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Faecal microbiota dynamics in *Cornu aspersum* during dietary change and antibiotic challenge

Parker N. Smith[1], Sarah M. Boomer[2] and Michael J. Baltzley[2]

[1]*Department of Microbiology, Oregon State University, 226 Nash Hall, Corvallis, OR 97331, USA and*
[2]*Department of Biology, Western Oregon University, 345 Monmouth Ave, Monmouth, OR 97361, USA*

Corresponding author: S.M. Boomer; e-mail: boomers@wou.edu

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ABSTRACT

Despite its widespread geographic distribution and applications, the herbivorous land snail *Cornu aspersum* represents an understudied animal host. In this study, we used a culture-based approach, in combination with a population analysis based on 16S rRNA amplicon sequencing, to describe changes in the faecal microbiota of wild-caught snails that were fed a nonsterile plant diet over a period of 21 days. At the beginning of the experiment, wild-caught snails harboured diverse populations of Gammaproteobacteria. The two most abundant genera of Gammaproteobacteria were soil-associated *Pseudomonas* and *Buttiauxella*; the latter is an understudied genus often reported in snails and slugs. During penicillin treatment, the diversity of Gammaproteobacteria in experimental snails bottlenecked, resulting in *Pseudomonas* takeover. Following penicillin treatment, experimental snails began to recover, with faecal samples containing pre-treatment Gammaproteobacteria groups by day 21. There was a significant difference in the colony-forming units/g across days and across treatments. Additionally, there was a significant interaction between day and treatment. The food source was not sterile and supported its own population of Gammaproteobacteria, primarily *Pseudomonas*, which would have been taken up by the snails daily. Given that *Buttiauxella* was not observed on the food source, we hypothesize that it was present and maintained within the digestive tract of *C. aspersum*, and this is consistent with the idea that *C. aspersum* maintains an endogenous gut microbiome.

INTRODUCTION

The brown garden snail, *Cornu aspersum* (Müller, 1774) (formerly known as *Helix aspersa*), is one of the most widely distributed land snails in the world. Native to the Mediterranean region, *C. aspersum* is an invasive species in locations with temperate and subtropic climates on six continents, the result of plant and vegetable movement and snail farming (Guiller *et al.*, 2012; Sherpa *et al.*, 2018). *Cornu aspersum* is a generalist herbivore that feeds on a variety of living and dead plants (Iglesias & Castillejo, 1999). This species acquires the additional calcium needed for shell development by consuming soil, resulting in the gut of *C. aspersum* being colonized, in part, by soil bacteria (Gomot *et al.*, 1989; Watkins & Simkiss, 1990). *Cornu aspersum* may also serve as a host for important parasites (Chan *et al.*, 2015; Helm *et al.*, 2015; Colella *et al.*, 2016, 2017; Gracenea & Gallego, 2017). Due to its macroscopic size, slow dispersal rate, habit of consuming soil and the relative ease with which it can be marked and tracked over time, *C. aspersum* has been used to study environmental pollution (Beeby & Richmond, 2003; Regoli *et al.*, 2006; de Vaufleury *et al.*, 2006; Angeletti *et al.*, 2013). Transcriptome analysis in aestivating *C. aspersum* has effectively shown both the promise of this interesting animal model and the challenges faced when annotating transcriptomes in this understudied group of organisms (Parmakelis *et al.*, 2017).

Previous studies of the bacterial microbiota of *C. aspersum* have involved a variety of feeding regimes, with the majority using a culture-based approach. The earliest study showed that whole, blended Moroccan *C. aspersum* snails carry *Salmonella* (Andrews *et al.*, 1975). Two studies, which involved dissections of the alimentary canals of *C. aspersum* from different localities and with differing diets, demonstrated the presence of an endemic population of Gammaproteobacteria and Firmicutes, including antibiotic-resistant isolates (Watkins & Simkiss, 1990; Charrier *et al.*, 2006). Importantly, one of the Gammaproteobacteria isolated by Charrier *et al.* (2006) was *Buttiauxella*, which was first described in 1981 (Ferragut *et al.*, 1981). *Buttiauxella* had previously been characterized from nearly 170 unidentified wild-caught snails and slugs from around the world (Muller *et al.*, 1996). Recent studies with *C. aspersum* aimed at describing the intestinal microbiota dynamics in farm-raised/fed *C. aspersum* found varying abundance of

Gammaproteobacteria and Firmicutes over time (Caullan *et al.*, 2014; Koleva *et al.*, 2014, 2015). However, these studies did not use methods that were sufficiently specific to determine whether *Buttiauxella* was present. Of all cited studies, only three employed parallel 16S rRNA gene-based methods to identify bacteria (Caullan *et al.*, 2014; Koleva *et al.*, 2014, 2015).

Most of the studies described above used dissected intestines to assess the gut microbiota of *C. aspersum* (Watkins & Simkiss, 1990; Charrier *et al.*, 2006; Koleva *et al.*, 2014, 2015), which prevents the assessment of changes in the microbiota over time. In the only study that used faecal samples to assess the gut microbiota (Caullan *et al.*, 2014), the researchers did not investigate changes over time. In our study, we examined the gut microbiota of wild-caught snails fed a nonsterile plant diet while challenging them with penicillin, in order to track changes in the faecal microbiota over time. We used a culture-based approach in combination with a population analysis based on 16S rRNA amplicon sequencing, a method not previously used for studying the microbiota of *C. aspersum*. We chose this regime because we ultimately hope to compare faecal microbiota profiles from isolates of *C. aspersum* from different locations in Oregon to determine whether environmental conditions, particularly antibiotics, affect microbiome composition.

MATERIAL AND METHODS

Snail collection, growth and treatment

This investigation was performed using eight individuals of *Cornu aspersum* that were collected in October 2016 from one home garden (total area of <2000 ft^2) in Monmouth, Oregon, USA (44°50′48″N, 123°12′44″W). Each snail was placed in a sterile wide-mouthed quart-sized glass canning jar (Ball Corporation) with no food for 24 h. Faecal samples were collected from each snail after 24 h to identify the pretreatment faecal microbiota. Two control snails ($n = 2$, designated C1 and C2) were individually transferred to new sterile glass jars, each containing 2 g organic baby lettuce (Earthbound Farms) and 10 ml of water. This transfer was repeated daily for the duration of the 21-day experiment. Each of the experimental snails ($n = 6$, designated P1-P6) was transferred to a new sterile glass jar with 2 g of lettuce and 10 ml of water containing 20 μg/ml penicillin (FisherBiotech). The experimental snails received this dose of antibiotics for 2 days (penicillin-treated), followed by the control diet for the remainder of the experiment (recovery). Faecal samples from all snails, if present, were collected on days 1, 2, 3, 4, 7, 10, 14 and 21. Each faecal sample was homogenized using a plastic, sterile pestle in Tris EDTA (TE) buffer (FisherBiotech) and made up to a concentration of 100 μg faeces/ml TE buffer. A portion of each sample of faecal homogenate was stored at −80 °C for DNA analysis.

Sample inoculation and bacterial culture

Immediately after collection, faecal homogenates containing 10 μg of faeces were serially diluted and spread on MacConkey agar plates (Difco). Four treatments were studied: day 1 (pre treatment), day 2 (penicillin-treated or control), day 7 (recovery or control) and day 21 (recovery or control). The snails used for three of these treatments (day 2 P3 and P5, day 21 P6) did not produce faeces. Plates were incubated aerobically at 37 °C for 24 h prior to counting colonies. Colonies were analysed on the basis of visual appearance on MacConkey agar and metabolic tests, which included oxidase activity (BD BBL™ Taxo™ N Discs, Becton and Dickinson and Company) and citrate utilization (Simmons Citrate Agar). Oxidase-positive colonies were grown on LB media (Difco) and inspected for fluorescence using a Fotodyne™ FOTO/Phoresis™ UV Transilluminator (Lysenko, 1961).

DNA analysis of cultured isolates

Representatives of the four observed colony types were subjected to 16S rRNA gene analysis. DNA was isolated from the four colony types using the freeze–thaw method (Kawai *et al.*, 2002). The 16S rRNA gene fragment was amplified using the PCR Master Mix (Promega) and the universal primers, 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (Lane, 1991; Turner *et al.*, 1999). Following isopropanol precipitation, DNA sequence data were generated using a Sanger approach at the Oregon State University Center for Genome Research and Biocomputing. Specifically, purified PCR product was combined with the universal 16S rRNA primer 357F (5′-CTCCTACGGGAGGCAGCAG-3′) (Turner *et al.*, 1999) and amplified using the BigDye™ Terminator v. 3.1 Cycle Sequencing Kit (ABI). Sequencing was performed using a 48-capillary genetic analyzer AB3130xl automatic sequencer (Hitachi Applied Biosystems) with the BigDye Terminator v. 3.1 cycle sequencing kit (ABI). Sequence data (all 850–1100 bp in length) were analysed using BLAST (Altschul *et al.*, 1990) and submitted to GenBank (acc. nos MK256261–MK256266).

DNA analysis of snail faeces and lettuce

DNA was extracted from the frozen faecal homogenates (0.1 g each) of four of the experimental snails (P1, P2, P4 and P6) on day 1 (pre-treatment), day 3 (penicillin-treated), day 7 (recovery) and day 14 (recovery), with faecal homogenates for day 14 being pooled into two groups (P1 + P2, P4 + P6) because only a limited amount of faecal material (<0.1 g) was produced by P2 and P6. DNA was extracted from the frozen faecal homogenates of control snail C1 on specific days as indicated: day 1, 0.049 g; day 3, 0.072 g; day 7, 0.066 g; and day 14, 0.051 g. DNA from individual faecal homogenate samples was extracted with a DNeasy PowerSoil Kit (Qiagen). Each PowerBead sample was disrupted with a Mini-beadbeater (Biospec Products), with the power set to 42 Hz twice for 30 s. Tubes were chilled on ice for 1 min after each beadbeating. Subsequent extraction procedures followed the manufacturer's instructions (Qiagen). All DNA samples were eluted in a final 100 μl of Solution C6 and stored at −80 °C.

The microbial community present on lettuce (the food source) was harvested by incubating 2 g of lettuce in sterile water at 4 °C on a shaker table for 3 h. Microbial samples were concentrated using a 0.45 μm filter (Millipore) with a glass microanalysis filter assembly (Fisher Scientific). Microbial samples were removed from the filter by vortexing for 2 min in wash buffer with 0.2% Tween 20 (Epicentre) and pelleted for 2 min at 14,000 g. Pelleted samples were subjected to PowerBead sample disruption (extraction procedures were as described for the faecal homogenates).

All isolated DNA samples were amplified and analysed by the Research and Testing Laboratory (RTL) using Illumina MiSeq analysis. Amplicons of the V1/V2 hypervariable regions of the 16S rRNA gene were generated using the primers 28F (5′-GAGTTTGATCNTGGCTCAG-3′) and 388R (5′-TGCTGCCTCCCGTAGGAGT-3′) (Sundquist *et al.*, 2007; Turnbaugh *et al.*, 2009). Raw 16S rRNA amplicon-sequence data were processed by RTL to reduce noise, remove chimeras and select operational taxonomic units (OTUs). Specifically, de-noising was performed using the PEAR Illumina paired-end read merger (Zhang *et al.*, 2014). Reads were then run through an internally developed quality-trimming algorithm that truncates reads having an average quality >25. Prefix de-replication and grouping into clusters (4% dissimilarity among sequences of the sample cluster) were performed using the USEARCH algorithm (Edgar, 2010). OTU selection was made using the UPARSE OTU selection algorithm (Edgar, 2013). OTUs were chimera-checked using the UCHIME software (Edgar *et al.*, 2011). The centroid sequence from each cluster was then run against the USEARCH algorithm

Table 1. Groups of Gammaproteobacteria identified using 16S rRNA gene-sequence analysis and culture-based methods.

Gammaproteobacteria group 16S rRNA gene analysis (GenBank acc. no., % similarity, source)	Phenotype	Oxidase	Citrate	Fluorescence
Pseudomonadales	White	+	+	+
Pseudomonas koreensis (KY203805, 98%, soil)				
Pseudomonas reinekei (KU350591, 98%, soil)				
Enterobacteriales *Buttiauxella*	Dark purple	–	+	–
Buttiauxella agrestis (DQ440549, 99%, dairy)				
Buttiauxella izardii (JX294882, 99%, snail)				
Xanthomonadales	White	–	–	–
Stenotrophomonas sp. (MF103764, 97%, soil/plant endophyte)				
Other Enterobacteriales	Pink	–	–	–
Kluyvera (KR189865, 89%, water)				

(Edgar, 2010), using a database of high-quality sequences derived from the NCBI database. The output was analysed using an internally developed Python programme that assigns taxonomic information to each sequence. The 16S rRNA sequence data have been deposited in GenBank's Sequence Read Archive, under the BioProject 'The Faecal Microbiota of *Cornu aspersum*' (acc. no. PRJNA509477).

We analysed the 16S rRNA amplicon sequence data in two ways. Preliminary analyses consisted of estimating the percentage of each bacterial OTU for each sample or timepoint, providing relative abundance information based on the relative numbers of reads for each sample or timepoint. While ten bacterial groups were initially identified from combined snail and/or over-time samples, only four phyla (Cyanobacteria, 2%; Bacteriodetes, 4%; No Hit, 7%; and Proteobacteria, 87%) represented more than 99% of all retrieved OTUs. For the purposes of presenting and discussing our results, we used the following family or genus-level categories (based on the culture-based findings) for Gammaproteobacteria recorded in our study: *Pseudomonadales* and *Xanthomonadales* for Gammaproteobacteria at the family level, *Enterobacteriales* (*Buttiauxella*) for genera of Gammaproteobacteria such as *Buttiauxella* and 'other Enterobacteriales' for Gammaproteobacteria genera other than *Buttiauxella* (abundant members of this group included *Rahnella*, *Hafnia* and *Klebsiella*). Proteobacteria groups, which were not retrieved using MacConkey-selected culture methods, are as follows: Alphaproteobacteria, Betaproteobacteria and unclassified Proteobacteria. Three other groups were also retrieved (i.e. only reported for 16S rRNA gene data). These were Bacteriodetes, Cyanobacteria and 'no hit' OTUs; the last were characterized by sequences that were not similar to any of those from the NCBI database.

Statistical analyses

In order to determine whether antibiotic treatment affected the density and diversity of bacteria in snail faeces, we performed several analyses. For Gammaproteobacteria retrieved using MacConkey agar, we log-transformed the number of colony-forming units per gram (CFU/g) of faeces, then performed a PERMANOVA using Bray–Curtis dissimilarities. We performed a full factorial analysis, treating both day and bacterial species as fixed factors. We also performed a principal component analysis (PCA) of the CFU/g data. To determine if the variables were significantly correlated prior to data reduction, we used Bartlett's test of sphericity. We assessed if we had a large enough sampling for the PCA by performing a Keiser-Meyer-Olkin (KMO) measure of sampling adequacy. For the log-transformed data of CFU/g, the KMO measure of sampling adequacy was 0.774 and Bartlett's test of sphericity was significant ($\chi^2 = 91.9$, df $= 6$, $P < 1.0 \times 10^{-16}$).

Additionally, we performed PERMANOVAs using population dataset OTUs to compare community composition over time for snails treated with antibiotics and to compare the bacterial community composition of snail faeces with the bacterial community composition of the food source. The OTU data did not meet the assumptions for PCA, so instead we performed a non-metric multidimensional scaling (NMDS) analysis of the data using Horn–Morisita indices to calculate the distance matrix. Using population dataset OTUs, we calculated the Shannon diversity index at each timepoint for the antibiotic-treated snails and performed an ANOVA with a post-hoc Tukey's HSD test to compare species diversity.

All statistical analyses were performed in R (R Core Team, 2017), and the packages used were as follows: PERMANOVAs and NMDS, *vegan* (Oksanen, 2018); PCA, *stats* (R Core Team, 2017); KMO, *REdaS* (Maier, 2015); and Bartlett's test, *psych* (Revelle, 2018).

RESULTS

Bacterial populations in the faeces of wild-caught Cornu aspersum

Eight wild-caught snails were collected from a single home garden in Oregon's Willamette Valley. The eight snails (C1–2, P1–6) were put on a 24-h fast, following which individual 10–µg samples of faeces were collected from all the snails. The bacterial colonies in these samples were counted and compared using selective MacConkey agar. Four colony types of Gammaproteobacteria were observed, namely Pseudomonadales, Xanthomonadales, Enterobacteriales (including *Buttiauxella*) and 'other Enterobacteriales' (Table 1). Using 16S rRNA-gene-based identification, we found that *Pseudomonas* represented the majority (>50%) of MacConkey-selected colonies observed across all snails, with most colonies being similar to unpublished soil isolates of *P. koreensis* or *P. reinekei* (Table 1, Fig. 1A). Enteric *Buttiauxella* was the second most-observed colony type, with most colonies being similar to *B. agrestis* (Park *et al.*, 2006) or *B. izardii* (Stalder, Loncaric & Walzer, 2014). Bacteria, which were similar to the groundwater-derived Enterobacteriales *Kluyvera* (Zhang *et al.*, 2015) and the soil/plant-endophytic Xanthomonadales *Stenotrophomonas* (unpublished observations), were the least abundant.

Parallel population studies using 16S rRNA amplicon sequence were performed on the faecal homogenates obtained over time from five wild-caught snails (C1, P1, P2, P4 and P6). Our analysis returned a total of 457,450 bacterial sequences belonging to 209 OTUs, with the mean amplicon library size being 21,783 sequences. Population distributions of the ten most common groups (representing 98% of observed diversity) are shown in Figure 1B. Across all five amplicon libraries, the predominant bacterial sequences observed prior to antibiotic treatment were those

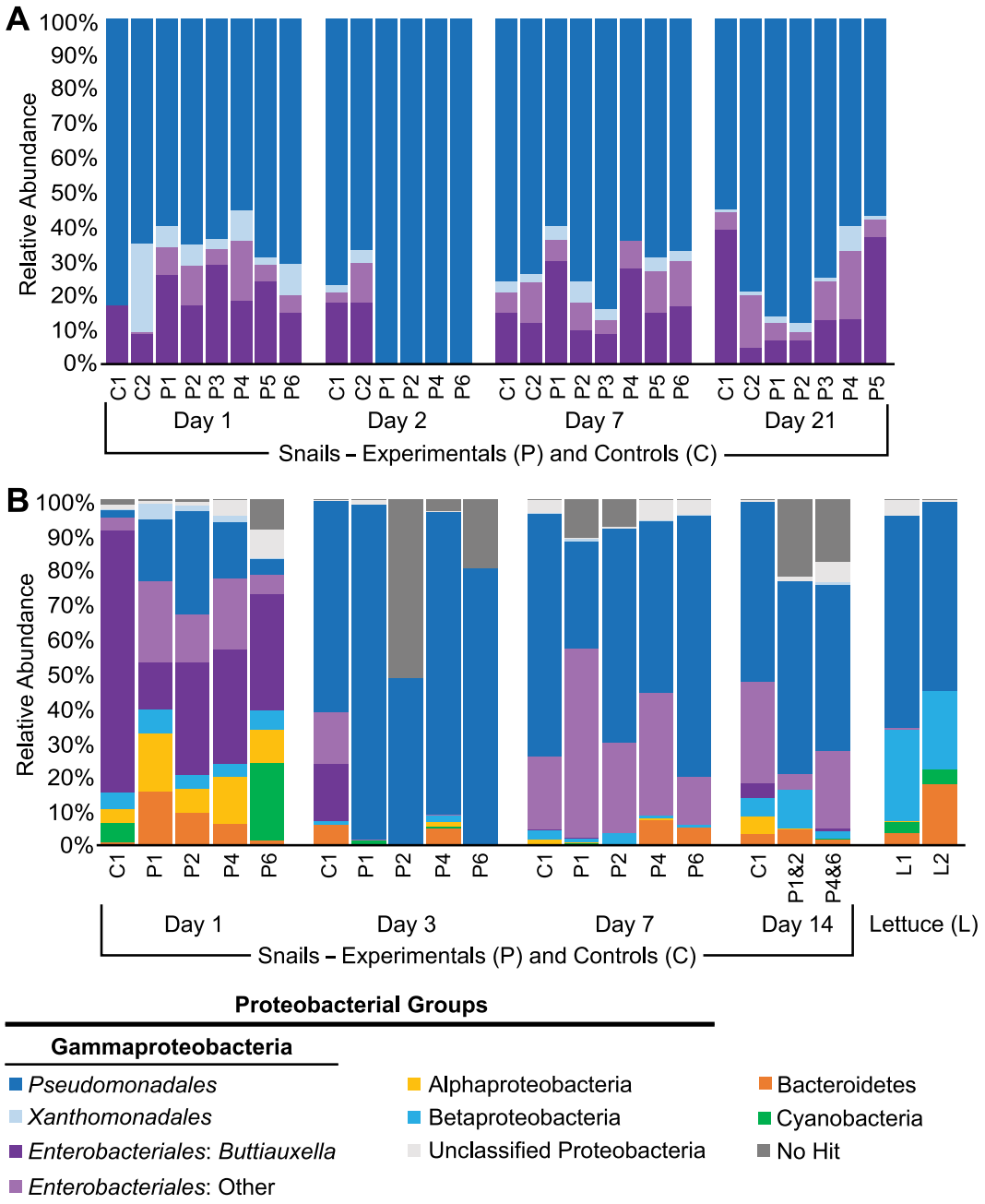

Figure 1. Relative abundance of total MacConkey-selected bacterial groups (**A**) and the ten most abundant bacterial groups obtained via 16S rRNA amplicon sequencing (**B**) retrieved from wild-caught snail faeces following a 24-h fast (day 1) and over time (days 2–21). In a PERMANOVA, there was a significant effect of day ($P < 0.0001$) and treatment ($P < 0.01$) on CFU/g, as well as an interaction between day and treatment ($P < 0.0001$); for experimental snails, there was a significant effect of day on OTUs ($P < 0.0005$).

most similar to Gammaproteobacteria. *Buttiauxella* was the most abundant group observed, with a relative abundance of 10–70% in snail faeces at day 1 (Fig. 1B). *Pseudomonas* was variably abundant (relative abundance of 2–30%). Several groups that would not have grown on MacConkey agar, including Cyanobacteria, Bacteriodetes, Alphaproteobacteria and Betaproteobacteria, were also retrieved (relative abundance of 1–20%).

Population analysis during antibiotic treatment and recovery

Both culture-based and 16S rRNA gene-based population analyses showed that wild-caught snails harboured bacteria from multiple Gammaproteobacteria orders prior to penicillin treatment. During penicillin treatment, the retrieved Gammaproteobacteria diversity from all experimental snails bottlenecked, resulting in population distributions that were dominated by *Pseudomonas*. Meanwhile, control snails continued to harbour diverse groups of Gammaproteobacteria, including *Buttiauxella*. Using the 16S rRNA gene data, the effect of day on community composition was significant (PERMANOVA: $P < 0.0005$, Figs 1, 2), but the effect of antibiotics was not significant ($P = 0.65$).

Following penicillin treatment, experimental snails began to recover, with faecal samples containing pre-treatment Gammaproteobacteria groups by day 21. There was a significant difference in CFU/g across days (PERMANOVA: $P < 0.0001$) and across treatments ($P < 0.01$) as well as significant interaction between day

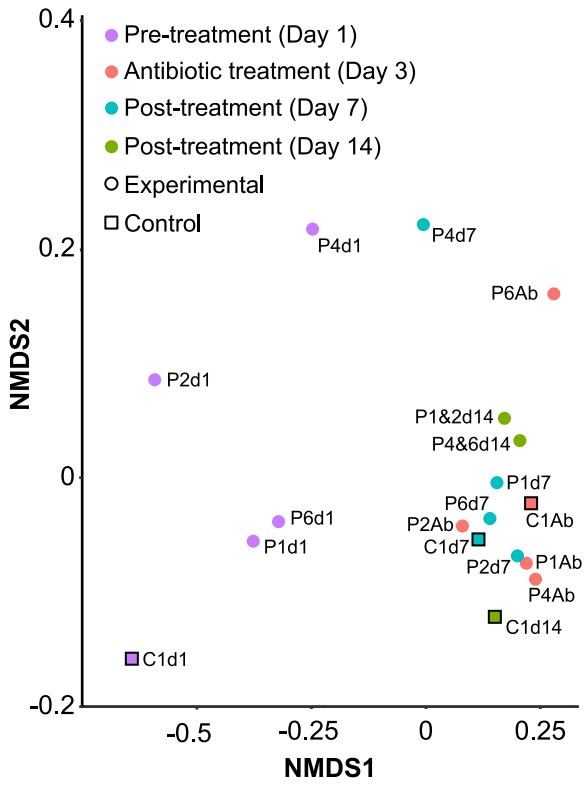

Figure 2. NMDS of OTU data from faecal samples before, during and after treatment with penicillin (stress value = 0.006, NMDS solution based on two axes). P1–P6 are the penicillin-treated experimental snails; C1 and C2 are the two control snails. Day 1 (d1) is the start of the experiment; antibiotic treatment occurred after this on day 2 (d2). The faecal sampling for the antibiotic treatment (Ab) occurred on day 3 (d3) of the experiment. Day 7 (d7) and day (d14) correspond to the phase of the experiment following antibiotic treatment.

and treatment ($P < 0.0001$). A PCA of the CFU/g data showed that the snails receiving penicillin formed a distinct cluster relative to the pre-treatment, post-treatment and control snails (Fig. 3). Our analysis of the microbiota populations based on the Shannon's diversity index yielded similar results. During antibiotic treatment, the H value significantly decreased from 2.7 ± 0.1 (mean $\pm$ SEM) to 0.7 ± 0.2 (ANOVA: $P = 0.0001$; post-hoc Tukey test: $P = 0.0001$; Fig. 4). The H value increased by day 7 to 1.5 ± 0.2 (antibiotic treatment *vs* day 7, $P = 0.04$) and remained unchanged on day 14 (day 7 *vs* day 14, $P = 0.92$). However, even by day 14, the H value was still significantly different from the baseline ($P = 0.005$).

Bacterial population on lettuce

Based on the 16S rRNA amplicon sequence data, we found that although the bacterial community composition on lettuce was significantly different from that in snail faeces prior to antibiotic treatment (PERMANOVA: $P = 0.05$), it was not significantly different from the bacterial community found in snail faeces 7 ($P = 0.07$) or 14 ($P = 0.33$) days after antibiotic treatment. Importantly, with lettuce as the food source, the bacteria in the faeces of the control snails appeared to show temporal change in community composition, with the abundance of *Pseudomonas* showing a significant increase and *Buttiauxella* showing a significant decrease over time (Fig. 1B).

DISCUSSION

Bacterial microbiota studies of *Cornu aspersum* have been largely culture-focused, with limited work having been carried out using 16S rRNA gene-based methods (Andrews *et al.*, 1975; Watkins & Simkiss, 1990; Charrier *et al.*, 2006; Caullan *et al.*, 2014; Koleva *et al.*, 2014, 2015). As our aim was to study the microbial populations within individual snails over time, we analysed the faecal microbiota from wild-caught snails. While we cannot reach definitive conclusions about the core gut microbiome of *C. aspersum* using these approaches, we made several important observations on the population dynamics of the microbiota in snail faeces.

First, we found that *Buttiauxella* was present in all wild-caught snail faecal samples. This is consistent with early reports of this intriguingly gastropod-associated genus (Muller *et al.*, 1996). Using combined culture-based and 16S rRNA gene approaches, *Buttiauxella* was present in all wild-caught snail faecal samples, was maintained in controls throughout the experiment and re-emerged (following *Pseudomonas* takeover) in all penicillin-treated snails. Retrieval of *Buttiauxella* was more consistent using MacConkey retrieval (Fig. 1A) than 16S rRNA amplicon-sequence population assessment (Fig. 1B). Given that *Buttiauxella* was not observed on the lettuce but was found in the snail faeces, we hypothesize that it was present and maintained within the gut microbiome of the snails, supporting the idea that *C. aspersum* maintains an endogenous gut microbiome (Charrier *et al.*, 2006).

After Muller *et al.* (1996) characterized *Buttiauxella* isolates from snails and slugs from around the world, *Buttiauxella* has been identified from a number of sources. In 2014, genome-sequencing efforts were completed for key *Buttiauxella* species, including *B. agrestis* ATCC 33320 (from an unidentified snail, unpublished), *B. brennerae* ATCC 51605 (from an unidentified snail, unpublished), *B. noackiae* ATCC 51607 (from an unidentified snail, unpublished), *B. ferragutiae* ATCC 51602 (from soil, unpublished) and *B. gavinae* ATCC 51604 (from an unidentified snail, unpublished). *Buttiauxella* has also been retrieved from contaminated or decomposing food (Ercolini *et al.*, 2009; He *et al.*, 2009; Mace *et al.*, 2012; Sade, Murros & Bjorkroth, 2013; Benson *et al.*, 2014; Castelo-Branco *et al.*, 2016; Masiello *et al.*, 2016), surface water (Jothikumar *et al.*, 2014), bird faeces (Agnew *et al.*, 2015; Murugaiyan *et al.*, 2015), gall midge guts (Bansal *et al.*, 2014) and human disease samples (Antonello *et al.*, 2014; De Baere *et al.*, 2002). Of these—few of which represent large-scale population assessments—*Buttiauxella* was only noted at high abundance from mallard faeces (15%), gall midge microbiomes (9%) and some stages of sausage spoilage (5%). While it is surprising that no recent studies have revealed high abundance similar to those observed in our study of snails, this may reflect the paucity of snail-focused studies. Taken together, these studies indicate that focused studies of *Buttiauxella* diversity and biogeography are needed. In this respect, efforts to investigate whether environments like soil and water represent thriving reservoirs for this genus, or whether such habitats are contaminated by host-endemic microbiome material, are of particular importance.

Second, we found that treating *C. aspersum* with penicillin significantly reduced the diversity of the faecal microbiota, allowing *Pseudomonas* to become the dominant taxon. This was the case in our culture-based study as well as our 16S rRNA gene-based population analysis. Chronic exposure to antibiotics can disrupt healthy microbial communities and can potentially cause an alternate stable state that is dominated by a drug-resistant strain of bacteria. In human cystic-fibrosis patients, for example, opportunistic soil-borne *Pseudomonas aeruginosa* dominates lung microbial communities as patients age, reflecting the cumulative exposure to antibiotics (Cystic Fibrosis Foundation Patient Registry, 2016). While *Pseudomonas* are typically not associated with healthy human microbiomes, they have been observed at high abundance in the gut of wild-caught zebrafish (Roeselers *et al.*, 2011) and the coffee-boring beetle *Hypothenemus hampei*, where they likely play a role in caffeine breakdown (Ceja-Navarro *et al.*, 2015). While it is likely that *Pseudomonas*

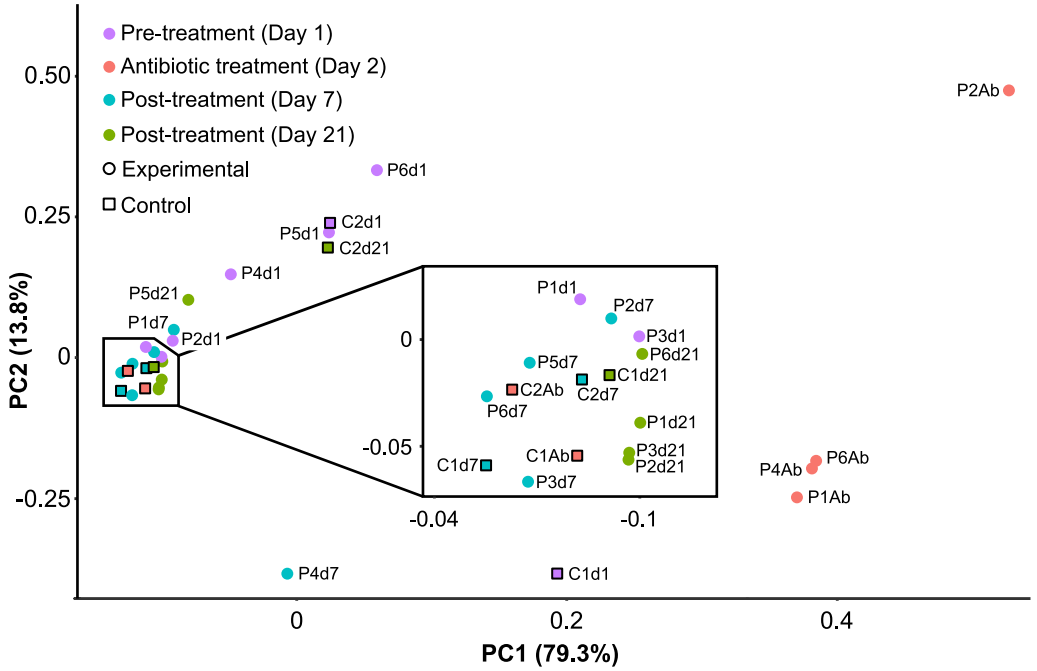

Figure 3. PCA of bacterial colony-forming units obtained from faecal samples before, during and after treatment with penicillin. PCA axes 1 and 2 explain 79.3% and 13.8%, respectively, of total variation in the data. P1–P6 are the penicillin-treated experimental snails; C1 and C2 are the two control snails. Day 1 (d1) is the start of the experiment; antibiotic treatment occurred on day 2 (d2), followed by faecal sampling for the antibiotic treatment (Ab) later on the same day. Day 7 (d7), day 14 (d14) and day 21 (d21) correspond to the phase of the experiment following antibiotic treatment.

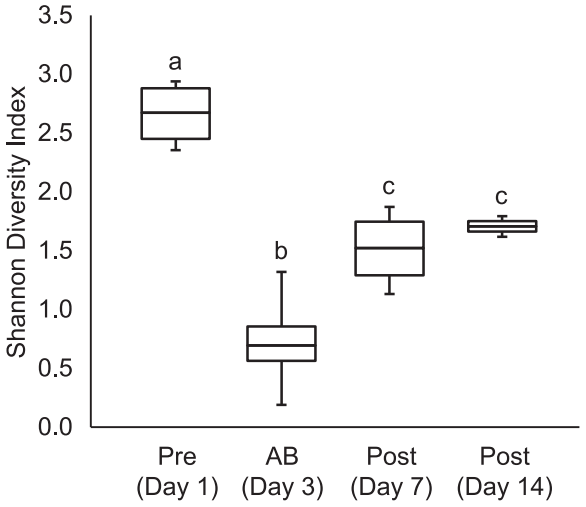

Figure 4. Shannon diversity index of faecal microbial communities at the OTU level. The box represents the median and first and third quartiles, with whiskers showing the maximum and minimum values. Groups with different letters are significantly different. Abbreviations: AB, treatment with penicillin; Pre, Post, phases of experiment before and after antibiotic treatment, respectively.

are present in higher abundance in other comparable animal microbiomes (e.g. cockroaches, Tinker & Ottesen, 2016), data resolution is often limited to phylum-level OTU presentation and does not include parallel culture studies.

Third, our work suggests that *C. aspersum* maintains a dynamic gut microbiome of which endemic *Buttiauxella* and *Pseudomonas* are important constituent taxa. The fact that *Buttiauxella* abundance did not fully return to wild-caught levels in treatment snails and was reduced even in control snails fed *Buttiauxella*-lacking lettuce suggests that exposure to exogenous faecal microbes may be necessary to maintain the gut microbiota of *C. aspersum*. This has, for example, been shown in studies using the fruit fly *Drosophila melanogaster*, which has to consume faecal bacteria to maintain its gut microbiome (Blum *et al.*, 2013). In the study by Blum *et al.* (2013), the incubation regime allowed developing flies to inoculate the sterile food source with faeces for 3 days prior to the flies passing into a new sterile environment and with a new sterile food source; this resulted in the development of a more stable gut microbiome population over time. In contrast, our feeding regime involved passing individual snails into a new sterile environment every 24 h. This likely provided insufficient time to maintain bacterial populations in faecal-inoculated food, thus contributing to the observed reduction in *Buttiauxella* abundance and overall microbial diversity over time in both treatment and control snails. Further testing of this hypothesis using different feeding and transfer timelines and housing multiple snails together would better address this issue.

Our findings show that the faecal microbiota of *C. aspersum* is dominated by Gammaproteobacteria, particularly *Pseudomonas* and *Buttiauxella*, confirming some findings of previous culture-based studies (Watkins & Simkiss, 1990; Charrier *et al.*, 2006; Caullan *et al.*, 2014; Koleva *et al.*, 2015). However, most previous studies plated dissected alimentary canal material on a broader variety of media, often under both aerobic and anaerobic conditions; as a result, more gram-positive and/or lactic-acid bacteria were observed (Charrier *et al.*, 2006; Koleva *et al.*, 2014, 2015). It is possible that our methods, which analysed whole faeces that were deposited up to 8 h prior to plating or freezing, were biased against these kinds of bacteria. We note, however, that a previous study demonstrated that preservation methods do not alter the ability to discern individual microbiome differences in faecal matter for up to a week (Song *et al.*, 2016). Although using faeces represents a potential limitation, the approach of analysing microbiome changes over time is the only ethical option in some systems (e.g. humans). Indeed,

our *C. aspersum* study closely resembles human faecal microbiota research, in which cohorts consume different diets, including whole food sources containing live exogenous microbes (Human Microbiome Project, 2012; David *et al.*, 2014). Studying faeces is also the only option for many field-based studies where faecal collection and comparison is the primary way of assessing host microbiomes or distributions (Ley *et al.*, 2008; Muegge *et al.*, 2011; Delsuc *et al.*, 2014).

We have shown that the faecal microbiota of *C. aspersum* changes in response to penicillin exposure, shifting from a diverse population that includes *Buttiauxella* to a more homogeneous population dominated by *Pseudomonas*. We hypothesize that these conditional microbiome changes may be used to compare faecal microbiota profiles from isolates of *C. aspersum* from different locations to determine whether environmental conditions, specifically antibiotics, affect microbiome composition. Although environmental antibiotics are recognized as a potential source for antibiotic resistance, few studies have addressed their impact on complex natural populations (Andersson & Hughes, 2014). Two recent studies have shown that even sublethal concentrations of antibiotics impact the composition of microbial communities (Beka *et al.*, 2018; Murray *et al.*, 2018). This has been observed for sewage-derived microbial populations (Murray *et al.*, 2018). The impact of antibiotics on microbial composition has also been observed in the microbiomes of invertebrates such as medicinal leeches, which can transmit infection to patients (Beka *et al.*, 2018). *Cornu aspersum* thrives throughout Oregon's Willamette Valley, a region that includes areas of antibiotic-heavy agricultural industry. Given this observation, we hypothesize that the distribution of snails and their faecal microbiome population profiles for the Willamette Valley may reflect the relative amount of antibiotics in the environment, with *Pseudomonas* being favoured where antibiotic concentrations are higher.

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