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The Gut Microbiota of Cornu aspersum

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Abstract
The "Brown Garden Snail," *Cornu aspersum* is a widely distributed land snail, whose consumption of soil to acquire calcium results in soil microbes colonizing their gut. While previous work has utilized culture-based approaches to analyze the gut microbiota of *C. aspersum*, no previous studies have used high throughput DNA sequencing, or examined the changes in the gut microbiota over time in response to antibiotics exposure. In this study, we use 16S amplicon sequencing and fecal plating to characterize the gut microbiota of *C. aspersum*, and to monitor changes in their microbiota over time after a short penicillin treatment. We found that the natural gut microbiota of *C. aspersum* is dominated by Gammaproteobacteria, and that a two day penicillin treatment reduces Gammaproteobacteria to one representative family, the *Pseudomonadales*. While the gut microbiota recovers some of its diversity following cessation of penicillin treatment, the incomplete recovery suggest that *C. aspersa* may depend on soil consumption, and potentially coprophagy, to exogenously maintain a diverse gut microbiota.

Keywords
Cornu aspersum, Helix aspersa, snail, gut microbiota, Buttiauxella, Pseudomonas

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The "Brown Garden Snail," *Cornu aspersum* is a widely distributed land snail, whose consumption of soil to acquire calcium results in soil microbes colonizing their gut. While previous work has utilized culture-based approaches to analyze the gut microbiota of *C. aspersum*, no previous studies have used high throughput DNA sequencing, or examined the changes in the gut microbiota over time in response to antibiotics exposure. In this study, we use 16S amplicon sequencing and fecal plating to characterize the gut microbiota of *C. aspersum*, and to monitor changes in their microbiota over time after a short penicillin treatment. We found that the natural gut microbiota of *C. aspersum* is dominated by Gammaproteobacteria, and that a two day penicillin treatment reduces Gammaproteobacteria to one representative family, the *Pseudomonadales*. While the gut microbiota recovers some of its diversity following cessation of penicillin treatment, the incomplete recovery suggest that *C. aspersa* may depend on soil consumption, and potentially coprophagy, to exogenously maintain a diverse gut microbiota.

Keywords: Cornu aspersum, Helix aspersa, snail, gut microbiota, Buttiauxella, Pseudomonas

Introduction

The "Brown Garden Snail," *Cornu aspersum* Müller, 1774 (Syn. *Helix aspersa* Müller) is one of the most widely distributed land snails in the world. Originating in the Mediterranean region, *C. aspersum* is now present as an invasive species in locations with Mediterranean, temperate, and sub-tropic climates on six continents (Guiller *et al*. 2012). *C. aspersum* are generalist herbivores that feed on a variety of living and dead plants (Iglesias and Castillejo 1999). Gut colonization of *C. aspersum* by soil bacteria (Watkins and Simkiss 1990) occurs as a consequence of the snail's consumption of soil to acquire the additional calcium needed for shell development (Gomot *et al*. 1989). Because of their soil consumption, macroscopic size, slow dispersal rate, and ability to be marked and tracked over time, researchers have begun using *C. aspersum* as a "sentinel organism" for environmental pollution (Regoli *et al*. 2006), including metals (Beeby 1985, de Vaufleury and Pihan 2000, Beeby and Richmond 2002, Beeby and Richmond 2003, Scheifler *et al*. 2003, Viard *et al.* 2004) and pesticides (de Vaufleury and Pihan 2000, Snyman *et al*. 2000).

In the past, the gut microbiota of *C. aspersum* has been analyzed using microscopy (Charrier 1990) and by the culturing of bacterial gut isolates (Watkins and Simkiss 1990, Charrier *et al*. 1998, Charrier *et al*. 2006, Villena *et al.* 2010, Caullan *et al*. 2014, Koleva *et al.* 2014, Koleva *et al.* 2015). While early studies found a transient gut microbial community (Charrier 1990, Watkins and Simkiss 1990), more recent work indicates that the gut of *C. aspersum* supports an endogenous bacterial population (Charrier 1990). The goals of this study were to non-invasively characterize the gut microbiota of *C. aspersum* by using 16S amplicon sequencing to analyze the fecal microbiota, and to look at the effects of penicillin on that microbiota. This study also aimed to establish the use of *C. aspersum* as a model organism for future studies on the effects of antibiotics on gut microbial communities.

Material and Methods

Snail collection and sampling

Eight C. aspersum were collected on two different days over two weeks from a home garden in Monmouth, OR, USA. All snails were placed individually in a sterile, glass jar (Wide-Mouthed Quart-Sized Canning Jars, Ball Corporation, Broomfield, CO) with no food for 24 hours and were then assigned to either the control or treatment group. Fecal samples were collected from the jars after the 24-hour fast to determine the pre-treatment fecal microbiome. After the 24-hour fast, control snails (n = 2) were each transferred to a new sterile, glass jar containing 2 g of organic lettuce (Earthbound Farms Organic Baby Lettuce) and

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10 ml of sterile, deionized water. Subsequently, each control snail was transferred to a new sterile, glass jar containing 2 g of organic lettuce and 10 ml of sterile, deionized water each day for the duration of the 21-day experiment. After the 24-hour fast, each treatment snail (n = 6) was transferred to a new sterile jar containing 2 g of organic lettuce to which 10 ml of sterile, deionized water containing 20 µg/ml penicillin (Fisher Biotech, Fair Lawn, NJ) had been applied. The treatment snails received this dose of penicillin on the first two days of the trial and were then given the same diet as the control snails for the remainder of the 21-day experiment. Fecal samples were collected on days 1-4, 7, 10, 14, and 21. Each sample was homogenized using a plastic, sterile pestle in Tris EDTA (TE) buffer, at a concentration of 100 µg feces/ml TE buffer. Aliquots of the fecal solution were used for plating, and the remaining fecal homogenate was stored at -80°C for future DNA isolation and 16S amplicon sequencing.

Culturing methods

Immediately after collection and homogenization, 10 µg of feces was suspended in 100 ml of sterile water and then serially diluted prior to plating on MacConkey agar plates (Difco, Sparks, MD) that select for Gammaproteobacteria. Plates were incubated at 37°C for 24 hours prior to counting colonies. Colonies were classified using their visual appearance on the MacConkey plates (lactose usage, colony size, and capsid formation). Individual colonies were subsequently isolated on nutrient agar plates and further classified based on their appearance on citrate plates (citrate usage, Simmons Citrate Agar, Oxoid, Hampshire, England), oxidase production (oxidase test, BD BBL™ Taxo™ N Discs, Becton and Dickinson and Company, Sparks, MD), and fluorescence under UV light on nutrient agar. One representative colony from each classification group was subjected to DNA-based identification to assign identities to the observed phenotypes.

DNA extraction for PCR amplification from cultured isolates

DNA was extracted from pure culture-isolated bacterial colonies using the freeze-thaw method (Kawai et al. 2002). The 16S gene region was PCR amplified using the Promega PCR Master Mix (Promega Corporation, Madison, WI) and the universal 16S primers, 8F and 1492R. The amplified DNA was purified via isopropanol precipitation and sent to the Center for Genome Research and Biocomputing (CGRB) at Oregon State University for Sanger sequencing (Sanger et al. 1977). The returned 16S sequences were assigned at the level of genera using BLAST.

DNA extraction from snail fecal samples and lettuce

For the 16S amplicon sequencing analysis, fecal samples from five of the snails (four treatment, one control) were analyzed from days 1, 3, 7, and 14. Two samples of the untreated, organic lettuce food source taken from the original package were selected for DNA extraction. The DNA was extracted using DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). To sample the microbial community in fecal samples, 0.1 g of feces from the fecal homogenate in TE buffer was placed directly into PowerBead tubes. Because 0.1 g of feces was not available from all of the treatment snails on day 14, the fecal samples from two snails were combined yielding two pooled samples of which 0.1 g was used for DNA extraction. The microbial community present on the lettuce was sampled by incubating 2 g of lettuce in sterile water at 4°C on a shaker table for 3 hours. The suspension was filtered through a 0.45 µm filter (Millipore, Billerica, MA) to concentrate the bacteria. The bacteria were removed from the filter by vortexing for two minutes in wash buffer with 0.2% Tween 20 (Epicentre, Madison, WI), and then pelleted and placed in a PowerBead tube. Fecal and lettuce samples were agitated twice, for 30-second intervals, in a BioSpec Mini-beadbeater with the power set to 42 Hz. Tubes were placed on ice for one minute after each bead beating treatment to prevent overheating the sample.

Illumina MiSeq analysis

The isolated DNA was sent to the Research and Testing Laboratory (RTL; Lubbock, TX) for Illumina MiSeq analysis (Bentley et al. 2008). The universal 16S primers 28F and 388R were used to amplify the first and second variable regions of the 16S gene. Raw 16S sequence amplicons were processed by RTL to remove low quality reads, reduce noise, and remove chimeras. RTL assigned sequence reads to operational taxonomic units (OTU's) by clustering sequences at 4% dissimilarity, and then assigned identities to the OTU's using the USEARCH global alignment algorithm against the NCBI database as previously described by Minervini et al. (2015).

Statistical analysis

Shannon's Index (Shannon and Weaver 1949) was calculated in Excel for the 16S amplicon sequencing data at the OTU level and analyzed using an ANOVA and post-hoc paired-t-tests in Excel. Shannon's Index accounts for species richness and abundance when measuring the diversity of a community. The value produced by this index is Shannon's H, with high H values representing high diversity. Colony counts from the MacConkey plates were used to determine the colony forming units (CFUs) per gram of feces. These numbers were then log(x+1) transformed because the data was not normally distributed. Changes in CFUs of Buttiauxella and Pseudomonas per gram of snail feces in the culturing study were analyzed using ANOVAs and post-hoc two sample t-tests on the log(x+1) data in Excel.

Results

Fecal plating study

Colonies were assigned to one of four groups based on metabolic testing and 16S rRNA analysis. The groups were Pseudomonas, Buttiauxella, "Other enterics" (genera included Klebsiella, Kluyvera, and Hafnia), and Stenotrophomonas. On day 1, all pre-treatment fecal samples contained members of all four groups (Fig. 1). Over the course of the 21-day experiment, the CFUs/g of Buttiauxella (ANOVA, p = 1.09 x 10-15) and Pseudomonas (ANOVA, p = 0.029) in the fecal samples changed significantly in the treatment snails, but not in the control snails (ANOVA, all p-values > 0.25).

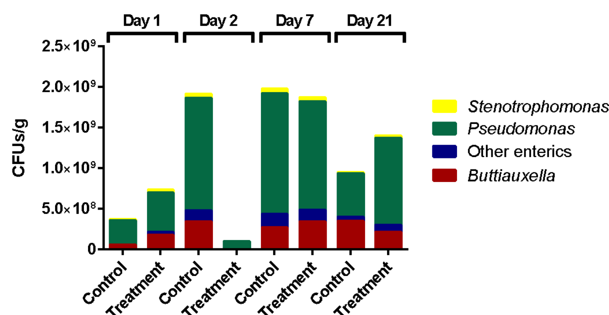

Figure 1: Fecal Plating Study. CFUs/g for two control and six treatment snails. Colonies were classified using the visual phenotype displayed on the MacConkey agar and further metabolic tests. A subset of each classification group was identified by sequencing the 16S gene of isolated pure cultures. The day 1 fecal samples were collected at the end of a 24-hour fast, after which treatment snails were exposed to penicillin on two consecutive days. The day 2 fecal samples were collected 24 hours after the treatment snails were initially exposed to penicillin.

On day 2, the fecal microbiota of treatment snails were reduced to Pseudomonas monocultures with a significant drop in Buttiauxella CFUs/g when compared to the day 1 fecal microbiota (t-test, p = 9.94 x 10-09; Fig. 1 and Fig. 2D). On day 7, fecal samples from treatment snails showed recovered levels of the four groups compared to the day 7 control (Fig. 1), with no significant difference in Buttiauxella CFUs/g of feces between day 7 and day 1 samples (t-test, p > 0.05; Fig. 2D). There was a significant increase in Pseudomonas CFUs/g feces in the treatment snails on day 7 compared to day 1 (t-test, p = 0.028), but levels on other days were not significantly different (Fig. 2B). There were no significant changes in Buttiauxella or Pseudomonas in the control snails (t-tests, all p-values > 0.05; Fig. 2A & C). On day 21, fecal samples from treatment and control snails showed a decrease in CFUs/g; however, this decrease was not statistically significant when compared to day 1 and day 7 fecal samples (t-tests, p > 0.05; Fig. 2).

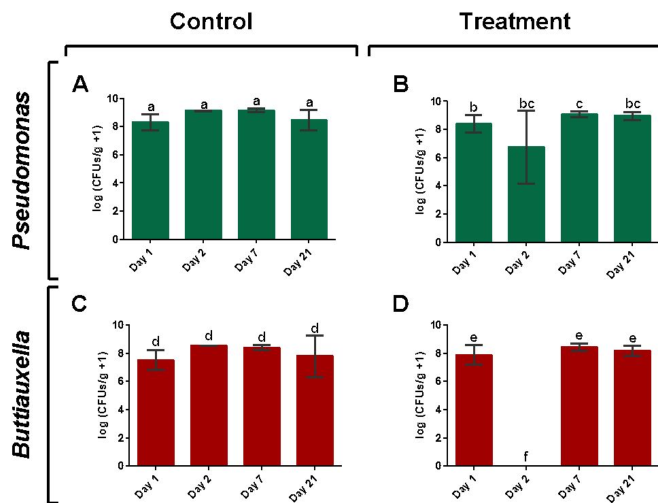

Figure 2: Change in *Pseudomonas* and *Buttiauxella* on MacConkey plates over time. Log(x+1) transformed colony counts of *Pseudomonas* from fecal samples of control **(A)** and treatment **(B)** snails and *Buttiauxella* from fecal samples of control **(C)** and treatment **(D)** snails on MacConkey agar plates. Error bars represent standard deviations for two control and six treatment snails. Bars with same letters(s) within a graph are not significantly different (two-tailed, two sample t-tests assuming equal variance, $p > 0.05$). Bars labeled "bc" are not significantly different from bars labeled either "b" or "c".

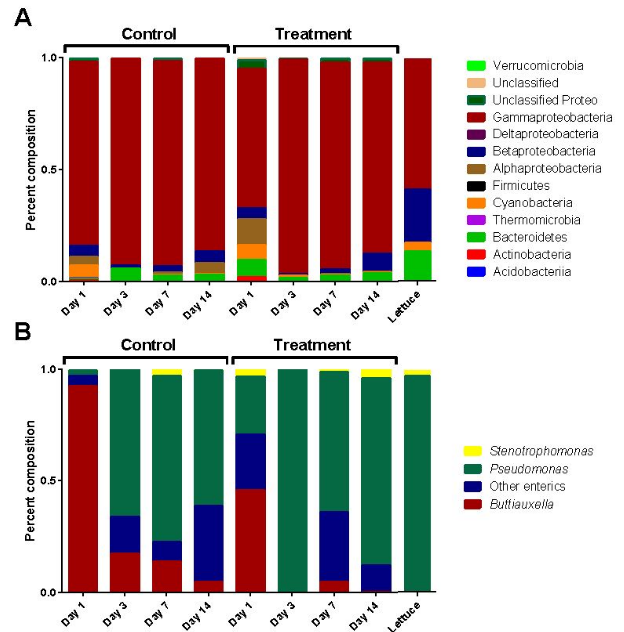

Figure 3: Percent composition of the fecal microbiome of *C. aspersum* based on 16S amplicon sequencing. The fecal community based on the 16S amplicon sequencing is displayed at the phylum level **(A)** and Gammaproteobacteria that encompass the four groups seen in the plating study **(B)**. Day 1 fecal samples were collected after a 24-hour fast prior to exposure to antibiotics and lettuce or lettuce only. Treatment snails ($n = 4$) were exposed to penicillin on two consecutive days after the day one sample was collected, while the control snail ($n = 1$) was not. The microbial community on the lettuce food source was sampled ($n = 2$) to see its effect on the change in the microbial community in the control and treatment snails.

16S amplicon sequencing

The 16S amplicon analysis of day 1 (pre-treatment) fecal samples showed diverse gut microbiomes that were dominated by Gammaproteobacteria (Fig. 3A).

The 16S amplicon data for pre-treatment snails showed that the feces were dominated by Buttiauxella. On day 3, 99.8% of Gammaproteobacteria found in the fecal samples of treatment snails were Pseudomonas, and 66.1% of Gammaproteobacteria found in the fecal samples of control snails were Pseudomonas (Fig. 3B). Shannon's H value for treatment snails recovered to the level of the control snail by day 7 (Fig. 4), with Pseudomonas being the most common OTU in both the control and treatment snails (Fig. 3B). "Other enterics" recovered more quickly than Buttiauxella in the treatment snails (Fig. 3B). By day 14, the H value for control and treatment snails was similar (Fig. 4), with Pseudomonas dominating all fecal samples (Fig. 3B). Buttiauxella decreased in the treatment samples, and was surpassed by "Other enterics" in the control sample, (Fig. 3B). The 16S amplicon data for the microbial community of the untreated lettuce food source was dominated by Pseudomonas (57%), Betaproteobacteria (24%), and Bacteroidetes (13%; Fig. 3A).

Prior to treatment, the average Shannon's H value for treatment snails was 2.6 while the H value of the pre-treatment control was 1.4 (Fig. 4). The H value for the control snail stayed constant for the duration of the experiment. The H value for the treatment snails changed significantly during the experiment (ANOVA, p = 2.46 x 10-6), falling from 2.6 to 0.28 after exposure to penicillin (paired t-test, p = 6.67 x 10-6) before recovering to 1.43 by day

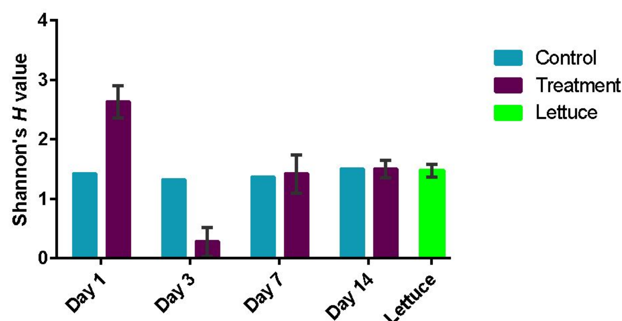

Figure 4: Shannon's diversity for the 16S amplicon fecal microbiota. Shannon's diversity was calculated for the treatment snails (n=4), the control snail (n=1), and the lettuce (n=2) using the 16S amplicon data at the species (OTU) level. Error bars represent standard deviation for the treatment snails (n=4) and the lettuce (n=2). The *H* value for the treatment snails changed significantly during the experiment (ANOVA, p = 2.46 x 10^{-6}), with a significant drop from day 1 to day 3 (paired t-test, p = 6.67 x 10^{-6}) and a significant increase from day 3 to day 7 (paired t-test, p = 6.45 x 10^{-4}). The treatment group *H* values for day 1 and day 14 were significantly different (paired t-test, p = 0.0032).

7 (paired t-test, p = 6.45 x 10-4). Though the fecal population did recover in the treatment snails the H values for day 1 and day 14 were significantly different (paired t-test, p = 0.0032). The average H value for the lettuce food source was 1.48, which was significantly lower than the H value for the treatment group prior to exposure to antibiotics (paired t-test, p = 0.0027), but was not significantly different from the H value of the treatment group on day 14 (paired t-test, p = 0.434).

Discussion

Our studies of untreated snails indicate that their fecal microbiota is dominated by Gammaproteobacteria (Fig. 3A). These findings confirm previous culture-based studies (Watkins and Simkiss 1990, Charrier et al. 1998, Villena et al. 2010), but do not support others that found low levels of Gammaproteobacteria (Caullan et al. 2014, Koleva et al. 2015).

The partial recovery of Buttiauxella in the fecal microbiota of C. aspersum (Fig. 3B) when it was not present on the lettuce food source (Fig. 3B) supports the previous finding that C. aspersum maintains an endogenous gut microbiota (Charrier 1990). However, the fact that Buttiauxella did not recover to former levels in the treatment snails and was reduced in the control snail (Fig. 3B) suggests that exposure to exogenous microbes may be necessary to maintain the gut microbiota of C. aspersum. This has been shown to be the case in the fruit fly, Drosophila melanogaster, whose consumption of feces maintains a beneficial gut microbiome (Blum et al. 2013).

Our study design impacted the final microbial community of the snails by changing the feeding regimen of the snails and housing them in sterile enclosures without soil or feces. The change in the control community and the stabilization of control and treatment snails at the same H value suggests that exposure to environmental microbes and a diverse diet could be important factors in C. aspersum's development and maintenance of a diverse microbiota. These effects could be explored further with future studies utilizing more control snails and housing the snails in environmental microcosms to explore the role of diet and environmental microbes in the shaping of the gut microbiota of C. aspersum.

Treating snails with penicillin significantly reduced the diversity of the fecal microbiota (Fig. 4) and allowed Pseudomonas to become the dominant taxon (Fig. 1 and Fig. 3B). Due to the change in diet and the limited duration of the experiment, it is difficult to predict whether the diversity in the treated snails' fecal microbiota would recover completely. Also complicating interpretations of these results is the low initial diversity in the control snail used for 16S amplicon sequencing and the way that Shannon's diversity is calculated. Because Shannon's diversity takes into account species richness and evenness, the dominant species in an uneven community can change without changing the H value. In the control snail, the dominant species switched from Buttiauxella to Pseudomonas but the H value did not change.

Chronic exposure to antibiotics can disrupt healthy microbial communities with the potential to cause an alternate stable state dominated by a drug resistant strain of bacteria. In a host-microbe system, a disease state can arise from the proliferation of a bacterial species normally present in small numbers, or through the colonization of available space by an opportunistic pathogen. In humans, the repeated disruption of the gut microbiota through antibiotic use can allow Clostridium difficile to dominate, resulting in a disease state

(Slimings and Riley 2014). Similarly, in cystic fibrosis patients, multi-drug resistant Pseudomonas aeruginosa begins to dominate lung microbial communities as patients age (Cystic Fibrosis Foundation Patient Registry 2016). P. aeruginosa is a soil bacteria and opportunistic pathogen of humans and snails that adapts to its environment and the presence of antibiotics (Govan and Deretic 1996, Oliver et al. 2000, Chmiel et al. 2014).

While the penicillin treatment suppressed the levels of other microbes in the snail fecal microbiota, it did not result in Pseudomonas competitively excluding other microbes and developing an alternate stable state similar to Clostridium difficile colitis in humans. The following are among the possible explanations why the penicillin treatment did not result in a complete takeover of the microbiota of C. aspersum by Pseudomonas. One explanation is that while penicillin suppressed other gut microbes, it did not completely eliminate them. Watkins and Simkiss (1990) found that a combination of two broad spectrum antibiotics was necessary to eliminate the microbes in the gut of C. aspersum. A second reason that may have prevented Pseudomonas from competitively excluding other microbes from the gut of C. aspersum may have been the amount of time that the snails were treated with antibiotics. In both cystic fibrosis and Clostridium difficile colitis cases, a pathogenic bacterium (P. aeruginosa and C. difficile, respectively) dominates the system and competitively excludes other microbes after repeated or long-term antibiotic use (Slimings and Riley 2014, Cystic Fibrosis Foundation Patient Registry 2016). Our study exposed the snails to penicillin for only two days, and while this was enough to suppress microbes normally present in the gut of C. aspersum, it may not have been enough to completely alter the balance of the gut microbial community of C. aspersum.

The goals of this study were to further the understanding of the gut microbiota of C. aspersum and to establish C. aspersum as a model organism for microbiology research. Using 16S amplicon sequencing and fecal plating, we showed that the fecal microbiota of C. aspersum is dominated by Gammaproteobacteria. We demonstrated that a two-day treatment with penicillin altered the fecal microbiota, with partial recovery occurring within two weeks. Future work should be carried out to determine if the gut microbiota can recover completely and if increasing the length of time that the microbiota is disrupted affects its ability to recover.

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