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Wenyi Shao April 06, 2023

Effect of Clove Oil on the Bean Beetle *Callosobruchus maculatus* and Its Gut Microbiome

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Abstract

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Bean beetles (*Callosobruchus maculatus*) are one of the pests that attack bean crops. Due to the drawbacks of traditional insecticides, alternatives are being explored for pest control. Plant essential oils have shown promising results in protecting stored bean crops against bean beetles, and among them, clove (*Syzygium aromaticum*) essential oil is easy to access and has exhibited insecticidal properties. It is also known that clove oil can act as an antimicrobial, and insects rely on their microbiome to detoxify compounds harmful to them. As a result, it is possible that the bean beetle microbiome changes in response to clove oil exposure. Previous studies have not examined the possibility of bean beetles developing resistance to clove oil and the role that the beetle gut microbiome might play in the process. To explore these questions, a multi-generation study was conducted. Bean beetles were reared under no, low, or high clove oil treatments for four generations. The results suggested that only high clove oil exposure significantly decreased bean beetle populations, but the effect did not last throughout the study. Analysis of bacteria cultured from beetles showed that clove oil could affect the beetle's microbial community, but next-generation sequencing data from the last generation of beetles showed no difference in the microbial community between the three treatments. In addition, the dominant bacteria strains showed no difference in resistance to clove oil. However, due to the temperature variation during the beetle rearing and the limited span of the experiment, further investigation is necessary to confirm these findings.

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Introduction:

Pulses, including beans, are the edible seeds from legumes. Pulses contain protein content varying from 15.5 – 42% and are rich in dietary fiber and low in saturated fat (Singh et al., 2022). Their high nutritional content makes them an essential source of food across the world, and their importance in diet varies depending on factors including consumption of animal-based products. Pest damage on stored pulse products causes significant losses, especially in developing countries (Mssillou et al., 2022). Bean beetles (*Callosobruchus maculatus*) are among the pests that attack bean crops in the field and in storage facilities (Jairoce et al., 2016). They are endemic to Asia and Africa but are also found in tropical and subtropical areas (Mssillou et al., 2022).

While the main hosts of *C. maculatus* are black-eyed peas (*Vigna unguiculata*) and mung beans (*Vigna radiata*), they can feed on a variety of beans including adzuki beans (*Vigna angularis*) and pigeon peas (*Cajanus cajan*) (Beck & Blumer, 2019; Tuda et al., 2005). The beetle's life cycle begins when the female lays eggs on beans following insemination by a male. The egg hatches in about two days, and the larva burrows into and feeds on the same bean until it develops into an adult beetle. The beans decrease in quality and quantity as the larvae feed. The generation time from egg to adult varies depending on temperature, humidity, and host bean type. Under ideal conditions in an incubator, the range is usually within 3-5 weeks. If kept at about 22 °C, the generation time could increase to 7 weeks. The adult beetles live for about two weeks and do not feed.

Synthetic pesticides are frequently used in pest prevention and control for legume products (Jairoce et al., 2016). Despite the efficiency, this method has side effects, including environmental pollution, impacts on human health by residual pesticide, and the evolution of pesticide resistance. More recently, the possibility of using plant essential oils (EOs) as an alternative to insecticides

has gained increasing attention. The idea of using botanical products in crop protection has been recorded in ancient civilizations (Mssillou et al., 2022). EOs are plant secondary compounds (PSCs) that plants produce in response to the environment, which includes signaling another stage in their life cycle, acting as antimicrobials, and attracting or defending against insects (Teoh, 2016). Therefore, EO could potential be natural pesticides (Afroz et al., 2021).

Modern technology has made extraction of biologically active compounds from plants at high purity relatively accessible. The prospect of pest repellents without the drawbacks of synthetic pesticides is appealing, and a wide range of EOs have been assessed and showed promising results (Kiran et al., 2017; Mssillou et al., 2022; Viteri Jumbo et al., 2014). The EOs could be applied to beans through fumigation, direct/indirect contact, and seed dressing. Several of the commonly used parameters to measure efficacy include the concentration needed to reach LD_{50} (lethal dose; the subscript indicates the percent of the population killed) and above of the adult in a given amount of time, decrease in oviposition, and decrease in bean mass loss after one beetle generation. For example, EO from cinnamon (*Cinnamomum zeylanicum*) showed LD_{50} toxicity against the bean weevil, *Acanthoscelides obtectus* at 46.8 μL/kg (Viteri Jumbo et al., 2014). EO from *Boswellia carterii* showed LD_{100} toxicity against pulse beetles (*C. maculatus* and *C. chinesis*) at 0.10 μL/mL air and significantly reduced their reproductive development at a lower concentration (Kiran et al., 2017).

EOs from plants such as *Matricaria chamomilla L.*, *Pistacia khinjuk, Chrysanthemum* sp, *Cinnamomum aromaticum L.*, *Cymbopogon schoenanthus*, and *Syzygium aromaticum L.* have shown effective insecticidal and repellent activities against *C. maculatus* (Mssillou et al., 2022). Among them, extract from the bud of *Syzygium aromaticum L.*, commonly known as clove, is easy to access. The main volatile compounds in clove oil are eugenol and caryophyllene, making up at

least 80% of the volatile compounds present, although the individual composition could differ depending on the source of clove buds and the extraction process (Mssillou et al., 2022). Eugenol is the compound most credited for insecticidal effects in clove oil. It can change the activity of insect neurons, and at high concentrations, it leads to hyperactivity followed by death (Jankowska et al., 2017). In previous studies, beans treated with clove oil resulted in insecticidal activity, insect repellency, reduced oviposition and decreased loss in bean weight (Noël et al., 2021). For *C. maculatus*, 10 µL of clove oil in a 500 mL jar could lead to 100% beetle mortality within 72 hours. In another study, it was found that 67.6 µL of clove oil per kg of black-eyed peas has LD_{60} toxicity (Viteri Jumbo et al., 2018). In addition, clove oil can significantly decrease the number of beetle offspring emerging, and at sublethal concentrations, it reduces oviposition as well.

Another notable property of EOs is that they can act as antimicrobials. Taking clove oil as an example, it inhibits growth of certain species of both gram-positive and gram-negative bacteria, including *Staphylococcus aureus*, *Serratia marcescens*, *Escherichia coli*, and *Enterococcus faecalis* (Hammer, 1999). As insects have open circulatory systems, the gut microbiome of bean beetles will likely be exposed to the volatile compounds in the air as they breathe. This means that if bean beetles were to survive clove oil exposure, their microbiome could be impacted. Previously, Akami et al. (2019) found a shift in the microbiome composition of bean beetles due to exposure to the EO from the plant *Lippia adoensis.*

Past research has also shown that the insect's microbiome is involved in a range of aspects in insect's survival, such as food digestion, fecundity, immunity, and detoxification or elimination of certain toxins from their system (Afroz et al., 2021; Berasategui et al., 2021; Li et al., 2021; Muhammad et al., 2019). *Enterobacter* and *Staphylococcus* are two genera of bacteria commonly found in bean beetles' gut microbiome, and one strain of bacteria, *Staphylococcus gallinarum*,

might be responsible for providing the beetle larva with essential nutrients (Berasategui et al., 2021). As a result, a shift in the microbiome composition due to EO exposure could negatively impact bean beetles, and the microbiome has shown limited adaptation to the EO from *Lippia adoensis* (Akami et al., 2019). Change in the bean beetle's gut microbiome could give insight into the beetle's susceptibility to clove oil.

Past research on the effects of clove oil on *C. maculatus* often exposed beetles to clove oil for several days or during different stages in its life cycle for only one generation (Mssillou et al., 2022). However, it is crucial to track the response of bean beetles to find out if adaptation could occur, as resistance to clove oil would decrease its efficiency in managing beetle infestations. In addition, in natural conditions beans are harvested and stored in large quantities, in contrast to a relatively small and controlled environment in the laboratory. If EOs are used as pest control but fail to distribute evenly, bean beetles may have a chance to move to an area with lower EO concentration. As a result, it is important to explore the impact of sublethal concentrations of EOs on the beetles and their gut microbiome.

We chose clove oil for its wide accessibility and promising results from previous research (Mssillou et al., 2022). In our experiment, bean beetles were reared in the absence of clove oil and under two sublethal concentrations of clove oil for four generations. Each treatment was replicated four times. To explore the potential of bean beetles to adapt to clove oil exposure, cohort life history traits including total number of emerged adults and emergence time were recorded. To monitor the microbiome of the beetles over time, bacteria from the gut microbiome of beetles from each replicate were cultured and identified by colony morphology and 16s Sanger sequencing of a subset of colonies. For the last generation, whole microbiome community profiling was

performed by MiSeq. In addition, the resistance of a subset bacteria colonies to clove oil was measured with disc diffusion assays.

We hypothesized that bean beetles were capable of responding to clove oil exposure, meaning the experimental groups treated with clove oil would likely have fewer offspring compared to the control at first, but within a few generations, this gap could decrease. While our experiments cannot be used to identify the exact mechanism of a response, we hypothesized that a response could be due to a shift in the community structure of the beetle gut microbiome or due to change in resistance of some bacterial strains to clove oil.

Materials and Methods

Bean Beetle Selection Lines

The stock culture bean beetles used were from replicate stock cultures maintained on organic black-eyed peas at 30 °C for over 20 years. They were originally obtained from infested beans from a grocery store in Columbus, OH.

We created four replicate selection lines for each of three clove oil treatments, adding up to 12 plates of beetles total (Figure 1). Each replicate had 20 beetles, 10 male and 10 female. Beetles from two bottles of stock culture were selected and randomly assigned to the 12 plates as Generation 0 (Gen 0) beetles. They were reared in large petri dishes (14 cm diameter, 2 cm height) with 25 ± 0.1 g organic black-eyed peas. Different concentrations of clove oil were added once all the beetles and beans were present in the dish (Figure 1). The dish was immediately sealed with parafilm. The dead beetles were removed after about 3 weeks after the trial started. Once the new generation emerged, the first 20 (10 males and 10 females) beetles were removed within 48 hours

from the original dish and transferred to a clear petri dish, where they were kept until all 20 beetles emerged. They were then transferred to a new, large petri dish with black-eyed peas. The process was repeated for a total of four generations. Due to the limitations of available incubator space, the beetles in the experiment were reared at room temperature.

In our experimental design, beetles were exposed to no clove oil, a low concentration of clove oil, or a high concentration of clove oil (Figure 1). As clove oil is volatile, we calculated the concentration as µL clove oil per cm^3 of container volume. We found during the pilot experiments that 0.003 µL/cm^3 and 0.014 µL/cm^3 may have an effect on bean beetle population parameters and the beetle microbiome (Shao & Beck, 2022). The group with no clove oil exposure was used as control. The interior volume of the petri dish used was about 334 cm^3, which converted to about 4.4 µL clove oil per dish for the higher concentration, and 1 µL clove oil per dish for the lower concentration. The clove oil used was purchased from rareEssence Aromatherapy and diluted to 50% in DMSO to measure the volume accurately. To control for the potential impact of DMSO on bean beetles, 4.4 µL of DMSO was added to the control group. The solutions were added onto 1 cm^2 filter paper disks placed in the middle of the petri dish under the beans.

Bean Beetle Cohort Life History Traits

The beetle emergence time (first beetle emergence date minus replicate start date) and number of successful emergences were recorded for all 12 plates across all four generations. As temperature fluctuations occurred in between Gen 3 and Gen 4 beetles, the beetles were moved to 30 °C incubators when room temperature dropped below 17 °C. A drastic decrease in the number of successful emergences and an increase in emergence time were seen. As these changes were most likely due to the lower temperature, not clove oil treatment, the data from Gen 4 was not included in analysis. Two-way ANOVAs were performed to analyze emergence time and number

of emergences with generation and clove oil concentration as independent variables. A significance level of $\alpha = 0.05$ was used. Figures 2 – 5 in this paper were generated using *ggplot2* and *dplyr* packages in R.

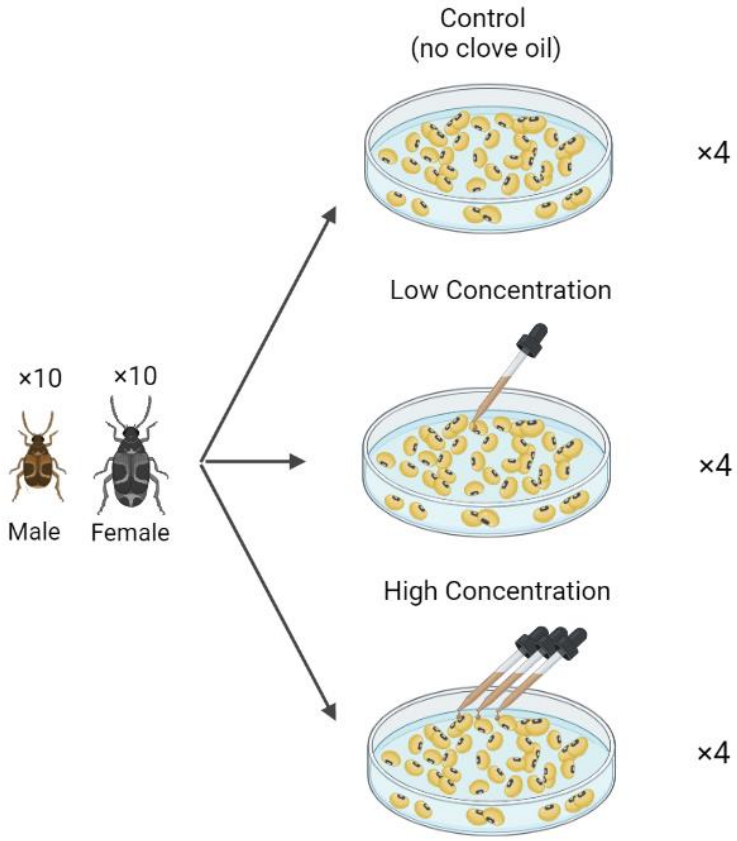


Figure 1. Illustration of the experimental setup.

Culturing Beetle Microbiome

For each generation, within 10 days of the first emergence, five beetles were removed from each plate. Depending on the order of first emergence time, the five beetles removed alternated between three females/two males and two females/three males. For each treatment, a total of 10 female and 10 male beetles were removed across the four replicates. The beetles were first killed through freezing for five minutes at -80 °C and surface-sterilized through soaking in the following solutions in the given order: 10% bleach (3 seconds), deionized (DI) water (10 seconds), 70% ethanol (5 seconds), DI water (10 seconds). They were then ground in 200 µL sterile phosphate-

based saline (PBS) solution in 250 µL tubes with a sterile pestle. The whole beetle homogenate was then serially diluted 10^3-fold in PBS. The 10^{-1} and 10^{-3} dilutions were plated on nutrient agar (NA) plates. The NA plates were then kept at room temperature.

Analysis and Identification of Cultured Microbes

To identify a potential shift in microbiome composition, the number of bacterial colonies and the morphology of bacterial colonies that were cultured was recorded. The colony counts were converted to an estimated CFU in the beetle's gut. The colony phenotype traits recorded include the color, form (e.g., circular/irregular), gloss (e.g., shiny/matte) and elevation (e.g., raised/flat). A considerable number of the NA plates showed no growth, and the distribution of the data was right skewed. When the zeros were not taken into consideration, the distribution was close to normal (Figure 2). To analyze the trends in zero counts, the data was first transformed into a binomial model depending on whether the colony count was zero or positive. Then, the positive counts were plotted alone.

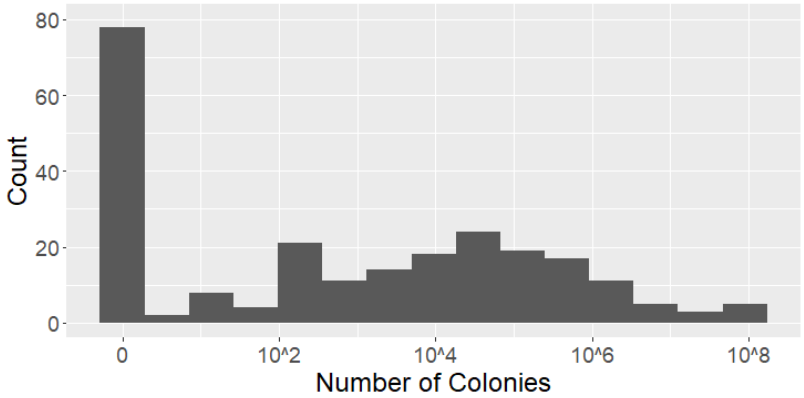


Figure 2. Distribution of cultured colony count data. x-axis is in logarithmic scale.

To identify cultured bacteria, 16s rRNA PCR (27F/1492R primers) was performed on colonies of distinct morphology. A small amount of the colony was suspended in 100 µL molecular grade water. 7.5 µL was added to 17.5 µL master mix, which was made from OneTaq Hot Start

Quick-Load 2X Master Mix according to the product's instructions. Each PCR cycle was set with 10 minutes of denaturation at 95 °C in the beginning. Then, 36 cycles of the following were done: denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds and extension at 72 °C for 90 seconds. An extra four minutes of extension at 72 °C was performed at the end of the run. The product was kept at 4 °C in the PCR machine and then frozen at -20 °C until sequencing. PCR products were confirmed through DNA gel electrophoresis and then sequenced using the 27F primer for Sanger sequencing by Azenta/Genewiz. The sequencing result was processed by trimming off low quality nucleotide reads and identified to the genus-level through BLAST. A stacked bar graph was generated based on percent composition for each unique genus. The total number of CFU across four generations was taken into account. *Enterobacter* was not included in the graph, as its predominance masks the presence of other less common genera.

The 16s rRNA sequences of *Enterobacter* from the three clove oil treatments and four generations were compiled. A multiple sequence alignment was performed through Clustal Omega to generate two phylogenetic trees using the neighbor-joining and unweighted pair group method with arithmetic mean (McWilliam et al., 2013). The results allowed comparison of the genetic variation between different *Enterobacter* strains. There were 43 total samples, with 18 samples from Generation 1, seven from Generation 2, 11 from Generation 3 and seven from Generation 4. Fourteen of these samples were from the control group, 19 from low treatment and 10 from high treatment.

Disc Diffusion Assays

To explore whether there was a difference in resistance to clove oil for the bacteria from the three treatments, three bacterial colonies (two *Enterobacter* colonies and one *Enterococcus* colony) that were isolated from each replicate of beetles in the last generation (Generation 4) were

selected for disc diffusion assays. Two lab strain *Escherichia coli* colonies were chosen as positive controls (grown from live tryptic agar tube cultures at room temperature; purchased from Carolina Biological). Each selected bacterial colony was suspended in two separate sterile glass tubes containing 5 mL nutrient broth and grown overnight, shaken at 150 rpm at room temperature. For each tube, the resulting OD600 absorbance was measured using a spectrophotometer and diluted to between 0.300 to 0.400. One tube from each colony was selected for the disc diffusion assay, and two replicate plates were used for each tube. After swabbing the surface of an entire NA plate with a sterile cotton swab, a 6 mm diameter diffusion disc was placed in the center, and 10 µL of clove oil was subsequently added. The plates were immediately sealed with parafilm and kept at room temperature. The inhibition zone diameter (in cm) was measured after 24 hours. The results were compared by taking the means and 2 * SEM of the measurements.

Whole-Community Microbiome Sequencing

For the last generation (Generation 4), we sampled the entire microbial community to determine if exposure to clove oil resulted in a shift in the bean beetle microbiome. DNA was extracted from five female beetles from each treatment using the QIAGEN DNA Miniprep procedure according to the manufacturer's protocol with one modification. Molecular grade water was used as the elusion buffer at the end. The samples were stored at -20˚C until sequencing. A blank control was created with the same extraction procedure but no beetle at the beginning. The quality of the extraction was confirmed with the A260/A280 value being in between 1.75 – 2.1 and having a clear peak at 260 nm. The nucleic acid concentration was adjusted to values between 20 – 30 ng/µL. DNA samples were sequenced by Illumina MiSeq (2 x 300 Paired End) for the V4 region of the 16s rRNA gene with the 515F (GTGYCAGCMGCCGCGGTAA) and 806R

(GGACTACNVGGGTWTCTAAT) primers to a depth of at least 20,000 base pairs per sample by Mr. DNA Lab.

The abundance and identity of bacterial taxa was identified through the DNA Subway bioinformatics pipeline that runs QIIME2 (Bolyen et al., 2019) in the background. Sequences (N = 10,000) were checked for quality and demultiplexed sequences with the bottom 25th percentile of quality score recurring under 30 were trimmed off. In the DADA2 step, trim length for forward reads was 0 bp and reverse reads was 234 bp. The minimum number of reads per sample was 29227, which was used as the maximum sequencing depth for alpha rarefaction. The classifier chosen to assign bacteria taxa was Greengenes (515F/806R). The level-5 (family level) taxonomic diversity file was used for further analysis. Before analysis, the archaea, chloroplast, mitochondria, and unassigned types were removed. In addition, as the blank control sample returned a number of reads comparable to over half of the samples, these reads were subtracted from each sample. If subtraction resulted in a negative number, it was adjusted to zero. The resulting file was analyzed and graphed on the RShiny Bean Beetle Microbiome Analysis App (Huang et al., 2022). In particular, the alpha diversity was measured using rarified data at both the phylum and the family levels. The richness and the Shannon diversity of the samples were calculated and compared across treatments with a one-way ANOVA. Beta diversity was also measured using rarified data at both the phylum and the family levels. Bray-Curtis distance measure and NMDS ordination method were used. Differences in community structure across treatments were determined using PERMANOVA.

The data was also adjusted by removing reads from the most predominant family Enterobacteriaceae so that less common reads would be more visible. The beetle replicate labelled “High.3.B1” had no reads other than Enterobacteriaceae, so it was removed to ensure the rest of

the data could be processed normally. The same tests mentioned above were run on the adjusted data on the RShiny app.

Results:

Bean Beetle Cohort Life History Traits

Number of Successful Emergences

In general, the number of beetle emergences was significantly affected by generation and clove oil treatment but not the interaction between the two (Table 1). Significantly fewer beetles emerged in Generation 1 as compared to Generation 2 ($p = 0.030$) and Generation 3 ($p = 0.007$) (Figure 3). In comparison to the control, significantly fewer beetles emerged from the high clove oil treatment ($p = 0.006$) but not from the low clove oil treatment ($p = 0.52$) (Figure 3a). Trends for individual replicates within each treatment did not follow the general trends completely (Figure 3b). For example, in the control group, one replicate line of beetles had a lower number of emergences in Generation 2 than Generation 1, and another had lower number of emergences in Generation 3 than Generation 2. In both low and high clove oil treatments, one replicate had a lower number of emergences in Generation 3 than Generation 2.

Table 1. ANOVA table for number of successful beetle emergences.

	df	F	P
Generation	2	24.85	< 0.001
Treatment	2	4.45	0.021
Generation : Treatment	4	1.02	0.414

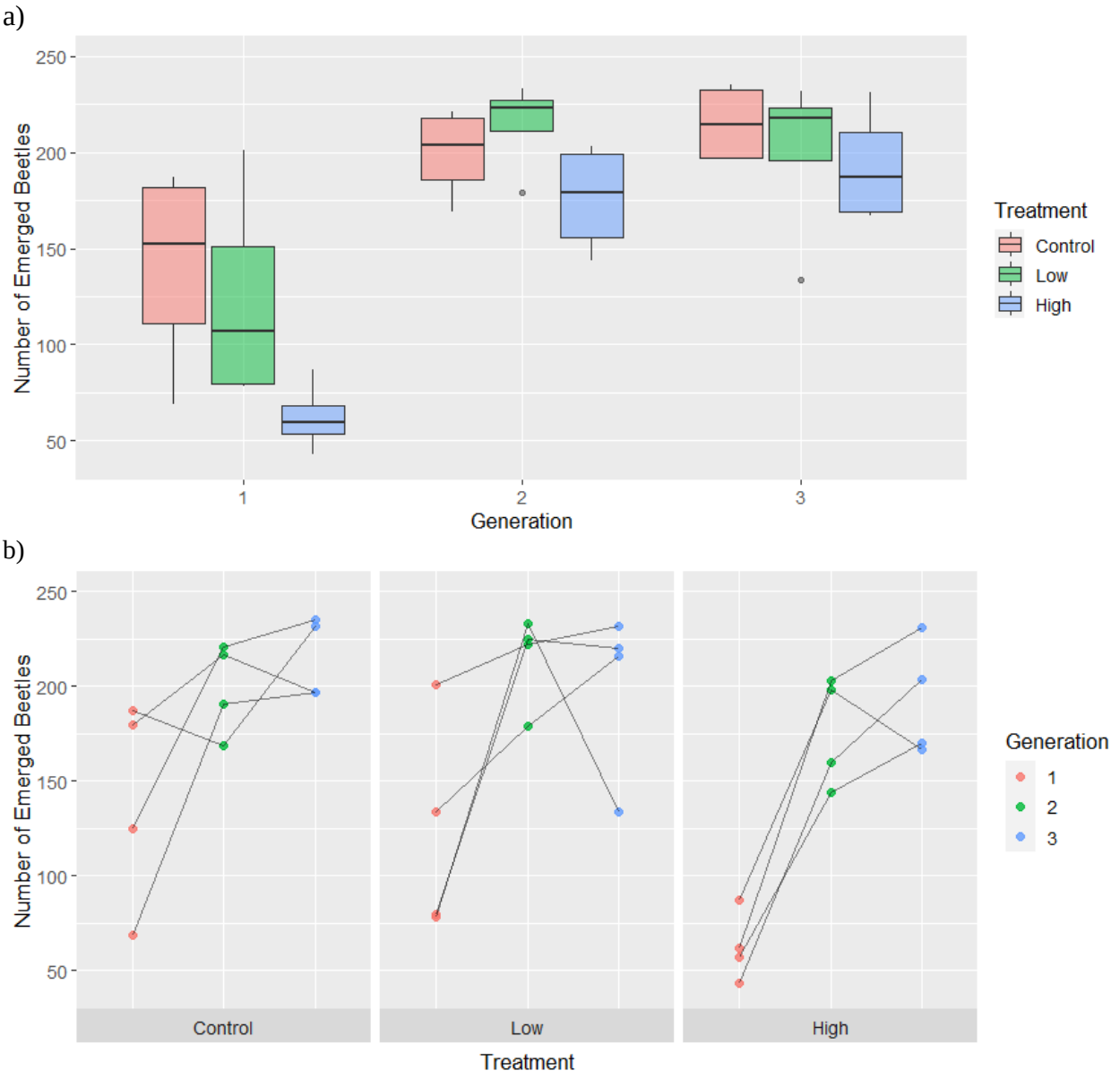


Figure 3a – b. a) Differences in successful beetle emergence between the three clove oil treatments across three generations. b) The number of emerged beetles each replicate from control, low and high clove oil treatments across three generations.

Time to Emergence

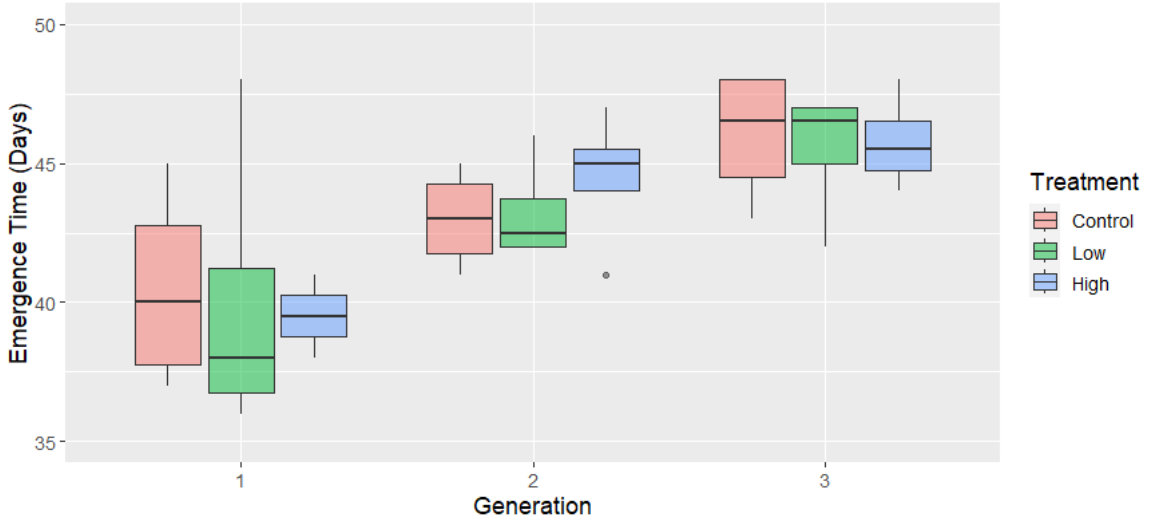
In general, time to emergence was significantly influenced by the generation but not the treatment or the interaction between the two (Table 2). Beetles from Generation 3 showed significantly longer emergence times compared to those from Generation 1 ($p = 0.011$) but not Generation 2 ($p = 0.23$) (Figure 4a). Like the data on the number of emerged beetles, trends for

individual replicates within a treatment did not follow the general trends completely (Figure 4b). For example, one line of beetles from control and low clove oil treatment showed shorter emergence times in Generation 2 than Generation 1. Similarly, one line of beetles from low and high clove oil treatment showed shorter emergence times in Generation 3 than Generation 2.

Table 2. ANOVA table for time to emergence.

	df	F	P
Generation	2	12.45	< 0.001
Treatment	2	0.04	0.96
Generation : Treatment	4	0.21	0.93

a)



b)

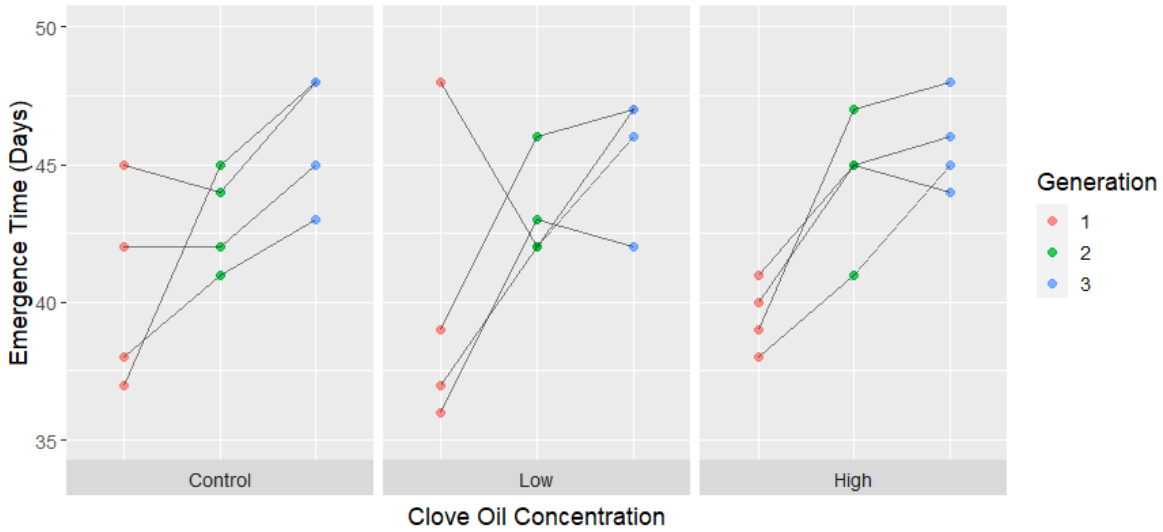


Figure 4a – b. a) Differences in time to emergence between the clove oil three treatments across three generations. b) Time to emergence of each replicate from three clove oil treatments across three generations.

Cultured Bean Beetle Microbiome

Colony Counts

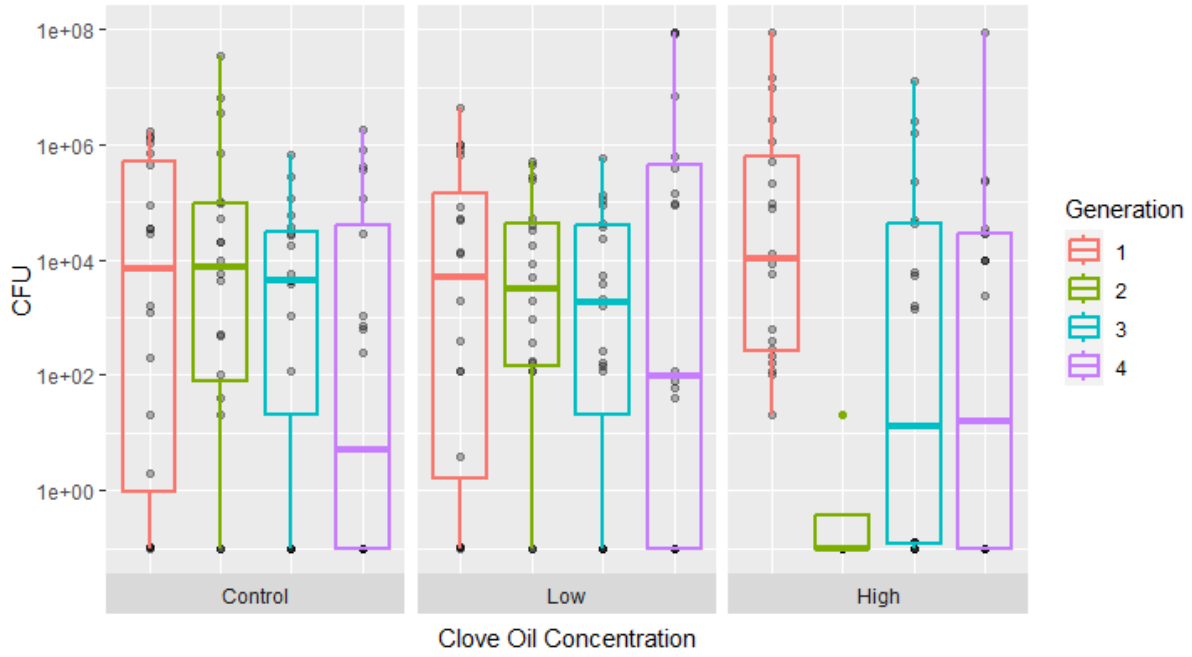
A total of 32.5% of the 240 replicates from cultured beetle gut microbiome showed no colony growth. Of the replicates that showed colonies, CFU in the beetles' guts were calculated to vary between 10^1 and 10^8 (Figure 5a). The mean number of CFU for control and low clove oil treatment lines of beetles did not show consistent patterns, but there was a uniform decrease in mean CFU for all four lines of beetles from the high clove oil treatment from Generation 1 to 2. The CFU of beetles from each of beetle lines was shown in Figure 5d.

The number of zero colony counts varied depending on the generation. In Generation 1, we were able to culture bacteria from 83.3% of beetles. In Generation 2, the number dropped to 65%, and it slightly increased to 66.7% in Generation 3. However, in Generation 4, we only cultured bacteria from 55% of the beetles. The three clove oil treatments also showed different patterns of change over four generations (Figure 5b). For control and low clove oil treatment groups across the four generations, with a slight increase in the number of replicates with nonzero colony counts from Generation 1 to 2, and then a constant decrease from Generation 2 to 4. However, for high clove oil treatment, there was a drastic decrease in the number of replicates with nonzero colony counts from Generation 1 to 2, an increase from Generation 2 to 3, and the number remained the same in Generation 4.

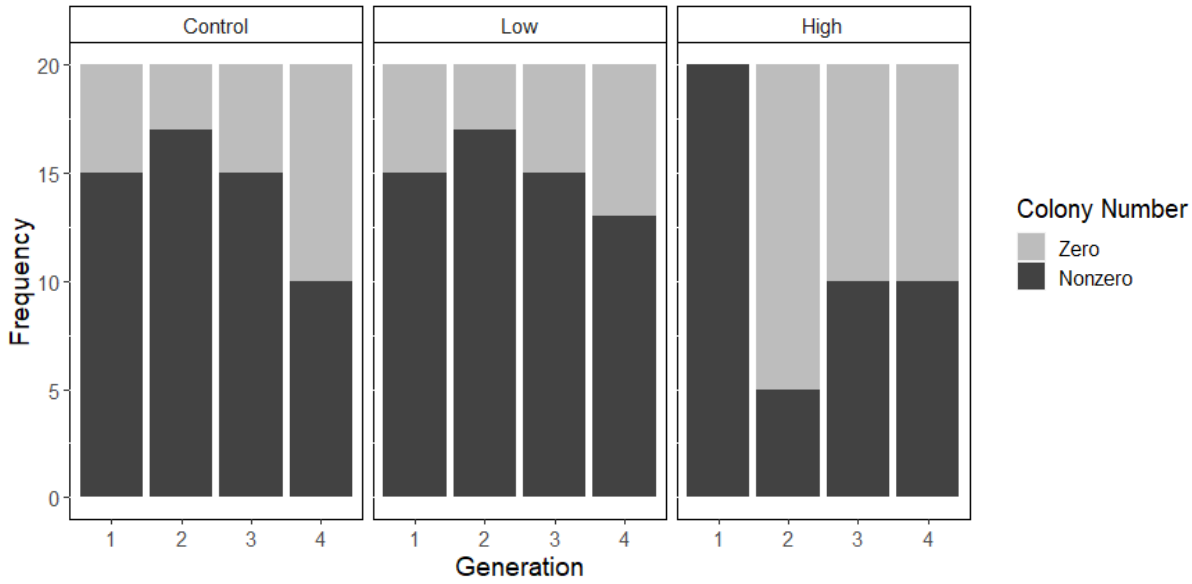
When only nonzero CFU was taken into consideration, in Generation 1, the CFU in the control and low clove oil treatment were higher than high clove oil treatment (Figure 5c). This

remained the same in Generation 2. In Generation 3, the high treatment showed a higher mean CFU than the control and low treatment. In Generation 4, the mean for high treatment was again lower than the control and low treatment.

a)



b)



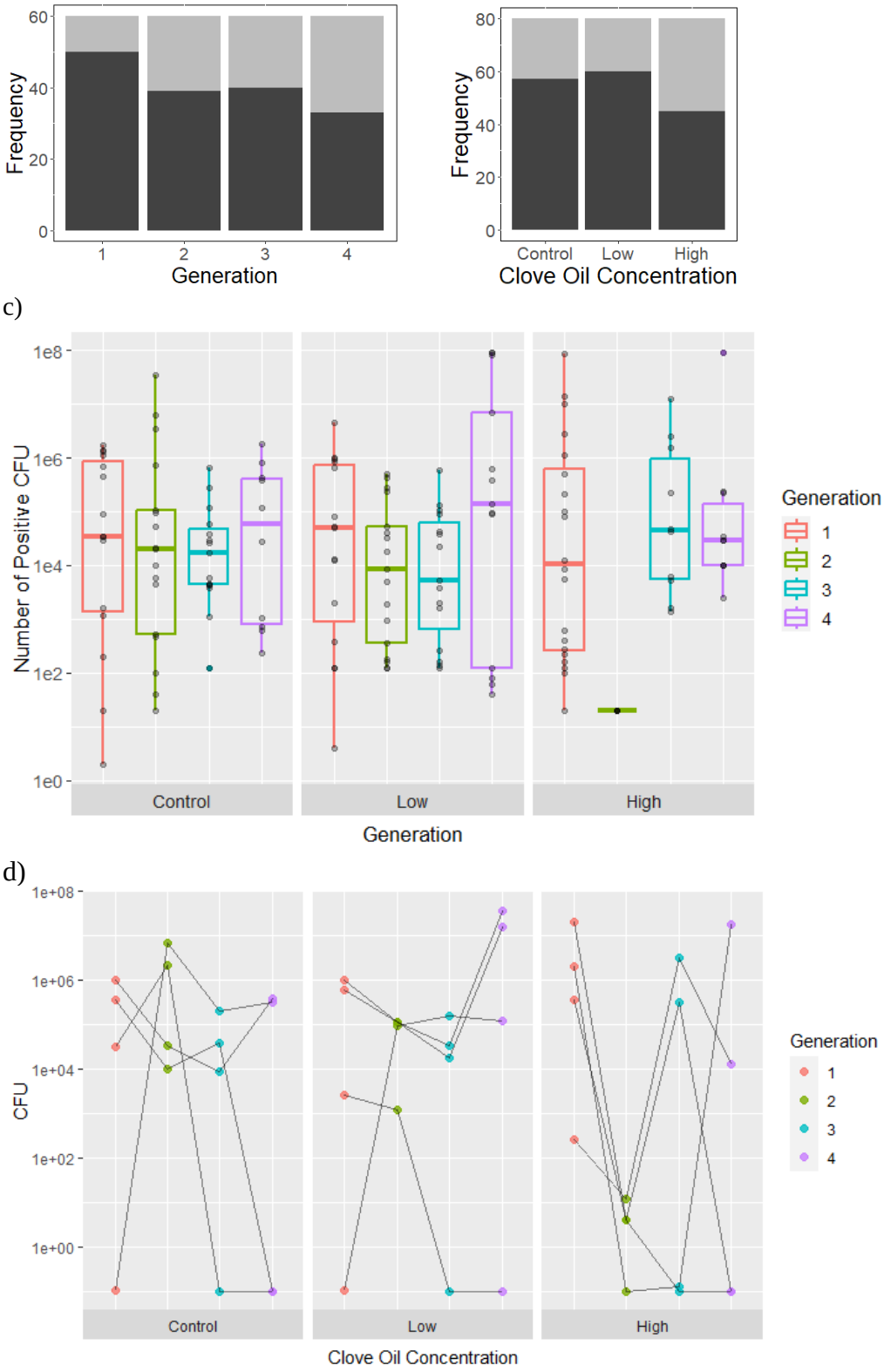


Figure 5a – d. a) Colony counts from the cultured microbiome from each beetle overlaid with box plot by treatment and generations. b) Stacked bar graphs representing the effect of clove oil treatment, generation, and the interaction between the two on the colony number cultured from bean beetle's gut being zero. c) Nonzero colony counts from the cultured microbiome from each

beetle overlaid with box plot by treatment and generations. d) Mean colony count from each replicate for three clove oil treatments in four generations.

Colony Diversity

Distinct colony morphologies were recorded in Table 3. The most common genus of bacteria present throughout the four generations of beetles for all three clove oil treatments was *Enterobacter*, which was present in all replicates except in high clove oil treatment in Generation 2 (Table 4). The total colony count of *Enterobacter* made up 98.60% of all colonies in the entire experiment. It should be noted that in Generation 2, *Brevibacterium*, *Gordonia*, one type of *Staphylococcus* colony, *Paenibacillus*, and *Pseudarthrobacter* were observed on only one replicate plate. Among them, *Brevibacterium*, *Gordonia*, and *Pseudarthrobacter* have not been recorded in the bean beetle culture line (Blumer & Christopher W. Beck, 2021). If these rare occurrences were excluded, the species diversity remained mostly uniform for the beetle lines during the first two generations and all treatments, before *Enterococcus* appeared. The percentage of *Enterobacter* was between 98 to 100% of all colonies. In Generation 3, *Enterococcus* colonies increased the species diversity, as it made up 81%, 24% and 9% of all colonies for control, low and high treatments, respectively. In Generation 4, these numbers dropped to 46%, 1% and ~0% for the three treatments, respectively.

The stacked bar graph shows that during the four generations the majority of the beetle lines showed only one dominant genus of colonies in addition to *Enterobacter* (Figure 6). There was no clear trend across the three treatments. The 16s rRNA gene samples collected from *Enterobacter* isolates during the entire experiment were compared, as shown in the phylogenetic trees. No clear clustering for generations or treatments was found (Figure 7).

Table 3. List of distinct colony morphology.

Genus	Morphology			
	Color	Form	Gloss	Elevation
Brevibacterium	white	circular	shiny	flat
Enterobacter	off-white	circular	shiny	raised
Enterococcus	white	circular	shiny	raised
Gordonia	pink	irregular	matte	raised
Microbacterium	orange	circular	shiny	raised
Paenibacillus	off-white	circular	shiny	raised
Pseudarthrobacter	off-white	circular	shiny	raised
Staphylococcus	white	irregular	matte	raised
Staphylococcus	white	circular	shiny	flat
Staphylococcus	yellow	circular	shiny	raised

Table 4. List of the genera of bacteria found in plated bean beetle microbiome across 4 generations from 3 treatments. Names arranged alphabetically. Distinct colony morphology was included.

Generation	Genera of Bacteria		
	Control	Low	High
1	*Enterobacter* *Staphylococcus*	*Enterobacter*	*Enterobacter* *Staphylococcus*
2	*Enterobacter* *Staphylococcus*	*Brevibacterium* *Enterobacter* *Gordonia* *Microbacterium* *Staphylococcus* (×2)	*Microbacterium* *Paenibacillus* *Pseudarthrobacter*
3	*Enterobacter* *Staphylococcus* *Enterococcus*	*Enterobacter* *Enterococcus*	*Enterobacter* *Enterococcus*
4	*Enterobacter* *Enterococcus*	*Enterobacter* *Enterococcus*	*Enterobacter* *Enterococcus*

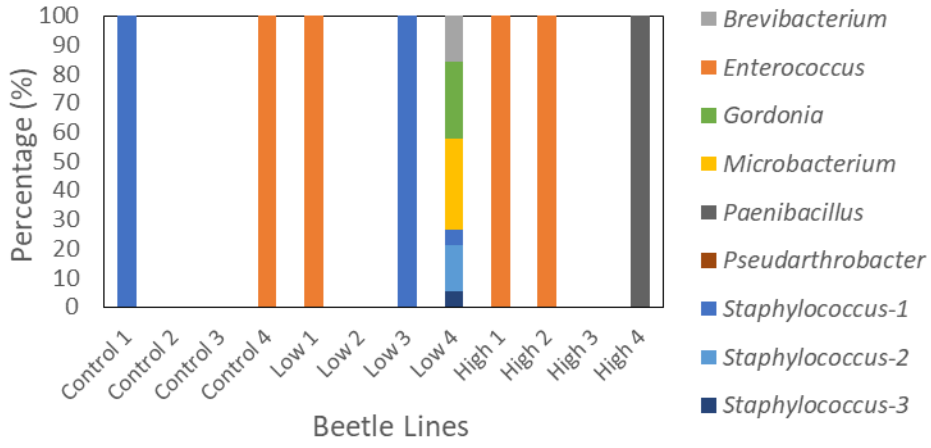


Figure 6. Total colony diversity from four generations in the 12 beetle lines without *Enterobacter*.

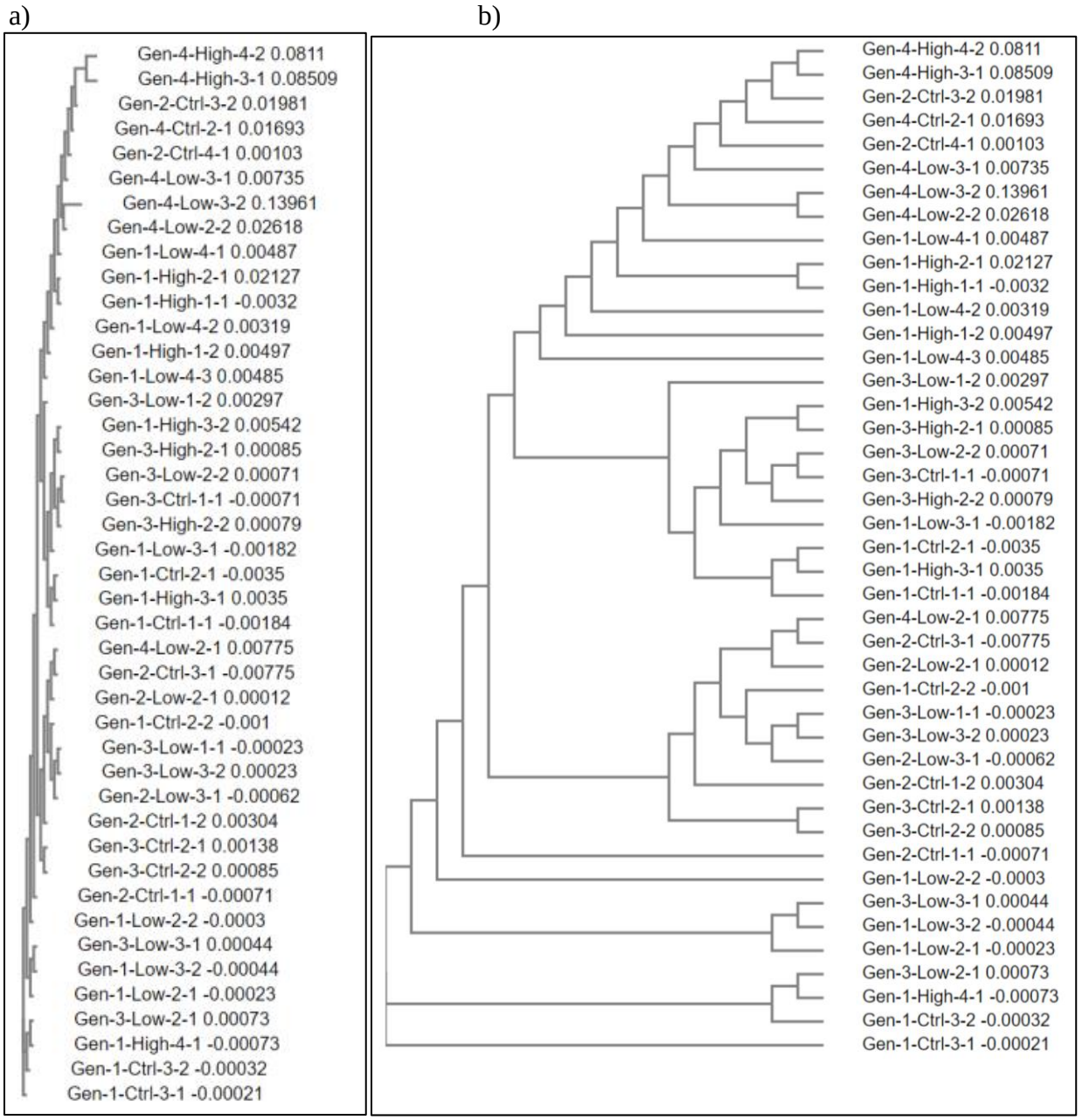


Figure 7a – b. Phylogenetic trees of *Enterobacter* strains from three clove oil treatments collected from four generations. The samples were labelled as the following: "Gen"-Generation Number-Treatment-Treatment Line-Replicate Number". The sample IDs were followed by the numbers indicating the evolutionary distance between the sequences (McWilliam et al., 2013). a) Phylogenetic tree without distance corrections. b) Cladogram of the same samples.

Disc Diffusion Assays

There was no significant difference between the level of resistance in the bacteria from the three treatments, as error bars overlapped (Figure 8). The difference between the level of resistance

in *Enterobacter* and *Enterococcus* was not significant, either, as error bars had overlap as well. However, the mean diameter of the inhibition zones of *Enterococcus* was lower than that of *Enterobacter*, which reflected potentially stronger resistance from the former. Overall, the bacteria isolates from bean beetle's gut showed stronger resistance than lab strain *E. coli*.

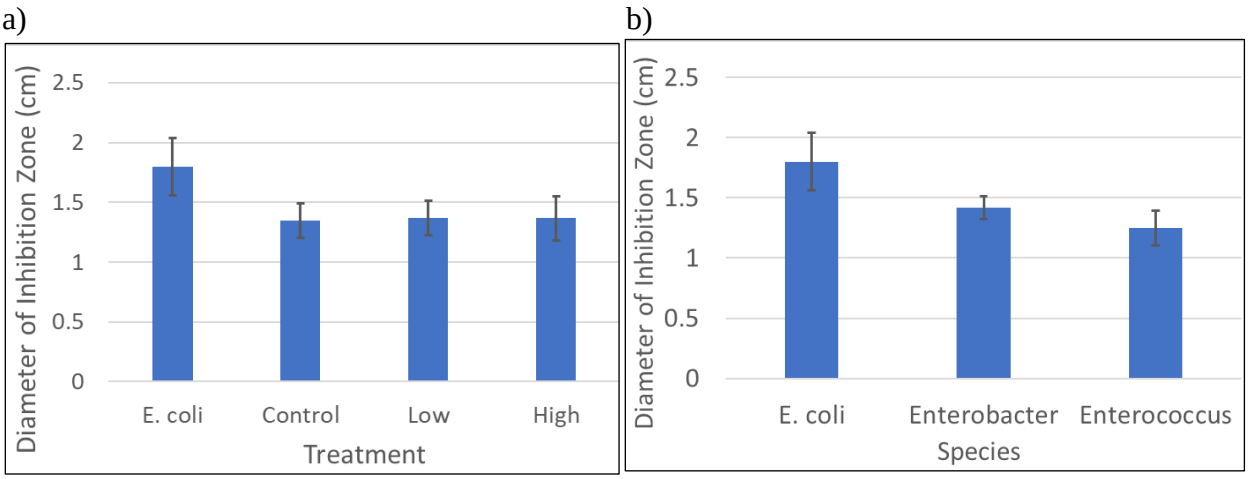
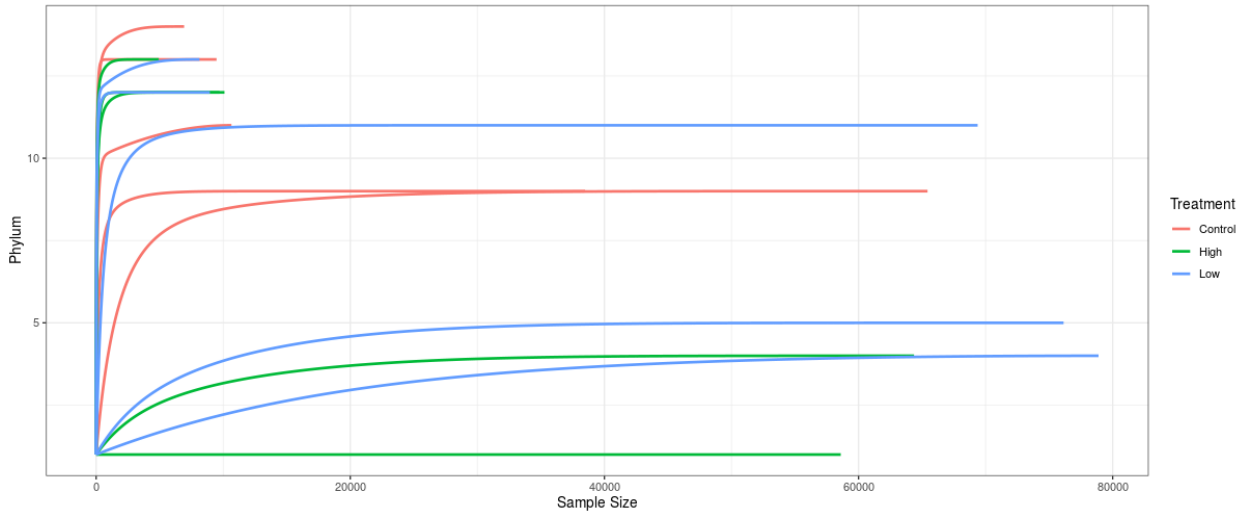


Figure 8a – b. Height of the bars represented the mean diameter of the inhibition zone, and error bars represent 2 * SEM. a) Bacteria isolates from the three clove oil treatments. n = 4 for *E. coli*; n = 12 for each treatment. b) *Enterobacter* and *Enterococcus* strains from the treatments. n = 4 for *E. coli*; n = 12 for *Enterobacter*; n = 6 for *Enterococcus*.

Whole-Community Microbiome Sequencing

The rarefaction curves were shown in Figure 9. No clear difference was found between the bean beetle gut microbiome according to the next-generation sequencing results. The beetle lines with zero colony counts in cultured microbiome in Generation 4 often corresponded to lower absolute abundance in comparison to the lines with nonzero readings (Figure 10a, d). There was more variation in bacterial phyla and families in beetles with lower absolute abundance compared to ones with higher abundance (Figure 10b, e). Proteobacteria was the most abundant phylum in those with high absolute abundance except one control group, where Firmicutes was the most abundant (Figure 10a). Beetles from the same lines have similar microbiome composition.

a)



b)

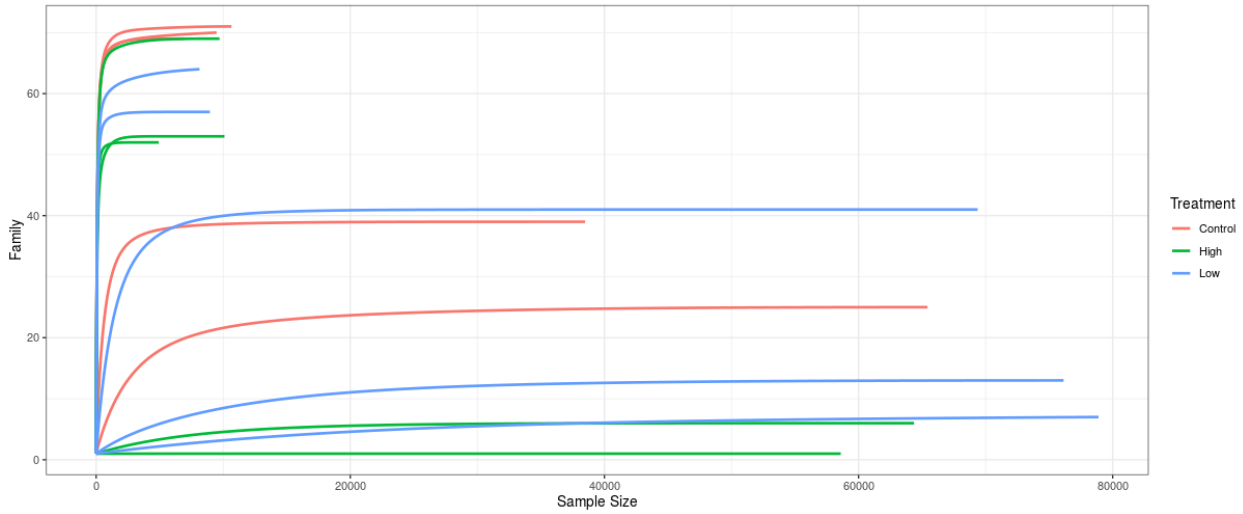
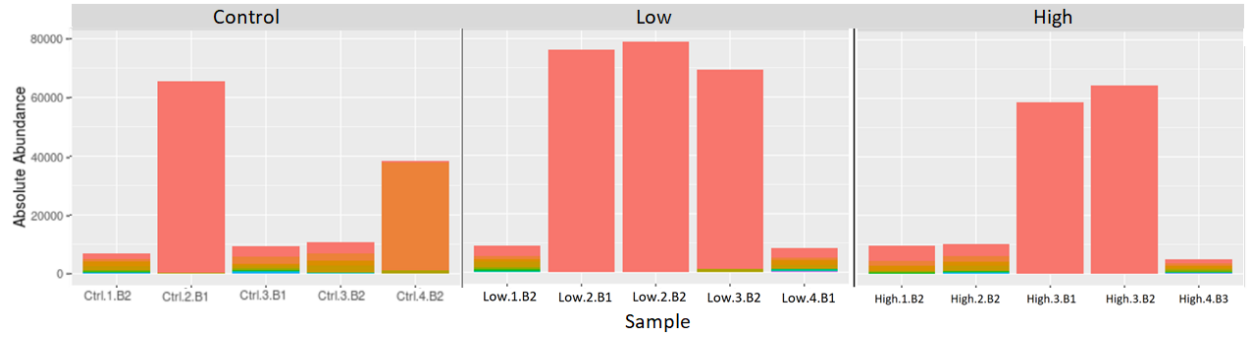
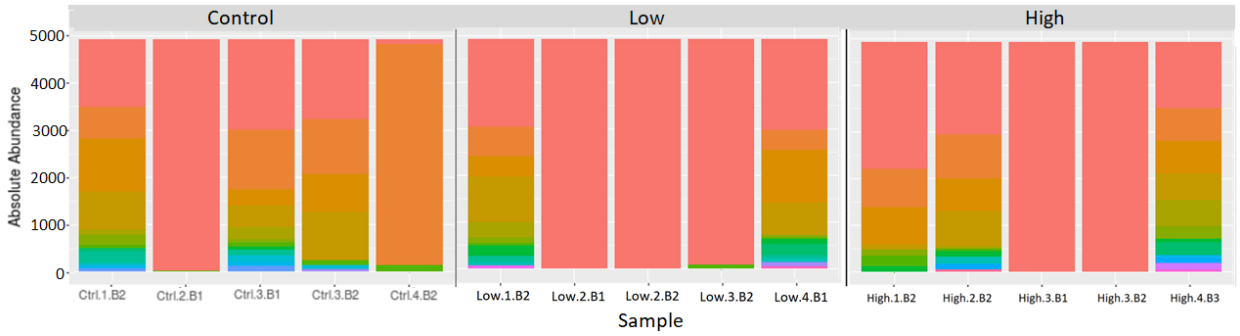


Figure 9a – b. Rarefaction curves for samples grouped by treatment. a) Data at phylum level. b) data at family level.

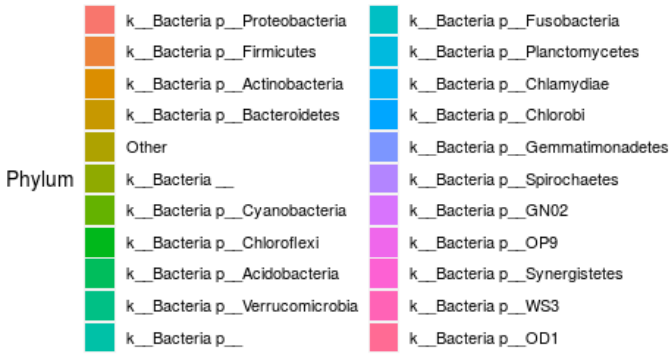
a)



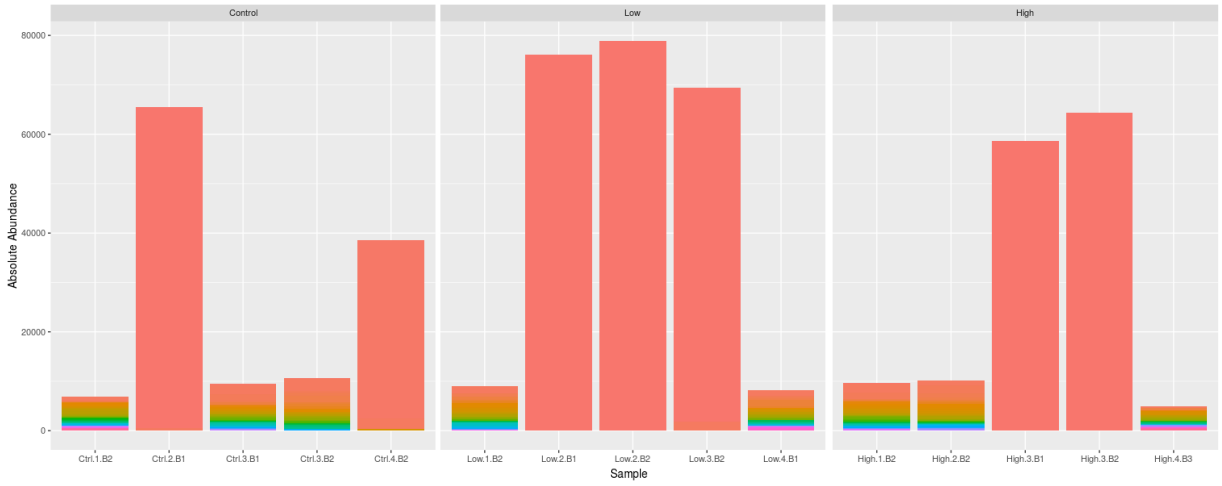
b)


Control
Low
High
Absolute Abundance
5000
4000
3000
2000
1000
0
Ctrl.1.B2
Ctrl.2.B1
Ctrl.3.B1
Ctrl.3.B2
Ctrl.4.B2
Low.1.B2
Low.2.B1
Low.2.B2
Low.3.B2
Low.4.B1
High.1.B2
High.2.B2
High.3.B1
High.3.B2
High.4.B3
Sample

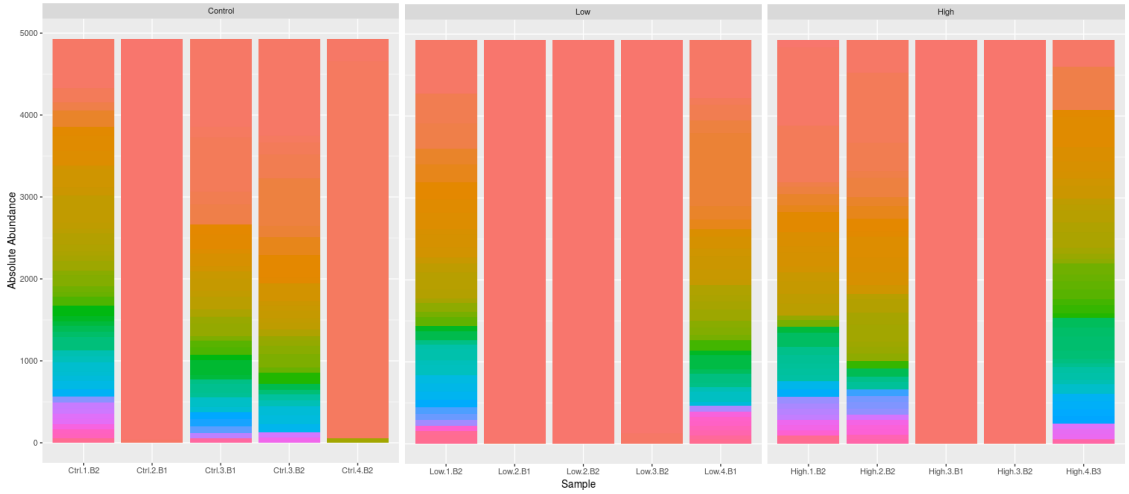
c)


Phylum
k__Bacteria p__Proteobacteria
k__Bacteria p__Firmicutes
k__Bacteria p__Actinobacteria
k__Bacteria p__Bacteroidetes
Other
k__Bacteria __
k__Bacteria p__Cyanobacteria
k__Bacteria p__Chloroflexi
k__Bacteria p__Acidobacteria
k__Bacteria p__Verrucomicrobia
k__Bacteria p__
k__Bacteria p__Fusobacteria
k__Bacteria p__Planctomycetes
k__Bacteria p__Chlamydiae
k__Bacteria p__Chlorobi
k__Bacteria p__Gemmatimonadetes
k__Bacteria p__Spirochaetes
k__Bacteria p__GN02
k__Bacteria p__OP9
k__Bacteria p__Synergistetes
k__Bacteria p__WS3
k__Bacteria p__OD1

d)


Control
Low
High
Absolute Abundance
80000
60000
40000
20000
0
Ctrl.1.B2
Ctrl.2.B1
Ctrl.3.B1
Ctrl.3.B2
Ctrl.4.B2
Low.1.B2
Low.2.B1
Low.2.B2
Low.3.B2
Low.4.B1
High.1.B2
High.2.B2
High.3.B1
High.3.B2
High.4.B3
Sample

e)



f)

Family

k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Enterobacteriales f__Enterobacteriaceae
k__Bacteria p__Firmicutes c__Bacilli o__Lactobacillales f__Enterococcaceae
Other
k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Pseudomonadales f__Pseudomonadaceae
k__Bacteria p__Bacteroidetes c__Bacteroidia o__Bacteroidales f__Bacteroidaceae
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Lachnospiraceae
k__Bacteria __ __ __ __
k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Pseudomonadales f__Moraxellaceae
k__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Corynebacteriaceae
k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Aeromonadales f__Aeromonadaceae
k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Neisseriales f__Neisseriaceae
k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Oxalobacteraceae
k__Bacteria p__Bacteroidetes c__Bacteroidia o__Bacteroidales f__Rikenellaceae
k__Bacteria p__Bacteroidetes c__Bacteroidia o__Bacteroidales f__S24-7
k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Caulobacterales f__Caulobacteraceae
k__Bacteria p__Firmicutes c__Bacilli o__Bacillales __
k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Sphingomonadales f__Sphingomonadaceae
k__Bacteria p__Firmicutes c__Bacilli o__Lactobacillales f__Carnobacteriaceae
k__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Streptomycetaceae
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__
k__Bacteria p__Firmicutes c__Erysipelotrichi o__Erysipelotrichales f__Erysipelotrichaceae
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Ruminococcaceae
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__[Tissierellaceae]
k__Bacteria p__Bacteroidetes c__Flavobacteriia o__Flavobacteriales f__Flavobacteriaceae
k__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Microbacteriaceae
k__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Actinomycetaceae
k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Rhizobiales __
k__Bacteria p__Bacteroidetes c__[Saprospirae] o__[Saprospirales] f__Chitinophagaceae
k__Bacteria p__ c__ o__ f__
k__Bacteria p__Bacteroidetes c__Cytophagia o__Cytophagales f__Cyclobacteriaceae
k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Rhizobiales f__Bradyrhizobiaceae
k__Bacteria p__Fusobacteria c__Fusobacteriia o__Fusobacteriales f__Fusobacteriaceae
k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Rhizobiales f__Methylobacteriaceae
k__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Mycobacteriaceae
k__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Dermabacteraceae
k__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Geodermatophilaceae
k__Bacteria p__Proteobacteria c__Deltaproteobacteria o__Desulfovibrionales f__Desulfovibrionaceae
k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Rhodospirillales f__Rhodospirillaceae
k__Bacteria p__Bacteroidetes c__Bacteroidia o__Bacteroidales f__[Paraprevotellaceae]
k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Oceanospirillales f__Halomonadaceae
k__Bacteria p__Bacteroidetes c__[Saprospirae] o__[Saprospirales] f__Saprospiraceae
k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Rhodobacterales f__Rhodobacteraceae
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Clostridiaceae
k__Bacteria p__Firmicutes c__Bacilli o__Gemellales f__
k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Sphingomonadales f__Erythrobacteraceae
k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Alteromonadales f__[Chromatiaceae]
k__Bacteria p__Proteobacteria c__Betaproteobacteria o__ f__
k__Bacteria p__Proteobacteria c__Deltaproteobacteria o__Desulfovibrionales f__
k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Methylococcales f__Crenotrichaceae
k__Bacteria p__Cyanobacteria __ __ __
k__Bacteria p__Firmicutes c__Clostridia o__Natranaerobiales f__ML1228J-1
k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__RF32 f__
k__Bacteria p__Chloroflexi c__Anaerolineae o__SJA-15 f__
k__Bacteria p__Actinobacteria c__Thermoleophilia o__Gaiellales f__AK1AB1_02E
k__Bacteria p__Cyanobacteria c__4C0d-2 o__YS2 f__
k__Bacteria p__Proteobacteria c__Deltaproteobacteria o__Desulfovibrionales f__Desulfohalobiaceae
k__Bacteria p__Proteobacteria c__Deltaproteobacteria o__Desulfuromonadales f__Geobacteraceae
k__Bacteria p__Proteobacteria c__ o__ f__
k__Bacteria p__Firmicutes c__Bacilli o__Turicibacterales f__Turicibacteraceae
k__Bacteria p__Gemmatimonadetes c__Gemm-1 o__ f__
k__Bacteria p__Acidobacteria c__Acidobacteria-6 o__iii1-15 f__
k__Bacteria p__Acidobacteria c__Acidobacteria-6 o__iii1-15 f__mb2424
k__Bacteria p__Firmicutes c__Bacilli o__Bacillales f__[Exiguobacteraceae]
k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Rhizobiales f__Beijerinckiaceae
k__Bacteria p__Proteobacteria c__Betaproteobacteria o__IS-44 f__
k__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__ACK-M1
k__Bacteria p__Actinobacteria c__Thermoleophilia o__Solirubrobacterales f__
k__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Intrasporangiaceae
k__Bacteria p__Actinobacteria __ __ __
k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Alteromonadales f__211ds20
k__Bacteria p__Chloroflexi c__Anaerolineae o__SBR1031 __
k__Bacteria p__Actinobacteria c__Rubrobacteria o__Rubrobacterales f__Rubrobacteraceae
k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Rhizobiales f__Brucellaceae
k__Bacteria p__Actinobacteria c__Actinobacteria o__ f__
k__Bacteria p__Chloroflexi c__Chloroflexi o__[Roseiflexales] f__
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Peptococcaceae
k__Bacteria p__Proteobacteria c__Deltaproteobacteria o__Myxococcales f__
k__Bacteria p__Spirochaetes c__[Brevinematae] o__[Brevinematales] f__Brevinemataceae
k__Bacteria p__Proteobacteria c__Betaproteobacteria o__ASSO-13 f__
k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Oceanospirillales f__Endozoicimonaceae
k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__HTCC2188 f__HTCC2089
k__Bacteria p__GN02 c__BD1-5 o__ f__
k__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Nocardiaceae
k__Bacteria p__Bacteroidetes c__Flavobacteriia o__Flavobacteriales f__Cryomorphaceae
k__Bacteria p__Firmicutes c__Bacilli o__Lactobacillales f__Lactobacillaceae
k__Bacteria p__OP9 c__OPB46 o__OPB72 f__TIBD11
k__Bacteria p__Chlorobi c__Ignavibacteria o__Ignavibacteriales f__Ignavibacteriaceae
k__Bacteria p__Actinobacteria c__Acidimicrobiia o__Acidimicrobiales f__AKIW874
k__Bacteria p__Chlamydiae c__Chlamydiia o__Chlamydiales f__Parachlamydiaceae
k__Bacteria p__Firmicutes c__Bacilli o__Lactobacillales f__Streptococcaceae
k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Methylophilales f__Methylophilaceae
k__Bacteria p__Synergistetes c__Synergistia o__Synergistales f__Thermovirgaceae
k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Rhizobiales f__Methylocystaceae
k__Bacteria p__Acidobacteria c__RB25 o__ f__
k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Alteromonadales f__
k__Bacteria p__Proteobacteria c__Deltaproteobacteria o__Desulfobacterales f__Desulfobacteraceae
k__Bacteria p__Verrucomicrobia c__Verrucomicrobiae o__Verrucomicrobiales f__Verrucomicrobiaceae
k__Bacteria p__Cyanobacteria c__Synechococcophycideae __ __
k__Bacteria p__Bacteroidetes c__Bacteroidia o__Bacteroidales f__[Odoribacteraceae]
k__Bacteria p__Acidobacteria c__Acidobacteria-6 __ __
k__Bacteria p__Firmicutes c__Bacilli o__Bacillales f__
k__Bacteria p__Chloroflexi c__Anaerolineae o__SBR1031 f__A4b
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Peptostreptococcaceae
k__Bacteria p__Proteobacteria c__Deltaproteobacteria o__Bdellovibrionales f__Bacteriovoracaceae

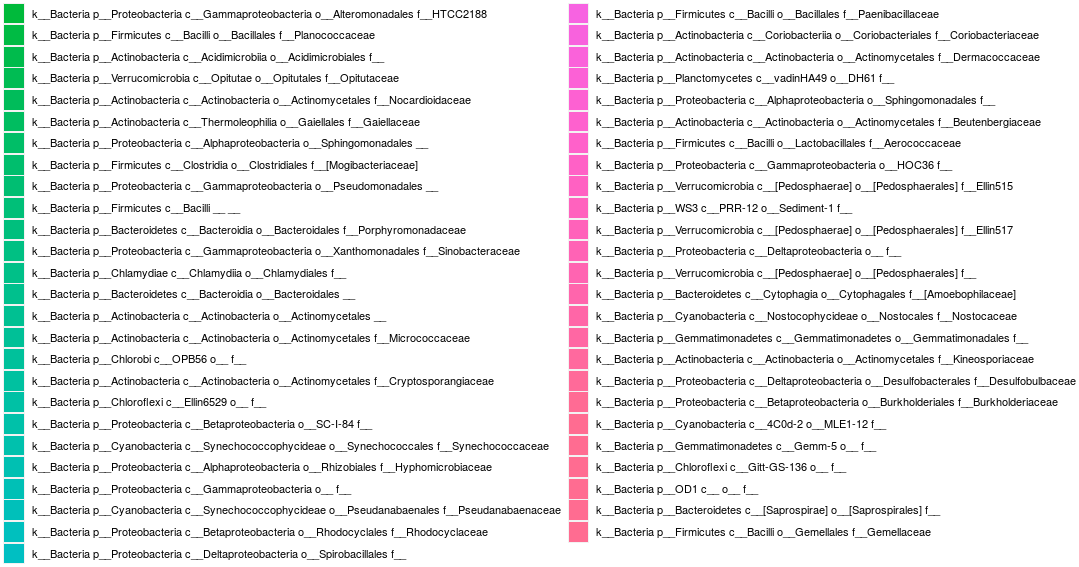


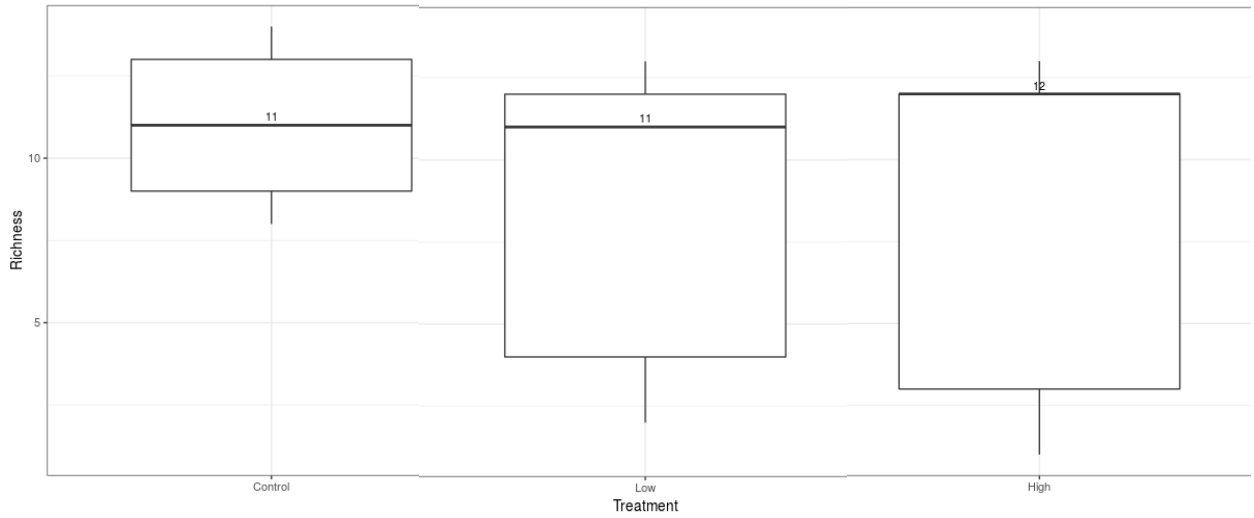
Figure 10a – f. Absolute and rarified abundance level of 15 beetles' gut microbiome samples from three clove oil treatments from Generation 4. The samples were labelled as the following: Treatment.Beetle line.Beetle replicate number. Note that for each treatment, there was one line of beetles that had two replicates. "Other" indicated the reading was less than 1% of any of the samples. a) Absolute abundance of reads at phylum level. b) Rarified abundance of reads at phylum level. c) Legend at phylum level. d) Absolute abundance of reads at family level. e) Rarified abundance of reads at family level. f) Legend at family level.

Clove oil treatment had no significant effects on the Shannon diversity or richness of the microbial community in bean beetles at either the phylum or family level (Table 5). This indicated similar community structures between the treatments. All four alpha diversity visualizations showed large overlapping areas in the resulting graphs (Figure 11a, b).

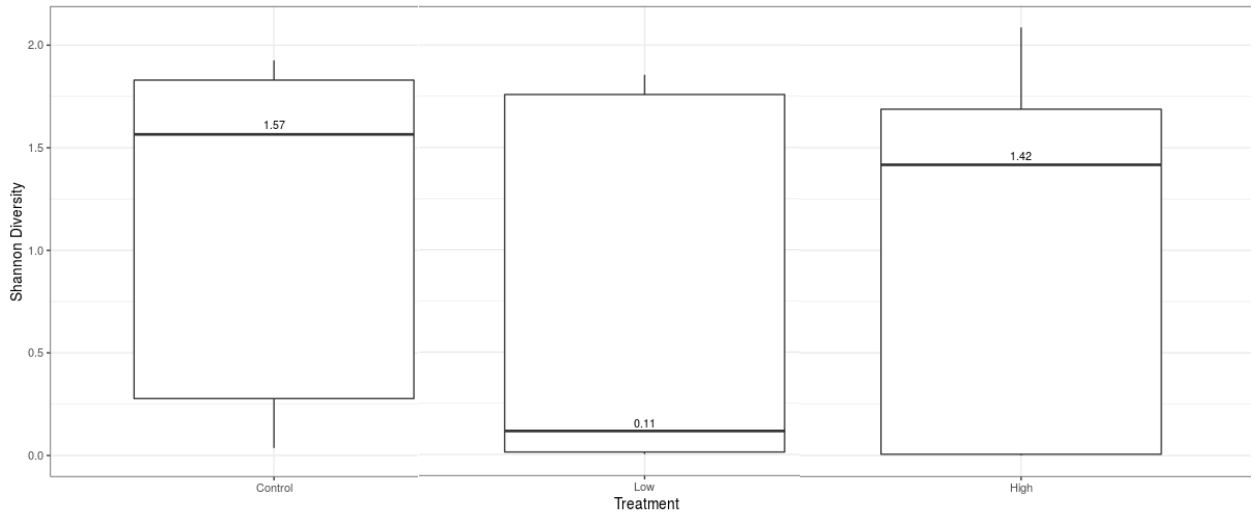
Table 5. ANOVA table for Shannon diversity and richness of bacterial communities in bean beetle's gut at phylum and family levels.

Diversity Measure	Taxonomical Level	df	F	P
Richness	Phylum	2	0.57	0.58
Shannon Diversity	Phylum	2	0.22	0.81
Richness	Family	2	0.75	0.49
Shannon Diversity	Family	2	0.27	0.77

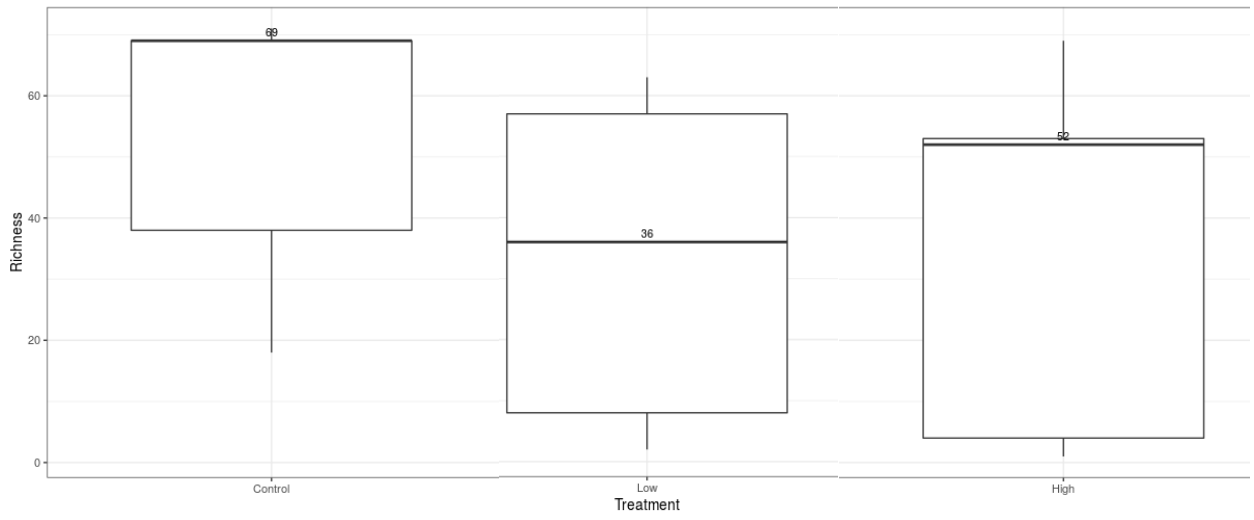
a)


Richness
10
5
11
11
12
Control
Low
High
Treatment

b)


Shannon Diversity
2.0
1.5
1.0
0.5
0.0
1.57
0.11
1.42
Control
Low
High
Treatment

c)


Richness
60
40
20
0
69
36
52
Control
Low
High
Treatment

d)

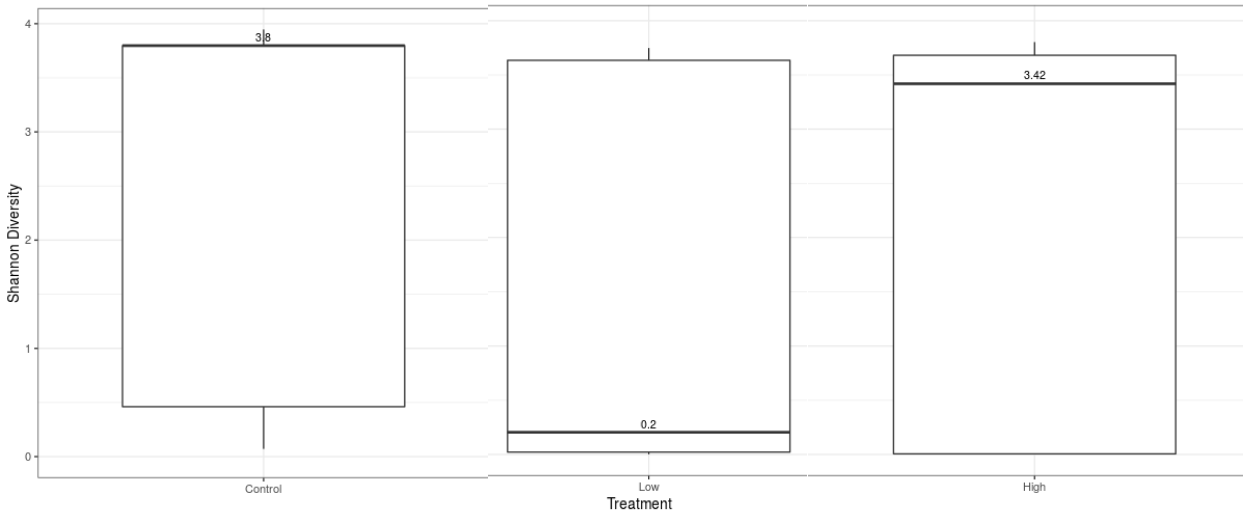
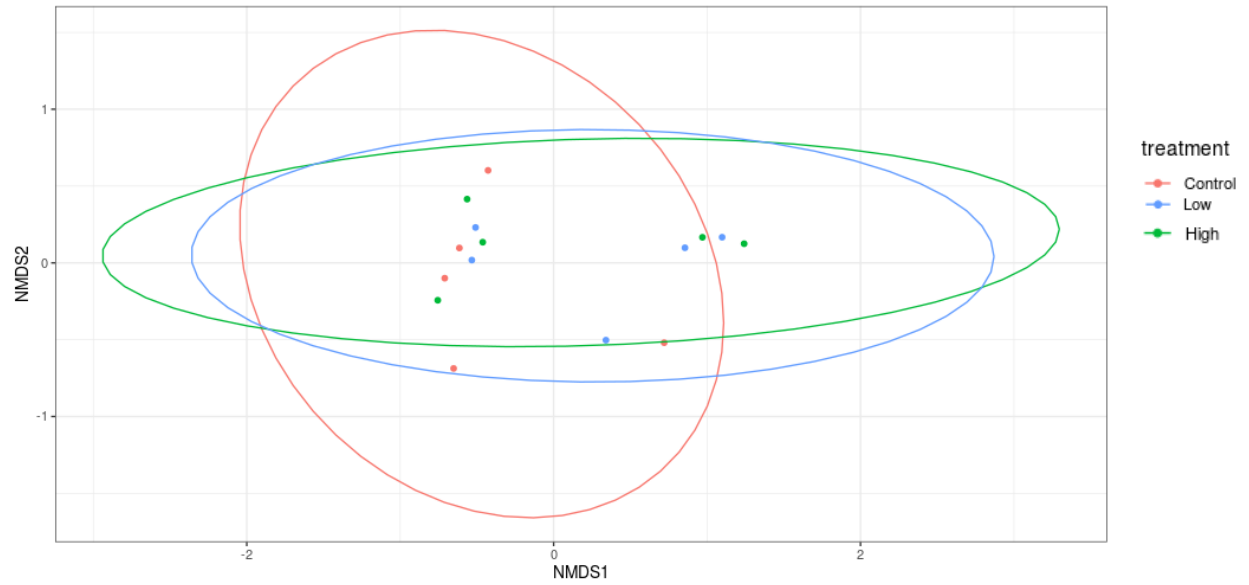


Figure 11a – d. Alpha diversity visualizations of 15 beetles' gut microbiome samples from three clove oil treatments from Generation 4. Rarified data was used. a) Richness at phylum level. b) Shannon diversity at phylum level. c) Richness at family level. b) Shannon diversity at family level.

Similar to the conclusions from the alpha diversity results, beta diversity showed that there was no significant difference in the microbial community structure between clove oil treatments at phylum level (PERMANOVA: df = 2, F = 1.08, P=0.30) or at family level (PERMANOVA: df = 2, F = 1.08, P = 0.28). Visualizations of the beta diversity showed that microbial communities from different treatments were often very close to one another, indicating similarity (Figure 12). The control showed slightly different distribution compared to low and high treatments. This may be due to the one control sample with high abundance in Firmicutes instead of Proteobacteria (Figure 10).

a)



b)

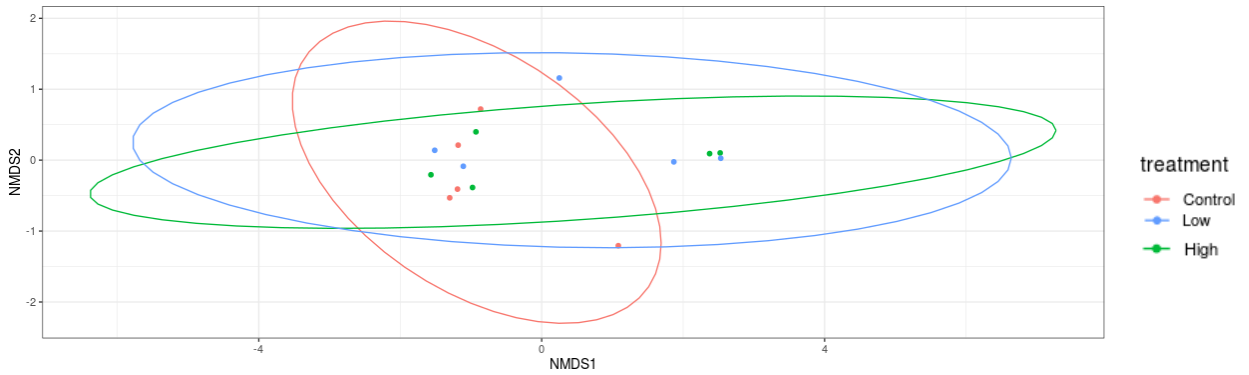
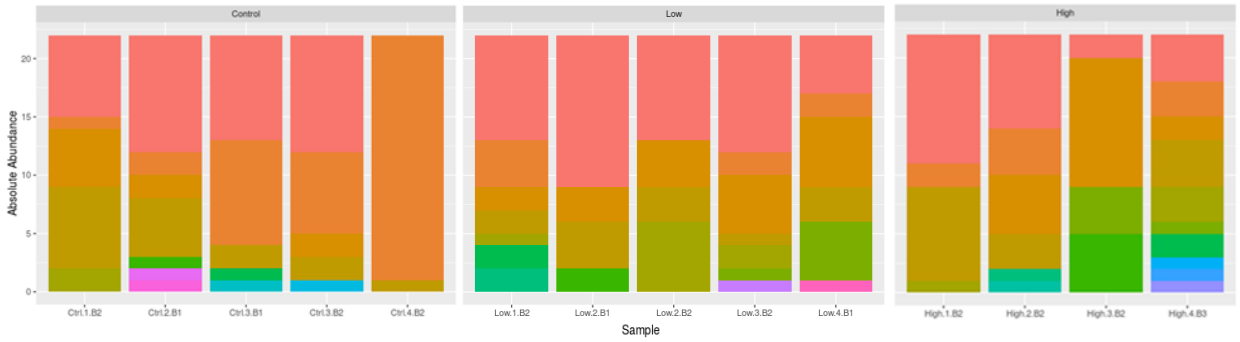


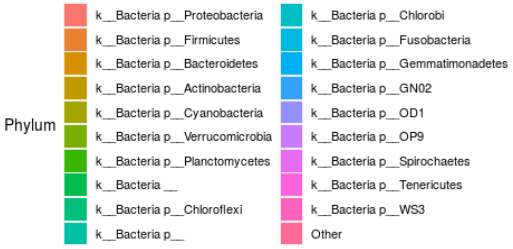
Figure 12a – b. Beta diversity visualizations of 15 beetles gut microbiome samples from three clove oil treatments from Generation 4. Rarified data, Bray-Curtis distance measure and NMDS ordination method were used. a) At phylum level, stress = 0.0552. b) At family level, stress = 0.067.

The modified data without Enterobacteriaceae and one High treatment replicate better visualized the less common bacteria (Figure 13). It appeared that although Enterobacteriaceae was dominant, it seldom occupied the entire beetle microbiome. Still, no consistent trends across the different treatments were present for phylum or family level. The alpha diversity, including richness and Shannon index, showed no difference across treatments. The results from beta diversity of the treatments showed higher similarity.

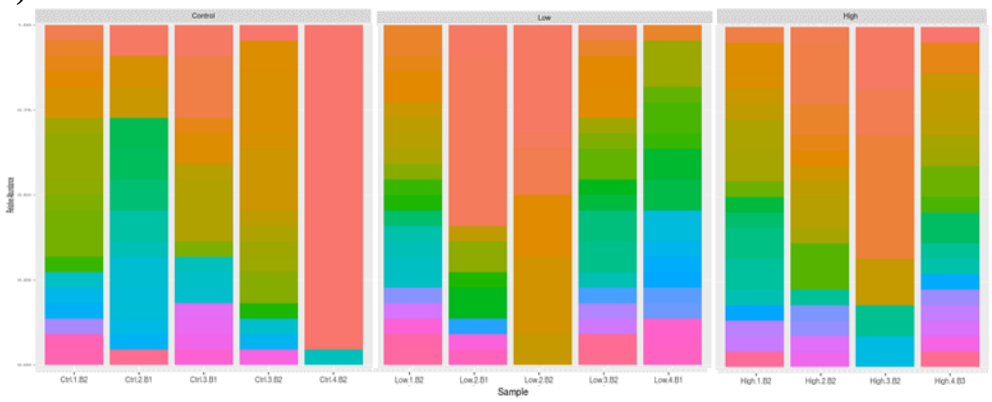
a)


Control
Low
High
Absolute Abundance
Sample
Ctrl.1.B2
Ctrl.2.B1
Ctrl.3.B1
Ctrl.3.B2
Ctrl.4.B2
Low.1.B2
Low.2.B1
Low.2.B2
Low.3.B2
Low.4.B1
High.1.B2
High.2.B2
High.3.B2
High.4.B3

b)


Phylum
k__Bacteria p__Proteobacteria
k__Bacteria p__Firmicutes
k__Bacteria p__Bacteroidetes
k__Bacteria p__Actinobacteria
k__Bacteria p__Cyanobacteria
k__Bacteria p__Verrucomicrobia
k__Bacteria p__Planctomycetes
k__Bacteria __
k__Bacteria p__Chloroflexi
k__Bacteria p__
k__Bacteria p__Chlorobi
k__Bacteria p__Fusobacteria
k__Bacteria p__Gemmatimonadetes
k__Bacteria p__GN02
k__Bacteria p__OD1
k__Bacteria p__OP9
k__Bacteria p__Spirochaetes
k__Bacteria p__Tenericutes
k__Bacteria p__WS3
Other

c)


Control
Low
High
Sample

d)


k__Bacteria p__Firmicutes c__Bacilli o__Lactobacillales f__Enterococcaceae
k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Neisseriales f__Neisseriaceae
k__Bacteria p__Proteobacteria __ __ __
k__Bacteria p__Bacteroidetes c__Cytophagia o__Cytophagales f__Cyclobacteriaceae
k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Pseudomonadales f__Pseudomonadaceae
k__Bacteria p__Bacteroidetes c__Bacteroidia o__Bacteroidales f__Prevotellaceae
k__Bacteria p__Proteobacteria c__Betaproteobacteria o__Burkholderiales f__Oxalobacteraceae
k__Bacteria __ __ __ __
k__Bacteria p__Bacteroidetes c__Bacteroidia o__Bacteroidales f__Rikenellaceae
k__Bacteria p__Cyanobacteria c__Synechococcophycideae o__Pseudanabaenales f__Pseudanabaenaceae
k__Bacteria p__Firmicutes c__Erysipelotrichi o__Erysipelotrichales f__Erysipelotrichaceae
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Lachnospiraceae
k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Rhizobiales __
k__Bacteria p__Proteobacteria c__Deltaproteobacteria o__Myxococcales f__Cystobacteraceae
k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Aeromonadales f__Aeromonadaceae
k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Alteromonadales f__[Chromatiaceae]
k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Enterobacteriales f__Enterobacteriaceae
k__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Streptomycetaceae
k__Bacteria p__Bacteroidetes c__[Saprospirae] o__[Saprospirales] f__Chitinophagaceae
k__Bacteria p__Bacteroidetes c__Bacteroidia o__Bacteroidales f__Bacteroidaceae
k__Bacteria p__Chlorobi c__OPB56 o__ f__
k__Bacteria p__Fusobacteria c__Fusobacteriia o__Fusobacteriales f__Fusobacteriaceae
k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Caulobacterales f__Caulobacteraceae
k__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Rhizobiales f__Bradyrhizobiaceae
k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Methylococcales f__Crenotrichaceae
k__Bacteria p__Proteobacteria c__Gammaproteobacteria o__Pseudomonadales __
k__Bacteria p__Spirochaetes c__[Brevinematae] o__[Brevinematales] f__Brevinemataceae
k__Bacteria p__Tenericutes c__Mollicutes o__Acholeplasmatales f__Acholeplasmataceae
k__Bacteria p__Verrucomicrobia c__[Pedosphaerae] o__[Pedosphaerales] f__Ellin515
k__Bacteria p__Verrucomicrobia c__[Pedosphaerae] o__[Pedosphaerales] f__Ellin517
k__Bacteria p__Acidobacteria c__Acidobacteria-6 o__CCU21 f__
k__Bacteria p__Acidobacteria c__RB25 o__ f__
k__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Cryptosporangiaceae
k__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Geodermatophilaceae
k__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Gordoniaceae
k__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Microbacteriaceae
k__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Micrococcaceae
k__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Nocardioidaceae
k__Bacteria p__Actinobacteria c__Coriobacteriia o__Coriobacteriales f__Coriobacteriaceae
k__Bacteria p__Actinobacteria c__Thermoleophilia o__Solirubrobacterales f__Conexibacteraceae
k__Bacteria p__Actinobacteria c__Thermoleophilia o__Solirubrobacterales f__Patulibacteraceae
k__Bacteria p__Bacteroidetes c__Bacteroidia o__Bacteroidales f__[Paraprevotellaceae]
k__Bacteria p__Bacteroidetes c__Bacteroidia o__Bacteroidales f__S24-7
k__Bacteria p__Bacteroidetes c__Flavobacteriia o__Flavobacteriales f__Flavobacteriaceae
k__Bacteria p__Chlamydiae c__Chlamydiia o__Chlamydiales f__Parachlamydiaceae
k__Bacteria p__Chloroflexi c__Anaerolineae o__SJA-15 f__
k__Bacteria p__Chloroflexi c__Chloroflexi o__[Roseiflexales] f__
k__Bacteria p__Chloroflexi c__Gitt-GS-136 o__ f__

Figure 13a – d. Rarified abundance level of 14 beetles' gut microbiome samples from three clove oil treatments from Generation 4. The Enterobacteriaceae readings were removed. The samples were labelled as the following: Treatment.Beetle line.Beetle replicate number. Note that for control and low treatments, there was one line of beetles that had two replicates. "Other" indicated the reading was less than 1% of any of the samples. a) Rarified abundance of reads at phylum level. b) Legend at phylum level. c) Rarified abundance of reads at family level. d) Legend at family level.

Discussion:

The drawbacks of traditional insecticides have led to the exploration of plant essential oils as an alternative solution. However, long term studies on the effects of EO on pests and on the role their gut microbiome plays in the process remain limited. This study examined these questions using bean beetles and clove oil for their wide presence and easy accessibility. We hypothesized that the bean beetle would be able to adapt to clove oil exposure, and its microbiome would either shift in structure or show increased resistance to the toxin. Our results showed that bean beetles

were capable of adaptation to clove oil exposure, but we did not find evidence that the beetle's gut microbiome played a role in the process.

We found that high clove oil exposure and the generation of the beetles could lead to changes in beetle life cohort history. Generation significantly impacted both the number of emergences and time to emergence (Table 1). As the general trend of increased emergence time over the three generations applied to the control group as well, the source of impact could likely be traced to factors unrelated to clove oil treatment. Emergence time and the number of emergences could be used as parameters to measure whether the beetles adapted to the environment (Adenekan et al., 2018; Hausch et al., 2020). The expected pattern indicating adaptation would be that over the generations, the beetles showed decreased emergence time and increased number of emergences. However, the general trend in this experiment was increased emergence time and increased number of emergences. This may be due to the fluctuation in the temperature beetles were reared under, as temperature has been reported as one of the major factors that changes beetle cohort life history traits, including female fecundity and developmental period (Adenekan et al., 2018). The Generation 0 stock beetles were moved from a 30 °C environment to about 23 °C at room temperature. Over the course of the experiment, room temperature slowly decreased from about 23 °C in summer to about 17 °C in winter. The impact of temperature variation could lead to comparisons between generations being less valid. As a result, comparisons within the same generation would better support the relative effects of clove oil treatment on bean beetle's cohort life history traits.

Our results aligned with the multiple previous findings that clove oil, at high concentration, effectively decreased bean beetle population (Mssillou et al., 2022). When treated with high clove oil concentration, the number of successful bean beetle emergences was significantly decreased

compared to the beetles that received no clove oil exposure or low exposure (Table 1). Low clove oil treatment did not reduce the number of emergences significantly. The general trend of each generation's replicates showed increased emergences on average, but the number from individual replicates fluctuated (Figure 3). For example, a higher number of emergences could occur in a replicate line in Gen 2 than Gen 3, or in Gen 1 than Gen 2. This occurred at least once in all three clove oil treatments. These results indicated that it would be important to ensure a high enough concentration of clove oil was used to decrease a bean beetle population, as the mere presence of clove oil did not always perform as an insecticide.

Interestingly, while beetle lines constantly reared under high clove oil exposure showed the lowest mean emergence across all generations and for all treatments, there was more overlap between the counts in Generation 2 and 3 (Figure 3). This could be evidence for bean beetles adapting to clove oil exposure. Previously, Afroz et al. (2021) suggested that insects are capable of adapting to plant secondary compounds. However, the findings from Akami et al. (2019) showed *C. maculatus* had limited adaptation to the EO from *Lippia adoensis*. It is possible that the beetles are more capable of adapting to certain PSC than others.

Our experiment did not find an impact of clove oil treatment on the length of beetle emergence time, but due to the temperature variance mentioned before, no definitive conclusions could be made. Previous research showed that clove oil could decrease the rate at which beetles emerge, and the first beetle emergence time may be slightly delayed as well (Viteri Jumbo et al., 2018). However, if beetle adaptation were to occur to clove oil treatments in our experiment, which meant the emergence time being longer at Generation 1 than Generation 3, the effect may have been complicated by temperature change. There was little consistent trend in emergence time over the three generations, but it could be concluded that in Generation 1 and 3, no significant difference

was observed between the beetles in the three treatments (Figure 4). Whether clove oil had an impact on beetle emergence time or not during the exposure, this effect likely diminished after a few generations. These results suggested that beetles were potentially capable of adapting to clove oil exposure as well.

Different clove oil treatments could have potential impacts on the microbiome composition in bean beetles. Due to the uncertainties due to sampling effects, including how well the beetle was homogenized and the process of serial dilution, it was difficult to draw definitive conclusions. The CFU trends observed over the generations could provide some insight into the question. For the cultured microbiome, different trends were observed between high concentration and low concentration or the control group, such as the frequency of beetle replicates that showed no culturable colonies (Figure 5b). When exposed to low or no clove oil concentrations, the number of colonies in the beetle's gut fluctuated with similar trends. When exposed to high clove oil concentration, the trend somehow reversed that of low or no clove oil concentrations. For all high treatment beetle lines, the CFU in the beetle's gut likely decreased from Generation 1 to 2 (Figure 5a, d). However, the CFU increased in Generation 3, and in Generation 4, the percentage of replicates with no colonies was the same for control and high concentration (Figure 5b). Of the replicates that showed colony growth, trends similar to that of the presence of bacteria were observed (Figure 5c). The microbiome cultured from beetles in the high treatment had the lowest average CFU at Generation 2, but the counts increased in Generation 3 and 4 and showed no difference compared to the other two treatments.

The species richness of cultured microbiome was more difficult to analyze, as *Enterobacter* comprised the vast majority of colony counts. However, it did seem that *Enterobacter* remained the most abundant genus during the experiment for the three treatments. Interestingly, at both

Generation 1 and 4 for high clove oil treatment, *Enterobacter* comprised ~100% of the beetle microbiome that we cultured. Considering this was not the case for Generation 2 and 3 for high treatment (~0% and 91%, respectively), different concentrations of clove oil may have caused different fluctuations in the microbial community in the beetle's gut, but eventually the differences leveled out. The appearance of *Enterococcus* as a common genus in beetle's microbiome was likely related to variation across generations. The dominant taxa of bacteria isolated from bean beetles used in other experiments may drastically differ from the bean beetles used in this experiment (Akami et al., 2019; Berasategui et al., 2021; Sevim et al., 2015). In addition, while the beetle line High-2 showed a high abundance of *Enterococcus* when bacteria were cultured, no *Enterococcus* sequences were reported in the beetle sample from the same replicate line used for next-generation sequencing (Figure 10). These results could be due to the random variation in the bean beetle microbiome.

Next-generation sequencing results supported the claim that there was no clear difference in community structure in Generation 4 beetles, as no consistent trends across the three clove oil treatments was found (Figure 11, 12). The relative abundance and number of taxa were mostly uniform. Two control beetles, two from low treatment, and one from high treatment showed high abundance, while the rest of the replicates showed low abundance. A wide range of families were detected for each treatment, and the diversity indices confirmed there was no significant difference in diversity across treatments (Table 5). In addition, the overall microbial community structure was not different for control and treatment groups (Figure 15, 16). As no conclusions could be made from the cultured microbiome, and next-generation sequencing results showed no shift in bean beetle microbiome, we cannot conclude that clove oil had an impact on the beetle's microbial community structure. Still, it is possible that the time span of four generations was not sufficient

to detect shifts in the beetle's microbiome. It also should be noted that temperature can change the composition of insect microbiome (Raimondi et al., 2020). As the whole-genome sequencing was performed on Generation 4 beetles that went through a considerable temperature drop, the results would likely differ from the case where they were reared under constant temperature.

Some of our results suggest the possibility that clove oil changed the microbial community in bean beetles during the course of the experiment. Past research has shown that essential oils are capable of disrupting the efflux/influx pumps, membrane (Willing et al., 2018). Clove oil has exhibited effective antimicrobial activities against *Enterobacter* and *Enterococcus* isolates (Faujdar et al., 2020; Hammer, 1999), the most common genera in the majority of beetles in this study. The low CFU in cultured beetle microbiome from high treatment in Generation 2 could be related to clove oil exposure killing a significant portion of the *Enterobacter* population present in the beetle's gut (Figure 5a). The reason why this did not occur in Generation 1 is unknown. As the CFU in high treatment rebounded in Generation 3 and 4, the microbial community in the beetle's gut could have developed some kind of method to counter clove oil exposure.

However, we cannot conclude that this change was due to the bacteria's increase in resistance to clove oil. The disc diffusion assay results indicated no difference between clove oil resistance in *Enterobacter* and *Enterococcus* strains isolated from beetles treated with or without clove oil (Figure 8). This aligned with the results from past research on EO's effect on bacteria. As EO's chemical composition was much more complex than antibiotics, it could be more difficult for bacteria to develop resistance (Becerril et al., 2012). Existing research on bacteria developing resistance to plant essential oil is limited. One study found that lab strain *Enterobacter cloacae* was susceptible to and had limited adaptation to EOs from cinnamon and oregano. However, other strains of gram-negative bacteria, including *Serratia marcescens*, could develop resistance after

50 passages under EO treatment. It should be noted that in our study, since the main components of clove oil are largely nonpolar, it might not diffuse effectively across the nutrient agar medium (Balouiri et al., 2016). The main purpose in this section of experiment was to find out whether there would be a difference in clove oil resistance between the bacteria of the three treatment groups, not obtaining a minimal inhibition concentration (MIC). As the disc diffusion assays cannot reveal the nuances between treatments, we could not make definite conclusions on whether a difference in clove oil was present in the bacteria strains.

In addition to the results from the disc diffusion assays, a phylogenetic tree of *Enterobacter* isolates based on 16s rRNA sequences suggested that the bacteria likely did not develop resistance to clove oil (Figure 7). The same generations or treatments did not cluster together. If the level of clove oil resistance were to be different among the *Enterobacter* strains, this change may be reflected in the genetic composition of the bacteria, meaning each generation and treatment would spread across the phylogenetic tree in an ordered manner. Colony isolates from the same beetle lines in the same generation were neighboring. As all beetles had the microbiome community from the same two stock cultures, and Generation 1 *Enterobacter* isolates were relatively evenly dispersed, it was likely that there was plenty of natural variation in the bacteria strains in the beetles. The variation could occur vertically from adult beetles mating or the bacteria could be acquired from the environment (Berasategui et al., 2021). These results, together with the conclusion that the community structure of the beetle microbiome did not change significantly, indicate that the beetle's adaptation to clove oil was likely not dependent on its microbiome. Therefore, the adaptation could be related to the beetle's own defense mechanisms. Past research revealed that essential oils could target a range of receptors in the beetle neural system, including octopaminergic receptors (Mssillou et al., 2022), and they can change the activities of enzymes,

including those involved in detoxification or gene expression (Gao et al., 2020). The resistance to clove oil may thus originate from changes in these aspects in the bean beetle itself.

If similar experiments were conducted in the future, it would be important to rear the beetles at a constant temperature to minimize confounding variables. For clove oil alone, to determine the effects of clove oil concentration on beetle cohort life history traits, higher concentrations of clove oil would be needed. To make more conclusive claims regarding the impact of clove oil on the beetle microbiome, experiments involving more generations of beetles would be needed. The clove oil resistance in bacteria strains would require methods such as liquid culture assays to reveal the potential nuances between treatments, and transcriptomic studies could help explore origin of the beetle's adaptation mechanisms to clove oil. Overall, to find the ideal EO candidate to replace traditional insecticides against bean beetles, it is important to compare the efficacy of a wide range of EOs on the pest in the long term.

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