarro et al., 2015; Zhang et al., 2020). Without the microbial detoxification, these secondary metabolites would otherwise hinder insect development, reproduction or survival (Berasategui et al., 2016; Boone et al., 2013; Ceja-Navarro et al., 2015; Zhang et al., 2020).

Presently, the gut microbial communities of *Gonipterus* and the mechanisms underlying tolerance to its host's secondary metabolites is poorly understood. To determine if *Gonipterus* sp. n. 2 gut microbial communities are likely to be involved in the detoxification of secondary metabolites, examples of gut microbial detoxification in other Curculionid species is explored below.

1.8 Microbes: The hidden players in the insect gut

A complex of microbial communities was first described by Lederberg (2001) as a "microbiome", representing the "ecological community of commensal, symbiotic and pathogenic microorganisms that share the body space of various organisms". Many of these microbial communities have also been recognised as hidden players in interactions between insects and their environment (Engel and Moran, 2013). Insect gut microbiomes consist of various fungi, eubacteria, archaea, protists and viruses (Engel and Moran, 2013). Of the microorganisms inhabiting the gut of insects, bacteria of the Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes phyla are in the highest abundance (Douglas, 2015; Engel and Moran, 2013).

Although the gut habitat is nutrient rich and provides protection from external threats, it remains a highly unstable environment for the majority of ingested microbial communities (Douglas, 2015). Microbial members need to be highly adaptable to changing physiochemical conditions, such as redox potentials, pH, limiting oxygen and anti-microbial peptides (Vallet-Gely et al., 2008). Furthermore, insect moulting severely compromises the gut microbiome through repeated shedding of the hindgut, foregut and the replacement of the peritrophic membrane (Engel and Moran, 2013; Moll et al., 2001). Insect gut microbial colonisation is thus highly complex, as the structures, physiochemical conditions and resources vary with location and life stage (Dillon and Dillon, 2004; Douglas, 2015).

For some insects, particularly in Diptera, the foregut is a region that houses a large community of microbes (Ben-Yosef et al., 2015; Schauer et al., 2014). In social insects that perform trophallaxis (oral exchange of nutrients), the oesophagus of the foregut is enlarged to house several communities of microbial symbionts (Ben-Yosef et al., 2015). However, because the crop functions as a temporary storage organ in most insects, food material is concentrated and discharged regularly, leading to the possibility that microbes may collect but only persist for a short amount of time (Douglas, 2015).

Early studies by Douglas and Beard (1996) indicated finding microbial symbionts in specialised cells called mycetocytes found in the caeca of the midgut in Coleoptera and Hemiptera. However, for the majority of food associated microbes, the midgut is a highly unstable environment generally less suitable for microbial colonisation (Chapman et al., 2013; Douglas,

2015). Microbial communities must contend with secreted digestive enzymes and anti-microbial compounds (Vallet-Gely et al., 2008). These conditions are thought to inhibit or kill microbes associated with the ingested food (Douglas, 2015). Furthermore, the peritrophic membrane inhibits microbial colonisation by preventing movement of microbes between the endo- and ecto-peritrophic space (Chapman et al., 2013; Douglas, 2015). Consequently, a large population of dormant microbes in the midgut are cleared by peristalsis (Vallet-Gely et al., 2008).

The hindgut of insects is a relatively stable environment for microbial colonisation and commonly bears the highest population of microbial communities (Chapman et al., 2013; Engel and Moran, 2013). The lack of digestive enzymes and antimicrobial compounds maintains the stability of this environment (Douglas, 2015; Engel and Moran, 2013). In xylophagous insects, the hindgut is modified to serve as an enlarged fermentation chamber housing a large community of microbes responsible for degrading complex plant polysaccharides (Chapman et al., 2013).

1.9 Microbial transmission

Gut microorganisms may exist extracellularly in the environment of the insect, or intracellularly within the insect host (Salem et al., 2015). The routes in which these microbes are acquired are referred to as horizontal and vertical transmission (Sugio et al., 2015). Horizontal transmission may involve contact with the environment or the related or non-related community of insect hosts (Engel and Moran, 2013; Salem et al., 2015). *Riptortus pedestris* (Hemiptera: Alydidae) harbour *Burkholderia* symbionts in specialised cells along the midgut (Salem et al., 2015). In a study by Kikuchi et al. (2011) on axenic lab-reared *R. pedestris, Burkholderia* was absent, while *Burkholderia* was present in field-collected *R. pedestris*. This suggests that *Burkholderia* is acquired from the environment (Kikuchi et al., 2011).

Vertical transmission entails microbial transfer from one generation to the next (Engel and Moran, 2013). Some Crysomelidae and Staphylinidae appear to acquire microbial symbionts through vertical transmission (Kellner, 2002; Kleinschmidt and Kolsch, 2011). In the case of transmission through the egg stage, microorganisms need to persist in the environment before ingestion and colonisation of the hatching larvae (Brinkmann et al., 2008; Paniagua

Voirol et al., 2018). For this to be achieved, microorganisms need to be deposited as an inactive form, or need to be nourished while actively growing (Brinkmann et al., 2008; Paniagua Voirol et al., 2018). Bacterial symbionts of reed beetles (Coleoptera: Chrysomelidae) are responsible for insect development and require reliable transfer to the progeny (Kleinschmidt and Kolsch, 2011). The mechanism of transmission is performed by female beetles (Kleinschmidt and Kolsch, 2011). During oviposition, bacteria are deposited on the egg surface. Over the hatching period, the larvae ingest the deposited bacteria from the egg surface for further bacterial infection and proliferation in the larval gut (Kleinschmidt and Kolsch, 2011). The same mechanism of transmission was observed in *Paedarus sabaeus* (Coleoptera: Staphylinidae) (Kellner, 2002). *Pseudomonas* populations were deposited on the surface of the eggs (Kellner, 2002). Once ingested, these *Psuedomonas* populations have been thought to play a role in the production of a toxin called perderin (Kellner, 2002).

Gut microbial communities may be distinguished as transient, temporary members of the gut acquired from the environment, or resident, successful permanent inhabitants of the gut (Douglas and Beard, 1996; Engel and Moran, 2013). A small proportion of transient microbial communities may have pathogenic interactions with the insect host; while a large percentage of transient members associated with food are cleared from the gut (Engel and Moran, 2013). However, some food associated microbes may facilitate digestion or nutrient supplementation (Douglas, 2009; Franzini et al., 2016). Numerous studies in Lepidoptera illustrate the absence of a resident microbiome due to hostile physiochemical conditions and repeated shedding events within the gut (Acevedo et al., 2017; Hammer and Bowers, 2015; Paniagua Voirol et al., 2018). It can be deduced that microorganisms present in Lepidoptera are acquired from the environment of the insect host, occurring transiently in the gut as dead or dormant cells (Hammer and Bowers, 2015).

The resident microbiome can be further categorised into facultative or obligate members. Obligate symbionts are strictly vertically transmitted and have been theorised to offer nutrient provision essential to the insect host (José Gosalbes et al., 2010). The insect host derives these nutrients from specialised microbial-insect cells referred to as bacteriocytes or mycetocytes (José Gosalbes et al., 2010). Obligate symbionts are characterised by a reduced genome, retaining only genes needed to associate with the insect host (Hansen and Moran, 2014; Moran, Nancy A. et al., 2008). In aphids, a well-studied obligate symbiont is *Buchnera*

aphidicola (Moran, Nancy A. et al., 2008; Munson et al., 1991). *B. aphidicola* occurs in high concentrations within mycetocytes of the haemocoel (Moran, Nancy A. et al., 2008; Munson et al., 1991). Microbial transfer between aphid generations was discovered to occur vertically through transovarial transmission (Wilkinson and Douglas, 1995). Without the endosymbiont, *B. aphidicola*, larvae grew slowly, producing smaller adults with little to no offspring (Wilkinson and Douglas, 1995). The adverse effects indicate that *B. aphidicola's* nutrient contribution (tryptophan and other amino acids) are necessary for the development, reproduction and survival of the insect host (Wilkinson and Douglas, 1995).

Facultative symbionts are acquired largely through vertical transmission, but can, in specific instances be transmitted horizontally (Moran, Nancy A. et al., 2008). Furthermore, because facultative symbionts do not form direct associations with the host, they retain larger dynamic genomes (Engel and Moran, 2013; Hansen and Moran, 2014; Moran, N. A. et al., 2008). In aphids, the facultative symbiont, *Hamilotenella defensa*, is a bacterium with a dynamic genome and resides externally from the insect's cells (Oliver et al., 2003). *H. defensa* is transferred to progeny primarily through vertical transmission and provides a means of protection against entomopathogenic fungi and parasitoid wasps (Oliver et al., 2003).

1.10 The nutritional role of the gut microbial communities in the Curculionidae

The nutritional value of plants does not always fulfil the needs of insects (Douglas, 2009). Insects survive on plant tissues that contain small amounts of nitrogen, amino acids, and vitamins (Douglas, 2009; Engel and Moran, 2013). Furthermore, plants reduce or inhibit insect herbivory with physical and chemical defences. Microbial communities of insect guts facilitate in enhancing the survival of insects on low nutrient food sources by providing biosynthetic and degradative capabilities (Douglas, 2009, 2015). Both resident and transient gut microbial communities have been found to facilitate in secreting essential vitamins, digestive enzymes and detoxifying plant secondary metabolites (Hansen and Moran, 2014).

The diet of saprophagous, phytophagous and xylophagous herbivores lack the needed nitrogen to produce physiologically essential nitrogen containing compounds (Douglas, 2009). Many of these insects rely on mutualistic members of the gut microbiome for available nitrogen. Microbes can fix atmospheric nitrogen, recycle nitrogenous products and produce essential nitrogenous compounds required by the insect host (Douglas, 2009). Experimental

studies in Curculionidae indicate that *Dendroctonus valens* and *Dendroctonus rhizophagous*, two saprophagous weevils, contain *Raoultella terrigena*, a nitrogen-fixing bacterial member of the gut (Morales-Jiménez et al., 2013). In the study by Morales-Jiménez et al., (2013), the bacterium had a high activity of acetylene reduction (Morales-Jiménez et al., 2013). By sequencing the *nif* genes, *R. terrigena* was found to harbour genes *nifD* and *nifH* (Morales-Jiménez et al., 2013). Furthermore, bacterial members, *Pseudomonas fluorescens, Serratia proteomaculans*, *Rahnella aquatilis* facilitated in the recycling of uric acid (Morales-Jiménez et al., 2013).

The synthesis of vitamin B by gut microbial communities has previously been mentioned in many different studies (Douglas, 2009). Nevertheless, because vitamins are needed in small amounts, the nutritional interaction becomes difficult to demonstrate with the insect host (Douglas, 2009; Salem et al., 2015). In the Curculionidae, the rice weevil, *Sitophilus oryzae* (Coleoptera: Curculionidae), contains an obligate symbiont, *Sodalis pierantonius*. It has been suggested that the obligate symbiont provides the insect host with as many as five different B vitamins (Heddi and Nardon, 2005; Wicker, 1983). Studies on a phylogenetically close relative of *Sodalis*, *Nardonella*, also presented similar symptoms in *Nardonella*-deprived weevil hosts compared to *Sodalis*-deprived rice weevils (Kuriwada et al., 2010). Therefore, the provision of vitamins by *Nardonella* and *Sodalis* highlights the significance of microbial vitamin provisioning required in insects.

The physical defence of the plant cell wall also presents a challenge to herbivorous insects. Plant cell walls are lined with various recalcitrant polymers consisting of cellulose, hemicellulose, pectin or lignin (Hochuli, 1996). Many insects contain plant cell wall degrading enzymes (PCWDE). Degradation of cellulose is a slow process, requiring many enzymes (Douglas, 2009; Paniagua Voirol et al., 2018). Many xylophagous insects house microorganisms in special paunches or fermentation chambers, that facilitate in the metabolism of cellulose and hemicellulose into simple sugars (Engel and Moran, 2013). Additionally, phylogenetic analysis of PCWDE genes in insects indicates that a large proportion are acquired and integrated horizontally from gut microbial communities (Shelomi et al., 2014).

The ability of microbial communities to detoxify plant secondary metabolites is also related to the nutritional roles of the gut microbiome (Nishida, 2014). Plants produce an abundance

of biochemical defences (Nishida, 2014). Plant defences like proteinase inhibitors and plant secondary metabolites impair digestion or have toxic and repellent effects towards insect herbivores (Engel and Moran, 2013; Hammer and Bowers, 2015). Some gut symbionts have a greater influence on insect-plant interactions by aiding insect hosts in the detoxification of plant chemical defences (Engel and Moran, 2013; Hammer and Bowers, 2015). Because microbial symbionts can generate a diversity of enzymes that aid in detoxification, microbes are ideal detoxifiers (Mason et al., 2014). Plant secondary metabolites consist largely of lipophilic compounds (Panini et al., 2016). The general mechanism of plant secondary metabolite detoxification is the conversion of a lipophilic molecule into easily excretable, water-soluble compound (Panini et al., 2016). This is achieved either through oxidation and/or conjugation with a lipophilic molecule (Panini et al., 2016). Example studies of plant secondary metabolite detoxification by gut-associated microbes in the Curculionidae will be discussed below.

1.11 Compositional and functional studies of plant secondary metabolite detoxification in members of Curculionidae

1.11.1 Coffee berry borer (*Hypothenemus hampei)*

The *Coffea* species are known to produce an alkaloid, caffeine, that has been demonstrated to provide protection against insect herbivory (Ceja-Navarro et al., 2015; Guerreiro Filho and Mazzafera, 2003). Caffeine acts on the insect's nervous system (Guerreiro Filho and Mazzafera, 2003). In large concentrations, caffeine paralyses the insect host and has negative effects on DNA recombination and repair systems (Ceja-Navarro et al., 2015; Guerreiro Filho and Mazzafera, 2003). In addition to its toxicity, caffeine deters insect herbivores with its bitter taste. Regardless of the elaborate chemical defences of this genus, a persistent pest of *Coffea* species is the coffee berry borer, *Hypothenemus hampei* (Coleoptera: Curculionidae) (Guerreiro Filho and Mazzafera, 2003). The coffee berry borer is capable of feeding and completing its lifecycle within the caffeine-rich environment of the *Coffea* beans (Guerreiro Filho and Mazzafera, 2003).

A large diversity study investigated the gut microbiome's involvement in caffeine metabolism (Ceja-Navarro et al., 2015). Antibiotic-treated insects demonstrated a significant increase in

faecal caffeine concentrations mirroring the same concentrations amended into the artificial media (Ceja-Navarro et al., 2015). Furthermore, disruption of the gut microbiome halted the insect's ability to degrade caffeine, reduced reproduction and negatively affected its development to pupal or adult stages (Ceja-Navarro et al., 2015). A culture independent method was employed to investigate the bacterial diversity in the microbiome of the insect. In addition to this, a culture dependent method was performed to identify bacterial species capable of growth on media amended with caffeine (Ceja-Navarro et al., 2015). Fourteen caffeine tolerant colonies grew on the amended media consisting of orders Pseudomonales, Actinomycetales, Turicibacteriales, Enterobacteriales and Alteromonadales (Ceja-Navarro et al., 2015). The bacterial isolates persisting on caffeine were screened for *ndma*, (Alpha unit of caffeine demethylase) (Ceja-Navarro et al., 2015). From the screening, *Pseudomonas fulva* was found to possess and express *ndma* (Ceja-Navarro et al., 2015). Caffeine detoxification in this bacterium was confirmed with the reinfection of previously antibiotic-treated coffee berry borers, which restored caffeine degradation in *H. hampei* (Ceja-Navarro et al., 2015).

1.11.2 Bark beetles, *Dendroctonus ponderosae* and other *Dendroctonus* species

The gut bacterial associates of *Dendroctonus ponderosae;* a significant North American pine pest, was studied through a community metagenomics approach. Adams et al. (2013) investigated the ability of *Dendroctonus ponderosae* gut symbionts to assist in overcoming pine defences through the detoxification of terpenes. Gut bacterial communities of *Dendroctonus ponderosae* were dominated by *Pseudomonas*, *Rahnella*, *Serratia*, *Erwinia*, *Stenotrophomonas*, and *Pantoea* (Adams et al., 2013). Furthermore, genera from *Rahnella* and *Pseudomonas* were found to possess a substantial percentage of genes involved in the breakdown of pine terpenes (Adams et al., 2013). The implication of *D. ponderosae* associated with bacterial communities containing genes involved in terpene metabolism suggests that *Dendroctonus* bark beetle bacterial communities facilitate in the detoxification of tree chemical defences (Adams et al., 2013; Hernández-García et al., 2017).

Furthermore, the core gut bacteriome of thirteen *Dendroctonus* sp. (*D. adjunctus, D. approximates, D. brevicomis, D. frontalis, D. mesoamericanus, D. jeffreyi, D. mexicanus, D. parallelocollis, D. ponderosae, D. pseudotsugae, D. rhizophagus, D. valens, D. vitei*) was highly similar, consisting loosely of Enterobacteriaceae and Pseudomonadaceae (e.g., *Enterobacter*,

Pantoea, *Pseudomonas*, *Rahnella*, *Raoultella*, and *Serratia*) (Hernández-García et al., 2017). Bacterial diversity within samples was highly homogenous, indicating that there are several selective pressures structuring the bacterial community (Hernández-García et al., 2017).

In addition to the metagenomic studies, a functional culture dependent method was performed by Boone et al. (2013) on *D. ponderosae*. The new study investigated whether the gut microbiome of *D. ponderosae* was capable of reducing the concentrations of plant secondary metabolites (3-carene, -*α*-pinene, +-*α*-pinene, -*b*-pinene) *in vitro* on amended media (Boone et al., 2013). *Serratia marcescens* reduced the concentration of the majority of monoterpenes, whilst *Brevundimonas vesicularis* metabolised abietic acid and *Rahnella aquatilis* degraded any residual (-)- and (+)-*α*-pinene (Boone et al., 2013).

1.11.3 Pine Weevil *Hylobius abietus*

Hylobius abietus is a significant pest that causes extensive damage of several conifer species (Berasategui et al., 2016). The adults consume above and belowground parts, whilst the larvae are specific to the bark of roots (Berasategui et al., 2016). Throughout the lifecycle, the pine weevil is in contact with high concentrations of terpenes, suggesting that it has adapted to these metabolites (Berasategui et al., 2016). A bacterial diversity study investigating the gut microbiome of the pine weevil sampled across Europe indicated that the most common gut microbial communities consisted of *Wolbachia, Enterobacteria* and *Firmicutes* (Berasategui et al., 2016). Furthermore, a piCRUST (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) functional inference analysis found that various Enterobacteriaceae found in the gut harboured terpene detoxification genes (Berasategui et al., 2016).

Following the discovery of possible microbial detoxification mechanisms in the guts of *H. abietus*, a second functional study by Berasategui et al. (2017) was performed to investigate the role of the gut microbiome in plant secondary metabolite detoxification through a broad metagenomic survey and bioassays. The gut microbiome of the pine weevil was largely dominated by Gammaproteobacteria with genera from *Erwinia*, *Rahnella* and *Serratia*, differing only slightly from the above study (Berasategui et al., 2017). Furthermore, metagenomic analysis successfully found 10 *dit* (diterpene degradation) genes, implicated in diterpene degradation, from Enterobacteriaceae members (Berasategui et al., 2017).

Pine weevil fitness bioassays demonstrated reduced rates of hatching in eggs of antibiotically treated weevils, however, adult mortality remained comparable to that of control weevils (Berasategui et al., 2017). It was therefore hypothesized that pine weevils may benefit from gut microbial communities. However, the lack of evidence indicating an effect on the adult weevils may suggest that gut microbial communities are not involved in the detoxification of plant secondary metabolites (Berasategui et al., 2017). Instead, gut microbial communities of the pine weevil may perform terpene degradation to ensure their own survival and improve weevil development by providing essential nutrients (Berasategui et al., 2017).

1.12 Conclusion

Despite the importance of the *Eucalyptus* snout beetle and its ability to tolerate varying secondary metabolite contents from different *Eucalyptus* hosts, little is known about the diversity and functions of its gut microbiome. Likewise, *Gonipterus* behavioural and biological studies are lacking, and limit the discovery of potential microbial transmission routes. However, based on diversity and functional research on other members of the Curculionidae feeding on hosts with high secondary metabolite concentrations, we conclude that it is likely that microbes also assist in detoxifying secondary metabolites in the *Eucalyptus* snout beetle.

Chapter 2: Research Aims and Objectives

2.1 Background

Gonipterus sp. n. 2 feeds on *Eucalyptus* hosts that contain high concentrations of essential oils (Tooke, 1955; Dhakad et al., 2018). *Eucalyptus* essential oils function as repellents, digestive inhibitors, or are harmful towards insect herbivores (Boone et al., 2013; Mason et al., 2016). Nevertheless, *Gonipterus* sp. n. 2 is able to overcome high levels of *Eucalyptus* essential oils thereby revealing that the beetle has detoxification mechanisms to negate the harmful effects. Recently, gut microbial communities have been observed to play a role in the detoxification of plant secondary metabolites (Adams et al., 2013; Berasategui et al., 2017; Ceja-Navarro et al., 2015). However, knowledge on the effectiveness and acquisition of insect gut microbial communities is still lacking. I, therefore, hypothesize that the acquired bacterial communities from the host plants and the bacterial communities of the gut of *Gonipterus* assist the insect in overcoming the deleterious effects of these toxic chemical defence compounds.

2.2 Aims

The overall aim of the current study was to determine the diversity of bacterial communities in the intestinal tract of *Gonipterus* sp. n. 2, how the bacteria are acquired by the insect and how their metabolic activity influences the detoxification of plant chemical defences. To achieve this aim, the bacterial communities of the leaves from two different *Eucalyptus* genotypes, as well as the bacterial communities in the gut and frass of *Gonipterus* beetles fed on the leaves of these *Eucalyptus* genotypes were catalogued using sequenced. Secondly, to investigate the metabolism of *Eucalyptus* essential oils in the *Gonipterus* gut, terpene profiles of intact *Eucalyptus* leaves were compared to terpene profiles of *Gonipterus* frass. Lastly, to determine if the gut microbial community of *Gonipterus* aids in the detoxification of *Eucalyptus* essential oils, a comparative biochemical analysis was conducted on the frass from beetles with and without a disrupted bacterial gut community.

2.3 Objectives

2.3.1 Bacterial diversity Analysis of *Gonipterus* sp. n. 2 gut communities

- To catalogue the bacterial communities in the intestinal tract and frass from *Gonipterus* beetles reared on *Eucalyptus dunni* and *Eucalyptus grandis* x *Eucalyptus urophylla* genotypes.
- To compare the gut and frass microbial communities of beetles to the resident communities present on the host plants (*E. dunni* and *E. grandis* x *E. urophylla*).

2.3.2 Essential oil detoxification potential of *Gonipterus* sp. n. 2 gut microbial community.

- To compare the essential oil profile of *Eucalyptus* leaves with that of *Gonipterus* frass.

- Analysis of the changes in the metabolic profile of *Gonipterus* frass after amending the diet of beetles with antibiotics.

The results of this study are intended to increase the knowledge on the diversity and functional role of gut microbial communities in Curculionidae beetles feeding on a diet rich in essential oils. Insights into potential detoxification mechanisms of essential oils by *Gonipterus* beetles may result in a better understanding of the beetle's biology, its behaviour and control.

Chapter 3: Materials and Methods

3.1 Sample Collection

3.1.1 Propagation of *Eucalyptus* genotypes

Various pure and hybrid genotypes of *Eucalyptus*, such as *Eucalyptus dunnii* (BE0068962), *Eucalyptus nitens* (STNH09), *Eucalyptus grandis* x *Eucalyptus urophylla* (STGU12), and *E. grandis* x *E. urophylla* (STW13) were maintained in 5L pots with pine bark potting mix (Culterra, Johannesburg, South Africa). All plants were maintained under natural light conditions in a greenhouse at 23 ± 5 ˚C at the University of Pretoria Experimental Farm (25.7472° S, 28.2588° E).

Five sets of eight leaves from four different plants (n=20) were picked randomly from each of the four-year-old *Eucalyptus* genotypes. The mid-rib vein and the petiole were removed from all the leaves and the leaf blades were flash frozen using liquid nitrogen. Thereafter, all the collected leaf tissue samples were stored at -80°C for metabolite and DNA extraction.

3.1.2 Field collection of *Gonipterus* sp. n. 2 feeding on *Eucalyptus* leaves

Adult *Gonipterus* sp. n. 2 beetles were collected from a private *Eucalyptus* plantation in Seven Oaks, Kwa-Zulu Natal (29.1934795° S 30.6082423° E) in June 2019 and in October 2020. Adult *Gonipterus* beetles were collected and stored in pillowcases at room temperature. In the laboratory, groups of 100 individual beetles were transferred into 15 x 10 x 7 cm sized plastic containers. The beetles were reared on *E. dunnii* branches (genotype D01) in a BINDER Series KBW Model 240 incubator (Baden-Württemberg, Germany) at a temperature of 21 °C and with a 14:10 H day: night cycle.

To observe how diet associated bacterial communities and phytochemicals affect gut bacterial communities in *Gonipterus* sp. n. 2, beetles were transferred from *E. dunnii* (D01) to branches of *E. dunnii* (genotype BE0068962) and *E. grandis* x *E. urophylla* (genotype STGU12) for one week. Subsequently, beetles were starved for 24 hours, preserved, and stored at -20 °C for DNA extraction.

3.1.3 Collection of frass samples

Twenty beetles were immediately transferred onto branches of *E. grandis* x *E. urophylla* (genotype STGU12) and *E. dunnii* (genotype BE0068962) and frass was collected after five days. The collected frass samples were weighed into four 45 mg groups for each *Eucalyptus dunnii* and *E. grandis* x *E. urophylla* rearing groups and stored at -80°C for chemical analysis of essential oils.

Five groups consisting of 12 beetles from the corresponding rearing groups (*E. grandis* x *E. urophylla* and *E. dunnii*) were transferred to modified Petri dishes to collect frass samples for DNA extraction. The Petri dish bottoms were perforated with four drawing pins. Thereafter filter paper pieces (diameter = 95 mm) were mounted on the drawing pins and square pieces of leaves from the *Eucalyptus* genotypes *E. grandis* x *E. urophylla* and *E. dunnii* mounted on the drawing pins. Five sets of frass samples were collected from each of the corresponding rearing groups every day for five days and stored at -80°C. From the collected frass samples, ten samples were weighed to 20 mg and lyophilised using a VirTis AdVantage Pro Freeze Dryer (SP scientific, New York, USA) for 24 hrs at a pressure of 13.33 kPa. All freeze-dried frass samples were stored at -20 °C for DNA extraction.

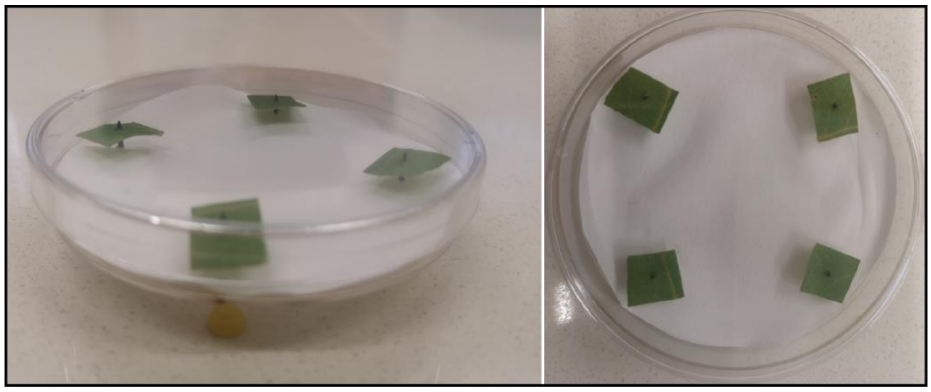

Figure 3: Modified Petri dish setup for frass collection

3.2 Dissection of *Gonipterus* guts

The elytra and wings were removed from 80 frozen beetles prior to dissection. All beetles were surface sterilised with 0.05% (v/v) sodium hypochlorite for 30 secs, 70% ethanol for 1 min and rinsed in deionised water. Thereafter, ten male and ten female beetles from each of the rearing groups (*E. dunnii* and *E. grandis* x *E. urophylla*) were fixed and dissected using a light microscope (Nikon SMZ645). Two guts were pooled in a sterile Nucleospin bead tube

(Macharey-Nagel, Düren, Germany), for each of the 20 samples, and stored at -80°C for DNA extraction.

3.3 Manipulation of the gut microbial communities of *Gonipterus* sp. n. 2

Artificial medium was prepared according to the recipe described by Joubert (2020). Each disk of artificial medium used in the experiment weighed approximately 80 mg. Four disks were mounted onto four needles over a filter paper in modified Petri dishes. Twelve beetles were placed in each of nine modified Petri dishes. Three treatment groups were included in this experiment: (1) beetles were fed for the entire duration of the experiment (6 days) on unamended artificial medium (control group); (2) beetles were fed for the entire duration of the experiment on artificial medium supplemented with the antibiotic Streptomycin (Sigma, Missouri, USA) (100 µg/ml); (3) beetles fed on antibiotic supplemented artificial medium for four days after which the antibiotic artificial media was replaced with artificial media supplemented with a solution containing 10 µl crushed *Gonipterus* guts for two days (re-infected group). This solution consisted of three *Gonipterus* guts in 1 ml dH_2O and crushed using a pestle mixer (VWR, Pennsylvania, Germany). To ensure feeding, beetles were starved for 24 hrs prior to initiating the experiment.

After the beetles were pre-conditioned for six days on a specific diet regime, beetles were transferred onto a diet setup consisting of two pieces of artificial media packed between a filter paper of a diameter of 10 mm. Filter papers were supplemented with 9 µl DMSO (Sigma, Missouri, USA) and 1 µl of *Eucalyptus* essential oils (Dischem, Pretoria, South Africa). Beetles from each treatment were maintained on this artificial medium for two more days and frass was collected every 24 hours. Collected frass was weighed to 5 mg, frozen and stored at -80°C.

3.4 Chemical Analysis of leaf and frass samples

3.4.1 Analyses of Eucalyptus leaf and frass samples

Plant samples belonging to four *Eucalyptus* genotypes (*E. dunnii*, *E. nitens,* and two different genotypes of *E. grandis* x *E. urophylla*) were pulverised using a mortar and pestle in liquid nitrogen (reported on in section 3.1.1). Ground plant tissue samples were weighed to 45 mg.

Ground plant tissue samples and frozen frass samples reported on in section 3.1.3 were extracted with 1.5 ml or 0.75 ml methanol (Sigma, Missouri, USA), respectively for 1 hr under continuous shaking. Frozen frass samples reported on in section 3.3 were extracted with 0.4 ml methanol. Homogenates were centrifuged at 10 000 rpm for 10 min and the supernatant was transferred into glass vials. Four separate leaf and frass extractions were performed for each plant genotype (sections 3.1.1 and 3.1.3) and six extractions were performed for antibiotic and control treatment groups and three were done for the re-infected treatment group (section 3.3).

The leaf and frass methanolic extracts (sections 3.1.1 and 3.1.3) were analysed using a Bruker Daltronics Esquire 3000 ion trap mass spectrometer coupled to an Agilent 1100 high pressure liquid chromatograph using the methods described by Hammerbacher et al. (2019). The same frass and plant extracts were analysed using an Agilent 7890 gas chromatograph equipped with a quadrupole mass spectrometer (Agilent, California, USA) (GCMS) using a ZB-WAX column (30 m x 0.25 mm x 0.25 µm) (Agilent, California, USA). One µl of the samples was injected directly using a split mode of 10:1. The GC oven was set to rise 10 °C min^{-1}, from an initial temperature of 50 °C to a maximum temperature of 250 °C. Flow rate of the carrier gas (helium) was set for 1.2 ml min^{-1}. MS settings were adjusted to scan a mass range between 40-350 mz^{-1}. Samples from beetles feeding on artificial diet (section 3.3) were analysed using the same settings, with the modification that a HP5 column (30 m x 0.25 mm x 0.25 µm) (Agilent, California, USA) was used.

3.4.2 Analyses of mass spectrometric data

Raw LCMS *Eucalyptus* genotype data was converted from .d to .mzXML using Proteowizard (Holman et al., 2014). Converted data was processed in XCMS online (https://xcmsonline.scripps.edu/) using multigroup statistical analysis according to *Eucalyptus* genotype (Smith et al., 2006). Base peak height, mass and retention times were extracted from the XCMS results table. To compare differences between chemical profiles of the *Eucalyptus* genotypes, extracted data was log transformed and applied to multivariate analysis using MetaboAnalyst 5.0 (Pang et al., 2021).

GC-MS data of the *Eucalyptus* leaves, frass from beetles fed on *E. dunnii* and *E. grandis* x *E. urophylla* genotypes and frass from the groups of *Gonipterus* beetles feeding from the artificial diet were processed in MassHunter Unknowns Analysis software (Agilent, California, USA). Peak data was deconvoluted with the following parameters, left m/z delta at 0.3, right m/z delta at 0.7 and a sharpness threshold of 25%. A peak filter was not added, and compound identification minimum match factor was set at 30. Deconvoluted data was exported, filtered and uploaded into MetaboAnalyst 5.0 (Pang et al., 2021). Uploaded data of *Eucalyptus* leaves and frass from *E. dunnii* were normalised through log transformation and mean-centring whereas *E. grandis* x *E. urophylla* data was normalised through square transformation and range-scaling. T-tests were generated to show significant differences in major terpene concentrations between *Eucalyptus* leaves and frass. Data from frass from the groups of *Gonipterus* beetles feeding from the artificial diet was normalised through log transformation and range scaling for univariate analysis.

3.5 Extraction of Genomic DNA

3.5.1 Extraction of DNA from leaf samples

Prior to plant genomic DNA extraction, frozen collected leaf material from the genotypes, *E. dunnii* and *E. grandis* x *E. urophylla*, was pulverised in liquid nitrogen using a mortar and pestle. Pulverised tissues from each sample were collected in 2 ml tubes and freeze dried using a VirTis AdVantage Pro Freeze Dryer and Lyophilizer (SP scientific, New York, USA) at a pressure of 13.33 kPa for 24 hrs. Each freeze-dried sample was weighed to 20 mg and stored at -20°C until plant DNA extraction.

Lyophilised plant tissue samples were washed using 1 ml of Sorbitol wash buffer ((100 mM Tris-HCl pH 8.0, 0.35 M Sorbitol (Sigma Aldrich, Missouri, USA), 5 mM EDTA pH 8.0 (Sigma Aldrich, Missouri, USA), 1% (w/v) PVP 40 000 (Sigma Aldrich, Missouri, USA), 1% β-Mercaptoethanol (Sigma Aldrich, Missouri, USA) to remove polysaccharides and polyphenols that would inhibit downstream reactions. Plant genomic DNA was extracted from the washed tissue using the Nucleospin Soil DNA Extraction kit (Macharey-Nagel, Düren, Germany) using the manufacturer's instructions.

3.5.2 Extraction of DNA from gut samples

Total genomic DNA was extracted from pooled dissected gut samples using the QIAamp PowerFecal kit (Qiagen, Hilden, Germany). The gut tissues were macerated in a two-step approach. In the first step, 200 µl of Powerbead solution was added to the tubes containing beetle guts, after which, the sample was macerated using a pellet mixer (VWR, Pennsylvania, USA) for 1 min. Thereafter, the remaining 400 µl of Powerbead solution and 150µl of Enhancer was added to each tube. All the tubes were incubated at 65°C. The final maceration step was performed using a Qiagen TissueLyser II for 2 min at 30/sec frequency. Thereafter, the genomic DNA was extracted following the manufacturer's protocol.

3.5.3 Extraction of DNA from frass samples

Total environmental DNA from frass samples was extracted with the Nucleospin Plant II kit (Macharey-Nagel, Düren, Germany). The frass samples were also macerated in a two-step approach. In the first step, five frass samples from each of the rearing groups (*E. dunnii* and *E. grandis* x *E. urophylla*) were macerated in 100 µl of PL 1 buffer using a pellet mixer (VWR, Pennsylvania, USA) for 2 min, after which, the remaining 300 µl of PL 1 buffer was added, and the mixture was incubated at 70°C for 15 min. The final maceration step was done using a Qiagen TissueLyser II for 5 min at 30/sec a frequency. Thereafter, the genomic DNA was extracted following the manufacturer's protocol. DNA concentration and quality from the gut, frass and the plant were assessed using the Nanodrop 1000 Spectrophotometer (Thermo Fisher, Massachusetts, USA).

3.6 Amplicon sequencing of environmental DNA samples

3.6.1 Library and preparation and sequencing

The bacterial diversity associated with each sample type (plant, beetle gut and frass) was catalogued using the Illumina MiSeq platform. The hypervariable region spanning between the V3-V4 region of the 16S rRNA gene was amplified using the primers 341F and 785R (Weisburg et al., 1991). In total, high-throughput amplicon libraries were constructed for 40 DNA samples representing three sample types (10 leaf, 20 gut, 10 frass). Library preparation and sequencing was outsourced to Inqaba Biotechnical Industries (Pty) Ltd, South Africa.

3.6.2 Bioinformatic analysis of diversity data

The raw data were demultiplexed by the sequencing facility. The demultiplexed 16s rRNA paired end reads were merged using BBMerge (v38.79) (Bushnell et al., 2017). For further analyses, merged reads were imported into Quantitative Insights in Microbial Ecology (QIIME2), (v2020.6) (Bolyen et al., 2019). Trimming, chimera removal, filtering and denoising was performed using 'q2-dada2' plugin (Callahan et al., 2016). High quality sequences with a Phred score of 33 were trimmed from the left at 18 bp and truncated at 250 bp. Representative sequences of each ASV (Amplicon sequencing variants) were aligned by MAFFT (Katoh and Standley, 2013). A phylogenetic tree was constructed using Fasttree (Price et al., 2009). Reads of a 232 bp length were assigned taxonomy using a 'q2-feature-classifier' plugin trained Naïve Bayes classifier of the Silva database (v132) (Glöckner, 2019). Sequences identified as Chloroplast and Mitochondria were discarded. QIIME 2 core-feature analyses were performed between bacterial families of the *E. dunnii* and *E. grandis* x *E. urophylla* guts and diet groups. From the data, Venn diagrams were generated using Venny (v2.1.0) (Oliveros, 2007).

3.6.3 Statistical analysis of diversity data

Statistical analysis of QIIME generated data was conducted in R (v4.05) with Rstudio (v1.4.1106) (R-Team, 2013). The ASV table, ASV taxonomy table and phylogenetic tree were converted into a merged phyloseq object. The observed species index and average relative abundance was calculated using the phyloseq package (v1.34.0) (McMurdie and Holmes, 2013; Oksanen et al., 2013). Absolute abundance of the bacterial communities associated with the *Eucalyptus* leaves, *Gonipterus* gut and frass was represented in a heatmap using the phyloseq package (McMurdie and Holmes, 2013). To compare the microbial communities between the diet treatments of the two gut groups, a Kruskal-Wallis test followed by a Wilcoxon post-hoc test was performed using R base (R-Team, 2013). For beta diversity analysis, data was filtered and normalised through the DESeq2 package (v1.30.1) by negative binomial Wald normalisation (Love et al., 2014; McMurdie and Holmes, 2014). With the normalised data, a principal coordinate analysis (PCoA) was generated using Bray-Curtis dissimilarity calculations. Graphical representations of relative abundance and alpha and beta diversity were generated in R using the ggplot2 package (v3.3.5) (Wickham et al., 2016).

3.7 Molecular cloning of bacteria associated with the gut and frass of *Gonipterus*

3.7.1 Molecular cloning of gut and frass DNA samples

While analysing the next generation sequencing data, there was a significant number of sequences belonging to the family Enterobacteriaceae. However, due to the size of the amplicons emerging from the short-read platform, genus and species identification of these taxa could not be achieved. Therefore, molecular cloning was used as an alternative strategy for resolving and confirming the taxonomy of this abundant taxa.

The 16s gene was amplified with DNA extracted from six gut and six frass samples using the universal primers 27F and 1492R (Weisburg et al., 1991). The PCR reaction included 5 µL of 5X MyTaq reaction buffer (25mM $MgCl_2$, 250µM dNTPs), 0.5 µL of each forward and reverse primers (10 µM), 0.3 µL of MyTaq DNA Polymerase (5 U μl^{-1}) (Bioline, London, UK), 1 µL of template DNA and volume was made up to 25 µl using nuclease free water. PCR amplifications were conducted with an initial denaturation at 95 °C for 2 min; followed by 35 cycles of 95 °C for 2 min, 55 °C for 30 sec, and 72 °C for 1 min; and final elongation at 72 °C for 7 min. PCR amplification was verified using gel electrophoresis.

Amplified PCR products of approximately 1500 bp were cleaned using the NucleoSpin Gel and PCR Clean-up kit (Macharey-Nagel, Düren, Germany) according to the manufacturer's instructions. Cleaned PCR products were ligated into the pGEM-T easy plasmid (Promega, Wisconsin, USA) and transformed into *Escherichia coli* TOP10 chemically competent cells following the manufacturer's protocols (Thermo Fisher, Massachusetts, USA).

3.7.2 Selection of clones and amplification of inserts

From the transformed colonies, fifteen clones from the gut and 10 clones from frass samples containing the 16s rRNA gene insert were selected through blue-white screening from each 16s rDNA cloning library. The 16s rDNA clones were amplified using pGEM-T easy specific M13 forward and reverse primers. PCR conditions were similar to those used for library preparation with the following modifications: 2.5 µl of 5X MyTaq reaction buffer (25 mM $MgCl_2$, 250 µM dNTPs), 0.25 µl of each of the forward and reverse primers, 0.15 µl of MyTaq DNA polymerase 5 U μl^{-1}, 1 µl of bacterial suspension made by adding one bacterial colony to 10 µl of dH_2O, and the volume was made up to 12.5 µL using nuclease free water. The inserts

were sequenced using Sänger sequencing at the DNA sequencing facility of the University of Pretoria using the Big Dye cycle termination kit (Applied Biosystems, Massachusetts, USA).

3.7.3 Taxonomic identification of inserts

Sequences were downloaded and the quality was determined in Bioedit (Alzohairy, 2011). The sequences were aligned with MAFFT and assembled in Bioedit (Alzohairy, 2011; Katoh and Standley, 2013), after which, quality sequences were taxonomically identified by comparison to the Genbank Nucleotide database using BLAST (Madden, 2013).

Chapter 4: Results

4.1 Chemical Analysis of *Eucalyptus* genotypes

The phytochemicals of four *Eucalyptus* genotypes were analysed using both LC- and GC-MS. The data from both platforms were combined. A principal component analysis showed that the biochemical profiles of the *E. dunnii* (BE0068962), *E. nitens* (STNH09) and *E. grandis x E. urophylla* genotypes (STGU12 and STW13) (Fig 4A) differed substantially from each other (Fig. 4A). PC1 explained 48% of the variability in the samples and PC2 explained 21% of the variability. Thus, there was a larger difference between *E. dunnii* (BE0068962) and the other genotypes than between *E. grandis x E. urophylla* (STGU12), *E. grandis x E. urophylla* (STW13) and *E. nitens* (STNH09) (Fig. 4A). Based on this data *E. dunnii* (BE0068962) and *E. grandis x E. urophylla* (STGU12) were selected for further investigations.

The essential oils in *Eucalyptus* genotypes (*E. dunnii*, *E. grandis x E. urophylla*) and the collected frass from beetles reared on these two *Eucalyptus* genotypes were analysed using GC-MS. Interestingly, most of the major mono- and sesquiterpenes in the *Eucalyptus* leaves declined significantly in the frass of the beetles (Supplementary Tables 1 and 2). For example, eucalyptol (1,8-cineole), a major secondary metabolite of the essential oil of *Eucalyptus* declined approximately 15.7-fold in *E. dunnii* frass and 15.4-fold in *E. grandis x E. urophylla* frass when compared to intact leaves of the two *Eucalyptus* genotypes ($p < 0.05$; Fig.4B, Fig 4C). Limonene, on the other hand, declined approximately 5.1-fold in the frass of beetles feeding on *E. grandis x E. urophylla* ($p < 0.05$; Fig. 4B) but did not significantly decrease in the frass of beetles feeding on *E. dunnii*. Additional declines were also recorded for the other major monoterpenes in *Eucalyptus* leaves, including *o*-cymene, α-terpinyl acetate, γ-terpinene, thymol and terpinen-4-ol as well as the major sesquiterpenes, aromandendrene and globulol (Supplementary Tables 1 and 2).

Instead, there was a significant increase of hydroxylated and oxygenated terpene products in the frass (Figs. 4B and 4C, Supplementary Tables 1 and 2). For example, three oxygenated derivatives of eucalyptol were identified (2-hydroxycineole, 2-hydroxycineole-acetate and 3-β-hydroxy, 1,8-cineole) which were detected in high levels in the frass but not the leaves. Biotransformation products from d-limonene (perillyl alcohol) and *α*-pinene (trans-verbenol) were also identified and appeared in significantly higher concentrations in the beetle's frass

than in the intact leaf ($p < 0.05$). For example, trans-verbenol showed an increase between 12.2-fold and 35.3-fold in the frass of the two rearing groups (*E. dunni*, *E. grandis* x *E. urophylla*).

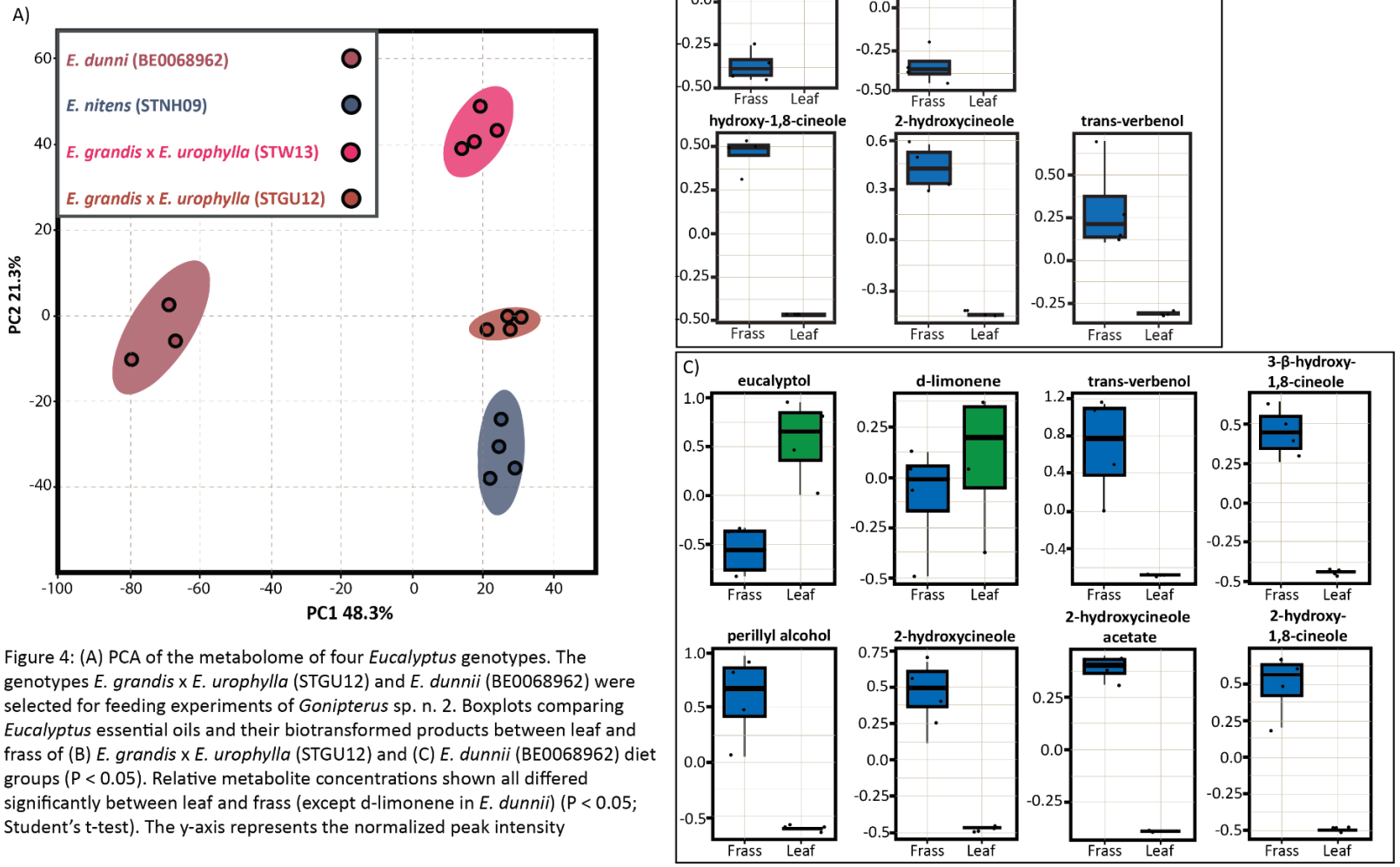

Figure 4: (A) PCA of the metabolome of four *Eucalyptus* genotypes. The genotypes *E. grandis* x *E. urophylla* (STGU12) and *E. dunnii* (BE0068962) were selected for feeding experiments of *Gonipterus* sp. n. 2. Boxplots comparing *Eucalyptus* essential oils and their biotransformed products between leaf and frass of (B) *E. grandis* x *E. urophylla* (STGU12) and (C) *E. dunnii* (BE0068962) diet groups ($P < 0.05$). Relative metabolite concentrations shown all differed significantly between leaf and frass (except d-limonene in *E. dunnii*) ($P < 0.05$; Student's t-test). The y-axis represents the normalized peak intensity

4.2 Antibiotic treatment of *Gonipterus* sp. n. 2

To determine the potential for *in vivo* gut microbial detoxification of *Eucalyptus* secondary metabolites, frass was collected from beetles that were either treated with antibiotics, treated with antibiotics and reinoculated with the gut microbial communities or not treated with antibiotics. The three treatment groups were allowed to feed on artificial medium amended with *Eucalyptus* oil, and their frass was subsequently analyzed using GC-MS to determine their essential oil content. Eucalyptol, *α*-pinene and the biotransformed product of eucalyptol (2-hydroxycineole acetate) were detected. D-limonene was not detected during peak deconvolution and compound identification. No significant difference in *α*-pinene, eucalyptol or hydroxycineole acetate contents were detected between antibiotic, control and reinfected treatments ($p > 0.05$; Student's t-test) (Fig. 5).

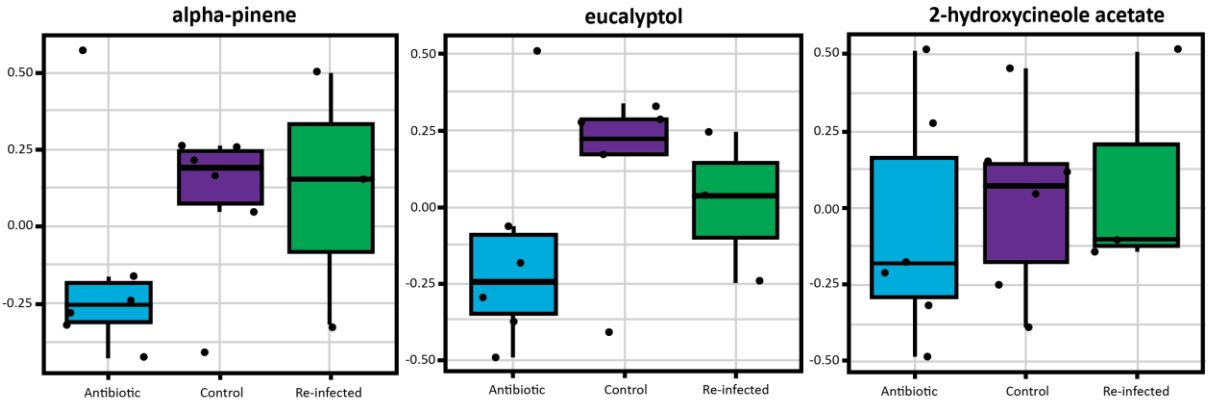

Figure 5: Boxplots showing statistically non-significant changes of secondary metabolites in collected *Gonipterus sp. n. 2* frass samples from three treatment groups of beetles, antibiotic, control and reinfected ($P > 0$; Student's t-test). The y-axis is the normalized peak intensity.

4.3 Metabarcoding of bacterial communities in *Eucalyptus*, *Gonipterus* gut and frass samples

The bacterial community compositions of four plant, ten gut and four frass samples for each *E. dunnii* and *E. grandis* x *E. urophylla* diet group, respectively, were obtained from sequencing. Four outliers, two from the frass and two from the plant were removed from the data set. From a total of 1 479 873 raw reads, 1 263 963 reads were generated after the removal of low quality and chimeric sequences. Furthermore, 694 837 remained after filtering out plant organelle DNA from plant, gut, and frass samples. Removing plant organelle DNA created a read variation between samples, most notably in the plant samples, resulting

in low read numbers. The remaining filtered 694 837 sequences were clustered into 2843 ASVs.

4.4 Bacterial diversity of the plant hosts, guts and frass of *Gonipterus* sp. n. 2

From the 2843 ASVs, 42 bacterial phyla were identified in the plant, gut and frass samples. The ten most abundant bacterial orders in the *Eucalyptus* leaves, *Gonipterus* gut and frass were Bacilliales, Bacteroidales, Clostridiales, Enterobacteriales, Entomoplasmatales, Micrococcales, Oceanospirralales, Oligosphaerales, Sphingobacteriales and Sphingomonadales (Fig. 6A). At the order level the most dominant bacterial communities in the *E. dunnii* plant host were Clostridiales at 52%. In the *E. grandis* x *E. urophylla* plant host, Sphingomonadales dominated at an average of 50%. The gut samples portrayed very similar bacterial abundances in both the *E. dunnii* and *E. grandis x E. urophylla* diet groups, where Enterobacteriales dominated on average by 78%. Similarly in the frass of both diet groups, on average, 69% of the sequences belonged to Enterobacteriales (Fig. 6A).

The data indicated that the *Gonipterus* beetles reared on *E. dunnii* and *E. grandis x E. urophylla* shared a significant number of gut bacterial communities. Between the *E. grandis x E. urophylla* and *E. dunnii* gut associated microbial communities 74.5% of bacterial communities were shared irrespective of the difference in diet (Fig. 6B). Diet groups of the *E. dunnii* and *E. grandis x E. urophylla* host, however also indicated shared bacterial families between the gut, plant, and frass. The *E. grandis x E. urophylla* host group shared a larger portion of the plant gut and frass ASVs compared to the *E. dunnii* host group (Figs. 6C and 6D).

To further investigate, the 16s amplicon sequencing data, a heatmap of absolute abundance was generated to genus level. From the heatmap, the gut and the frass were observed to exclusively be enriched with bacteria from the Enterobacteriaceae, *Hafnia* and *Carnimonas* (more accurately identified as *Halotelea* in the 16s rDNA cloning library sequence results) (Fig. 7). Additionally, a large number of bacterial communities were observed to be absent in *E. dunnii* frass when compared to the bacterial communities in the *E. dunnii* gut and *E. grandis x E. urophylla* frass (Fig. 7).

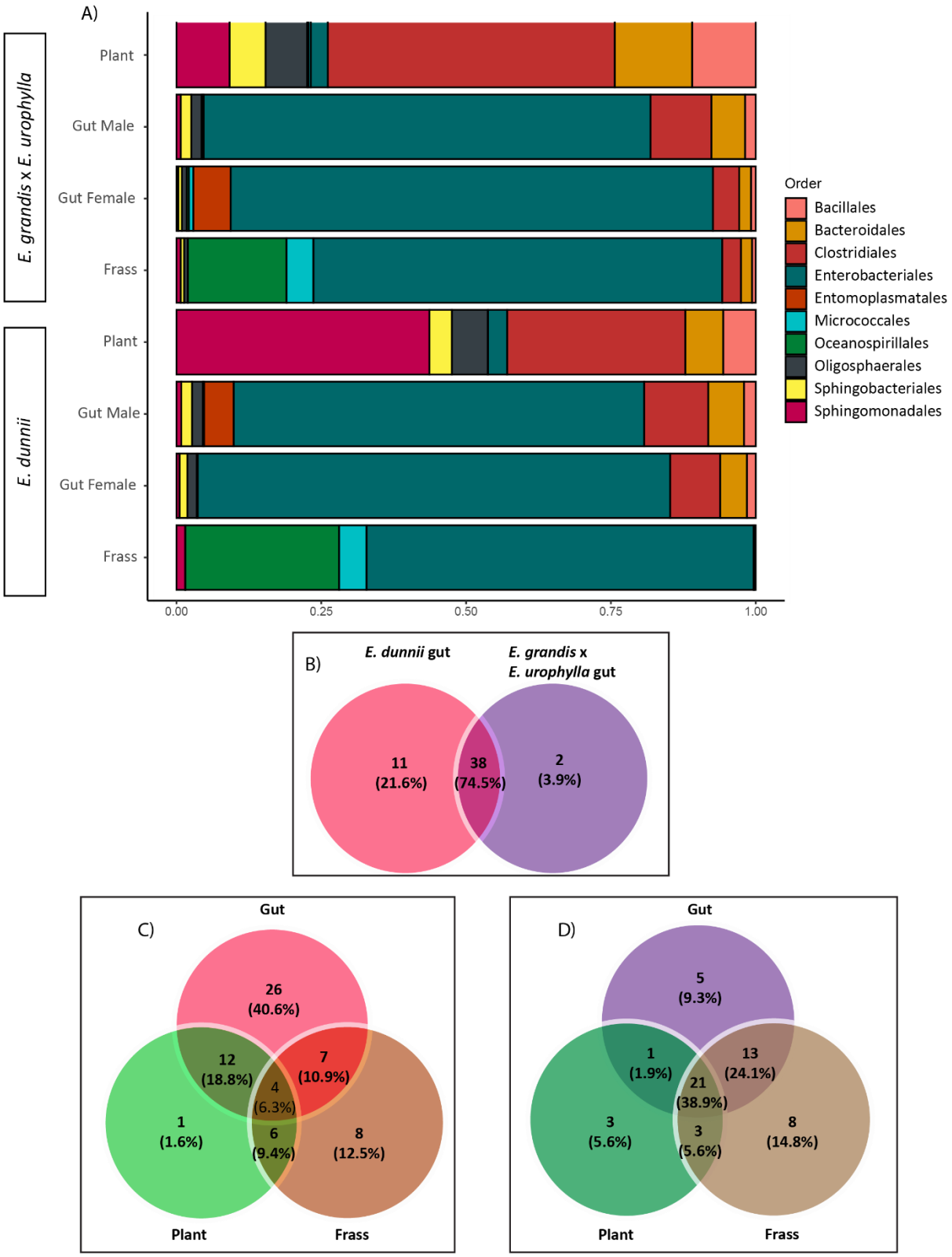

Figure 6: (A) The average relative abundance of bacteria (order level) associated with the plant, gut and frass of *Gonipterus* sp. n 2 fed on two different diet groups *E. dunnii* (BE0068962) and *E. grandis* x *E. urophylla* (STGU12). (B) Shared gut bacterial families between the two plant diet groups. (C) Shared bacterial families between the plant, gut and frass of the *E. dunnii* diet group. (D) Shared bacterial families between the plant, gut and frass of the *E. grandis* x *E. urophylla* diet group

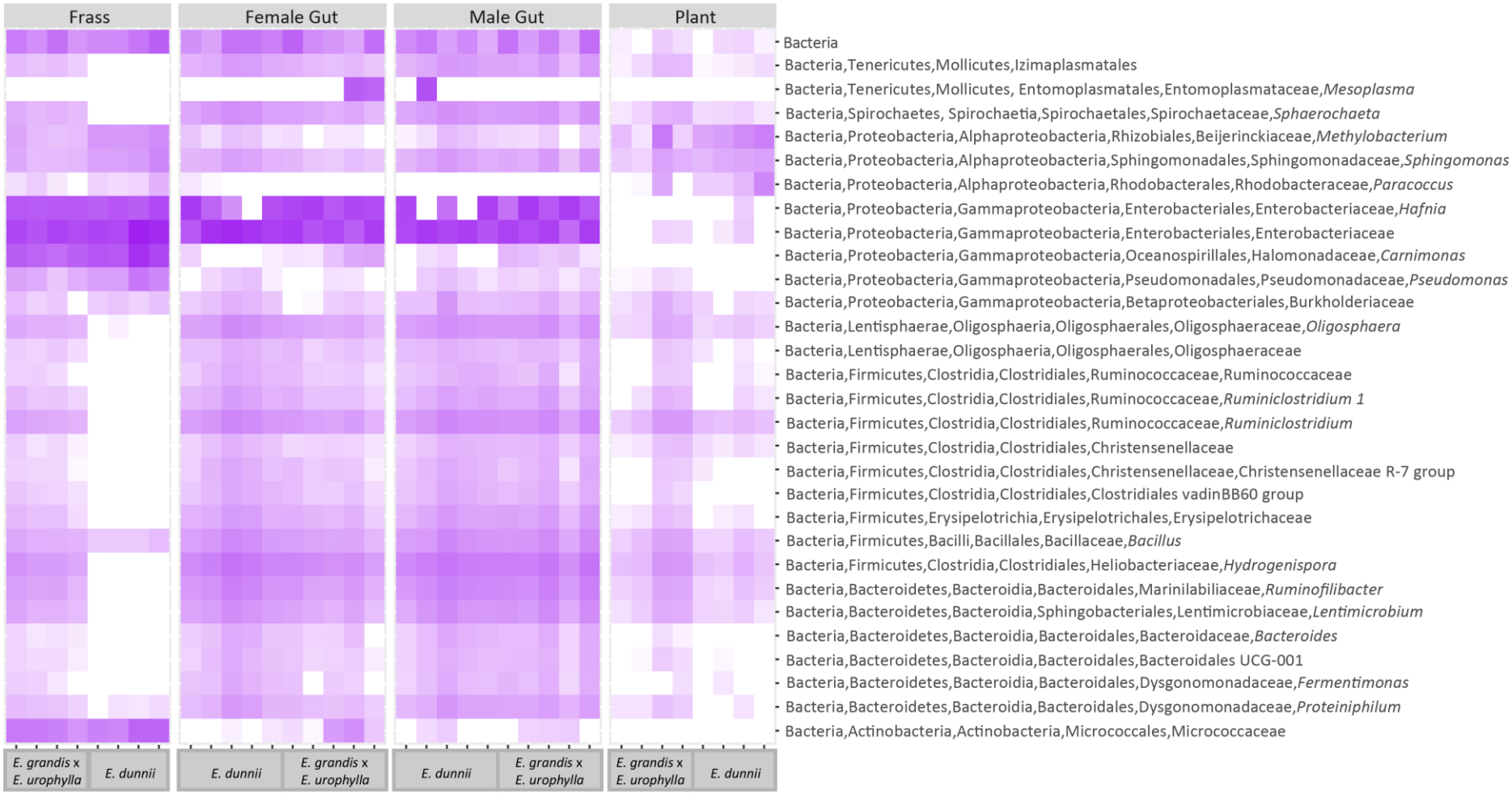

Figure 7: Bacterial communites associated with the host plants, gut and frass of *Gonipterus* sp. n. 2 . Thirty of the most abundant taxons are shown. Absolute abundances of bacterial taxa are displayed as a heatmap, with dark colours representing high abundances and white indicating absence.

4.5 Effect of plant genotype on *Gonipterus* gut bacterial diversity

The richness of the bacterial community in *Gonipterus* diet groups (*E. dunnii* and *E. grandis x E. urophylla*), guts and frass were determined by alpha diversity indices (Fig. 8A). The community richness was determined by the observed species index indicating that bacterial richness in the *Gonipterus* gut tissue was highly similar between plant host groups. No significant difference was observed in the richness between the gut bacterial communities belonging to the two rearing groups ($p>0.05$). Furthermore, taxa richness in plant samples was low whereas frass had a higher taxa richness compared to plant samples but lower than that of gut samples (Fig. 8A).

The beta diversity based PCoA plot calculated using a Bray-Curtis dissimilarity matrix revealed the dissimilarities of the bacterial communities associated with the plant, gut and frass from the two diet groups (Fig. 8B). Gut bacterial communities clustered together regardless of the plant diet groups. Unlike the gut bacterial communities, the frass and plant associated bacterial communities grouped according to plant host. Supporting the shared bacterial families (Figs. 6C and 6D), the *E. grandis x E. urophylla* host group of plant, gut and frass bacterial communities clustered separately but were more closely arranged to each other as opposed to the *E. dunnii* host group (Fig. 8B).

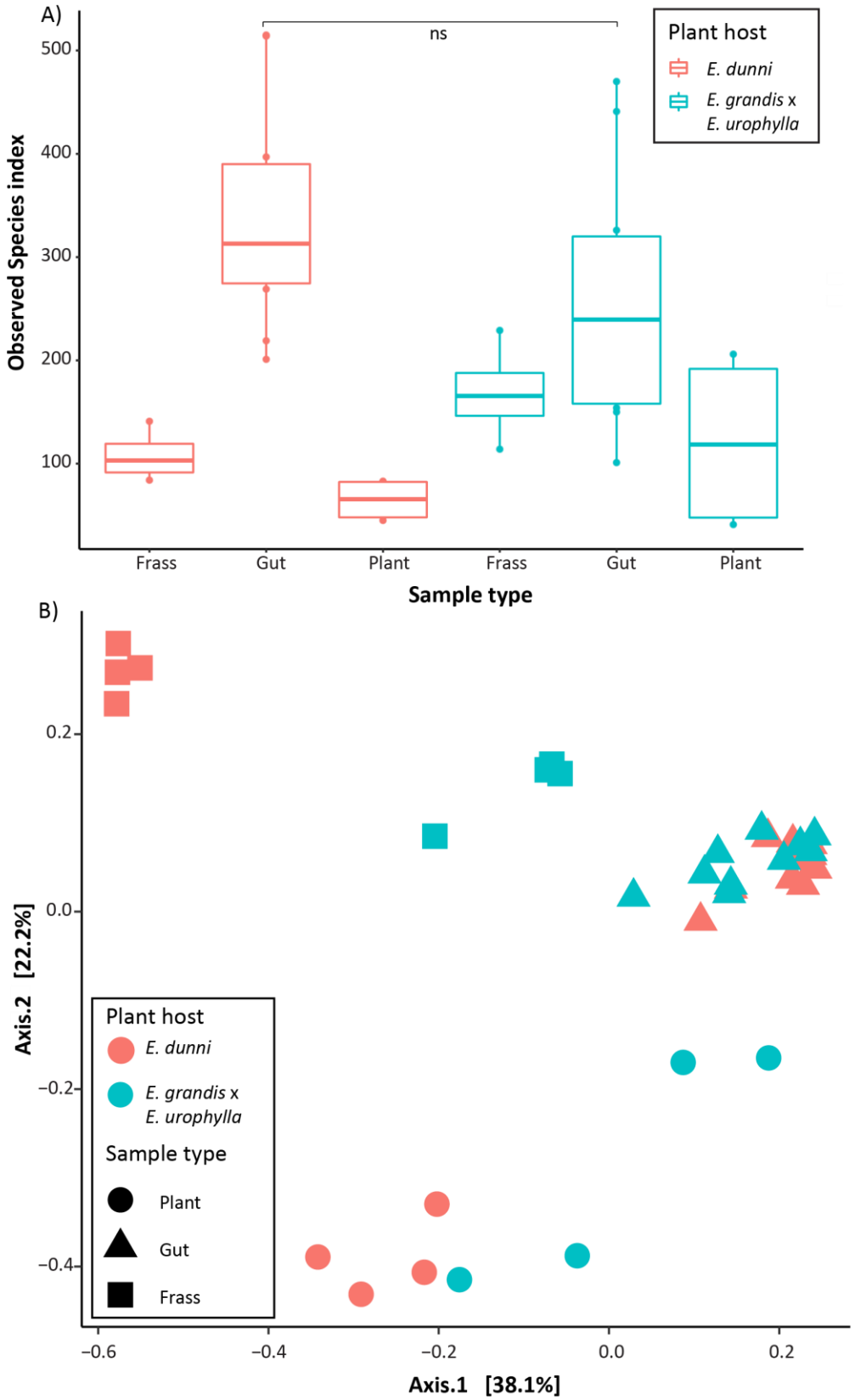

Figure 8: (A) Observed species index of the bacterial communities associated with the host plant, gut and frass of *Gonipterus* sp. n 2. Comparisons were performed using the Kruskal-Wallis test followed by a Wilcoxon post-hoc test. (B) PCoA plot based on Bray-Curtis distance matrix depicting differences in the composition of bacterial communities associated with the plant host, gut and frass of *Gonipterus* sp. n. 2.

4.6 Molecular cloning of bacteria associated with the gut and frass of *Gonipterus* sp. n. 2

Plasmids from a 16S rDNA cloning library with inserts of the correct length were sequenced using traditional Sänger long-read sequencing and potential chimeras were discarded. Sequences with a length of 1300 bp were aligned and identified through BLAST against the NCBI nucleotide database. Altogether, ninety reliable sequences were derived from the gut and sixty sequences from frass samples. Overall, *Hafnia alvei* sequences dominated both in the gut (53%) and frass (40%) bacterial communities irrespective of diet (Figs. 9a and 9b). Furthermore, *Obesumbacterium*, *Enterobacter*, *Klebsiella*, *Erwinia* and *Ewingenella* were found to be present in the gut (Fig. 9a). Frass bacterial communities were comparable to gut bacterial communities but included the unique genera *Halotelea* and *Bordetella* (Fig. 9b).

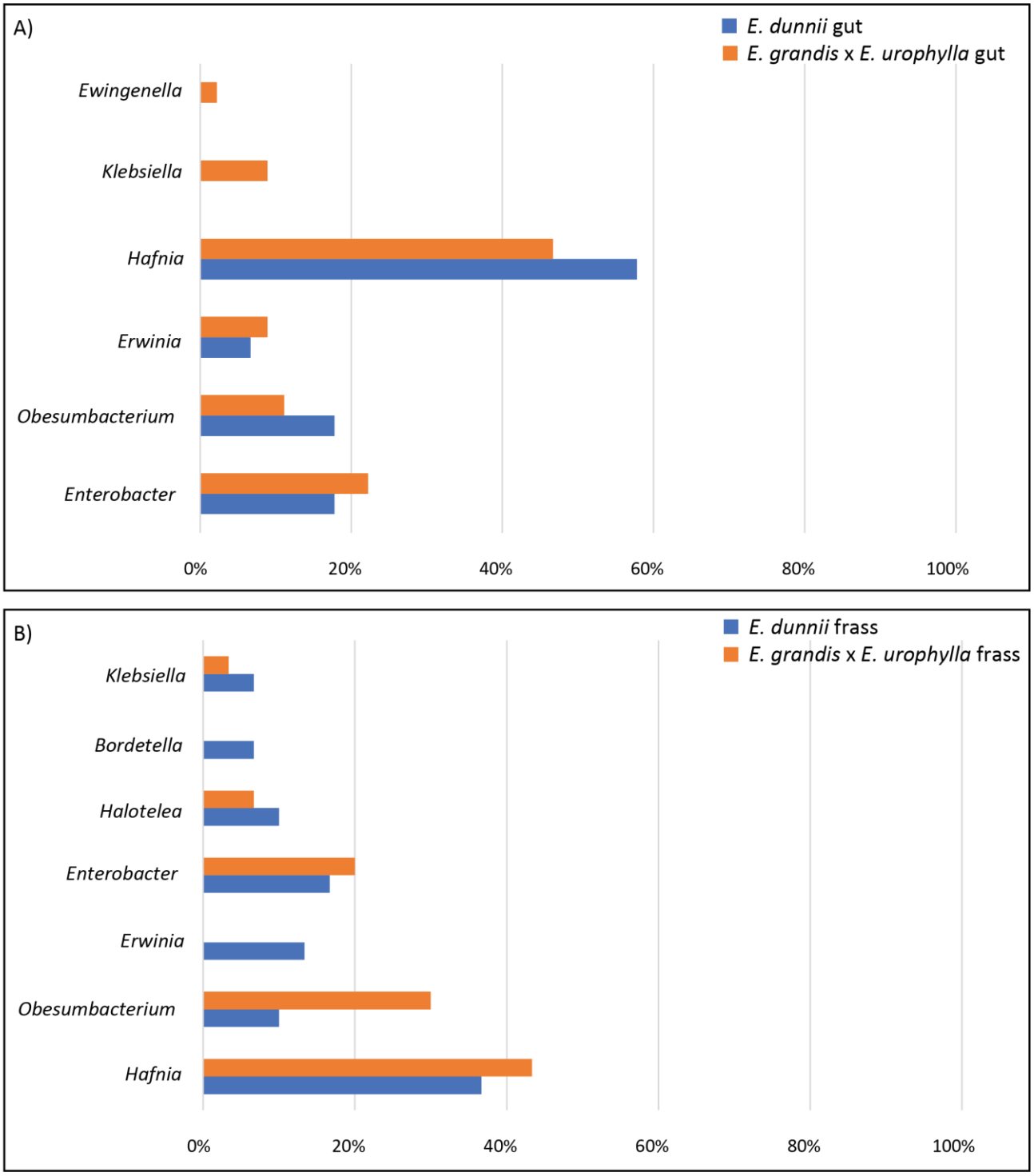

Figure 9: Percentage abundance of the bacterial genera determined by 16S rDNA clone library from (A) gut (90 sequences) and (B) frass (60 sequences) samples of *Gonipterus* sp. n. 2 fed on *E. dunni* and *E. grandis* x *urophylla* diet groups.

Chapter 5: Discussion

In the present study, a comparative analysis of the metabolic profiles between *Eucalyptus* leaves from two different hosts and the corresponding *Gonipterus* frass revealed a significant decline of essential oils in the frass compared to the intact leaf. Concomitantly, an increase in oxygenated terpenoids was detected in the frass. To investigate the possibility that *Eucalyptus* essential oils are detoxified by the microbial community in the beetle's gut, the bacterial communities associated with *Eucalyptus* leaves, the *Gonipterus* gut and frass were catalogued. Lastly, to test the hypothesis of whether the identified bacterial community of the *Gonipterus* gut was involved in the detoxification of *Eucalyptus* secondary metabolites, an attempt was made to disrupt the gut microbial communities in adult *Gonipterus* beetles.

5.1 Detoxification of plant secondary metabolites

A common secondary metabolite in *Eucalyptus* essential oils is eucalyptol (1,8-cineole). Eucalyptol functions as a feeding and ovipositional deterrent and has been shown to be toxic to agricultural, silvicultural, and medically important insect pests (Klocke et al., 1987; Moretti et al., 2015). However, *Gonipterus* sp. n. 2 is unaffected by the high concentrations of essential oils in its diet. From the GC-MS analysis of collected *Gonipterus* frass, several hydroxylated and oxygenated products of eucalyptol were putatively identified. This shows that the beetle has effective mechanisms for detoxifying this metabolite into less toxic oxygenated compounds.

Oxidation of eucalyptol may either occur through the beetle's native enzymes in the gut or may be mediated by microbial endosymbionts in the beetle's gut. Several studies on animals and insects feeding on a diet rich in eucalyptol suggested that microbes may play an important role in the detoxification of high eucalyptol concentrations (Foley et al., 1987; Francoeur et al., 2020; Pizzolante et al., 2017). Microbes (*Rhizopus*, *Aspergillus*, *Pseudomonas, Sphingobium, Bacillus, Rhodococcus*) are capable of metabolising eucalyptol to hydroxylated and oxygenated products (García et al., 2009; Mi et al., 2016; Rodríguez et al., 2006; Unterweger et al., 2016). However, very few studies have performed *in vivo* experiments of microbial eucalyptol detoxification in insects.

D-limonene is another metabolite in *Eucalyptus* essential oil that is toxic and repellent towards insects (Duetz et al., 2003). Comparative analysis between *Eucalyptus* leaves and *Goniperus* frass indicated a decrease in d-limonene content in the frass. Accordingly, a biotransformed product of d-

limonene (perilyll alcohol) was observed to exclusively increase in the frass of *Gonipterus* fed on the *E. dunnii* genotype. Insects, bacteria and fungi have been shown to be involved in hydroxylating d-limonene in the 7th position, producing perillyl alcohols which can then be further degraded to substrates of the tricarboxylic acid pathway (Duetz et al., 2003). The oxidation of limonene in the *Gonipterus* gut may therefore be another microbial detoxification product.

The loss of major monoterpene content from *Eucalyptus* leaves to *Gonipterus* frass thus suggested a potential role of microbes in the detoxification of *Eucalyptus* secondary metabolites. In order to confirm microbial detoxification of these plant secondary metabolites, *Gonipterus* beetles were subjected to an antibiotic treatment as a means to remove gut associated microbial communities. However, from the GC-MS frass analysis of antibiotic, control and reinfected treatments, monoterpene content (d-limonene, eucalyptol) did not significantly change. Similarly, no significant changes in biotransformed eucalyptol (2-hydroxycineole acetate) were observed. Several problems were encountered during these experiments. The beetles did not feed sufficiently on the artificial media and often did not survive the antibiotic treatment. Furthermore, volatiles were applied to the artificial medium through diffusion, leading to low concentrations in the diet and frass. All these factors may have led to the unsuccessful removal of gut microbial communities in the *Gonipterus* beetles and very low levels of essential oils in the beetles' frass. However, the fact that the antibiotic treatment significantly affected the beetles' survival allows the inference, that gut microbial communities play an essential role in the beetles' health and survival.

Microbial communities of the gut can play an important role in transforming secondary metabolites into components of insect pheromones (Blomquist et al., 2020; Xu et al., 2015). Plant monoterpenes and their metabolised products have gained recognition as important components of insect pheromones (Blomquist et al., 2020). For example, bacterial communities associated with the gut of *Dendroctonus valens* displayed an ability to transform the aggregation pheromone, cis-verbenol, into verbenone, an anti-aggregation pheromone (Xu et al., 2015). Suggested pheromones consisting of biotransformed monoterpenes (hydroxycineole) have been found in the faecal pellets of *Chrysolina herbacea*, *Pityrodia jamesii* and *Paropsisterna tigrine* (Carman and Klika, 1992; Cordero et al., 2012; Fletcher et al., 2000). Interestingly, the putatively identified biotransformation products of eucalyptol (hydroxylated and oxygenated cineole) and α-pinene (trans-verbenol) observed in this study in *Gonipterus* sp. n. 2 frass were tentatively identified as pheromone candidates in the closely related

Gonipterus platensis (Branco et al., 2020). In the study, hydroxycineole derivatives (2-α-hydroxy-1,8-cineole and 2-oxo-1,8-cineole) stimulated responses that attracted virgin males.

Interestingly, a number of studies have illustrated that microbial biotransformation products of host metabolites function as aggregation pheromones (Dillon and Charnley, 2002; Wada-Katsumata et al., 2015). For example, *Blattella germanica* (German cockroach) frass was shown to contain 12 major volatile carboxylic acid (VCA) associated semiochemicals that have been suggested to play a role in aggregation (Wada-Katsumata et al., 2015). Analysis of frass collected from axenic cockroaches indicated a loss in the 12 VCAs compared to the control frass (Wada-Katsumata et al., 2015). Results from the two choice sheltering assays indicated that first instar nymphs were attracted to extracts of control frass compared to axenic frass extracts (Wada-Katsumata et al., 2015). Furthermore, the inoculation of aerobic bacteria from untreated frass rescued the ability of the previously axenic cockroaches to produce the 12 major VCAs (Wada-Katsumata et al., 2015). Although little is known about the aggregation behaviour of *Gonipterus,* our field and laboratory observations indicated that the species displays gregarious behaviour. Therefore, I hypothesize that the *Gonipterus* gut microbial community could be involved in producing components of an aggregation pheromone.

5.2 Bacterial communities associated with *Gonipterus* sp. n. 2

The two plant diets (*E. dunnii* and *E. grandis x E. urophylla*) were observed to display significant differences in plant secondary metabolite content and small differences in bacterial composition. *Gonipterus* sp. n. 2 beetles from different diet groups exhibited similar bacterial species richness and largely overlapping bacterial communities. These results indicate that neither differences in plant chemical content nor plant bacterial communities caused variation in the gut bacterial communities of *Gonipterus* sp. n. 2.

Diet thus seems to have only a minor effect on the bacterial community structure in *Gonipterus* sp. n. 2. A study by Colman et al. (2012) also demonstrated that even vastly different diets do not significantly affect Coleopteran gut microbial communities. Correspondingly, studies on Cerambycid beetles showed a lack of variation in gut microbial communities after diet changes (Silver et al., 2021). A similar lack of variation has also been observed in the Lepidopteran species, *Bombyx mori* and *Lymantria dispar* (Chen et al., 2018; Mason and Raffa, 2014). The similarities in the gut microbial communities of *Gonipterus* sp. n. 2 feeding on different plant diets indicates that short-term diet

changes may not cause large structural changes in the gut bacterial communities in this beetle species. Furthermore, gut morphology and physiochemical conditions (i.e., oxygen availability, pH and production of anti-microbial peptides) may also play a role in shaping the structure and composition of gut-associated bacterial communities in *Gonipterus* sp. n. 2.

On the other hand, in other Coleopteran insects', diet was shown to have a major influence in the structure of the gut community (Chrostek et al., 2017; Hansen and Moran, 2014). In our study, bacterial communities of the gut had a minor resemblance to the plant bacterial communities. This may indicate that contact with or feeding of *Eucalyptus* associated bacterial communities is a method of horizontal transmission between the host and the beetle but may not cause a large change under conditions of short-term diet changes. However, due to the low bacterial counts in the plant samples, horizontal transmission of bacterial communities from the diet may be underestimated in this study and requires further investigation.

At the phylum level, the *Gonipterus* gut bacterial community consisted predominantly of Proteobacteria, with lower abundances of Firmicutes and Bacteroides. The dominance of Proteobacteria and Firmicutes has been demonstrated in many insect gut microbial community studies (Colman et al., 2012; Yun et al., 2014). At lower taxonomic levels, the most abundant and prevalent ASVs (Amplicon Sequencing Variants) belong to Enterobacteriaceae. Upon further inspection at the genus level, it was revealed that gut- and frass-associated bacterial communities were enriched with *Hafnia* and *Obesumbacterium.* Analysis of larger 16s rRNA segments confirmed the high abundance of *Hafnia* and *Obesumbacterium* co-occurring with lower abundances of *Enterobacter* (Figs 6A and 6B). *Hafnia alvei* has been mostly associated with the gut microbial communities of omnivorous and carnivorous insects but has also been associated with some herbivorous insects as a largely commensal microbe (Zheng et al., 2021). *Hafnia alvei* have been found in high concentrations in the guts of the honeybee, *Apis mellifera*, ground beetles, *Harpalus pensylvanicus* and *Anisodactylus sanctaecrucis*, the European firebug, *Pyrrhocoris apterus*, and the desert locust, *Schistocerca gregaria* (Dillon and Charnley, 1995; Gasper et al., 2017; Haas and König, 1987; Lundgren et al., 2007). In a study by Tian and Moran (2016), the genome of *H. alvei* from the *A. mellifera* intestinal tract was sequenced. Gene clusters associated with anti-microbial peptide and siderophore production were putatively identified. These putatively identified genes clusters are thought to enable *H. alvei* to be highly competitive by suppressing other microbial communities present in the insect gut (Tian and Moran, 2016). Additionally, *H. alvei* is known to produce chitinases

which may be useful in digesting gut fungal inhabitants but are also harmful to insects (Whitaker Jr et al., 2004). However, in the context of the gut of *Gonipterus* sp. n. 2, the role of *H. alvei* has not been elucidated yet.

Chapter 6: Conclusion

The Eucalyptus snout beetle, *Gonipterus* sp. n. 2, is a significant invasive insect pest of *Eucalyptus* species in South Africa. *Gonipterus* feed on *Eucalyptus* foliage that contain high concentrations of essential oils consisting of mono- and sesqui-terpenes in specialised oil glands (Tooke, 1955; Dhakad et al., 2018). These terpenoid essential oils have repellent, anti-digestive, and insecticidal activity towards insect herbivores (Boone et al., 2013; Mason et al., 2016). However, despite their potent bioactivity, *Gonipterus* sp. n. 2 can tolerate and overcome variable concentrations of *Eucalyptus* essential oils. Our results indicate that following passage through the gut, mono- and sesquiterpenes from *Eucalyptus* leaves are transformed into a diverse range of oxidised products. Many of these biotransformed products have been previously described as insect and microbial degradation products that are important in insect behaviour. Furthermore, despite differences in phytochemical and bacterial composition of the *Eucalyptus* diets, the gut bacterial community of *Gonipterus* remained unaffected. Future work should focus on the functional characterisation of these core microbial communities to elucidate how *Gonipterus* is able to survive and maintain on an otherwise very nutrient-limiting and toxic host.

Chapter 7: References

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Chapter 8: Supplemental Material

Table 1: Table showing the identified secondary metabolites in *E. dunnii* (BE0068962) leaves and *Gonipterus* sp. n. 2 frass

Retention time	Compound name	Leaf1	Leaf2	Leaf3	Leaf4	Frass1	Frass2	Frass3	Frass4	Mean Leaf	Mean Frass	Fold-change	p-value
6,6	*o*-cymene	300031,5	781905,0	18045,9	851207,0	6419,0	92105,8	33131,8	38221,7	487797,3	42469,60	1,1E+01	9,2E-01
5,7	eucalyptol	251043,9	88518,2	561070,2	754086,9	14954,7	13195,6	37135,9	40174,0	413679,8	26365,05	1,6E+01	3,6E-03
11,2	aromandendrene	179976,3	161637,1	110079,9	369695,3	78009,4	8015,5	146984,9	184812,0	205347,1	104455,44	2,0E+00	2,1E-01
6,3	γ-terpinene	167710,0	249217,6	4302,2	207639,8	10211,2	36496,3	87987,2	107041,6	157217,4	60434,09	2,6E+00	6,1E-01
16,2	(-)-globulol	66995,7	55382,8	54499,2	139405,0	22049,6	63443,5	55817,3	69394,9	79070,7	52676,32	1,5E+00	2,8E-01
12,2	α-terpinyl acetate	58650,1	31432,6	186027,9	102836,1	36685,3	111369,9	182665,6	233861,1	94736,7	141145,47	6,7E-01	5,0E-01
5,6	*d*-limonene	34921,0	13305,2	69969,6	74355,9	10025,6	26889,1	34142,4	42039,8	48137,9	28274,24	1,7E+00	4,0E-01
11,6	aromandendrene	30423,1	22548,2	27203,6	63681,8	25245,2	59609,9	47105,4	60257,8	35964,2	48054,60	7,5E-01	3,4E-01
11,0	β-gurjunene	28330,0	31897,4	25066,3	22931,1	1706,0	1352,4	60214,8	79517,5	27056,2	35697,68	7,6E-01	4,2E-01
11,1	terpinen-4-ol	17084,4	23506,3	5733,2	33291,2	0,0	0,0	0,0	0,0	19903,8	0,00	1,7E+01	4,3E-04
16,5	β-eudesmol	16787,8	13590,5	13347,5	40199,7	4765,1	15604,9	12094,0	15533,6	20981,4	11999,43	1,7E+00	2,1E-01
15,6	(-)-globulol	16559,5	14794,7	10929,6	34508,9	0,0	0,0	0,0	0,0	19198,2	0,00	8,8E+00	1,4E-04
17,1	thymol	16539,2	92360,1	0,0	105713,8	0,0	0,0	0,0	0,0	53653,3	0,00	1,6E+01	4,2E-02
12,3	β-maaliene	15962,4	12908,6	17067,2	37864,5	10835,2	25765,1	21837,8	27108,3	20950,7	21386,59	9,8E-01	8,7E-01
19,3	cadalene	15386,6	9672,4	8024,1	16239,2	0,0	0,0	0,0	0,0	12330,6	0,00	7,7E+00	2,6E-05
6,1	trans-β-ocimene	15161,1	2441,4	119756,3	0,0	26253,0	0,0	106427,0	132901,0	34339,7	66395,26	5,2E-01	5,5E-01
20,6	cyclohexane 1-(2,2-dimethylpropyl)-3,5-diethenyl-2-methyl-	14613,0	6598,8	13152,0	16058,0	0,0	0,0	0,0	0,0	6302,7	0,00	9,6E+00	3,5E-05
16,3	viridiflorol	13496,7	9234,2	14218,0	28528,9	7726,7	22123,9	18874,9	21187,9	16369,4	17478,37	9,4E-01	8,3E-01
12,9	γ-cadinene	13336,4	6987,3	7374,1	19426,6	0,0	0,0	0,0	0,0	11781,1	0,00	8,4E+00	1,7E-04
6,4	trans-β-ocimene	12291,6	14403,7	22025,7	23739,3	5437,1	1268,8	5498,9	5530,5	18115,1	4433,83	4,1E+00	8,9E-03
12,7	germacrene B	11736,8	3887,6	15047,8	10868,6	117449,9	221558,5	186246,3	218770,0	10385,2	186006,19	5,6E-02	1,1E-04

10,2	(-)-*α*-gurjunene	11183,6	3347,1	39948,1	3297,3	52934,3	0,0	113881,6	146584,5	14444,0	78350,08	1,8E-01	4,3E-01
13,8	trans-calamenene	8775,5	7216,8	4573,3	10041,0	0,0	0,0	0,0	0,0	7651,6	0,00	8,4E+00	1,9E-05
11,1	7-epi-*α*-eudesmol	7958,4	6871,0	5025,1	16347,5	0,0	0,0	0,0	0,0	9050,5	0,00	9,0E+00	2,6E-05
16,0	cubeban-11-ol	7605,3	6504,1	7107,0	18392,3	3461,8	9297,4	14099,5	68738,1	9902,2	23899,21	4,1E-01	5,8E-01
11,3	selina-5,11-diene	7032,8	6441,4	4875,1	15383,6	2837,0	6549,1	4722,9	6609,1	8433,2	5179,55	1,6E+00	2,1E-01
12,2	2H-2,4a-methanonaphthalene	6727,1	3665,1	6057,1	12025,8	13517,7	23686,1	8688,0	12147,8	7118,8	14509,88	4,9E-01	6,2E-02
5,1	*α*-phellandrene	6321,9	2824,5	26394,7	6287,4	7627,3	17205,0	56795,2	64374,1	10457,1	36500,39	2,9E-01	1,1E-01
4,3	*B*-pinene (-)	6101,3	0,0	14026,5	5505,0	4841,4	12330,7	12971,1	15501,5	6408,2	11411,17	5,6E-01	2,4E-01
9,4	isoledene	6049,0	3303,7	7280,3	9263,3	4297,1	11020,4	8367,1	10145,9	6474,1	8457,64	7,7E-01	4,1E-01
10,0	(-)-*α*-isocomene	5588,2	5846,9	1164,0	14547,1	0,0	0,0	0,0	0,0	6786,6	0,00	2,9E+01	1,2E-03
17,6	5-hydroxy-3-methyl-1-indanone	5325,0	2642,8	4847,2	6682,9	0,0	0,0	0,0	0,0	4874,5	0,00	9,2E+00	3,5E-05
12,6	*γ*-selinene	5253,9	4898,0	2674,6	8101,7	2251,2	6392,6	4804,5	6193,7	5232,1	4910,49	1,1E+00	8,5E-01
13,4	1H-cyclopropa[a]naphthalene	5137,6	2048,4	3143,3	4066,8	0,0	0,0	0,0	0,0	3599,0	0,00	8,8E+00	3,8E-05
16,4	1,2-dehydroviridiflorol	4693,5	0,0	4916,6	3824,4	3938,0	9773,8	7814,6	9714,6	3358,6	7810,24	4,5E-01	1,0E-01
17,3	*γ*-eudesmol	4427,7	3959,7	5776,7	10100,9	27963,1	61170,4	49966,2	57385,2	6066,3	49121,24	1,2E-01	2,5E-04
9,7	copaene	4255,9	3946,0	3304,9	9165,9	1991,3	1811,5	3662,8	4942,2	5168,2	3101,96	1,7E+00	1,7E-01
10,7	*δ*-guaiene	3667,1	3106,0	1786,3	7992,8	0,0	0,0	0,0	0,0	4138,0	0,00	1,2E+01	3,0E-04
13,0	silphiperfola-4.7(14)-diene	3660,2	0,0	0,0	0,0	2117,3	2473,7	3107,4	1900,8	915,1	2399,79	5,0E-01	7,2E-02
6,8	*α*-terpinene	3611,7	1370,1	3053,2	8477,3	0,0	0,0	0,0	0,0	4128,1	0,00	1,5E+01	2,9E-05
15,3	neophytadiene	3528,5	1869,0	7854,8	4185,1	3968,6	7401,8	7566,9	9948,2	4359,3	7221,38	6,0E-01	7,3E-02
11,8	*γ*-gurjunene	3475,6	2674,2	1537,4	6993,7	1114,9	2544,5	1920,2	2565,2	3670,2	2036,22	1,8E+00	2,3E-01
15,8	ledol	3420,0	2760,5	2563,7	6736,0	824,0	3875,0	3390,6	4861,7	3870,0	3237,81	1,2E+00	5,6E-01
17,7	*α*-cadinol	3124,9	2066,7	2767,7	5492,8	4202,7	5694,6	4345,7	5259,5	3363,0	4875,62	6,9E-01	9,6E-02
13,2	neoisolongifolene. 8,9-dehydro-	2696,1	1279,3	1363,7	3643,6	3438,4	8623,8	6925,3	9400,9	2245,7	7097,06	3,2E-01	1,4E-02
15,1	*α*-dehydro-ar-himachalene	2621,4	1294,6	2220,6	3883,0	3610,4	8514,2	6925,8	9456,8	2504,9	7126,78	3,5E-01	1,5E-02
5,3	*α*-terpinene	2603,5	1524,7	1464,1	2781,6	0,0	0,0	0,0	0,0	2093,5	0,00	7,1E+00	5,3E-04
16,8	dehydrolongifolene oxide	2429,5	356,3	2417,0	1546,9	8920,4	19967,4	18109,9	22724,0	1687,4	17430,42	9,7E-02	2,4E-03
17,9	neointermedeol	2379,9	2684,4	2212,5	4442,8	1703,4	1817,0	1660,3	2112,6	2929,9	1823,34	1,6E+00	3,9E-02
15,0	neophytadiene	2362,2	1294,8	5485,2	1688,8	3086,1	5792,7	5257,5	6661,3	2707,7	5199,41	5,2E-01	1,5E-01
11,8	3H-3aβ,7-methanoazulene	2338,1	1911,8	2511,3	5315,4	3203,6	7057,9	5167,6	5867,1	3019,1	5324,05	5,7E-01	7,2E-02

14,8	7-epi-*α*-eudesmol	2276,2	1715,6	3036,7	3880,4	0,0	0,0	0,0	0,0	2727,2	0,00	7,9E+00	1,5E-04
6,1	3,3,6,6-tetramethyl-1,4-cyclohexadiene	2253,7	1636,6	0,0	1863,8	0,0	0,0	0,0	0,0	1438,5	0,00	4,6E+00	2,5E-02
15,5	(+)-maaliol	2240,6	1839,1	1978,6	4887,8	0,0	0,0	0,0	0,0	2736,5	0,00	7,4E+00	1,4E-04
10,4	*α*-gurgujene	2189,8	1412,6	3092,8	4924,3	5653,4	141970,0	11694,0	13144,5	2904,9	43115,50	6,7E-02	3,9E-02
11,1	7-epi-*α*-selinene	0,0	0,0	0,0	0,0	3930,6	1190,3	6388,1	11206,9	0,0	5678,98	4,2E-02	9,2E-04
11,4	valerena-4,7(11)-diene	0,0	0,0	0,0	0,0	2668,2	6406,0	5056,7	6207,9	0,0	5084,71	1,0E-01	3,7E-05
11,9	trans-verbenol	0,0	0,0	0,0	0,0	6138,9	17143,9	69793,0	80223,2	0,0	43324,75	2,8E-02	2,2E-03
12,0	cadina-1(10),6,8-triene	0,0	0,0	0,0	0,0	7430,6	13453,3	9709,0	14210,6	0,0	11200,88	1,3E-01	1,2E-05
12,5	2-hydroxy-1,8-cineole	0,0	0,0	0,0	0,0	4749,3	9203,2	12549,2	14382,4	0,0	10221,04	9,3E-02	8,8E-05
12,9	*δ*-cadinene	0,0	0,0	0,0	0,0	6858,4	19753,3	11698,3	16863,3	0,0	13793,32	9,9E-02	7,7E-05
13,2	(-)-myrtenol	0,0	0,0	0,0	0,0	5393,0	15657,0	55226,4	64287,3	0,0	35140,91	1,2E-01	1,9E-03
13,9	2-hydroxycineole	0,0	0,0	0,0	0,0	218718,4	319894,8	505393,0	609734,8	0,0	413435,25	1,1E-01	8,2E-05
14,5	3β-hydroxy-1,8-cineole	0,0	0,0	0,0	0,0	4101,1	5147,7	7431,1	9799,0	0,0	6619,73	1,2E-01	4,3E-05
14,5	4,5-dehydro-Isolongifolene	0,0	0,0	0,0	0,0	6995,6	15590,4	13646,4	15766,8	0,0	12999,83	1,1E-01	2,9E-05
14,6	geranyl pentanoate	0,0	0,0	0,0	0,0	11076,3	16846,1	22453,0	31109,8	0,0	20371,29	1,1E-01	6,5E-05
15,5	*p*-mentha-1,8-dien-7-ol	0,0	0,0	0,0	0,0	2960,2	7503,6	16229,4	21060,2	0,0	11938,35	5,0E-02	7,7E-04
16,6	*p*-mentha-1(7),2-dien-8-ol	0,0	0,0	0,0	0,0	6465,4	23246,4	19463,4	25006,5	0,0	18545,41	7,0E-02	2,0E-04
16,7	acetic acid, 3-acetylcyclohexyl ester	0,0	0,0	0,0	0,0	3023,8	2816,2	3609,3	3419,8	0,0	3217,29	1,8E-01	7,9E-08
18,3	8-hydroxylinalol	0,0	0,0	0,0	0,0	3683,7	4798,5	8088,3	11389,3	0,0	6989,96	1,1E-01	1,5E-04
18,4	*p*-menth-6-ene-2,8-diol	0,0	0,0	0,0	0,0	6357,0	10291,7	8593,4	12245,6	0,0	9371,93	1,4E-01	8,1E-06
19,4	(+)-*β*-costol	0,0	0,0	0,0	0,0	5916,5	13705,5	11729,3	13445,7	0,0	11199,25	1,1E-01	3,2E-05
20,1	myrtenol	0,0	0,0	0,0	0,0	104576,4	166619,3	192002,3	246083,6	0,0	177320,37	3,1E-02	2,4E-05
20,3	p-menthadien-7-ol	0,0	0,0	0,0	0,0	8900,3	14407,0	13855,8	15206,5	0,0	13092,43	1,4E-01	3,7E-06
20,8	phytol	0,0	0,0	0,0	0,0	45313,3	76176,5	74882,9	101230,7	0,0	74400,85	1,2E-01	1,7E-05
21,8	2-hydroxycineole, acetate	0,0	0,0	0,0	0,0	8067,9	10589,2	9339,9	10857,4	0,0	9713,58	1,7E-01	2,0E-07
22,5	cedranoxide,8,14-	0,0	0,0	0,0	0,0	7650,9	11621,6	12306,1	15541,5	0,0	11780,02	1,3E-01	9,7E-06
23,3	isoaromadendrene epoxide	0,0	0,0	0,0	0,0	46700,9	76593,4	74191,0	91509,3	0,0	72248,66	1,3E-01	8,1E-06

Table 2: Table showing the identified secondary metabolites in the *E. grandis* x *E. urophylla* leaves and *Gonipterus* sp. n. 2 frass

Retention time	**Compound name**	**Leaf**	**Leaf2**	**Leaf3**	**Leaf4**	**Frass**	**Frass2**	**Frass3**	**Frass4**	**Mean Leaf**	**Mean Frass**	**Fold-change**	**p-value**
Retention time	Compound name	Leaf1	Leaf2	Leaf3	Leaf4	Frass1	Frass2	Frass3	Frass4	Mean Leaf	Mean Frass	Fold-change	p-value
6,6	*o*-cymene	582857,8	391681,2	362577,7	399163,8	107809,2	118508,7	38092,9	42522,0	434070,1	76733,2	5,7E+00	3,6E-04
5,7	eucalyptol	432240,9	315641,1	240486,0	283259,1	8347,0	11836,3	20517,9	41956,1	317906,8	20664,3	1,5E+01	6,7E-05
12,2	*α*-terpinyl acetate	97716,4	81184,8	54264,3	58224,1	5886,8	7784,7	9127,5	18963,4	72847,4	10440,6	7,0E+00	3,3E-04
5,6	d-limonene	43445,2	30057,9	24911,4	24892,9	3901,7	5202,8	5632,3	9333,2	30826,9	6017,5	5,1E+00	4,2E-04
6,3	*γ*-terpinene	42348,8	35275,6	19509,7	21753,4	19359,6	24470,7	39042,5	72640,5	29721,9	38878,3	7,6E-01	5,6E-01
17,5	thymol	24250,5	20095,1	15469,3	18722,4	0,0	0,0	0,0	0,0	19634,3	0,0	6,4E+00	1,0E-05
17,1	thymol	22842,8	18361,7	15261,0	16940,5	0,0	0,0	0,0	0,0	18351,5	0,0	6,3E+00	1,3E-05
13,8	trans-calamenene	20189,0	15859,0	14321,7	17360,9	7090,0	8058,4	5450,2	6925,8	16932,7	6881,1	2,5E+00	1,9E-04
17,4	*o*-cymen-5-ol	19067,6	16235,7	13577,8	15175,2	0,0	0,0	0,0	0,0	16014,1	0,0	5,9E+00	3,3E-06
11,1	caryophyllene	15303,1	7914,9	5901,7	6350,1	11847,5	16311,6	9417,2	12438,8	8867,4	12503,8	7,1E-01	1,8E-01
14,7	neophytadiene	13073,9	7749,2	6511,7	10515,9	7203,0	9310,4	10218,6	18823,5	9462,7	11388,9	8,3E-01	5,5E-01
16,8	1,4-cyclohexanediol	11208,9	7212,8	5951,8	7002,7	0,0	0,0	0,0	0,0	7844,0	0,0	6,6E+00	1,4E-04
12,7	bicyclogermacrene	9314,0	6487,3	2580,0	3909,4	33919,9	38773,3	20876,8	26255,0	5572,7	29956,2	1,9E-01	6,5E-04
17,2	thymol	7994,5	6653,0	5006,8	5874,6	0,0	0,0	0,0	0,0	6382,2	0,0	6,0E+00	1,8E-05
5,1	*α*-phellandrene	6629,4	3645,3	2030,5	2047,6	186215,9	257105,3	103538,3	126762,2	3588,2	168405,4	2,1E-02	1,8E-04
9,0	*m*-cymenene	5705,7	6049,1	4743,0	5851,0	4977,0	6348,2	1499,5	1355,2	5587,2	3545,0	1,6E+00	1,5E-01
15,3	neophytadiene	4928,3	2970,2	2376,2	3820,9	2630,1	3296,6	2952,0	5473,2	3523,9	3588,0	9,8E-01	9,5E-01
10,8	fenchol	4099,1	2158,6	2404,6	3037,4	0,0	0,0	0,0	0,0	2924,9	0,0	6,8E+00	1,5E-04
12,5	2-hydroxycineole, acetate	3894,1	2990,2	2477,7	1404,3	1411,1	1800,0	1373,4	1563,9	2691,6	1537,1	1,8E+00	1,1E-05
11,9	humulene	3674,6	1727,3	1420,2	1525,0	3795,9	4771,4	2657,6	3600,2	2086,8	3706,3	5,6E-01	5,0E-02
6,8	*α*-terpinene	3288,9	3699,0	1648,4	1935,5	737,0	986,1	844,9	1440,8	2643,0	1002,2	2,6E+00	1,2E-02
9,7	copaene	3111,3	2436,8	1848,5	1855,8	3825,9	4813,9	3628,1	5019,9	2313,1	4322,0	5,4E-01	4,6E-03
13,2	cubenene	2974,4	2346,1	1567,4	1752,5	5538,5	6054,2	4800,2	7358,0	2160,1	5937,7	3,6E-01	7,2E-04
15,8	(-)-gleenol	2883,7	1781,5	1610,9	2413,5	829,0	1552,5	1290,0	1977,4	2172,4	1412,2	1,5E+00	9,1E-02
16,1	1-10-epi-cubenol	2863,3	2431,1	2366,3	2296,6	2414,2	3334,7	3004,6	4897,2	2489,3	3412,7	7,3E-01	1,3E-01

4,3	*β*-pinene (-)	2155,6	0,0	1482,8	1727,5	8222,2	12140,2	7650,1	11210,7	1341,5	9805,8	1,4E-01	3,3E-04
13,3	*p*-mentha-1(7).8-dien-2-ol	1190,3	1000,5	1066,9	1458,0	0,0	0,0	0,0	0,0	1178,9	0,0	5,9E+00	8,5E-06
6,1	trans-*β*-ocimene	0,0	0,0	0,0	0,0	7003,1	8162,1	8325,7	13541,8	0,0	9258,2	1,5E-01	1,9E-04
10,2	*β*-maaliene	0,0	0,0	0,0	0,0	2193,8	1485,2	1041,1	2219,9	0,0	1735,0	1,2E-01	3,0E-04
11,2	selina-37(11)-diene	0,0	0,0	0,0	0,0	1710,3	758,7	364,4	1205,0	0,0	1009,6	7,2E-02	3,6E-03
11,6	aromandendrene	0,0	0,0	0,0	0,0	2437,9	2615,0	1326,0	1630,3	0,0	2002,3	1,3E-01	2,1E-04
11,9	trans-verbenol	0,0	0,0	0,0	0,0	915,4	1966,0	612,6	683,5	0,0	1044,4	1,2E-01	4,0E-03
12,5	2-hydroxy-1,8-cineole	0,0	0,0	0,0	0,0	913,1	1159,7	1172,0	1225,4	0,0	1117,6	1,6E-01	1,1E-05
14,0	2-hydroxycineol, acetate	0,0	0,0	0,0	0,0	49309,6	61723,6	46093,9	66725,7	0,0	55963,2	1,8E+00	6,8E-02
14,8	*α*-phellandrene dimer	0,0	0,0	0,0	0,0	100275,4	141133,2	35710,4	46274,9	0,0	80848,5	7,7E-02	4,9E-03
15,0	neophytadiene	0,0	0,0	0,0	0,0	7389,8	11628,5	4874,3	5801,8	0,0	7423,6	1,3E-01	6,5E-04
15,4	*α*-phellandrene dimer	0,0	0,0	0,0	0,0	12677,4	16294,1	3590,1	4661,5	0,0	9305,8	8,8E-02	7,6E-03
15,6	*α*-phellandrene dimer2	0,0	0,0	0,0	0,0	11334,6	16014,8	3775,8	5542,3	0,0	9166,9	8,2E-01	4,8E-03
16,0	*p*-mentha-1,4-dien-7-ol	0,0	0,0	0,0	0,0	3468,1	4654,0	3273,0	6066,9	0,0	4365,5	1,5E-01	1,6E-04
16,4	*p*-cymen-7-ol	0,0	0,0	0,0	0,0	4852,8	5258,2	1187,8	1629,2	0,0	3232,0	7,3E-02	7,5E-03
16,7	(+)-spathulenol	0,0	0,0	0,0	0,0	3314,0	1203,7	1256,3	1100,3	0,0	1718,6	1,3E-01	4,7E-03
19,6	ipsenone	0,0	0,0	0,0	0,0	8852,7	11409,2	10420,3	13065,0	0,0	10936,8	1,6E-01	6,3E-06
20,1	p-menth-6-ene-2,8-diol	0,0	0,0	0,0	0,0	13606,4	16534,9	17070,9	20648,4	0,0	16965,2	1,6E-01	8,2E-06
20,8	phytol	0,0	0,0	0,0	0,0	12893,9	23877,7	20339,1	28157,5	0,0	21317,1	1,2E-01	1,9E-04
22,5	arctiol	0,0	0,0	0,0	0,0	10468,0	17736,7	8670,9	12194,9	0,0	12267,6	1,4E-01	2,1E-04