Investigating the degradation of diterpene resin acids by the microbiome of the large pine weevil *(Hylobius abietis)*

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Abbreviations

AA-OH- Hydroxylated abietic acid

AA-OH Glucoside- Hydroxylated abietic acid Glucoside

ASV- Amplicon sequence variant

DHAA- Dehydroabietic acid

DHAA-OH- Hydroxylated dehydroabietic acid

DHAA-OH- Glucoside- Hydroxylated dehydroabietic acid Glucoside

DHAA Glucoside Ester- Dehydroabietic acid Glucoside Ester

DHAA-OH Glucoside Ester- Hydroxylated dehydroabietic acid Glucoside Ester

dit- Diterpene resin acid degradation gene cluster

DRA- Unidentified hydroxylated diterpene resin acid

LC-Q-TOF-MS-Liquid chromatography Quadrupole time-of-flight mass spectrometer

w/w- Weight/ Weight

w/v- Weight/Volume

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Abstract

The gut microbiome of herbivores can play an important role degrading the plant defensive chemical. *Hylobius abietis* (large pine weevil), is a significant pest of young conifer trees has able to overcome the chemical defences of conifers. Conifers possess different defensive chemical compounds, with diterpene resin acids representing a prominent class. Diterpenes such as abietic, dehydroabietic, isopimaric, neoabietic, palustric and pimaric, acid, which play a pivotal role in conifer ecological and biological defences. Previous research has shown that the gut microbiome of these weevils may play a role in the degradation of secondary metabolites present in conifers. This investigation delves into the symbiotic relationship between the large pine weevil and its gut microbiota in the context of overcoming plant defences and sheds light on the complex ecological interactions amongst plants, herbivores, and microbes.

In this study, I investigated changes in the degradation patterns of dehydroabietic acid and abietic acid following manipulations of the gut microbiome, as well as the impact of antibiotics on both the gut microbiome and the weevil (performance and mortality test). Furthermore, we assessed the dehydroabietic acid degradation capacity of a non-conifer feeding weevil, *Hypera postica* (alfalfa weevil). The experimental results reveal that pine weevils exhibit the capability to degrade dehydroabietic acid and abietic acid even after changes in the gut microbial community at the genus and family taxonomic levels. Notably, there were no differences in the degradation products of dehydroabietic acid and abietic acid between weevils after different types and concentrations of antibiotic treatments. Additionally, the antibiotic treatment did not appear to affect pine weevil performance and mortality in a significant manner. It was found that the ability of dehydroabietic acid degradation of the alfalfa weevil closely resembled that of the pine weevil. These findings collectively suggest that both pine weevils and other non-conifer feeding insects possess the capability to degrade diterpene resin acids independently. Furthermore, altering the gut microbiome does not appear to have a negative impact on weevil survival and performance. This research contributes towards our preliminary understanding of the degradation of diterpene resin acids by pine weevils and raises questions about the ability of non-conifer feeding insects to participate in the degradation of conifer defences.

1. Introduction

The complex relationship between plant secondary metabolites and insects has been a fascinating and important aspect in ecological research. More than 200,000 different plant secondary metabolites have been identified, and researchers continue to discover new ones each year (Hartmann, 2007). These compounds play a vital role in helping plants respond to various biotic or abiotic stress (Hartmann, 2007; Neilson et al., 2013). Initially, these compounds were perceived as wasteful products of plant metabolism (Seigler, 1998). However, it is now widely accepted that plants have well-defined metabolic pathways dedicated to producing these secondary metabolites (Seigler, 1998). Since then, there has been a surge of interest in studying these plant secondary metabolites as defensive mechanisms. These chemicals are important components of a plant's defence strategy and can exhibit toxicity or act as natural repellents towards herbivorous insects (Wink, 1988). Their mode of actions can include destruction of cell membranes, inhibiting the transportation of nutrients and ions, blocking communication signals, interfering with metabolism, or disruption of the hormonal control of physiological processes (Mithöfer and Boland, 2012).

Conifers have a variety of mechanisms to defend themselves against herbivores. They use both physical and chemical defences, and one particular group of defensive chemicals are called terpenes. The three types of terpenes in conifers are monoterpenes (C10), sesquiterpenes (C15), which are volatile in nature and diterpenes (C20) which are non-volatile. In most conifers, oleoresin has similar quantities of monoterpenes and diterpenes, and small amounts of sesquiterpenes (Kolosova and Bohlmann, 2012).

Recently the role of gut microbes has been recognised in facilitating herbivores in the degradation of plant defensive chemicals and influencing the complex interaction between insects and plants (Feldhaar, 2011; Douglas, 2013). Microbial symbionts play multiple roles, including the degradation of various plant compounds, supplementation of essential nutrients (Douglas, 2009) and degradation of plant defensive chemicals (Hammer, 2015). Microbes are effective in overcoming plant chemical defences due to their high diversity in catabolic pathways for processing chemical substances; microbes have a short generation time, enabling them to quickly adapt to plant chemicals. (Hammer, 2015).

1.1 Conifer oleoresins

Terpene-rich oleoresins are an important component of the conifer defences. This oleoresin is a complex blend of volatile monoterpenes (C10), volatile sesquiterpenes (C15), & non-volatile diterpenes (C20), and it acts as a strong defence against herbivores (Schmidt et al., 2010). It's stored in special structures in the tree, such as resin ducts, vesicles, blisters, glands, and cells; when these structures are damaged by herbivores, oleoresin is released and act as chemical and physical barrier against herbivores (Celedon and Bohlmann, 2019).

Terpenes in conifer oleoresin have ecological roles in interactions with insects. For instance, in the case of bark beetles (*Ips typographus*), these terpenes act as signalling molecules to identify their host trees; terpenes also influence various aspects of insect behaviour, including their eating habits, grouping tendencies, and reproductive activities (Raffa, 2014). Additionally, terpenes can either directly harm insects as toxins and feeding barriers or as attractants of parasitoids and predators (Seybold et al., 2006).

1.2 Diterpene resin acids

Diterpene resin acids are 20-carbon tricyclic carboxylic acids with various structural variations introduced through double-bond isomers, diastereoisomers, and additional functional groups. These chemicals are non-volatile, viscous in nature and many conifer species produce tricyclic diterpene acids like abietic, dehydroabietic, isopimaric, levopimaric, neoabietic, palustric, pimaric, and sandaracopimaric acid. (Figure 1) (Schmidt et al., 2005; Nagel et al., 2022).

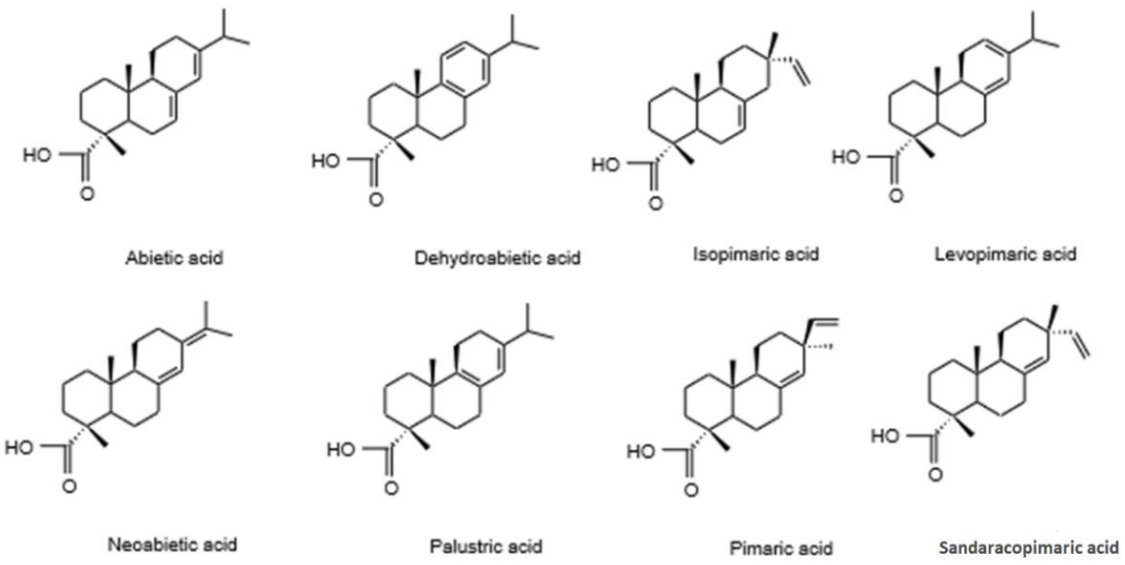

Figure 1. Examples of conifer diterpene resin acids.

Conifer diterpene resin acids act as defensive compounds against herbivores. There are many examples of this, such as the resistance of Sitka spruce (*Picea sitchensis)* and white spruce (*Picea glauca*) to the white pine weevil (*Pissodes strobi*) when they have higher diterpene resin acid concentrations (Byun-McKay et al., 2006; Keeling and Bohlmann, 2006). Certain species of spruce trees show increased resistance to pests when they have higher diterpene resin acid concentrations. When inducing terpenoids by methyl jasmonate in Norway spruce (*Picea abies*), inhibiting colonization by bark beetles (*Ips typographus*) (Erbilgin et al., 2006; Zhao et al., 2011). Inducing resin by methyl jasmonate in maritime pine (*Pinus pinaster)* showed negative effects towards large pine weevils (*Hylobius abietis)* (Sampedro et al., 2010). Similarly, when multiple terpenes were induced in ponderosa pine by methyl jasmonate (*Pinus ponderosa*), it negatively affected mountain pine beetle (*Dendroctonus ponderosae*) associated fungi (Keefover-Ring et al., 2015). Not much is known about the mode of action of these compounds but their toxicity might be due to loss of chemiosmotic control because of highly lipophilic nature of many of these compounds and targets cell membranes (Gershenzon & Dudareva, 2007).

Diterpenoids also exhibit antimicrobial properties. For instance, isopimaric acid has been found to inhibit the growth of multidrug-resistant and methicillin-resistant *Staphylococcus aureus* (Savluchinske-feio et al., 2006). Moreover, diterpene extracts from the leaves and twigs of *Chamaecyparis pisifera* have demonstrated antibacterial activity (Fukui et al., 1978; Savluchinske-feio et al., 2006). Moujir and Gutierrez-Navajo (1996) confirmed that the presence of the catechol group is crucial for this antimicrobial activity. Catechol is a type of benzenediol with a benzene core that has two hydroxy substituents positioned adjacent to each other. Its functions include acting as a genotoxin, an allelochemical, and a plant metabolite (PubChem (n.d.)).

1.3 *Hylobius abietis*

Hylobius abietis (Coleoptera: Curculionidae), or the large pine weevil, is a significant insect pest to young conifer forests (Figure 2). Its native range is predominantly in the western Palaearctic region. It is distributed throughout Europe, ranging as far south as Armenia and Turkey and as far north as Fennoscandia and the United Kingdom. However, there have been observations of the species in other regions extend to eastern Russia, China, Japan, and even New Zealand (UK Beetles 2023). The pine weevil feeds on the phloem and bark of young conifer trees and seedlings from various conifer species, resulting in substantial tree mortality (Suárez-Vidal., et al., 2017). They feed on several conifer species, including Scots pine *(Pinus sylvestris*), Sitka spruce *(Picea sitchensis),* Douglas fir *(Pseudotsuga menziesii*), western hemlock (*Tsuga heterophylla*), Corsican pine (*Pinus nigra*), lodgepole pine *(Pinus contorta*), and Norway spruce (*Picea abies*) (Forest Research 2023)

Figure 2. Large pine weevil (*Hylobius abietis)* feeding on a branch of young Norway spruce (*Picea abies)* tree. The pine weevil feed on the phloem and bark of young conifer trees and seedlings from various conifer species.

Pine weevil, develops in the conifer stumps and roots. Adult weevils lay eggs in the notches on roots, and the larvae go through four larval moults before pupating; depending on the quality of the conifer stumps and microclimate, pupation can occur within a year or can be delayed, with some weevils overwintering in pupal chambers; adults emerge in spring, feed on the conifer bark, and oviposit in the fresh stumps, with the oviposition period lasting from May to September (Leather et al., 1999). The average adult weevil lives for four years. (Leather et al., 1999).

This pest can destroy approximately 50% of newly planted conifer trees in an unprotected reforested site. In the most severe situations, it can destroy all of the young trees, even after use of insecticides (Forest Research 2023). In a 2019 study conducted by L*ó*pez-Villamor et al, it was observed that in *Pinus pinaster* and *Pinus radiata* plantations, 85% of newly planted seedlings were attacked by pine weevils causing a 45% mortality rate in both species. The pest is a significant obstacle to successful forest

regeneration in numerous European regions, particularly in areas where forest management involves clear-cutting followed by replanting (Björkman et al., 2015; Lalík et al., 2021). In a newly cleared area, stumps and logging debris release tree volatiles, attracting pests like the pine weevil. Pine weevils lay eggs in the soil and roots of conifer trees and their life cycle continues for several years after clear-cutting, making newly planted seedlings in clear-cut sites susceptible to pest attack (Rahman et al., 2018).

1.4 Degradation of diterpene resin acids by microbes

The initial concept of degradation of diterpene resin acids by microbes emerged from the microbes degrading on these compounds from toxic wastewater produced in paper manufacturing. It was observed that aerated and activated sludge treatment effectively removed diterpene resin acids (Stuthridge et al., 1991; Kostamo and Kukkonen, 2003). Bicho et al., 1995, investigated the degradation of resin acids in five bacteria isolated from a bleach mill effluent and found that these bacteria could degrade diterpene resin acids, and even use them as their sole carbon source for growth. In 1999, Martin and Mohn described a diterpenoid degradation pathway in *Pseudomonas abietaniphila* BKME-9. Many gene clusters associated with the catabolism of diterpene resin acids, known as *dit* gene cluster have been identified in bacteria capable of degrading these compounds. Studies have revealed the presence of gut bacteria with *dit* genes in conifer-feeding insects. The gut bacteria *Serratia, Pseudomonas,* and *Rahnella* found in *Dendroctonus ponderosae* were found to possess a significant number of terpene degradation genes (Adams et al., 2013). Another study showed that the gut microbial community in large pine weevils, which feed on spruce, contains 10 *dit* genes (Berasategui et al., 2017). A 2016 study by Berasategui et al. revealed that the gut microbial community in large pine weevils is similar to that of other conifer-feeding beetles but differs from closely related non-conifer-feeding weevils, indicating that the microbial community in these insects is influenced by their host plants.

1.5 Previous study on degradation of diterpene resin acid by large pine weevils

In a prior study conducted by Berasategui et al., 2017, the authors focused on investigating the role of the gut microbiome in large pine weevils in the degradation of diterpene resin acids. Their aim was to assess the capability their gut microbiota to break down diterpene resin acids. Their observations indicated that weevils and their gut microbes, when cultured on diterpene resin acid containing media, were capable of degrading these compounds. However, a significant increase in diterpene resin acids was observed in faeces after a 0.3% concentration antibiotic treatment with rifampicin. A metagenomic analysis of the gut bacteria in weevils revealed the presence of 10 *dit* genes. However, they observed only 1 *dit* gene after the antibiotic treatment. Although diterpene resin acids had no toxic effects on weevils' mortality, but a decrease in egg laying and egg hatching success was observed. This suggests that these gut microbes play a role in enhancing weevil fitness.

1.6 Potential degradation pathway and products by large pine weevils.

In an ongoing experiment on the degradation of diterpene resin acids by pine weevils in our research group, the potential degradation products of two diterpene resin acids i.e., dehydroabietic acid (DHAA) and abietic acid, have been identified (Kshatriya, unpublished data). Potential degradation pathways of DHAA and abietic acid have also been described (Figure 3).

DHAA undergoes hydroxylation into DHAA-OH and which further undergoes glycosylation to form DHAA-OH Glucoside. Another parallel degradation involves the conversion of DHAA into DHAA Glucoside Ester, which is further hydroxylated to form DHAA-OH Glucoside Ester. Abietic acid undergoes hydroxylation into two isomers of AA-OH. These two isomers then undergo glycosylation into AA-OH Glucoside.

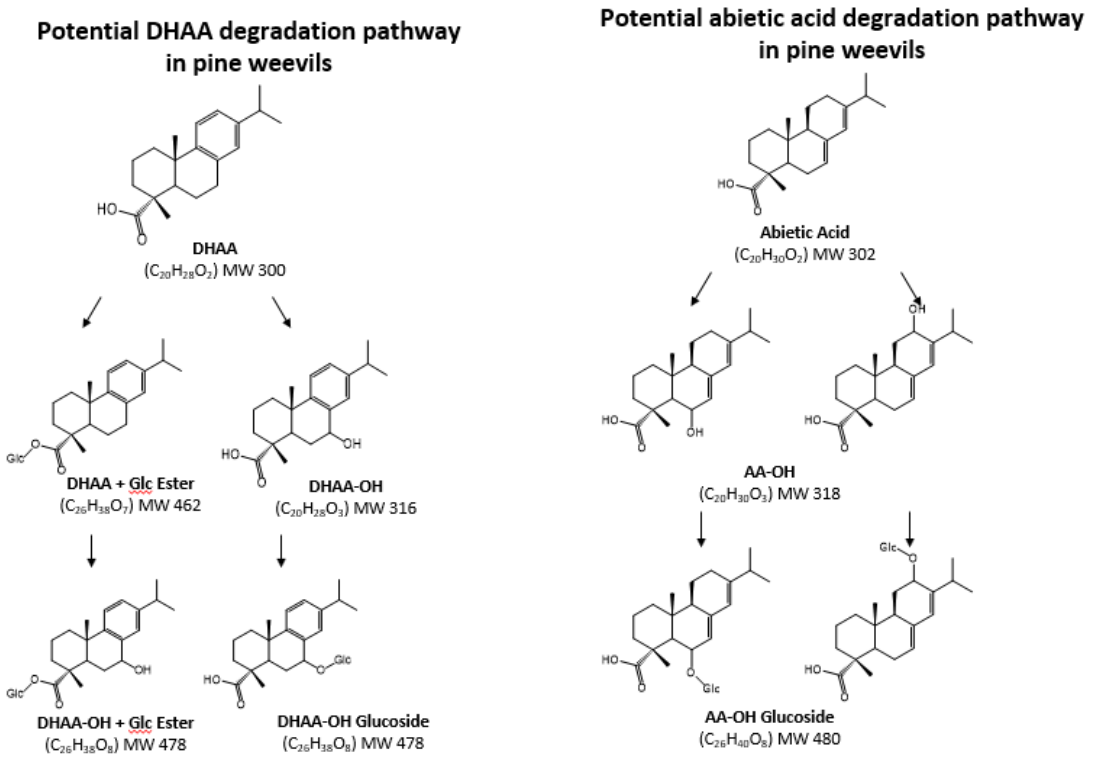

Figure 3. Predicted degradation pathways of dehydroabietic acid (DHAA) and abietic acid in large pine weevils (unpublished data by Kshatriya).

1.7 Aim of the study

The primary aims of this thesis are to investigate the role played by the gut microbiome in large pine weevils with regard to the degradation of conifer diterpene resin acids. The thesis sought to delve deeply into the symbiotic relationship between the large pine weevil and its gut microbiota in the context of overcoming the chemical defences of conifers. In this thesis, I am interested in potential changes in the degradation patterns of dehydroabietic acid and abietic acid following manipulations of the gut microbiome of the pine weevil. This included examining the impact of antibiotics on both the gut microbiome and the weevil's performance and mortality. This helps us better understand how the weevils gut microbiome contributes to their ability to withstand the natural defences of conifers. Furthermore, I am interested into the ability of insects that have never consumed oleoresin-containing plants to break down diterpene resin acids. This study will provide insights into how a wider range of insect species might handle these toxic compounds found in conifers.

2. Material and Methods

2.1 Chemicals

Name	Manufacture
Alpha-cellulose	Sigma-Aldrich, USA
Dextrose monohydrate	Sigma-Aldrich, USA
Dehydroabietic acid (DHAA)	Wako Pure Chemical Ltd, Japan
Disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$)	Carl Roth GmbH + Co. KG, Karlsruhe
Monopotassium phosphate (KH_2PO_4)	Carl Roth GmbH + Co. KG, Karlsruhe
Potassium chloride (KCl)	Carl Roth GmbH + Co. KG, Karlsruhe
Potato starch	Südstärke GmbH, Schrobenhausen
Rifampicin (used in antibiotic diet)	Duchefa Biochemie, Netherland
Rifampicin (used inspruce branch treatment)	Thermo Fisher Scientific Inc., USA
Sodium chloride (NaCl)	Carl Roth GmbH + Co. KG, Karlsruhe
Soyabean flour	Bauck GmbH, Rosche
Soybean oil	ThermoFisher GmbH, Bremen
Streptomycin sulphate	Duchefa Biochemie, Netherland
Sucrose crystallized	Duchefa Biochemie, Netherland
Vanderzant vitamin	Sigma-Aldrich, USA

2.2 Buffer solution

Name	Ingredients
0.5 L Phosphate Buffered Saline (PBS buffer) (1X) pH=7.2	4gm NaCl 100mg KCl 890mg $Na_2HPO_4.2H_2O$ 123mg KH_2PO_4

2.3 Instruments

Name	Manufacture
Autoclave	HP Labortechnik GmbH, Bruckmannring
Balance BP211D	Sartorius AG, Göttingen
Balance PG1003-S	Mettler-Toledo International Inc., Switzerland
Centrifuge 5417R	Eppendorf SE, Hamburg
Centrifuge 5910R	Eppendorf SE, Hamburg
LC-Q-TOF-MS	Bruker Corporation, USA
Micro-centrifuge GMC-060	Daihan Labtech Co., Ltd., South Korea
Millipore Milli-Q	Merck KGaA, Darmstadt
NanoDrop2000c Spectrophotometer	Thermo Fisher Scientific Inc., USA
pH meter 526	WTW, Dinslaken
Stereo microscope stemi 2000-C	Carl Zeiss Microscopy GmbH, Jena
Thermomixer comfort	Eppendorf SE, Hamburg
Vortex-Genie 2	Scientific Industries, Inc., USA

2.4 Software, website and database

Name	Purpose
Bruker Compass Data Analysis	Analysis of LC-Q-TOF-MS data
Brucker QuantAnalysis	Analysis of LC-Q-TOF-MS data
ChemDraw JS	Drawing of chemical structures
EZBioCloud	16s rRNA metagenomic database
Microsoft Office	Creation of figures
R-Studio	Statistical analysis and creation of figures

2.5 Beetle collection, maintenance and plant material

Adult *Hylobius abietis* (large pine weevil) were collected and provided by the Department of Ecology at the Swedish University of Agricultural Sciences. Weevils were collected in June 2022 from central Sweden, kept at 10°C and later were transported to Jena, Germany. Insects were fed with fresh spruce twigs every two weeks and stored at 10°C in ventilated plastic boxes (21x27x17 cm^3) that were changed every week. Spruce plants were bought from pflanzmich.de and kept outdoors. The weevils were allowed to adapt to room temperature for at least one week before each experiment was conducted. For quantification of diterpene resin acids from spruce twigs, needles were removed from the bark, the bark was pulverized in liquid nitrogen with a mortar pestle, and stored at -80°C.

2.6 Preparation of diet

The artificial diet used for our feeding and antibiotic experiments was prepared according to Salem et al. 2014, with slight modifications. The artificial diet was used to prepare antibiotic diet of different concentration, DHAA diet (1% w/w concentration) and abietic acid diet (1% w/w concertation).

Table 1. Composition of the artificial diet for large pine weevils

Component	Amount
Soybean flour	7.5 g
Cellulose	6.25 g
Wheat germ	5 g
Potato starch	3.75 g
Dextrose	3.75 g
Sucrose	1.25 g
Vanderzant vitamin	1 g
Soybean oil	5 ml
Double distilled water	20 ml

2.7 Gut microbiome manipulation of pine weevils and degradation of diterpene resin acid

2.7.1 Antibiotic treatment of 0.5 % (w/w) and degradation of DHAA

Two antibiotics, rifampicin and streptomycin sulphate, were used to prepare two antibiotic artificial diets at a concentration of 0.5% (w/w) by modifying the standard weevil artificial diet. Two groups of 21 weevils each were fed the respective antibiotic diets. Each group was further divided into three subgroups, and each group contained seven weevils that were fed the diet in a box for 12 days. The diet was changed every day and the boxes were changed every other day. To keep insects hydrated a 1.5 ml Eppendorf tube filled with water, plugged with paper towel and placed in each box. Dead insects were removed every day. After antibiotic treatment, the weevils were moved to a clean glass Petri dish and starved for two days. Then, they were fed an artificial diet containing DHAA at a concentration of 1% (w/w) for one day. Finally, they were transferred to a fresh glass Petri without any food for one day to collect insect faeces. The same was repeated two more times for a total of three replicates. Faecal samples were collected and weighed in 4-ml glass vials and stored at -20°C until metabolite analysis.

2.7.2 Antibiotic treatment of 1% (w/w) and degradation of DHAA, abietic acid and spruce bark

One control artificial diet and three modified diets with antibiotic addition at a concentration of 1% (w/w) were prepared using rifampicin, streptomycin sulphate and their combination (0.5% (w/w) each). Four groups of 36 weevils were fed one of the diets for eight days in a box (21x27x17 cm) that was cleaned every other day. A 50 ml Falcon tube with water, closed with paper towel was placed in each box to keep insects hydrated. Number of alive insects were counted and noted each day for survivorship analysis and dead weevils were removed every day. After day eight, the weevils were starved in a glass Petri plate for one day before being divided into three equal groups and fed with DHAA diet (1% (w/w)) for one day. To collect the faeces weevils were kept in different glass Petri dishes in the same three groups without any food source for a day. After that, weevils were fed their respective antibiotic diets for one day to

eliminate the effect of DHAA before being fed with abietic acid diet (1% (w/w)) for one day. The weevils were then starved for one day to collect faeces in a glass Petri plate. Later, the weevils were fed with spruce twigs in plastic box and after two days the insects were starved for another day to collect faeces in petri plate. Thenceforth, they were fed with spruce twigs. All faeces were collected in 4-ml glass vials, weighed and stored at -20°C until metabolite analysis. Final mortality was noted after day 8 of the last faecal collection.

2.8 DNA isolation and 16s rRNA sequencing

Four weevils that were consistently fed spruce, and four weevils from each antibiotic treatment (0.5% (w/w) and 1% (w/w)), were used for DNA isolation. DNA was also isolated from weevils that had been fed on spruce twigs for 10 days after receiving 1% (w/w) antibiotic treatment. A total of 44 weevils were dissected by following instructions from Ceja-Navarro *et al.* 2012. All weevils were killed by keeping them at -20°C for ten minutes. Dead insects were sterilised with 100% ethanol. After sterilisation, they were washed twice in sterile 1X PBS solution. The weevils were dissected under a microscope using dissecting forceps and scissors and the entire gut was extracted carefully. The gut sample was transferred to a 1.5ml Eppendorf tube in liquid nitrogen and later stored at -80°C until DNA isolation.

DNA was isolated in nucleus free water using DNeasy® Blood & Tissue kit by a protocol modified from DNeasy Blood & Tissue handbook supplementary insect protocol.

DNA concentration was measured using a Nanodrop 2000c spectrophotometer. DNA samples were sent for 16s rRNA amplicon metagenomic sequencing and bioinformatics analysis to by Novogene (UK) Company Limited. DNA samples underwent a quality check by Novogene before sequencing. The targeted regions (V3-V4) were amplified using PCR with specific primers linked to barcodes. The PCR products from each sample were pooled, end repaired, A-tailed and then further ligated with Illumina adapters. Libraries were sequenced on a paired-end Illumina platform to provide 250bp paired-end raw reads. The obtained raw data was cleaned up. The sequence of each of the generated ASVs was annotated to learn more about the associated species and the

frequency distribution based on the species. The species of each ASV are annotated with a pre-trained Naive Bayes classifier by the classify-sklearn algorithm of QIIME2. The results of the ASV annotations and the feature table were used for the abundance of the species at different phylogenetic levels. The provided ASV sequences results were compared with EzBioCloud database for final analysis.

2.9 Testing the impact of rifampicin on weevil feeding behaviour, weight, and survival

To test the effect of rifampicin on the weevils, a performance and survivorship study was performed. Three concentrations of rifampicin, i.e., 0% (w/v), 5% (w/v) and 10% (w/v) suspended in Milli-Q water were used for the following test. Forty-five weevils were used for the test in groups of fifteen weevils for each concentration. Similarly, fifteen fresh, four-centimetre-long spruce twigs were used as the experimental feeding source for each concentration. The twigs were dipped in the antibiotic suspension for 30 seconds and then air-dried on a paper towel for 30 minutes. The suspension was swirled each time before dipping the twigs. Insects were weighed at the beginning of the experiment. The individual weevils were then fed with a single piece of antibiotic treated twig in a ventilated small plastic box (10x11.5x7.5 cm) with a 1.5 ml Eppendorf tube filled with water and sealed with a paper towel to keep the insect hydrated. After the third day, the antibiotic treated twigs of the same size were replaced and fed for a further four days. On the seventh day, untreated twigs were given and the weevils were fed for another seven days. Weight, bark consumed and mortality were measured on the third, seventh and fourteenth days. Graph paper was used to calculate the bark consumed, using the same area as the consumed bark and later calculated the marked area. After fourteen days, all the weevils were grouped together according to same antibiotic concentration treatment and fed with non-treated branches in a larger box. After another week, mortality was noted. After the conclusion of experiment, all insects were coloured according to their treatment and moved to same box.

2.10 Degradation of DHAA by *Hypera postica* (alfalfa weevil)

Alfalfa weevils were collected in Lucerne, Switzerland and received from Dr. Stefan Toepfer at the Centre for Agriculture and Biosciences International (CABI). The weevils were fed with *Medicago sativa* collected at the Beutenberg campus, Jena, Germany in plastic ventilated boxes (10x11.5x7.5 cm). Three rounds of weevil faeces were collected from the weevils fed with the control (alfalfa) and the DHAA-treated alfalfa. DHAA sodium salt solution (5 mg/ml) was added at a volume of 75 µl to each leaf and 200 µl to each five cm long stem of the fresh alfalfa. After the insects were fed with either the control food or DHAA-treated food, they were transferred to a glass Petri dish without a food source for one day and faeces were collected in 4 ml glass vials, weighed and stored at -20°C.

2.11 Chemical analysis

To test the amount of diterpene resin acids in the diet and the degradation of diterpene resin acid by insects, methanol extractions were prepared for chemical analysis. Pure methanol solution was added to five different diterpene resin acid diet samples and all weevil faecal samples collected in 4 ml glass vials during the experiment. The amount of methanol added to each sample was proportionate to the weight of the faeces to achieve a final concentration of 10 mg/ml and was thoroughly vortexed. All methanol samples were centrifuged at 3000 rpm for 15 minutes at room temperature. All methanol extracts from the glass vials except the diet or faecal particles was transferred to new 2 ml glass vials. If the amount of methanol extract was low in volume, a glass insert was placed inside the 2 ml glass vial and methanol sample was transferred into those inserts.

All methanol extractions and a methanol blank were tested for chemical analysis in the LC-Q-TOF-MS instrument. For the structural elucidation, high-resolution MS and MS/MS spectra were recorded using an ultra-high performance liquid chromatography-electrospray ionization-high resolution mass spectrometry system (Ultimate 3000 series RSLC (Thermo Dionex, MA, USA) linked to a Bruker timsTOF, Bremen, Germany). The UHPLC was equipped with a C18 reverse phase column (Zorbax Eclipse XDB-C18, 1.8 µm, 2.1 x 100 mm, Agilent Technologies, Boblingen, Germany) maintained

at 25 °C and operated at 0.3 ml/min with a gradient flow of 0.1% aqueous formic acid (solvent A) and acetonitrile (solvent B) with the following profile: 10% B from 0-0.5 min, 10-90% B from 0.5 to 11 min, 90-100% B from 11-11.1 min, and kept at 100% B until 12 min, then re-equilibrated at 10% B from 12.1 to 15 min. HRMS analyses were conducted independently for positive and negative ionisation using data-dependent MS/MS, an active exclusion window of 0.1 min, a reconsideration threshold of 1.8-fold change, and an exclusion after 5 spectra. Fragmentation was observed on the two most intense peaks after being triggered on an absolute threshold of 50 counts for MS/MS spectra acquired at 12 Hz. The source end plate offset was kept at 500 V, the capillary voltage at 4500 V, the nebulizer gas at 2.8 bar, the dry gas at 8 L/min, and the drying temperature at 280 °C. The quadrupole ion energy was kept at 4 eV (low mass 90 m/z), and ion transfer was carried out with a funnel 1 RF of 150 Vpp, a funnel 2 RF of 200 Vpp, a multipole RF of 50 Vpp, and a deflection delta of 70 V. The mass scan range was between 50 and 1500 m/z at a 12 Hz acquisition rate. Between a collision energy of 20 eV and 50 eV, respectively, collision energies were ramped in a 50:50 manner. In order to calibrate the mass spectrometer using the anticipated cluster ion m/z values, 10 L of a sodium formate-isopropanol solution (10 mM solution of sodium hydroxide in 50/50 (v/v%) isopropanol-water containing 0.2% formic acid) was injected into the dead volume of the sample injection at the start of each chromatographic analysis.

All results for the total amount peak of the compound of interest were analysed in Bruker QuantAnalysis and the chromatograph peaks were analysed in Bruker Compass Data Analysis. The results from only negative ion mode were analysed as the product of interests are better ionized in the negative ion mode.

2.12 Statistical analysis

All statistical analysis was performed in R-Studio. Normality of data was tested by Shapiro-wilk test. Skewness data was log transformed before every statistical tests. For statistical analysis one-way ANOVA was done. The post hoc analysis was done using Tukey test. For survivorship analysis a log-rank statistical test was done. The threshold for the significance level was set at 0.05 ($P < 0.05$). Non-metric multidimensional scaling (NMDS) ordination was used for beta diversity analysis of 16s rRNA results from Novogene.

3. Results

3.1 Diterpene resin acid degradation by large pine weevils

Early studies have showed that gut microbes of pine weevils may play an important role in the degradation of diterpene resin acids. In the following study the degradation of various diterpene resin acids was investigated after the manipulation of the gut microbial community of the large pine weevils. Preliminary experiments with two antibiotic cocktails of streptomycin sulphate, gentamicin, ampicillin and rifampicin at final concentrations of 0.05%(w/w) and 0.1%(w/w) showed that the degradation trends of DHAA between control and antibiotic-treated groups were similar (Figure S1, S2 and S3). In the main experiment, two antibiotics having different modes of action i.e., streptomycin sulphate and rifampicin at 0.5%(w/w) and 1%(w/w) concentration was used. Streptomycin inhibits protein synthesis of microbes in the ribosome whereas rifampicin inhibits bacterial DNA-dependent RNA polymerase.

Metabolites were analysis using LC-Q-TOF-MS from weevil faeces of the control group and the antibiotic-treated groups after consuming the DHAA diet, abietic acid diet, and spruce branch. The main focus of this study was on the breakdown of certain diterpene resin acids, specifically DHAA and abietic acid (Figure 3). To confirm the identity of the targeted metabolites the retention times and mass spectra of the peaks in the chromatograph were compared that of previously identified compounds from the weevil faces (Figure 3). All the degradation metabolites of DHAA and abietic acid were observed in all the samples and similar trend of degradation were observed in the control and antibiotic-treated groups.

The weevils were fed with DHAA 1% (w/w) artificial diet for one day and faeces was collected for metabolite analysis. Chromatography results showed the presence of DHAA and all four potential degradation products of DHAA by pine weevil i.e., DHAA-OH, DHAA-OH Glucoside, DHAA Glucoside Ester and, DHAA-OH Glucoside Ester (Figure. 4).

The weevils were fed with abietic acid 1%(w/w) artificial diet for 1 day and faeces was collected for metabolite analysis. Both hydroxylated compounds of abietic acid i.e., AA-OH and their further glycosylated modification were observed i.e., AA-OH Glucoside (Figure 5),

The observed metabolites from the faeces collected from weevils after being fed on spruce, were DHAA, DHAA-OH, DHAA-OH Glucoside, DHAA Glucoside Ester, both isomers of AA-OH and other unknown hydroxylated diterpene resin acids (DRA) (Figure 6).

In order to obtain more information of all the metabolite quantities, identified peaks were integrated using Bruker QuantAnalysis software to calculate their peak areas. Later, peak areas from the control group and the experimental antibiotic-treated group were compared in R Studio to analyse peak area differences in observed metabolites across different groups.

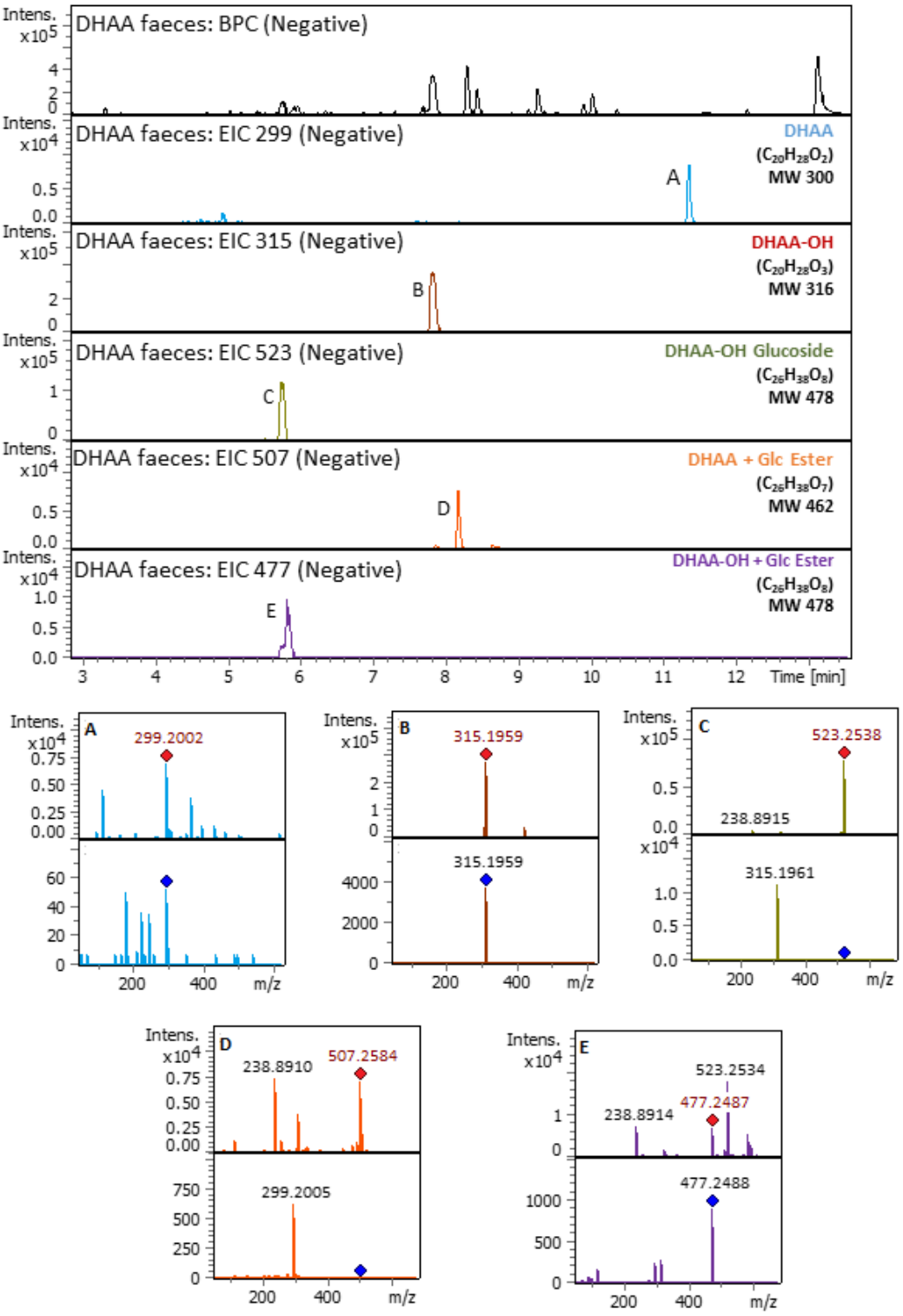

Figure 4. LC-QTOF-MS analysis of DHAA degraded products in large pine weevil faeces after feeding on artificial DHAA diet. Chromatograms and mass spectra of (A) DHAA, (B) DHAA-OH, (C) DHAA-OH Glucoside, (D) DHAA Glucoside Ester, (E) DHAA-OH + Glucoside Ester. BPC= Base peak chromatogram, EIC= Extracted ion chromatogram.

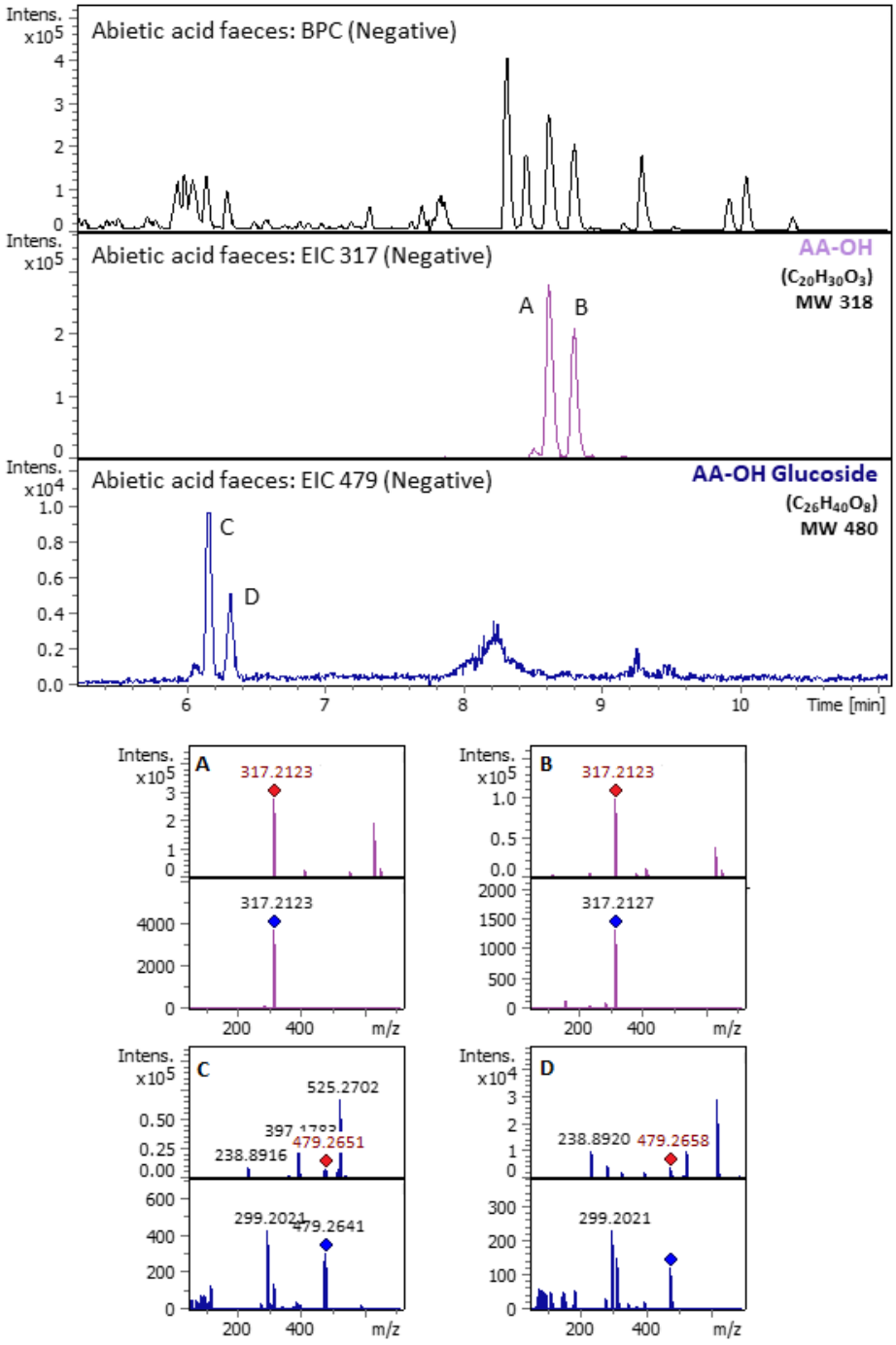

Figure 5. LC-Q-TOF-MS analysis of abietic acid degraded products in in large pine weevil faeces after feeding on artificial abietic acid diet. Chromatograms and mass spectra of (A) AA-OH-1, (B) AA-OH-2, (C) AA-OH Glucoside-1, (D) AA-OH Glucoside-2. BPC=Base peak chromatogram, EIC=Extracted ion chromatogram.

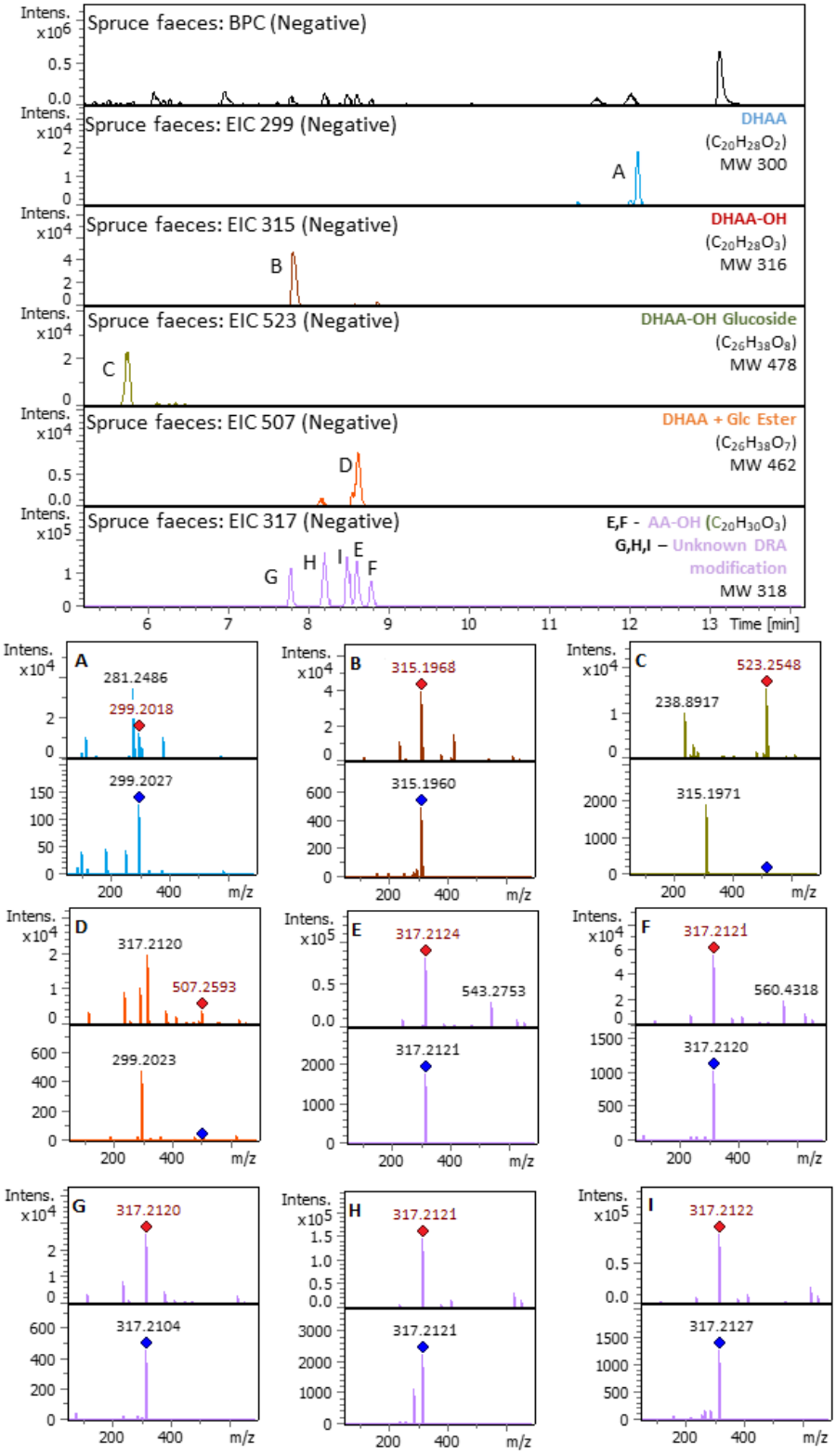

Figure 6. LC-Q-TOF-MS analysis of diterpene resin acid degraded products in in large pine weevil faeces after feeding on spruce branches. Chromatograms and mass spectra of (A) DHAA, (B) DHAA-OH, (C) DHAA-OH Glucoside, (D) DHAA Glucoside Ester, (E) AA-OH-1, (F) AA-OH-2, (G) DRA-1, (H) DRA-2, (I) DRA-3. BPC= Base peak chromatogram, EIC= Extracted ion chromatogram.

3.2 Quantitative analysis of differences in weevil faeces metabolites

The objective of this investigation was to determine the significance of the pine weevil gut microbiome in the degradation of diterpene resin acids. A comparative study of the degradation of DHAA and abietic acid between and antibiotic-treated weevil and non-antibiotic-treated weevil was done. A comparative analysis was conducted to assess variations in metabolite peak areas of present in the faecal samples between the groups.

To manipulate the gut microbial community, the weevils were treated with one of five different artificial diets containing antibiotics: streptomycin sulphate at a concentration of 0.5%(w/w) and 1%(w/w), rifampicin at a concentration of 0.5%(w/w) and 1%(w/w), and a combination of both at a concentration of 0.5%(w/w) each. The control group was fed with an artificial diet with no added antibiotics (see section 2.6). All insect groups studied were fed with DHAA (1%(w/w) concentration) artificial diet after antibiotic treatment and the peak areas of the degradation products in each group was measured using LC-Q-TOF-MS. The results showed no significant difference in the metabolites present in faeces samples between the control group and the antibiotic-treated group (Figure 5). The peak areas of DHAA and DHAA degradation products were compared with the original diet to confirm that degradation was taking place and that the degraded products detected in the weevils' faeces were not present in the original DHAA diet.

The DHAA total peak area was in all faecal samples 91% to 97% smaller than those in artificial DHAA 1% (w/w) diet (Figure 7A). None of the DHAA-modified products were detected in the diet, but they were present in all faecal samples. No significant difference in the peak area of DHAA degradation products was observed among the control group weevils treated with various antibiotic and concentrations ($F(5,12) = 1.654$, $p = 0.22$). The peak area for the hydroxylated modification of DHAA, DHAA-OH (Figure 7B), also did not show a significant difference between the antibiotic-treated and non-antibiotic-treated groups ($F(5,12) = 1.285$, $p = 0.333$). This trend extended to the total peak area of DHAA-OH Glucoside (Figure 7C), where no significant difference was observed among the antibiotic and non-antibiotic treated groups ($F(5,12) = 1.819$, $p = 0.183$). Similarly, for the other two metabolites, DHAA-Glucoside Ester (Figure 7D) and DHAA-OH-Glucoside Ester (Figure 7E), no

significant differences were found among the antibiotic and non-antibiotic treated groups (F (5,12) = 2.264, p= 0.114 and F (5,12) = 1.679, p= 0.07499, respectively).

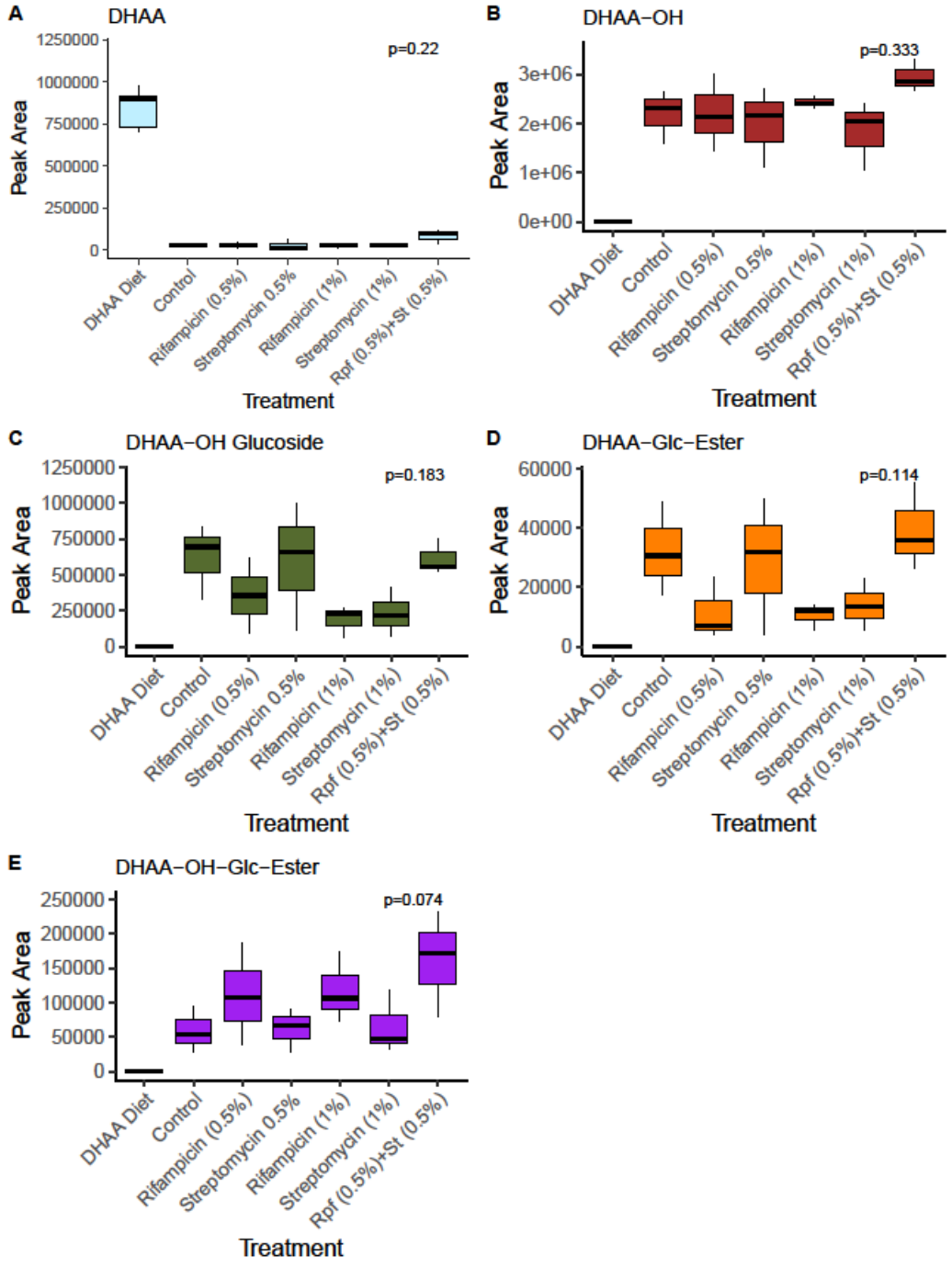

Figure 7. Peak areas of DHAA and its degradation products in faeces of antibiotic-treated pine weevils fed with artificial DHAA diet. Metabolites observed following LC-Q-TOF-MS analysis (A) DHAA, (B) DHAA-OH, (C) DHAA-OH Glucoside, (D) DHAA Glucoside Ester, (E) DHAA-OH Glucoside Ester. Statistical Analysis: One-Way ANOVA.

Another diterpene resin acid, which is easily commercially available as well as one of the most abundant diterpenes in spruce bark, is abietic acid. To investigate if the trend in DHAA degradation within the tested groups aligns with the degradation of another diterpene resin acid an experiment was conducted using abietic acid.

Abietic acid was fed to the control group and to weevils treated with streptomycin sulphate (1%(w/w)), rifampicin (1%(w/w)) and a combination of both at a concentration of 0.5%. Abietic acid and its degradation products peak area from the faecal samples of each group were compared to test for differences in the degradation pattern in each group (Figure 7). The results were also compared with the original diet to confirm that no degradation metabolites were present in the diet and all the modified compounds were processed within weevils. Abietic acid was not detected by LC-Q-TOF-MS in either the diet or the faecal samples, as abietic acid may not have ionised under these conditions. However, both isomers of hydroxylated abietic acid i.e., AA-OH and AA-OH Glucoside were present in all faecal samples (Figure 8), but not in the diet. No significant differences in the peak area of both isomer of AA-OH (Figure 8A and 8B) were observed in the faecal samples among the antibiotic and non-antibiotic treated groups (AA-OH-1 is $F(3,8)= 0.402$, $p= 0.755$ and AA-OH-2 is $F(3,8)= 0.439$, $p= 0.731$). Similarly, no significant differences were observed in peak area of isomers of AA-OH-Glucoside (Figure 8C and 8D) between faecal samples from weevils treated with different antibiotic types and concentrations (AA-OH-Glucoside 1 is $F(3,8) = 0.598$, $p= 0.634$ and AA-OH-Glucoside 2 is $F(3,8)= 0.374$, $p= 0.774$)

To investigate whether the degradation pattern of diterpene resin acids by weevils is similar after they are fed with their natural diet, spruce branches were fed to the weevils. After spruce twigs were fed to the control group and to weevils treated with streptomycin sulphate (1%(w/w)), rifampicin (1%(w/w)) and a combination of both at a concentration of 0.5% (w/w)each, the degradation products of the diterpene resin acids from the faecal samples were compared. No degradation products were present in the spruce bark, but they were present in all the faecal samples from the groups studied (Figure 9). 88-94% of DHAA was degraded in faeces samples of the control group and weevils treated with different antibiotic types and concentrations (Figure 9A). Three unknown products with the same molecular mass and retention time as AA-OH were

observed to have higher peak areas. These are predicted to be the hydroxylated forms of other diterpene resin acids, having identical molecular weight and chemical formula as abietic acid. However, I was unable to identify the specific diterpene resin acid that was being modified. (Figure 9G, 9H, 9I). DHAA-OH Glucoside (Figure 9C) and DHAA Glucoside Ester (Figure 9D) were significantly different from each other among antibiotic and non-antibiotic treated groups ($F(3,8) = 5.736$, $p = 0.0216$ and $F(3,8) = 5.701$, $p = 0.0219$, respectively). After post hoc analysis using Tukey test it was observed that these two metabolites were present in higher amounts in the faeces of the control group compared to faeces samples from weevils which were treated with a combination of streptomycin sulphate 0.5% and rifampicin 0.5% ($p > 0.05$).

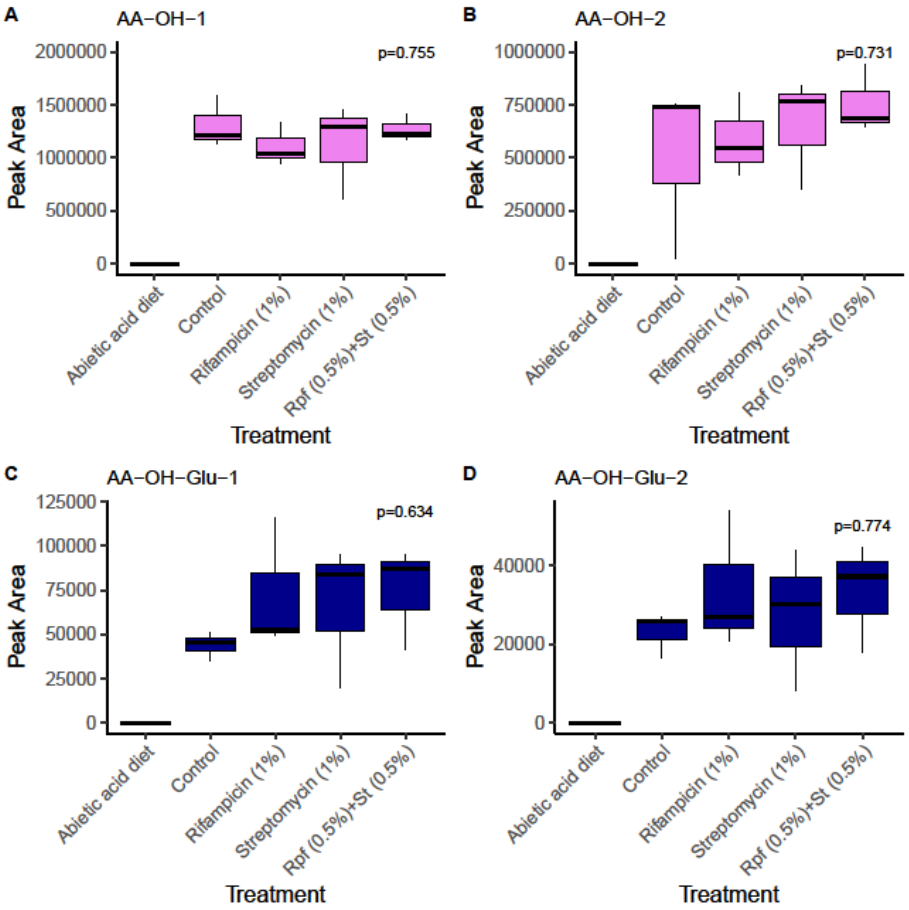

Figure 8. Comparison of peak areas for abietic acid and its degradation products in faeces of antibiotic-treated pine weevils after feeding with artificial abietic acid diet. Metabolites observed following LC-Q-TOF-MS analysis: (A) AA-OH-1, (B) AA-OH-2, (C) AA-OH Glucoside -1, (D) AA-OH Glucoside -2; Statistical Analysis: One-Way ANOVA.

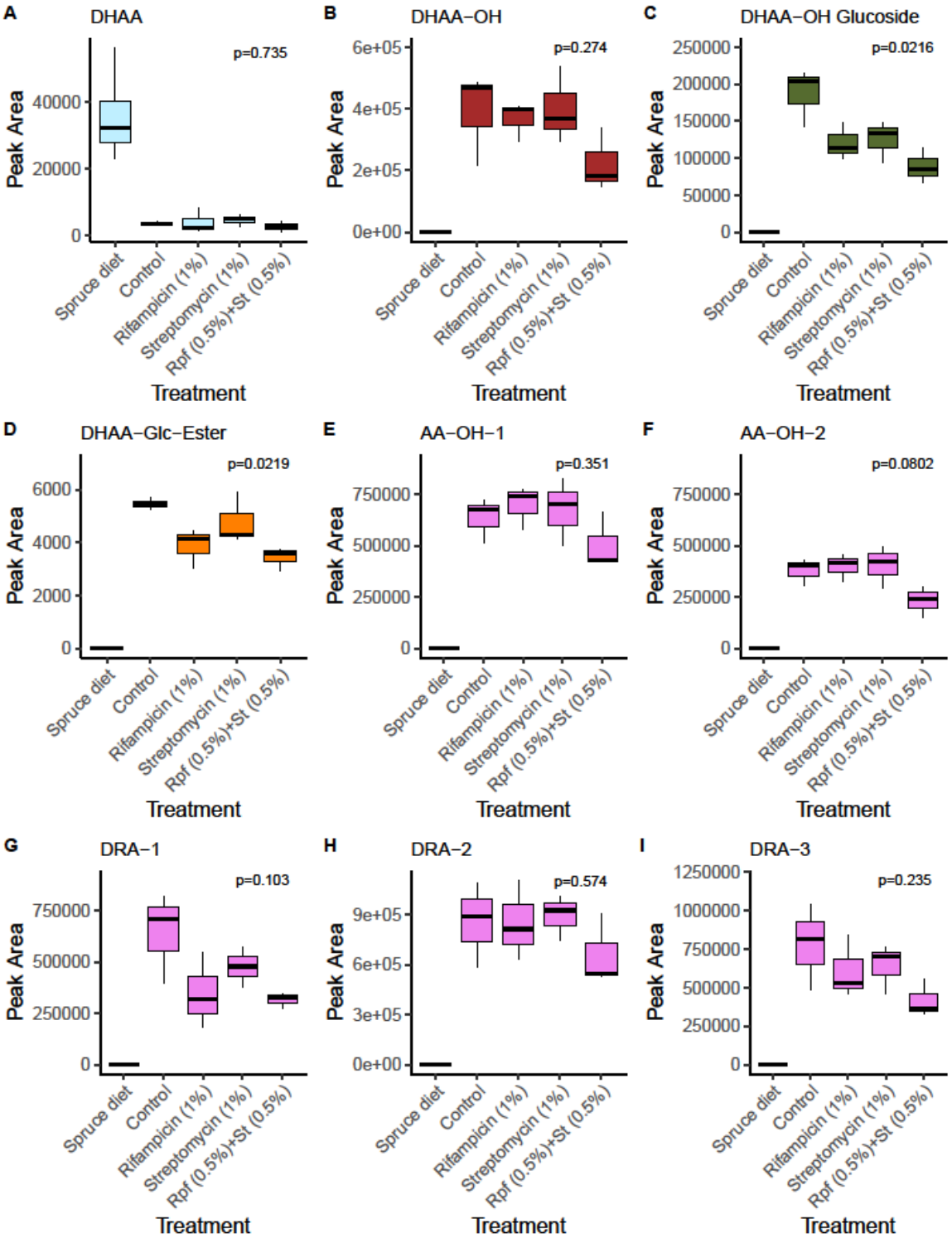

Figure 9. Comparison of peak areas for diterpene resin acids and their degradation products in spruce and faeces of antibiotic-treated weevils after feeding on spruce branches. Metabolites observed following LC-Q-TOF-MS analysis: (A) DHAA, (B) DHAA-OH, (C) DHAA-OH Glucoside, (D) DHAA Glucoside Ester, (E) AA-OH-1, (F) AA-OH-2, (G) DRA-1, (H) DRA-2, (I) DRA-3; Statistical Analysis: One-Way ANOVA.

3.3 Effect of antibiotics on the gut microbial community of large pine weevils

To investigate the impact of antibiotics on the pine weevil gut microbial community, 16S rRNA amplicon sequencing was used to compare the weevil gut microbiome. The study included four weevils that consumed their natural diet, four weevils on a control diet without antibiotics, and four weevils from each of the following antibiotic groups: streptomycin sulphate 0.5% (w/w) (administered for 12 days), rifampicin 0.5% (w/w) (administered for 12 days), streptomycin sulphate 1%(w/w) (administered for 8 days), rifampicin 1%(w/w) (administered for 8 days), and a combination of streptomycin sulphate 0.5%(w/w) & rifampicin 0.5% (w/w) (administered for 8 days).

The amplicon sequence variant (ASV) of the gut microbiome was compared by examining the closest taxonomic matches to assess differences in abundance between various groups. The taxonomic data was provided by Novogene and later was confirmed using the EzBioCloud 16S database. The relative abundance of bacterial taxa in weevil guts differed between weevils that consumed spruce and those that consumed the control artificial diet. The relative abundance was consistent in all samples from weevils fed on the artificial diet, with *Buttiauxella* sp. making up 32-60% of the total abundance. In contrast, the relative abundance of bacterial taxa varied among different individuals in spruce-fed weevils (Figure 10). This was also seen in the beta diversity analysis (Figure 13A), where the microbial communities of weevils fed with artificial diets clustered together whereas scattered in the case of spruce-fed weevils. However, the family Enterobacteriaceae had dominant relative abundance in both the control and spruce-fed weevils. After the weevils were treated with 0.5%(w/w) streptomycin sulphate, the microbial communities in each sample differed. The relative abundance of these communities differed compared to the gut microbial community in weevils that were feeding on their natural diet (Figure 10). Some weevils treated with 0.5%(w/w) streptomycin sulphate, had a higher relative abundance of *Lactococcus lactis*, while others had more *Pseudomonas sp.,* or an unclassified Enterobacterales, and *Wolbachia.* When the weevils were treated with rifampicin (0.5%(w/w)), a different microbial community was observed compare to the control group and weevils feeding on spruce, where *Wolbachia sp.* was most abundant (70-90%) (Figure 10). At both antibiotic treatments, i.e., 0.5%(w/w) streptomycin sulphate and 0.5%(w/w) rifampicin, the

dominance of the family Enterobacteriaceae was not observed as prominently as in weevils fed on spruce or on artificial diet. Dominance of the family Ehrlichiaceae was observed when weevils were treated with 0.5%(w/w) rifampicin, whereas Pseudomonadaceae, Streptococcaceae, Ehrlichiaceae (and in some individuals, Enterobacteriaceae) were observed when weevils were treated with 0.5%(w/w) streptomycin sulphate.

The beta diversity data (Figure 13A) showed differences in gut microbial communities between weevils fed on spruce and artificial diets to the weevils treated with 0.5%(w/w) streptomycin sulphate and 0.5%(w/w) rifampicin diets. Alpha diversity was also studied using Shannon index, Simpson index and species evenness. No significant difference in the alpha diversity of gut microbes between weevils fed on spruce and those on an artificial diet, to the weevils treated with 0.5%(w/w) streptomycin sulphate and 0.5%(w/w) rifampicin was observed (Figure 14) ($p>0.5$).

A noted difference was observed between weevils fed on spruce and those on an artificial diet to weevils treated with streptomycin sulphate (1%(w/w)) where only a few taxa were abundant in the gut microbial community. While there was some variation between individuals, *Wolbachia sp.* was one of the most abundant taxa across all weevils treated with streptomycin sulphate (1%(w/w)), with *Pseudomonas sp.3* also being highly abundant in two of the samples (Figure 11). Another difference was observed when weevils were treated with rifampicin (1%) to weevils fed on spruce, where a high abundance of *Buttiauxella sp.* (60-70%) and *Kluyvera sp*. (25-30%) was seen (Figure 11). The two most abundant microbial taxa, i.e., *Buttiauxella sp.* and *Kluyvera sp*. observed in weevils fed with artificial diet and rifampicin (1%(w/w)) were present in similar quantities. In three out of four samples of the artificial diet, the relative abundance of *Buttiauxella sp.* and *Kluyvera sp*. combined to account for 45% of the gut microbiota. and in one sample, these two species were notably more dominant, making up 80% of the gut microbiota. In contrast, in weevils treated with rifampicin (1%(w/w)), these two taxa constituted an average of 95% of all bacterial taxa observed (Figure 11).

Following a 1%(w/w) antibiotic treatment, an increase in the relative abundance of ASVs in the Enterobacteriaceae family was observed in the gut of pine weevils. When weevils were exposed to 1%(w/w) rifampicin, the gut microbial community closely resembled that of weevils fed a control artificial diet. In contrast, the 1%(w/w)

streptomycin sulphate treatment resulted in a gut microbial community similar to that of the other antibiotic-treated groups at the 0.5%(w/w) concentration (Figure 13A). No significant differences were detected in alpha diversity between the control groups and those treated with 1%(w/w) streptomycin sulphate or rifampicin (Figure 14) ($p>0.05$). The gut microbial community of weevils showed significant variation among samples when exposed to a combination of streptomycin sulphate (0.5%(w/w)) and rifampicin (0.5%(w/w)). Some individuals had a higher relative abundance of the Enterobacteriaceae family, while others had a greater abundance of the Ehrlichiaceae family. Genera such as *Buttiauxella, Wolbachia*, unclassified Enterobacterales, and *Kluyvera* exhibited higher relative abundances in response to the combination of both antibiotics. Beta diversity analysis also revealed varying results, with some weevils displaying gut microbial communities similar to the control group, while others exhibited communities resembling those of other antibiotic-treated groups (Figure 13 A). No significant differences in alpha diversity were observed between the weevils fed on their natural diets, the weevils fed on artificial control diets and the weevils treated with the combination of both antibiotics ($p>0.05$).

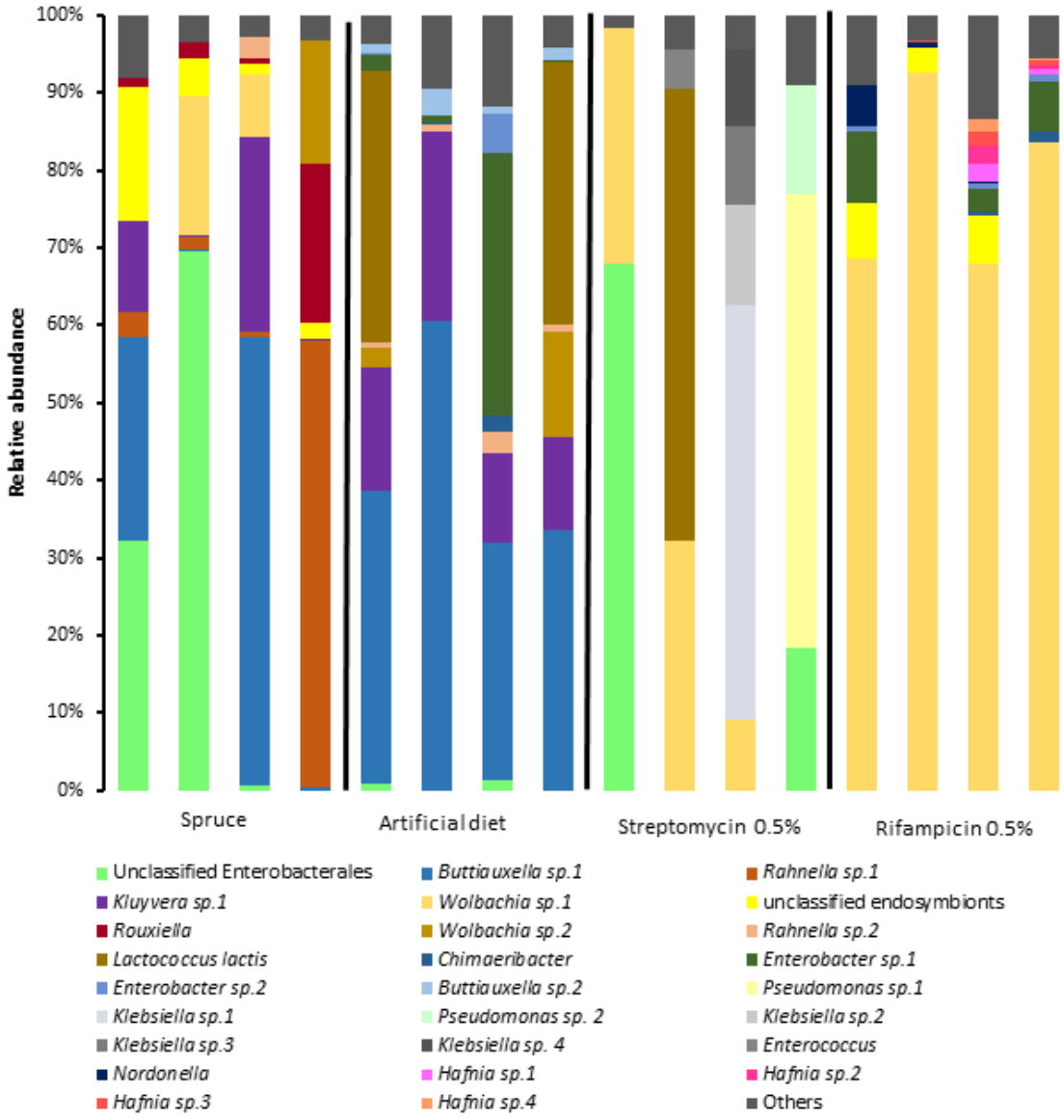

Figure 10. Comparative relative abundance of antibiotic (0.5% w/w) treated pine weevil gut microbiota at the closest taxonomic level. Individual weevils within the same groups were fed with four distinct diets: spruce, artificial diet, streptomycin sulphate (0.5%) diet and rifampicin (0.5%) diet.

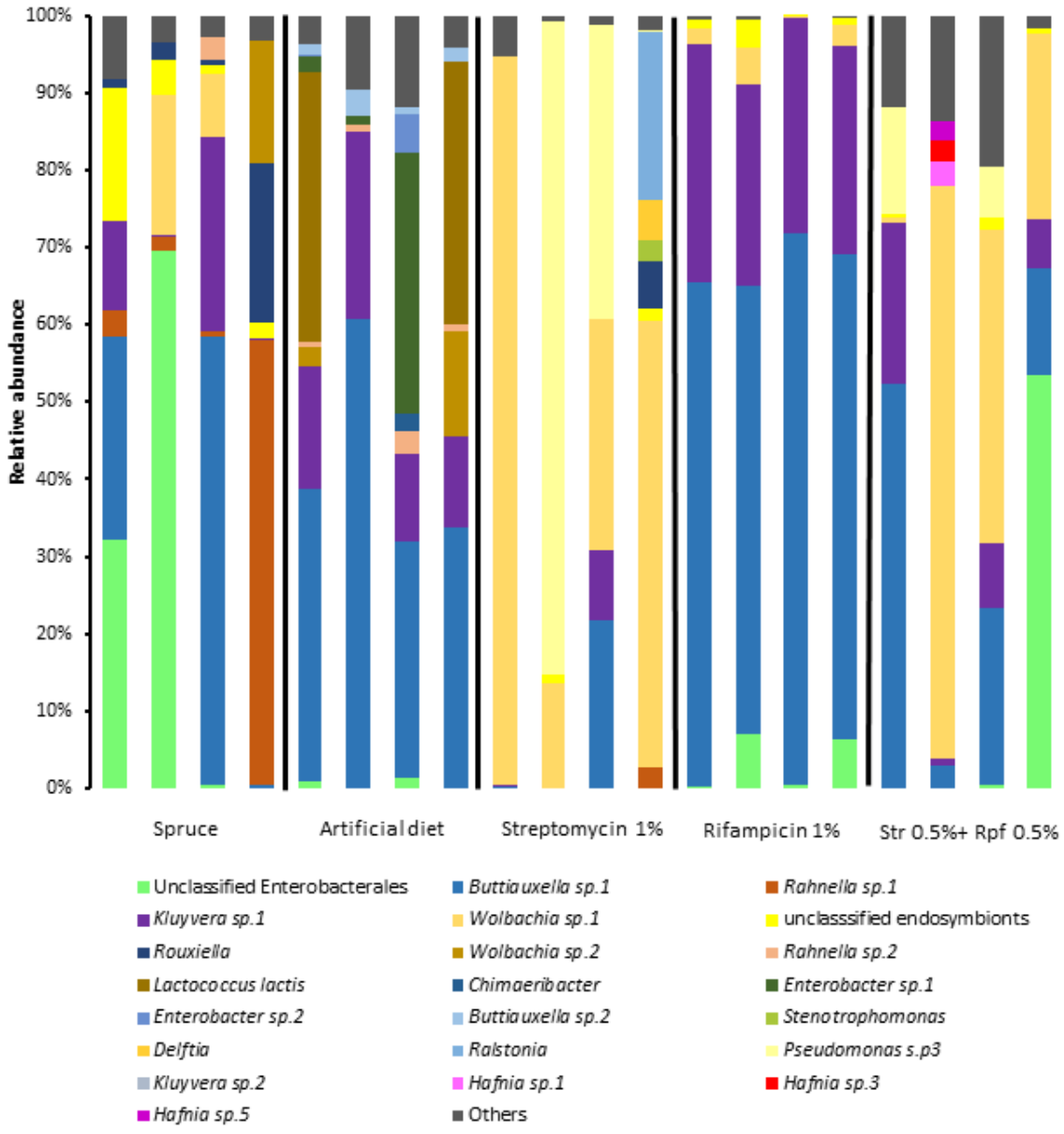

Figure 11. Comparative relative abundance of antibiotic (1% w/w) treated pine weevil gut microbiota at the closest taxonomic level. Individual weevils within the same groups were fed with five distinct diets: spruce, artificial control diet, streptomycin sulphate (1%(w/w)) diet, rifampicin (1%(w/w)) diet, streptomycin (0.5%(w/w)) + rifampicin (0.5%(w/w)) diet

3.4 Changes in gut microbial communities of antibiotic-treated weevils after feeding with spruce twigs

After concluding the antibiotic experiment, a 10-day recovery period was given during which the weevils were fed with their natural diet consisting of spruce branches. The purpose of this recovery period was to examine how the gut microbial community of antibiotic treated pine weevils changed after feeding on their natural diet for 10 days, and compare it to gut microbiome of weevils that exclusively consumed spruce.

Upon analyzing the microbial communities following this recovery period through 16S rRNA amplicon sequencing, significant differences were observed in the abundance of dominant bacterial taxa when compared to weevils that were fed only with artificial antibiotic or control diets.

Interestingly, it was found that weevils fed on the artificial control diet after the recovery period exhibited a more diverse microbial community similar to that of weevils consistently consuming their natural diet. (Figure 12). After the recovery period of weevils treated with streptomycin sulphate 1%(w/w), considerable variation among individuals was observed, with each weevil having different dominant microbial species. Although *Wolbachia* was notably dominant in all individuals in gut when weevils treated with streptomycin sulphate 1%(w/w) (Figure 12). The weevils treated with rifampicin 1%(w/w) and the combination of 0.5%(w/w) streptomycin and 0.5%(w/w) rifampicin showed a lasting impact on the weevils after recovery, having higher abundance of *Buttiauxella* and *Kluyvera* (Figure 12). After 10 days feeding on spruce, the micobial community in all the groups closely resembled that of spruce-fed weevils. (Figure 13B). In alpha diversity, statistical significance was observed only in the case of weevils treated with streptomycin sulphate 1%(w/w) during the recovery period, as indicated by the Shannon index ($p<0.05$) (Figure 14).

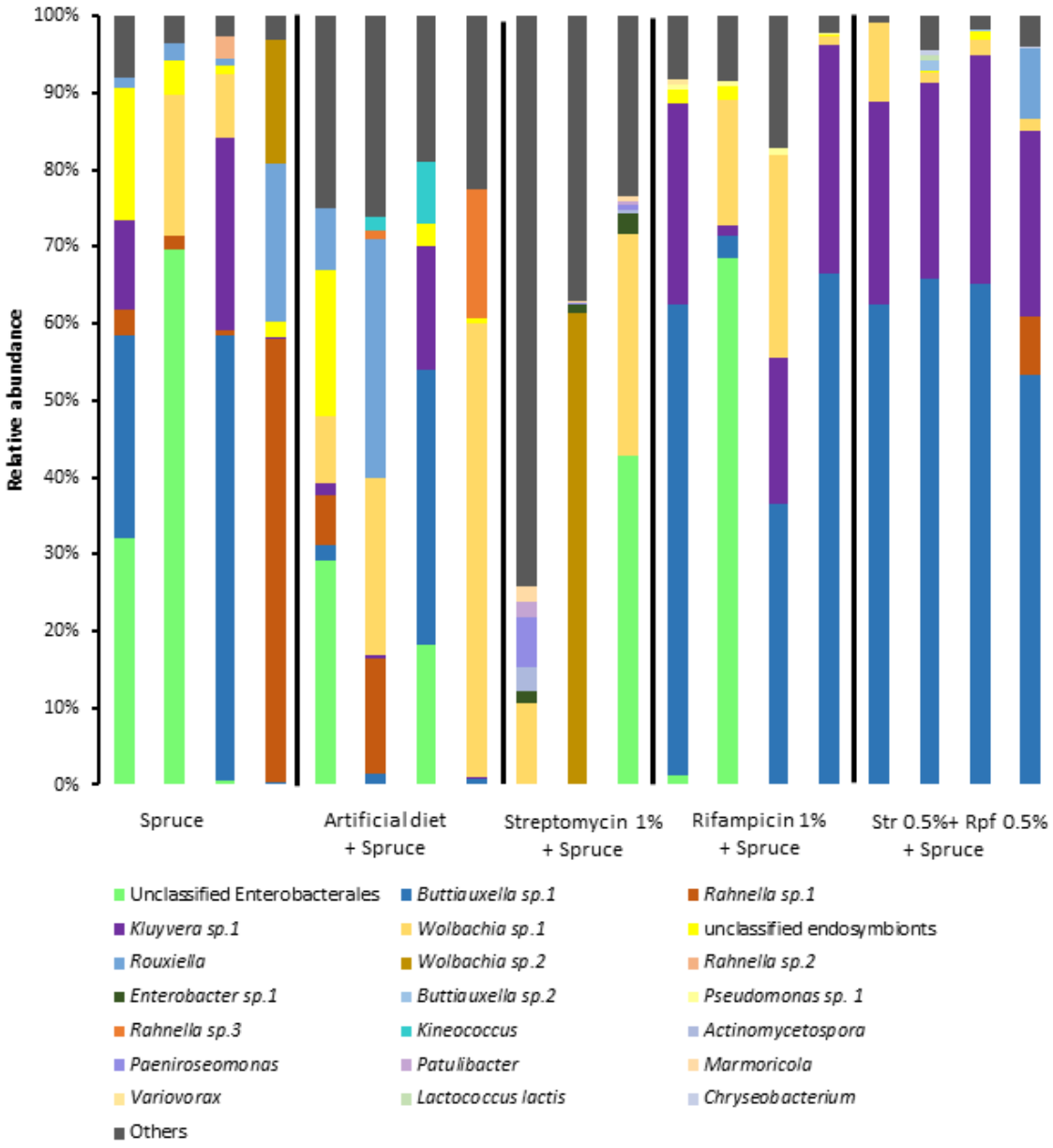

Figure 12. Comparative relative abundance of pine weevil gut microbiota in spruce fed weevils and post recovery period after 1% antibiotic treatment. Results are shown to the closest taxa level. During post recovery periods, weevils were fed with their natural diet for 10 days i.e., spruce to evaluate their response in their gut microbial community comparison to gut microbiome of weevils consistently feeding on spruce**.** Groups were treated with: control spruce, artificial control diet, streptomycin sulphate (1%(w/w)) diet, rifampicin (1%(w/w)) diet, streptomycin (0.5%(w/w)) + rifampicin (0.5%(w/w)) diet.

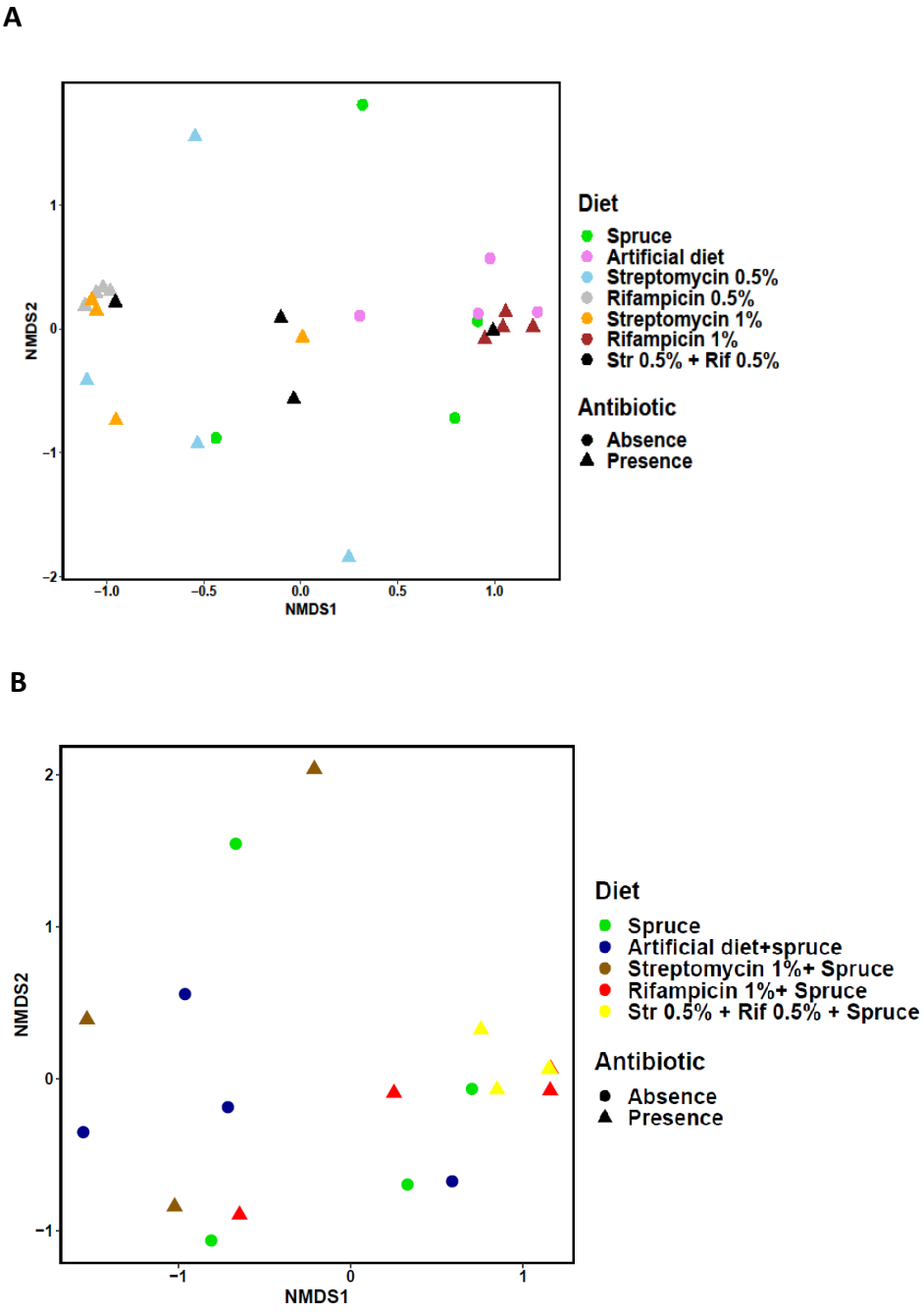

Figure 13. Beta diversity of gut microbes in antibiotic and non-antibiotic treated pine weevils fed on different diets. Non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis distances. Each symbol represents the bacterial community in a single gut sample and the colour represents the diet pine weevils; (A) Weevils after antibiotic treatment compared with weevils feeding on spruce and weevils feeding on non-antibiotic diet(B) Weevils after post recovery period after 1%(w/w) antibiotic concentration treatment where weevils fed with spruce for 10days compared with weevils feeding on spruce.

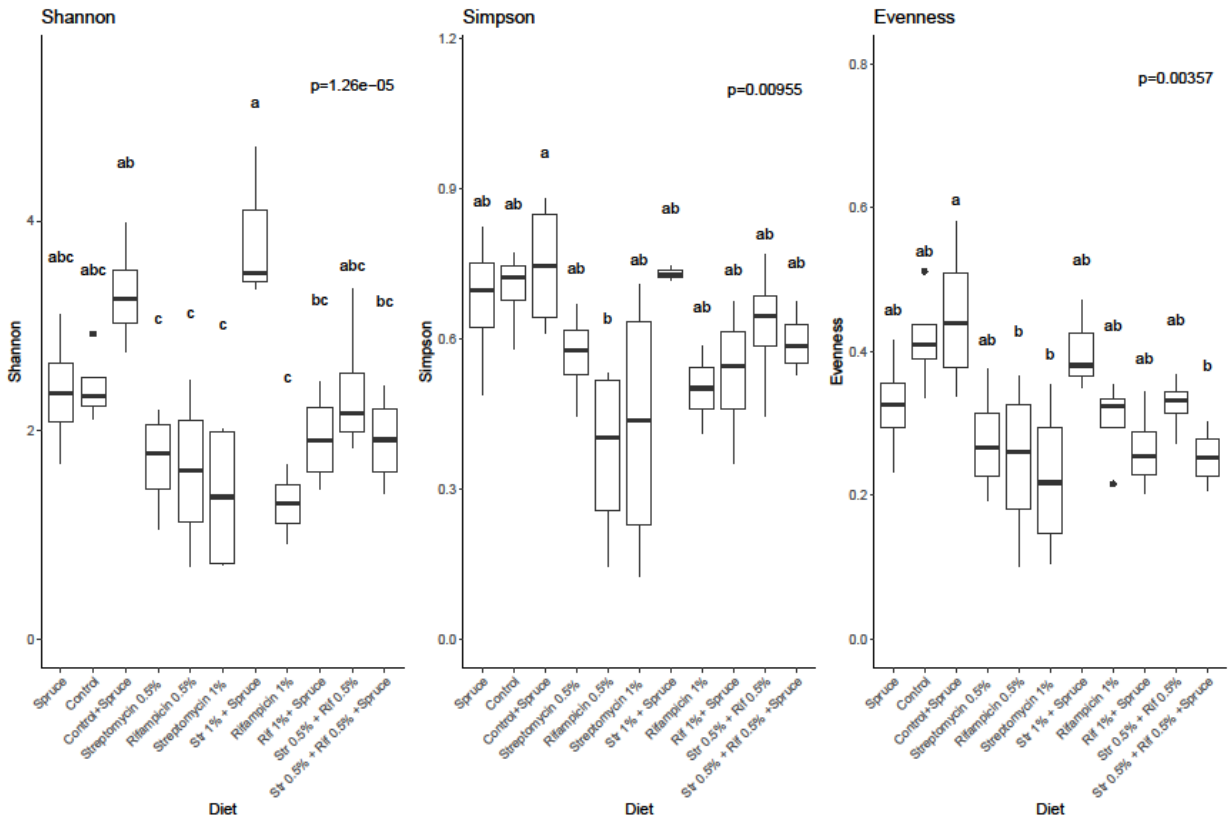

Figure 14. Alpha diversity of pine weevils' gut microbes in control group, antibiotic treated groups and after 10-day recovery period where weevils were fed with spruce for 10 days. Shown are alpha diversity measures with Shannon index, Simpson index and Species evenness; Statistical test: one way ANOVA. Letters are indicated significant differences according to post hoc Tukey test.

3.5 Effect of antibiotic treatment on weevil survivorship

To assess the effect of antibiotic treatment on weevil performance, the survival of weevils while they fed on artificial diets (control or antibiotic diets) and followed by spruce branches was tracked for 27 days. A significant difference was observed in survivorship rate among the antibiotic and non-antibiotic treated groups (Figure 15; p=0.028). After long-rank post-hoc test analysis, there were significant differences in survivorship between the control group and rifampicin 1%(w/w) treated weevils (p=0.023). No significance was observed among other groups. The survival of weevils treated with rifampicin 1%(w/w) showed early mortality compared to the other antibiotic-treated weevils. However, once the antibiotic treatment ended and the weevils began to feed on artificial diterpene resin acids diet, an unexpected increase in mortality was observed across all groups. This increase in mortality was more

pronounced in the rifampicin 1%(w/w) treated group and lowest in both the non-antibiotic treated and streptomycin sulphate 1%(w/w) treated groups. Furthermore, after a few days, when the weevils were transitioned to a spruce diet, there was no significant impact on the survivorship rate of any group of weevils.

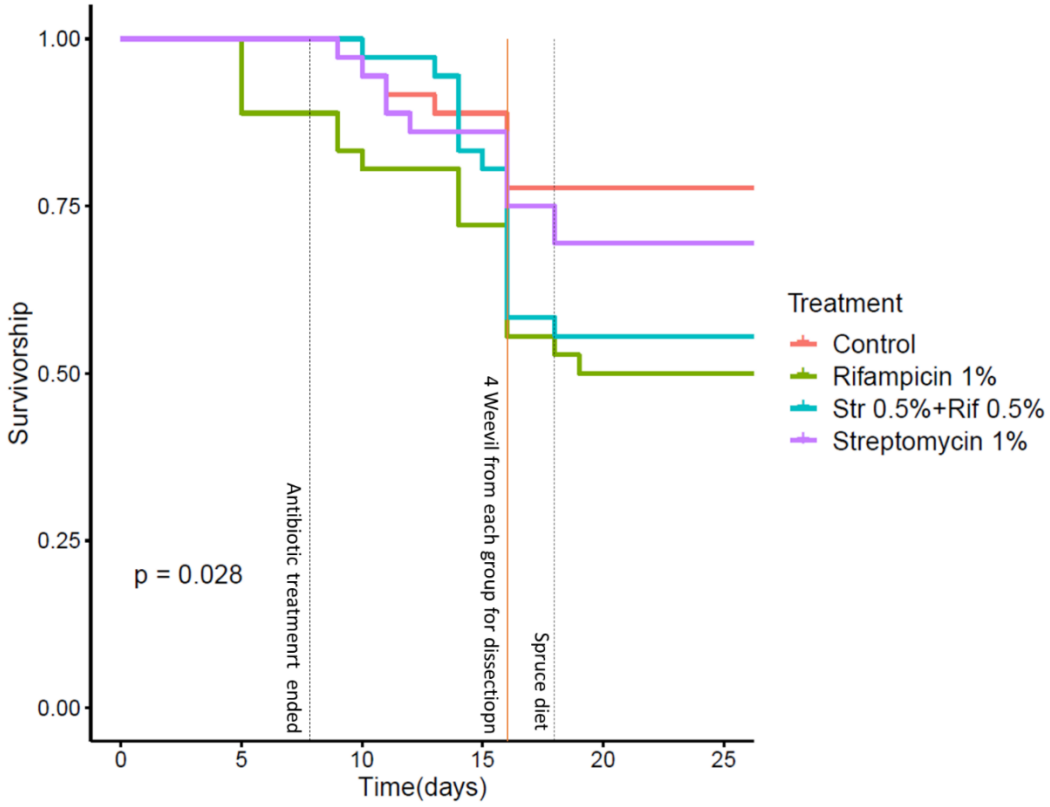

Figure 15. Survivorship of pine weevils after antibiotic treatment. Treatments included Control artificial diet, rifampicin 1%(w/w), streptomycin 0.5%(w/w) + rifampicin 1%(w/w), streptomycin 1%(w/w). Dashed lines represent the change in treatment: day 8 – end of antibiotic treatment, day 17 – the beginning of a 10-day recovery period during which spruce branches were fed to antibiotic-treated weevils to evaluate their response in their gut microbial community comparison to gut microbiome of weevils consistently feeding on spruce. Between day 8 and 17 weevils went through cycles of feeding on different diterpene resin acid diet and starvation for faeces collection. Solid line represents the day when four weevils were selected for dissection from each group. Statistical test: Log-rank test.

3.6 The effect of rifampicin on pine weevil feeding behaviour, weight and mortality

After a higher mortality rate was observed in the pine weevils after rifampicin (1%(w/w)) treatment, I was interested if the manipulation of the gut microbiome affects weevil feeding behaviour, weight and mortality. I tested the effect of two concentrations of rifampicin at 5%(w/v) and 10%(w/v) on pine weevils. The parameters tested were the area of spruce bark consumed for 14 days, weight change for 14 days and survivorship rate for 21 days. The amount of bark consumed was calculated using debarked area i.e., the area which weevils consumed (Figure 16). The mean area of bark consumed showed a significant difference among the different groups on the 3rd day ($F(2,38) = 3.467$, $p=0.0414$), 7th day ($F(2,38) = 7.166$, $p = 0.00229$), and 14th day ($F(2,38) = 4.882$, $p = 0.013$). The post hoc analysis, conducted using the Tukey test, showed that on the 3rd day, there was no significant difference ($p>0.05$) between weevils that were given branches without rifampicin treatment and those given rifampicin-treated branches (see Figure 16A). However, a general trend indicated that weevils feeding on non-rifampicin-treated branches consumed more food. This trend became significantly more pronounced on the 7th and 14th day, showing clear distinctions between the control group and the antibiotic-treated spruce branch treated groups (see Figure 16B and 16C; $p<0.05$). Importantly, there was no significant difference observed between the two antibiotic-treated groups on the 7th and 14th days ($p>0.05$) After seven days, weevils consumed twice as much bark from the non-rifampicin treated spruce branches when compared to the average branch consumed in both concentrations of rifampicin treated branches. Non-rifampicin treated branches were consumed an average of 129 mm^2 over four days, whereas the rifampicin treated branches were consumed an arear of 62 mm^2 during the same period. By the 14th day weevils continuing to prefer non antibiotic treated spruce branches. They consumed approximately 1.5 times more bark from non-rifampicin treated branches compared to antibiotic-treated branches. Non-rifampicin treated branches were consumed an average of 278 mm^2 over 7 days, while the treated branches were consumed average of 175 mm^2 during the same duration.

The weevils were weighed before the start of experiment, on the 3rd day, on the 7th day and on the 14th day to test the effects of the antibiotics on the change in body weight. No significant difference was found between all experimental groups on day 3 ($F(2,38) = 1.284$, $p=0.289$), day 7 ($F(2,38) = 3.246$, $p=0.05$) and day 14 ($F(2,38) = 0.41$,

p=0.667) (Figure 17A). The weevils in all groups gained weight, about 5% more, on the 3rd and 7th days compared to the weevil weight at start of the experiment. However, on the 14th day, their weight dropped by 2.5% from the start weevil weigh at start of the experiment. Survival rate was also observed for 21 days (Figure 17B), however no significant effect of rifampicin on the survival rate of weevils was found (p=0.85). The survival of pine weevils was not affected by rifampicin treatment. 80-85% of weevils survived after consuming the antibiotic-treated spruce branch. This survival rate was similar to that of weevils that fed on the spruce branches without rifampicin treatment.

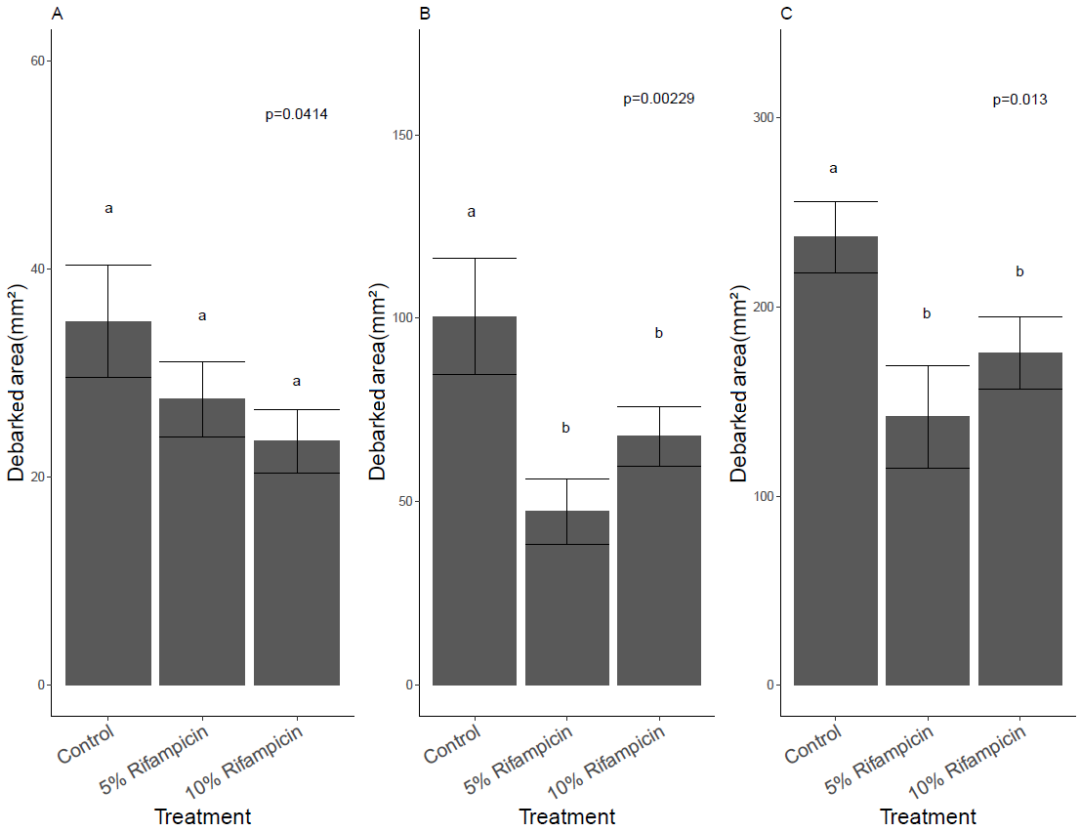

Figure 16. Area of rifampicin treated bark consumed by pine weevils. Experimental treatments include non-rifampicin treated spruce branches and spruce branches treated with two rifampicin concentrations (5%(w/v) and 10%(w/v)). (A) Bark consumption (mm²) during the initial 3 days of the experiment. (B) Bark consumption (mm²) during the 4 days following the 3rd day of the experiment. (C) Bark consumption (mm²) during the 7 days following the 7th day of the experiment. Statistical analysis: one way ANOVA. Different letters indicate significant differences according to post hoc Tukey test.

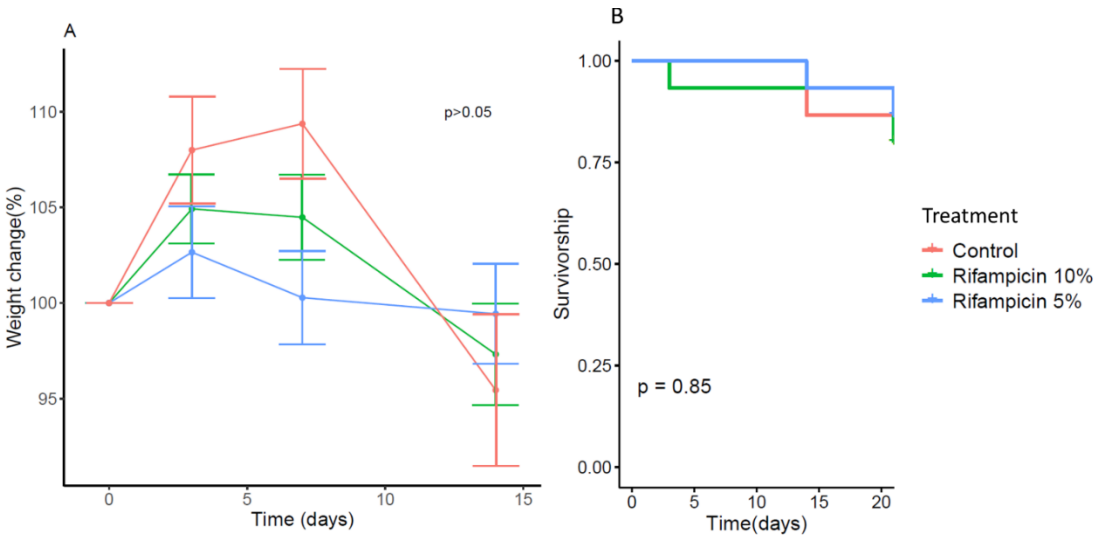

Figure 17. Impact of rifampicin on large pine weevil weight and survivorship. (A) Weight change of pine weevils in antibiotic twig experiment over the course of 14 days. Statistical test: one-way ANOVA. (B) Survivorship of pine weevils in antibiotic twig experiment over the course of 21 days. Statistical test: log-rank test.

3.7 Degradation of DHAA by *Hypera postica* (alfalfa weevil)

In the following experiment, the ability of alfalfa weevils to degrade DHAA was tested. It was shown in previous studies that microbiome communities in non-conifer feeding insects are different from that of conifer feeding insects (Berasategui et al., 2016). To test the DHAA degradation ability of alfalfa weevils, a non-conifer feeding weevil, I conducted the following experiment. The insects were fed with alfalfa (*Medicago sativa*) leaves and stems as the control or with DHAA-saturated *M. sativa* to test for DHAA degradation. Three faecal metabolite samples of both groups were analysed by LC-Q-TOF-MS. The base peak chromatographs of both samples were compared (Figure 18) and the presence of all potential DHAA degradation products was compared to DHAA degradation products by pine weevils. All the DHAA degradation products seen in pine weevil metabolites were found in alfalfa weevils after feeding on DHAA saturated alfalfa plants. A low peak area of DHAA was observed and DHAA-OH and DHAA-OH-Glucoside had higher peak areas, i.e., present in higher a relative abundance in faeces, while DHAA-Glucoside Ester and DHAA-OH-Glucoside Ester were present in low abundance. None of the target metabolites were present in the faeces from alfalfa weevils fed with untreated *M. sativa* plants.

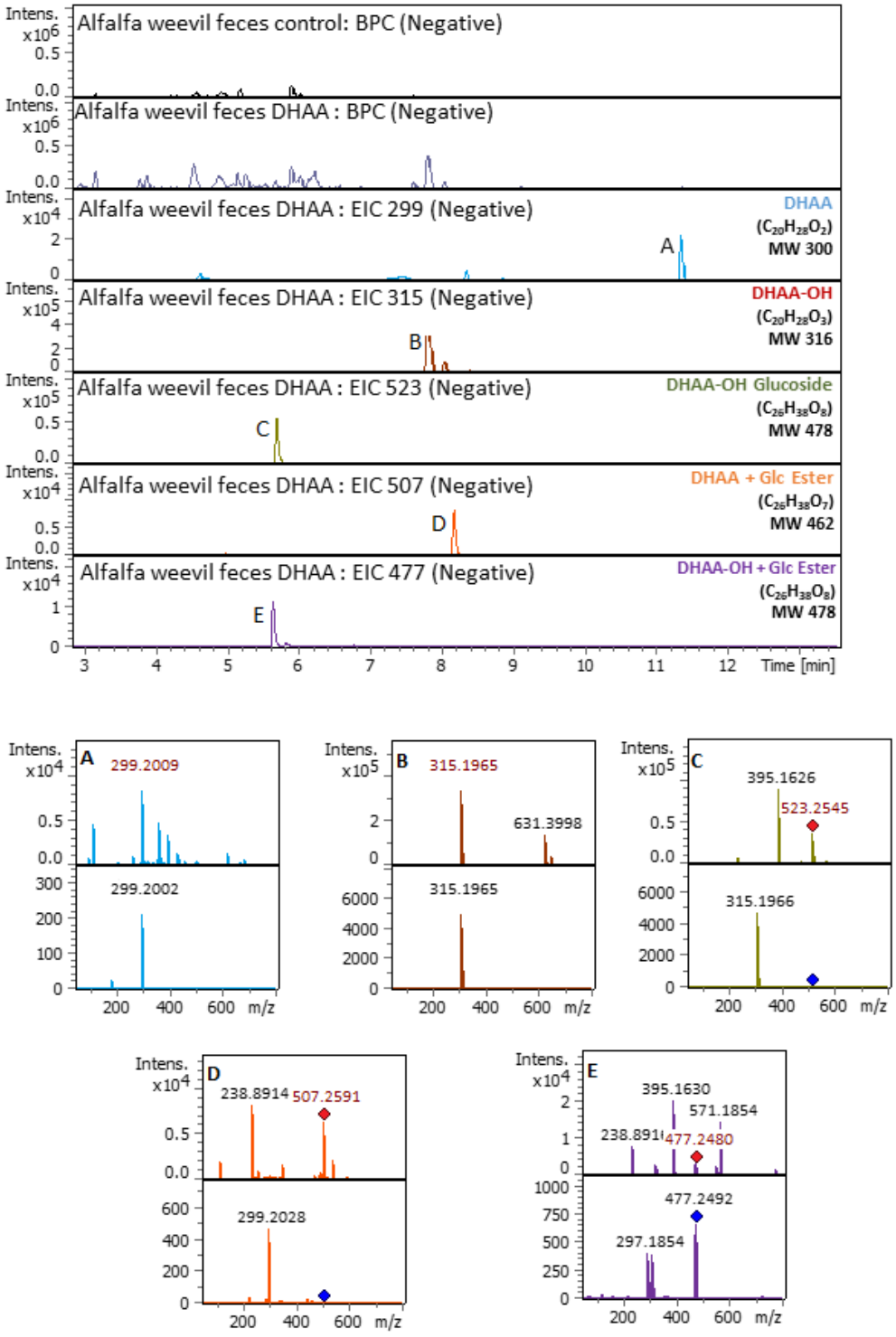

Figure 18. LC-Q-TOF-MS analysis of DHAA degraded products in in alfalfa weevil faeces after feeding on control plants and DHAA saturated plants; Chromatograms and mass spectra of (A) DHAA, (B) DHAA-OH, (C) DHAA-OH Glucoside, (D) DHAA Glucoside Ester, (E) DHAA-OH + Glucoside Ester; BPC= Base peak chromatogram, EIC= Extracted ion chromatogram.

4. Discussion

4.1 No significant difference in the degradation of diterpene resin acids by antibiotic-treated and untreated weevils

The aim of the following experiment was to investigate the role of the gut microbiome of large pine weevils in the degradation of conifer diterpene resin acids. A previous study by Berasategui et al. (2017) suggests that the gut microbiome plays an important role in improving the fitness of weevils by degrading diterpene resin acids. The authors concluded that the ability to degrade diterpene resin acids by large pine weevils decreases after antibiotic treatment (rifampicin w/w 0.3%) and suggested that the gut microbiome of weevils provides nutrients by degrading diterpene resin acids.

In my experiment, different types and concentrations of antibiotics were used: rifampicin (0.5% and 1%), streptomycin sulphate (0.5% and 1%), as well as a combination of both at a 0.5%. However, here, no significant differences were observed in the degradation of diterpene resin acids between the control weevils and the antibiotic-treated weevils. I tested the degradation of two of the diterpene resin acids found in spruce bark, DHAA and abietic acid.

In a study by Bicho et al. (1995), the consumption of various diterpene resin acids by five bacterial strains was studied These five bacteria were isolated from the enrichment of bleached kraft mill effluent and were capable of degrading DHAA. In their tests, they discovered that each three bacterial strains consumed nearly 95% of pure DHAA within 48 hours, while the remaining two bacterial strains consumed approximately 60-65% of DHAA in the same time frame. They also analysed that when these bacteria were exposed to the antibiotic tetracycline at the beginning of the experiment, the bacteria did not degrade the DHAA, but when exposed to it after four hours, this had no effect on the rate of degradation compared to the unexposed group. This suggests that if the activities of the DHAA-degrading bacteria are disrupted at the beginning of the experiment, no degradation will occur. In my experiment, weevils were treated with the antibiotic streptomycin sulphate, a protein synthesis inhibiter in bacteria as tetracycline, before being fed with DHAA. However, no significant difference was observed in the degradation capacity of DHAA by weevils. Even after administration of antibiotics intended to alter microbial communities in the gut, this did not affect the

degradation capacity of DHAA by pine weevils, suggesting that weevils might use a alternative mechanism to degrade the DHAA.

In the same experiment Bicho et al., 1995 also tested the consumption of different diterpene resin acids and observed the complete degradation of DHAA within 7 days by all bacterial strains. In contrast, similar degradation pattern of DHAA in faeces was observed from weevils with or without antibiotic treatment.

Another aim of the experiment was to compare the quantities of potential diterpene resin acid degradation products after the manipulation of the gut microbial community in pine weevils. All of the potential diterpene resin acid degradation products found in pine weevil faeces were previously identified in ongoing research in Conifer Defence group, Max Plank Institute for Chemical Ecology (Figure 1). The presence of the degradation products was observed by weevils in the faecal metabolites after the consumption of an artificial diet containing DHAA and an artificial diet containing abietic acid. However, no significant differences between the antibiotic-treated and non-treated control groups were observed. When the two groups were fed with spruce branches, a significant difference was observed only in the amount of DHAA-OH Glucoside and DHAA Glucoside Ester in the faecal metabolite samples. However, this significance was not observed when the weevils were just fed with DHAA artificial diet. After post hoc Tukey test, it was observed that the control group showed significance for both metabolites towards groups fed with a combination of rifampicin 0.5% + streptomycin 0.5%, and no significant difference was seen among other groups.

An abietinic diterpenoid degradation pathway in *Pseudomonas abietaniphila* BKME-9 was proposed by Martin and Mohn, 1999. According to their pathway, abietic acid undergoes a series of transformations. It begins with the conversion into dehydroabietic acid (DHAA), which is hydroxylated into 7-hydroxyDHAA. Further dehydrogenation led to the formation of 7-oxo-DHAA. This compound is then transformed into 7-oxo-11,12-dihydroxy-8,13-abietadien acid, which modified into dihydroxy-8,13-abietadien acid. This pathway has similarities to pathways found in other known diterpene resin acid degrading microbes, such as *Flavobacterium resinovorum* (Martin et al., 1999), *Alcaligenes eutrophus* and another *Pseudomonas sp.* (Martin et al., 1999), and an *Alcaligenes* sp. (Martin et al., 1999). Future modifications which were isolated are 3,7-

dioxo-11,12-diol and 2-isopropyl malic acid but the pathway of these modifications is currently unknown.

Previous work in Conifer Defence group, Max Plank Institute for Chemical Ecology has found different diterpene resin acid degradation products produced by weevils compared to the findings by Martin and Mohn in 1999 (Kshatriya, unpublished). In these observations DHAA is first hydroxylated, which is similar to the first modification proposed by Martin and Mohn (1999). However, in Martin and Mohn's study, DHAA-OH then undergoes a different modification into 7-oxo-DHAA whereas in the weevils it is glucosylated into DHAA-OH Glucoside. It has been suggested that DHAA undergoes another parallel modification leading to DHAA Glucoside Ester, which hydroxylated into DHAA-OH Glucoside Ester. Abietic acid hydroxylate into two isomers of AA-OH, which further undergo glycosylated into AA-OH Glucoside by weevils. These modified metabolites found in weevil faeces were also different from the previously described abietic acid degradation products by bacteria. These differences in the modifications of diterpene resin acids by weevils and microbes indicates that weevils may have an alternative mechanism in the degradation of diterpene resin acids.

4.2 Antibiotic treatment altered the pine weevil gut microbial community

To analyse the impact of antibiotics on the gut microbial community of pine weevils, I conducted 16S rRNA sequencing of weevil gut genomic DNA. The microbial gut community of weevils feeding on their natural food source (i.e., spruce branches), weevils consuming an artificial diet and weevils exposed to different concentrations of streptomycin sulphate and rifampicin (0.5% and 1%), as well as a combination of streptomycin sulphate (0.5%) and rifampicin (0.5%) were compared with each other.

Previous research by Berasategui et al. (2017) identified a dominant presence of the Enterobacteriaceae family in pine weevils feeding on natural sources and those on non-antibiotic diets. Within the Enterobacteriaceae family, the most dominant genera were *Erwinia*, *Rahnella*, and *Serratia*. My study observations showed a significant abundance of the Enterobacteriaceae family in weevils from these two groups. However, the dominant genera observed in both groups were distinct. Among weevils

feeding on spruce branches, different individuals had different dominant bacteria. Some individuals were dominated by *Rahnella*, others by *Buttiauxella*, or by an unclassified Enterobacterales. *Kluyvera, Rouxiella,* and an unclassified endosymbiont were also observed as other dominant genera. The microbial composition of each individual fed on artificial diet had a similar microbial composition at the genus level. Within this microbial community, the dominance of specific taxa, including *Buttiauxella, Kluyvera, Lactococcus, Enterobacter* was observed.

Berasategui et al. (2017) also observed a decrease in abundance of the order Enterobacterales, with some exceptions like *Escherichia* sp. and endosymbionts when they treated the weevils with a rifampicin (0.3%) antibiotic treatment. Instead, other bacterial taxa such as *Stenotrophomonos* sp., *Xanthomonas*, and *Wolbachia* increased in relative abundance. In my experiment with weevils treated with different antibiotics, varying relative abundances of bacteria was observed in different groups. When treated with a streptomycin sulphate 0.5% treatment, some weevils had a higher relative abundance of *Lactococcus lactis*, while others had more *Pseudomonas sp.*, or an unclassified Enterobacterales, or *Wolbachia*. With rifampicin 0.5% treatment, all individuals had a high relative abundance of *Wolbachia* bacteria, making up a significant portion of their microbial community. When I increased the streptomycin sulphate treatment at 1% concentration, some individuals had a higher relative abundance of *Wolbachia*, while others had more *Pseudomonas* taxa. When I treated weevils with rifampicin (1%), a higher relative abundance of the genera *Buttiauxella* and *Kluyvera* was observed in each individual, with these two bacterial taxa dominating 90 to 99% of the relative abundance. However, when weevils were treated with a combination of streptomycin sulphate (0.5%) and rifampicin (0.5%) a diverse microbial composition was observed in different individuals. Some had a greater relative abundance of *Wolbachia*, some had more *Buttiauxella*, and in one individual, a higher relative abundance of unclassified Enterobacterales. The unclassified Enterobacterales observed in the all groups belonged to the same taxon. While higher abundance of Enterobacterales was observed when treated with rifampicin (1%), in other antibiotic-treated weevils a lower abundance of taxa belonging to Enterobacterales, which was also observed by Berasategui et al. (2017). Previous studies have shown the presence of the bacteriome-localized *Nardonella* in weevils (Conord et al., 2008) and stays as dominant microbial gut species in antibiotic-treatment group (Berasategui et al. (2017).

However, *Nardonella* was not observed in current study as a dominant endosymbiont. However, another endosymbiont bacterium was observed, which has yet to be identified.

The presence of an unidentified *Pseudomonas* sp. was noted in some individuals after treatment with streptomycin at both concentrations. While some strains of *Pseudomonas* are known to contain genes with functions in diterpene resin acid degradation (*dit* genes) (Martin and Mohn, 1999), no significant differences were observed in the degradation of diterpene resin acids in streptomycin-treated weevils when compared to other antibiotic-treated groups. Berasategui et al. (2017) reported that pine weevil gut bacteria had the presence of 10 of the 19 *dit* genes that are known to degrade diterpene resin acids. After antibiotic treatment, this was reduced to one *dit* gene. The authors also mention that not all of these 19 genes are required for effective degradation of diterpene resin acids by bacteria. They also observe that most of these genes found in their experiment belong to bacteria in the Enterobacteriaceae. In contrast to the findings by Berasategui et al. (2017), where they observed significant differences in the degradation of diterpene resin acids between control and antibiotic-treated groups, my study observed different results. No differences in the quantity of diterpene resin acids and their degradation products in weevil faeces was observed. This suggests that pine weevils might use a different mechanism for the degradation of diterpene resin acids that does not rely on gut microbes.

While there was no significant difference in the alpha diversity of gut microbes, a distinct variation was observed in the trend of beta diversity among the control and antibiotic-treated weevil groups. It is likely that the antibiotics specifically influenced certain bacterial groups. A study conducted by Meyel et al., 2021 indicated that broad-spectrum antibiotics had a notable impact on older, more distantly related bacterial groups.

After completing the antibiotic treatment experiment, a 10-day recovery phase was implemented for weevils, during which they were fed with spruce branches. The aim of this recovery phase was to see how the reintroduction of their natural diet affected the gut microbial community of weevils following antibiotic treatment. Upon analysing the recovery phase, it was observed that there was a resemblance in the relative abundance of bacterial taxa to that seen after the antibiotic treatment, indicating a long-

lasting impact of the antibiotic on these insects. This suggests that, although the 10-day recovery period in weevils resulted in a community similar to weevils with a natural diet, they require a more extended recovery period to fully mitigate the effects of antibiotics on their gut microbial community.

There was no significant difference in alpha diversity between the control group and the antibiotic-treated group. However, upon recovery, only the streptomycin 1% group exhibited a significantly higher alpha diversity compared to the antibiotic-treated groups but not when compared to the control group. This could be attributed to antibiotics targeting specific taxonomic groups.

4.3 Altering the pine weevil microbiome did not affect insect mortality or performace

In our antibiotic experiment, I noticed that pine weevils treated with rifampicin had a higher mortality rate compared to those fed on an artificial control diet. This raised the question of whether the manipulation of the gut microbiome of pine weevils can cause any health or performance issues among weevils, especially when they are exposed to conifer defence compounds, such as the diterpene resin acids and other metabolites present in spruce bark.

In a prior study conducted by Tudoran et al., 2020 explored the potentially adverse effects of *Bacillus thuringiensis* (Bt) strains on pine weevils. Tudoran 's study examined bark consumption, survival rates, and change in body weight between the control and Bt treated weevil. Using the same research method as the Tudoran et al., 2020 study, I also aimed to investigate the same factors i.e., bark consumption, survival rates, and change in body weight between the control and antibiotic treated weevil. A significant difference in feeding behaviour was observed where weevils that were provided with control spruce twigs consumed more bark compared to antibiotic treated twigs. However, no significant effect on weight change and survivorship was observed among these groups.

The treatment of spruce twigs with rifampicin may have altered the taste, which may have affected their eating behaviour. As a consequence, the weevil may have chosen to eat less bark when treated with rifampicin. However, rifampicin did not affect weight

or survivorship after feeding antibiotic treated twigs. One limitation of this study is its emphasis on the short-term effects of rifampicin on weevils, while it's possible that the manipulation of the gut microbial community could have adverse long-term consequences.

4.4 Alfalfa weevils showed the ability to degrade DHAA

Not much is known about the ability of insects that do not feed on conifers to degrade diterpene resin acids. A number of studies have shown that the gut microbiota of herbivorous insects is shaped by their host plant (Berasategui et al. 2016., Chung et al., 2017., Jones et al., 2019). Gut microbes are believed to help pine weevils in breaking down diterpene resin acids, a process also observed in other conifer-feeding beetles, (Berasategui et al., 2017). While the microbial communities in other conifer feeding insects share similarities with pine weevils, they differ significantly from those found in closely related non-conifer-feeding beetles (Berasategui et al., 2016)

This study was conducted to test the DHAA degradation abilities of the alfalfa weevil (*Hypera postica*), an insect which never consumes diterpene acids. Alfalfa weevils are a pest of alfalfa plants (*Medicago sativa)* and closely related legumes (Byrne and Bickerstaff, 1968). When these insects were fed with DHAA-saturated *M. sativa* plant material and their faeces metabolites were analysed it was observed the degradation of DHAA and the presence of DHAA degradation products, suggesting that the alfalfa weevils have the capability to break down DHAA. The metabolites detected in alfalfa weevil faeces were the same as those found in pine weevil faeces. The similarities in the degradation products suggests that some insects might have other mechanisms that enable them to degrade diterpene resin acids themselves. As there was no change in the plant material the insects were feeding on, it is unlikely that they acquired diterpene resin acid degrading microbes. Previous studies have shown that microbial communities residing within herbivores are influenced by the plants they feed on (Berasategui et al. 2016., Chung et al., 2017., Jones et al., 2019). Therefore, the fact that a non-conifer feeding weevil is also able to degrade diterpene resin acids in the same way as the pine weevil provides support to the idea that some insects can play a more active role in breaking down diterpene resin acids without the assistance of bacterial symbiotic relationships.

5. Conclusion

The aim of this thesis was to study the role of the gut microbiome of the large pine weevil in the degradation of diterpene resin acids. The gut microbiome of the weevil was manipulated through the use of various antibiotics at different concentrations and combinations. After antibiotic treatment, it was observed that pine weevils retain similar diterpene resin acid degradation abilities when compared to pine weevils with an intact gut microbiome. The antibiotic treatment altered the gut microbial community in the weevils, however antibiotic treatment did not affect alpha diversity. This indicates that the richness and evenness of bacterial species within individuals, might not show significant differences. However, there are distinctions in the microbial communities among various antibiotic treatments suggesting that the microbial communities vary significantly across different treatment groups.

After a 10-day recovery period, during which the antibiotic-treated weevils were fed with their natural diet, it was observed that antibiotics have long-term impact on weevils. However, antibiotic treatment did not have any negative impact on weevil survivorship or performance, even when fed in conjunction with their natural diet of spruce bark. This implies that altering the gut microbial community does not increase weevil mortality when consuming spruce defensive compounds.

Another interesting finding in this study was the similar diterpene resin acid degradation ability observed in alfalfa weevils, a non-conifer herbivore, to pine weevils. The differences in the diterpene resin acid degradation products produced by weevils and diterpenoid degrading bacteria, combined with the fact that a non-conifer herbivore can break down these compounds, strongly indicate that some insects possess an inherent mechanism for degrading conifer defensive compounds.

The observed resilience of pine weevils to conifer defensive chemicals and their efficiency in degrading these compounds highlights their specialization and adaptation to this ecological niche. This underlines the important ecological interactions between insects and conifer trees and motivates for further research in this aspect.

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7. Appendix

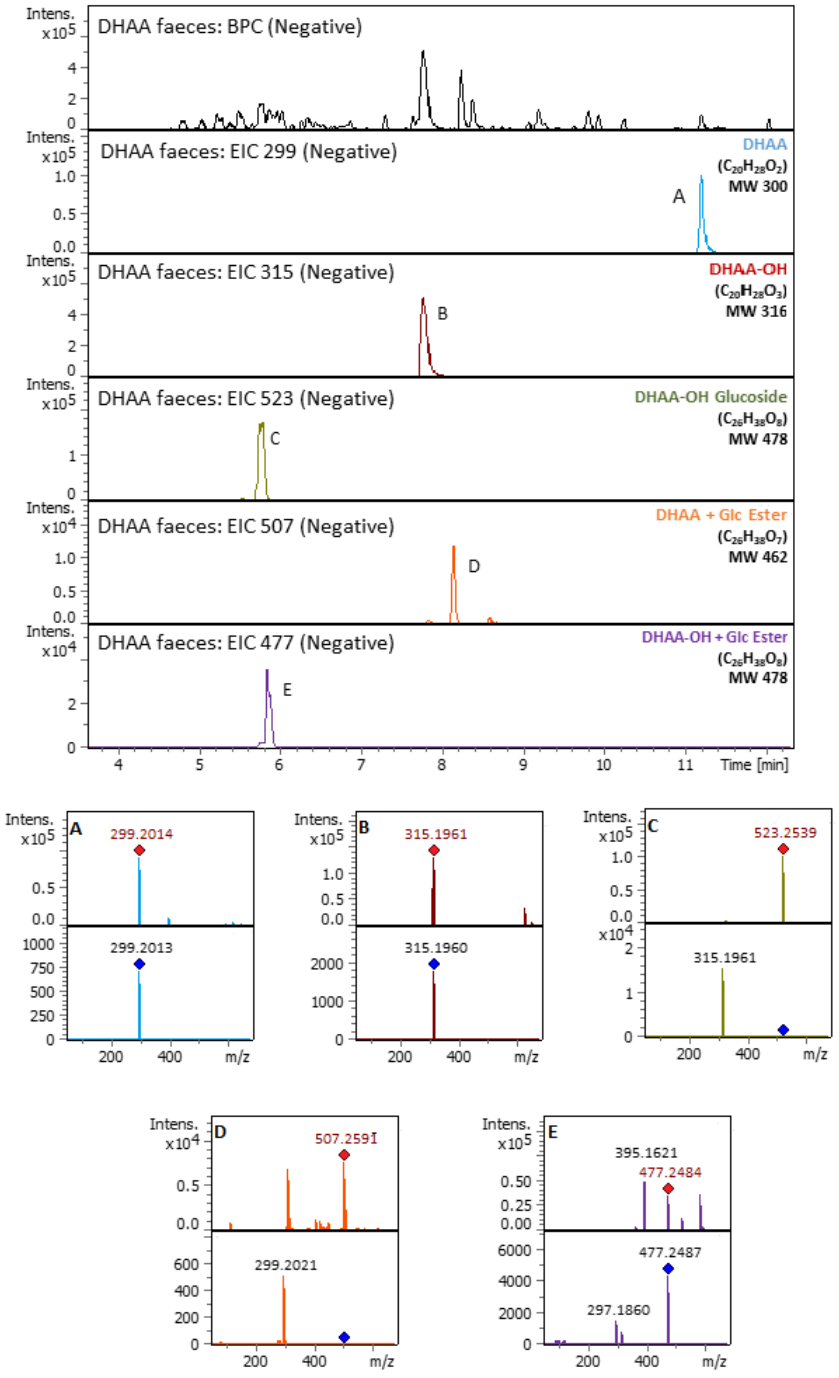

Figure S1. LC-Q-TOF-MS analysis of DHAA degraded products in non-antibiotic large pine weevil faeces in preliminary test; Chromatograms and mass spectra, (A) DHAA, (B) DHAA-OH, (C) DHAA-OH Glucoside, (D) DHAA Glucoside Ester, (E) DHAA-OH + Glucoside Ester; BPC= Base peak chromatogram, EIC= Extracted ion chromatogram.

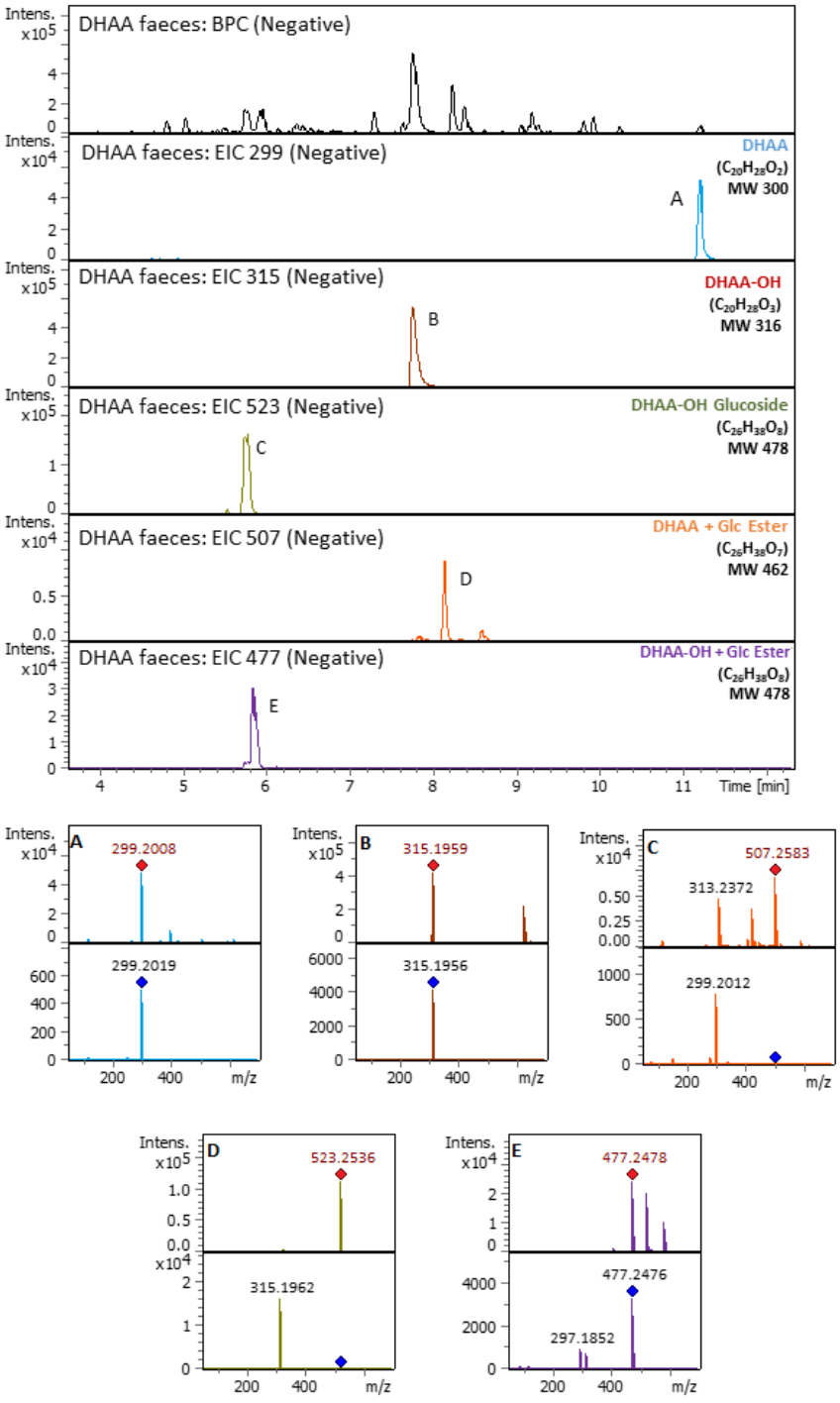

Figure S2. LC-Q-TOF-MS analysis of DHAA degraded products in antibiotic treated large pine weevil faeces in preliminary test. Chromatograms and mass spectra, (A) DHAA, (B) DHAA-OH, (C) DHAA-OH Glucoside, (D) DHAA Glucoside Ester, (E) DHAA-OH + Glucoside Ester. Weevils were tested with cocktail of streptomycin sulphate, gentamicin, ampicillin and rifampicin at total concentration 0.05%; BPC= Base peak chromatogram, EIC= Extracted ion chromatogram.

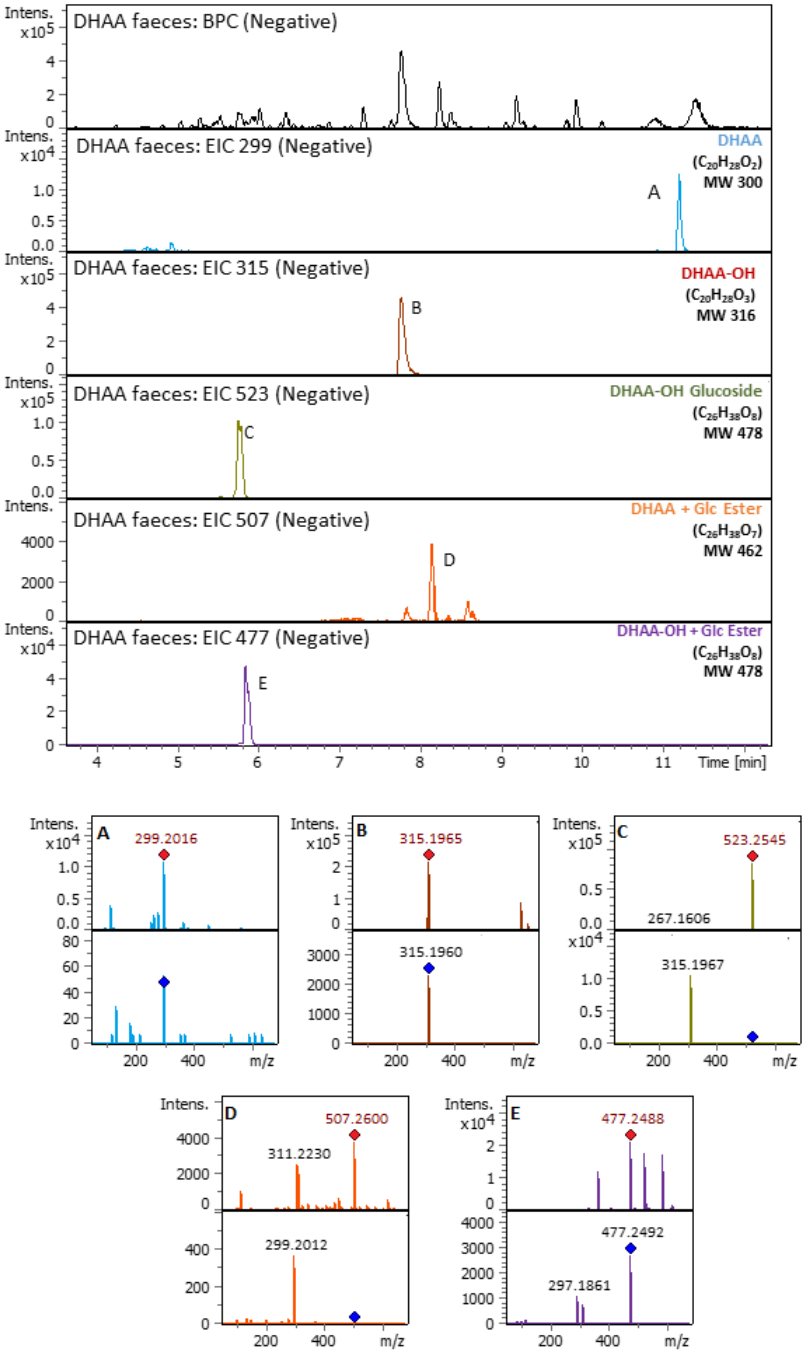

Figure S3. LC-Q-TOF-MS of DHAA degraded products in antibiotic treated large pine weevil faeces in preliminary test. Chromatograms and mass spectra, (A) DHAA, (B) DHAA-OH, (C) DHAA-OH Glucoside, (D) DHAA Glucoside Ester, (E) DHAA-OH + Glucoside Ester. Weevils were tested with cocktail of streptomycin sulphate, gentamicin, ampicillin and rifampicin at total concentration 0.1%; BPC= Base peak chromatogram, EIC= Extracted ion chromatogram.

Table S1. Peak area data of DHAA metabolities in weevil faeces. Treatment represent the diet weevils treated during expriment and diet represent the original artificial diet

	Peak area of DHAA metabolites				
Treatment /diet	DHAA	DHAAOH	DHAAOH Glucoside	DHAA Glucoside Ester	DHAAOH Glucoside Ester
Control 1	29595.76367	2315414	696041.4375	30673.71875	53817.45313
Control 2	31721.54297	1603434.125	328591.4375	17511.08398	28060.37109
Control 3	30947.13086	2648715.25	832943.125	48794.83203	95728.39844
Rifampicin (0.5%) 1	6646.478516	2146286	95077.42969	4084.544434	106757.7891
Rifampicin (0.5%) 2	42715.60156	1450485.5	357474.7813	7220.937988	39266.12891
Rifampicin (0.5%) 3	23913.69531	2996583.25	623007.25	23493.89258	186376.4688
Streptomycin 0.5% 1	64605.52344	2710099.75	1001272.25	49606.23047	67748.53906
Streptomycin 0.5% 2	9976.291992	2152590.75	659870.25	32091.21484	90159.35156
Streptomycin 0.5% 3	7763.849609	1122622.875	119174.1016	3988.905029	29296.76367
Rifampicin (1%) 1	31632.5957	2559413.5	62022.34375	5523.96582	174018.125
Rifampicin (1%) 2	13949.62402	2411073.5	268407.2188	13910.10742	73494.46094
Rifampicin (1%) 3	30341.26563	2322565.5	232453.5469	12198.33691	106405.5781
Streptomycin (1%) 1	36078.85156	1052688.875	78750.60938	5471.665527	33770.72266
Streptomycin (1%) 2	26869.60547	2400497	218532.7188	13553.80176	47597.57813
Streptomycin (1%) 3	27889.20508	2038155.125	409436.5625	22830.08398	117287.0391
Rpf (0.5%)+St (0.5%) 1	97540.69531	2675457.75	525483	26593.9082	80656.36719
Rpf (0.5%)+St (0.5%) 2	111865.9531	2861447.5	558642.625	36078.78906	172370.5469
Rpf (0.5%)+St (0.5%) 3	34473.55859	3299175.75	751704.3125	55165.47266	232193.2344
DHAA diet 1	975081.0625	0	0	0	0
DHAA diet 2	732339.5625	0	0	0	0
DHAA diet 3	910084.25	0	0	0	0
DHAA diet 4	897021.6875	0	0	0	0
DHAA diet 5	699454.5	0	0	0	0

Table S2. Peak area data of abietic acid metabolities in weevil faeces. Treatment represent the diet weevils treated during experiment and diet represent the original artificial abietic acid diet

	Peak area of abietic acid metabolites			
Treatment/diet	AAOH1	AAOH2	AAOH Glucoside 1	AAOH Glucoside 2
Control 1	1208182.5	754516.5	35405.72266	16297.18555
Control 2	1135308.25	739464.5	51309.13281	26701.53711
Control 3	1587586.875	21207.1875	45156.27734	25776.20703
Rifampicin (1%) 1	1039109.688	544128.8125	52654.79297	26876.38672
Rifampicin (1%) 2	945888.5	416650.7813	49117.84375	20833.4082
Rifampicin (1%) 3	1334955.625	803589.4375	116251.4453	53828.71484
Streptomycin (1%) 1	1458787.375	837366.5	95040.11719	43798.13672
Streptomycin (1%) 2	1295048.25	768245.8125	83977.32813	30043.38281
Streptomycin (1%) 3	612098.875	349782.375	19907.78516	8293.87793
Rpf (0.5%)+St (0.5%) 1	1227911	688580.6875	41657.32031	17733.57422
Rpf (0.5%)+St (0.5%) 2	1412823.25	939974.125	86844.13281	37134.6875
Rpf (0.5%)+St (0.5%) 3	1171749	643585.8125	95207.22656	44618.77734
Abietic acid diet 1	0	0	0	0
Abietic acid diet 2	0	0	0	0
Abietic acid diet 3	0	0	0	0
Abietic acid diet 4	0	0	0	0
Abietic acid diet 5	0	0	0	0

Table S3. Peak area of diterpene resin acid metabolites in weevil faeces. Treatment rpresent the diet weevils treated with. Diet represent to spruce bark

	Peak area of diterpene resin acids metabolites								
Treatment	DHAA	DHAAOH	DHAA OH Glucoside	DHAA Glucoside Ester	AAOH1	AAOH2	DRA 1	DRA 2	DRA 3
Control 1	3154.165527	217814.1719	142115.0938	5260.059082	512113.3438	304457.4063	399231.8438	588843.625	489962.7188
Control 2	4183.740234	469081.6875	214284.1875	5725.387695	675196.0625	402181.1875	709934.3125	892781.375	817499
Control 3	3541.141602	486254.8438	203420.7188	5434.450195	720178.1875	430369.5313	818911.375	1090290.75	1043931.625
Rifampicin (1%) 1	2426.968994	397654.0313	114255.25	3058.2771	742349.875	418985.3438	185531.6719	633531.1875	461941.3438
Rifampicin (1%) 2	1661.425537	294806.2188	99094.49219	4168.458008	576428.375	324427.0938	316727.9063	814473.5	530185.5
Rifampicin (1%) 3	8109.21582	406909.9063	148576.5625	4459.591309	775632.0625	455313.5938	546428.875	1110724.5	840914.4375
Streptomycin (1%) 1	5156.705078	368940.7813	133588.9844	4318.712402	703855.4375	422602.4688	478124.4375	1011456.5	699504.0625
Streptomycin (1%) 2	2571.702393	295018.25	94722.67188	4153.050781	498169.2813	294353.5625	379531.1875	746570.5	462108.625
Streptomycin (1%) 3	6229.433594	536295.375	147991.25	5927.169434	823545.6875	495301.875	569267.125	926298.9375	763627.9375
Rpf (0.5%)+St (0.5%) 1	1014.598755	182769.4375	67267.65625	3619.382568	430542.375	151823.8125	274642.8125	531366.9375	326499.375
Rpf (0.5%)+St (0.5%) 2	4433.624023	340373.25	114225.4141	3747.373047	666339.625	299506.5313	347092.1875	907650.8125	555430.4375
Rpf (0.5%)+St (0.5%) 3	2592.160156	148378.6406	85850.34375	2921.299805	423166.1875	242335.9375	328218.625	549603.125	362707.7188
Spruce diet 1	35039.58984	0	0	0	0	0	0	0	0
Spruce diet 2	56587.98438	0	0	0	0	0	0	0	0
Spruce diet 3	29380.64258	0	0	0	0	0	0	0	0
Spruce diet 4	22949.66602	0	0	0	0	0	0	0	0

Table S4. Rifampicin treated bark consumed (mm^2) by weevils during pine weevils performance and mortality test measured on third, seventh and fourteenth day.

		Bark consumed area (mm^2) by weevils		
Weevil	Treatment of spruce branch	3rd day	7th day	14th day
C1	Control	76	115	180
C3	Control	20	190	250
C4	Control	60	300	200
C5	Control	15	185	220
C6	Control	15	35	120
C7	Control	25	165	300
C8	Control	125	105	380
C9	Control	55	90	250
C11	Control	70	145	250
C12	Control	50	170	425
C13	Control	30	30	280
C14	Control	50	95	320
C15	Control	30	55	450
A1	5% Rifampicin	35	70	400
A2	5% Rifampicin	15	90	220
A3	5% Rifampicin	20	115	300
A4	5% Rifampicin	40	10	230
A5	5% Rifampicin	10	10	220
A7	5% Rifampicin	35	45	350
A8	5% Rifampicin	25	30	80
A9	5% Rifampicin	15	25	55
A10	5% Rifampicin	30	45	85
A11	5% Rifampicin	30	180	220
A12	5% Rifampicin	35	80	145
A13	5% Rifampicin	60	35	110
A14	5% Rifampicin	25	40	5
A15	5% Rifampicin	10	20	35
B1	10% Rifampicin	17	125	260
B2	10% Rifampicin	23	65	180
B3	10% Rifampicin	15	60	195
B4	10% Rifampicin	25	60	160
B6	10% Rifampicin	25	65	300
B7	10% Rifampicin	20	35	205
B8	10% Rifampicin	50	95	165
B9	10% Rifampicin	30	65	240
B10	10% Rifampicin	15	25	65
B11	10% Rifampicin	40	95	205
B12	10% Rifampicin	70	115	201
B13	10% Rifampicin	10	40	135
B14	10% Rifampicin	15	35	35
B15	10% Rifampicin	20	70	115

Table S5. Measured weight of weevils on third, seventh and fourteenth day during pine weevils performance and mortality test.

	Wight of weevils (mg)		
Treatment of spruce branch	3 day	7 th day	14th day
Control 1	117.401	114.2287	98.66432
Control 2	101.729	106.7956	107.6105
Control 3	112.8818	110.9668	88.06165
Control 4	85.89249	90.64429	77.02546
Control 5	108.1736	101.2109	71.4786
Control 6	103.3104	102.3581	98.05759
Control 7	108.2111	114.813	103.4261
Control 8	119.3762	121.1586	97.941
Control 9	113.3106	110.2304	83.02901
Control 10	105.788	117.9319	97.5792
Control 11	105.4225	104.2117	97.71686
Control 12	97.70253	98.20253	92.01899
Control 13	124.7392	129.0921	128.2292
5% Rifampicin 1	103.529	103.9074	97.02544
5% Rifampicin 2	87.42835	106.9184	101.7098
5% Rifampicin 3	86.87118	101.6639	100.26
5% Rifampicin 4	94.56771	85.85936	83.23016
5% Rifampicin 5	103.1916	97.70757	89.0482
5% Rifampicin 6	104.1441	100.8122	109.6071
5% Rifampicin 7	100.724	97.9983	94.79557
5% Rifampicin 8	111.9455	103.1907	98.17121
5% Rifampicin 9	105.2584	78.96627	104.8394
5% Rifampicin 10	105.6343	114.7628	111.1011
5% Rifampicin 11	109.5597	100.7441	116.5981
5% Rifampicin 12	107.4595	107.0272	99.27684
5% Rifampicin 13	97.11029	108.5221	103.1985
5% Rifampicin 14	119.7538	95.83822	83.23564
10% Rifampicin 1	105.1807	114.7967	98.2003
10% Rifampicin 2	105.8393	100.4699	82.40505
10% Rifampicin 3	104.5555	95.16882	88.04839
10% Rifampicin 4	101.5769	95.71489	106.2933
10% Rifampicin 5	103.3732	93.9184	80.33452
10% Rifampicin 6	103.5549	102.0917	91.08318
10% Rifampicin 7	97.29491	111.2173	95.63364
10% Rifampicin 8	97.76868	94.30976	109.8826
10% Rifampicin 9	113.6152	99.16071	92.80385
10% Rifampicin 10	111.7756	108.0155	98.57356
10% Rifampicin 11	110.6049	110.7944	106.8067
10% Rifampicin 12	117.4319	116.3768	114.0747
10% Rifampicin 13	92.6436	115.5136	100.5552
10% Rifampicin 14	103.677	105.202	97.78603

Table S5. No of alive weevils third, seventh, fourteenth and twenty first day during pine weevils performance and mortality test.

	Number of weevils alive		
Day	Control	5% Rifampicin	10% Rifampicin
0	15	15	15
3	14	15	14
7	14	15	14
14	13	14	14
21	12	13	12