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INVERTEBRATE MICROBIOLOGY

Direct Comparison of Fecal and Gut Microbiota in the Blue Mussel (*Mytilus edulis*) Discourages Fecal Sampling as a Proxy for Resident Gut Community

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Abstract

Bivalves have ecological and economic importance but information regarding their associated microbiomes is lacking. As suspension feeders, bivalves capture and ingest a myriad of particles, and their digestive organs have a high throughput of particle-associated microbiota. To better understand the complement of transient and resident microbial communities, standard methods need to be developed. For example, fecal sampling could represent a convenient proxy for the gut microbiome and is simple, nondestructive, and allows for sampling of individuals through time. The goal of this study was to evaluate fecal sampling as a reliable proxy for gut microbiome assessment in the blue mussel (*Mytilus edulis*). Mussels were collected from the natural environment and placed into individual sterilized microcosms for 6 h to allow for fecal egestion. Feces and gut homogenates from the same individuals were sampled and subjected to 16S rRNA gene amplicon sequencing. Fecal communities of different mussels resembled each other but did not resemble gut communities. Fecal communities were significantly more diverse, in terms of amplicon sequence variant (ASV) richness and evenness, than gut communities. Results suggested a mostly transient nature for fecal microbiota. Nonetheless, mussels retained a distinct resident microbial community in their gut after fecal egestion that was dominated by ASVs belonging to *Mycoplasma*. The use of fecal sampling as a nondestructive substitute for direct sampling of the gut is strongly discouraged. Experiments that aim to study solely resident bivalve gut microbiota should employ an egestion period prior to gut sampling to allow time for voidance of transient microbes.

Keywords Mussel · Gut microbiome · Resident · Transient

Introduction

Little is known about the microbial communities occupying marine invertebrates relative to what has been established for terrestrial species; marine invertebrate microbial communities warrant increased attention for a comprehensive understanding of animal-associated microbiomes. In particular, bivalve molluscs are a group of ecologically and economically important invertebrates, but information regarding their tissue-associated microbiomes is lacking. Bivalves are often dominant members of the macrobenthos in coastal ecosystems and provide numerous pivotal ecosystem services such as nutrient cycling, benthic-pelagic coupling, and exerting top-down control on phytoplankton communities [1–3]. Suspension-feeding bivalves can filter large quantities of water (e.g., 3–5 L/h/g dry mass) [4]; and therefore, their digestive organs encounter large numbers of both free-living and particle-associated microbiota. The anatomical arrangement of the digestive tract of bivalves includes digestive diverticula, gut tubules ending in blind sacs that are sites for phagocytosis and intracellular digestion. This complex morphological feature of the gut is an ideal location for resident microbes [5].

As efforts to study the microbial communities of bivalves continue to grow, methods for microbial community sampling should be evaluated and standardized to maximize comparability and reproducibility among research groups. For

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example, the sampling of bivalve feces could represent a convenient proxy for the gut microbiome because fecal collection is simple, nondestructive, and allows for the sampling of individuals through multiple time points. Fecal sampling is routine for microbial investigations of the human gut [6, 7]; similarities between human gut and fecal communities likely explain the efficacy of fecal transplantation therapies [8]. However, efforts to directly compare fecal communities with those of rectal tissue and mucosal swabs in humans [9], bats [10], and cane toads [11] repeatedly reveal stark differences between fecal and gut microbiomes.

It is currently unknown how the fecal and gut communities of marine bivalves, which have markedly different digestive physiology than vertebrates [12], relate or differ. Simons et al. [13] examined the effects of changes in microalgal diets on the microbiome of the Pacific oyster (*Crassostrea gigas*). They sampled oyster feces and claimed that it was representative of the "digestive microbiome" without any verification of gut community composition. Only one study has evaluated the effect of depuration (non-sterile) on the mussel gut microbiome [14]. In this study, two mussels were allowed to depurate for 15 h, and taxonomic composition of their guts was compared with three mussels that did not undergo depuration. Although depurated mussels showed a reduced alpha diversity compared with non-depurated ones, lack of sufficient replication in the study and absence of direct comparison to fecal microbiota justifies further experimentation.

The goal of the present study was to evaluate fecal sampling as a reliable proxy for gut microbiome assessment in the blue mussel (*Mytilus edulis*). Mussels are commonly used as model suspension-feeding bivalves in laboratory studies and have been the focus of some gut microbiome research efforts [15–17]. Animals were collected from the natural environment and placed into individual sterilized microcosms to allow for fecal egestion. Samples of feces and gut homogenates from the same individuals were subjected to 16S rRNA gene amplicon sequencing and analysis to directly compare alpha diversity and taxonomic composition of the different substrates. This design allowed for an effective comparison of autochthonous (resident) and allochthonous (transient) microbes associated with mussel guts under normal physiological conditions. Such comparisons are important because they can provide insight into the microbial information that can be gleaned from fecal sampling, and because they can enhance interpretation of other bivalve gut microbiome studies. The results are also important for advancing basic ecological and spatial understanding of the microbial communities that become ingested and egested by suspension-feeding bivalves. The tested null hypothesis was that fecal communities would resemble gut communities of the same individuals in terms of similarity metrics, alpha diversity, and taxonomic composition.

Materials and Methods

Mussel Collection

Eleven adult blue mussels were collected from a natural population in Long Island Sound at the University of Connecticut Avery Point campus (June 2019). All mussels were of similar size, ranging from 4 to 6 cm in shell length, and environmental seawater was 19 °C at time of collection. The salinity at Avery Point from June–July was approximately 30 ppt. Mussel valves were gently scrubbed and rinsed briefly with 70% EtOH to remove epiphytic invertebrates and microbes.

Microcosm Design

Microcosms were employed to isolate individual mussels during the fecal egestion period. The microcosms consisted of 1.9-L volume glass jars fitted with custom plastic lids that allowed an air supply to pass into the experimental seawater (Fig. 1). Air was supplied by means of aquarium pumps and

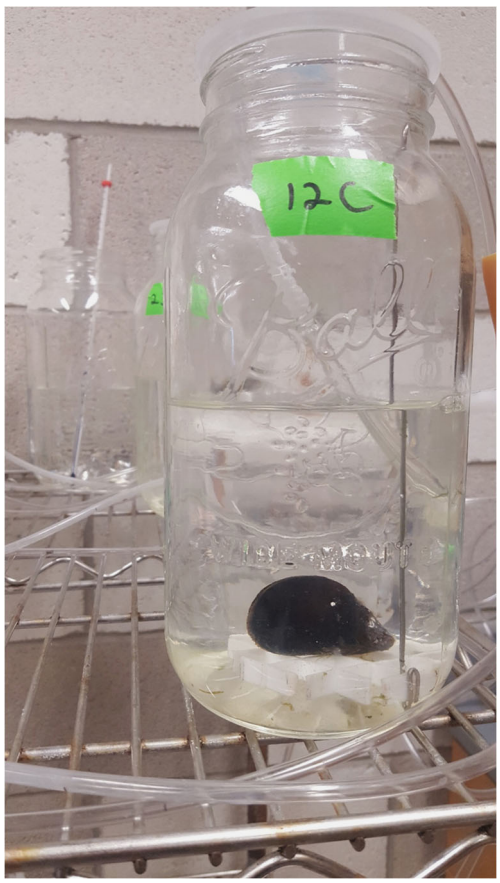

Fig. 1 One individual microcosm housing a mussel. Air was supplied by means of aquarium pumps, and directed through silicone tubing, 0.22-μm Whatman air filters, and glass pipettes (top). Each chamber was also outfitted with a plastic grid platform (bottom) upon which mussels could rest and remain elevated above biodeposits. All components of the microcosm system were sterilized prior to experimentation

directed through silicone tubing, 0.22-μm Whatman air filters, and glass pipettes. Every microcosm also was outfitted with a plastic grid platform upon which mussels could rest and remain elevated above biodeposits. All components of the microcosm system were sterilized prior to experimentation; glass jars and pipettes were autoclaved, while plastic lids, grid platforms, and air tubing were soaked overnight in 70% EtOH. Each sterilized microcosm was filled with 1.5 L of filtered (0.2-μm cartridge) and autoclaved seawater and housed within an environmental chamber held at a constant temperature of 19 °C. Seawater was sterilized in 20-L polycarbonate carboys; autoclaving increased pH by less than 0.02 and had no effect on salinity.

Egestion Period and Fecal Sampling

Mussels were placed into their individual microcosms and left to defecate for 3 h. This amount of time was sufficient to allow intestinal feces and some, but not all, glandular feces to clear from the gut. After 3 h, all mussels had deposited feces at the bottom of their jars, and these feces were collected with serological pipettes and transferred to 15-mL collection tubes. Collection tubes were centrifuged at 1500 ×*g* for 20 s to pellet the feces, and the overlying seawater was decanted down to a volume of 1 mL. Feces were resuspended in this 1 mL and transferred to 1.5-mL microcentrifuge tubes which were centrifuged at 10,000 ×*g* for 60 s to further pellet the feces. All remaining supernatants were removed by means of a micropipette. Fecal pellets were then resuspended in 200 μL of ZymoBiomics DNA/RNA Shield (Zymo Research Corp., Orange County, CA) to prevent nucleic acid degradation and homogenized uniformly with pestles. Homogenized fecal samples were then stored at − 80 °C until DNA extraction.

Mussel Dissections

Mussels remained in their aerated microcosms for an additional 3 h after the end of the egestion period while fecal samples were being processed. This additional time period allowed mussels to further clear glandular feces from the gut, but this second round of feces was not collected in the present study. Mussels were dissected individually following the aseptic techniques outlined by Greenberg and Hunt [18]. Each mussel was removed from its microcosm and its valves washed briefly with 70% EtOH to remove potentially contaminating microbes. The adductor muscles were cut, the valves were opened, and the pallial cavity rinsed with 10 mL of sterilized 3% NaCl solution to remove microbes associated with the pallial fluid. The gut (including stomach and digestive diverticula) was then removed using sterilized instruments over a cold surface to minimize nucleic acid degradation. The gut was transferred to a 1.5-mL microcentrifuge tube, suspended in 400–600 μL of ZymoBiomics DNA/RNA Shield (enough to cover the whole tissue), and homogenized uniformly with a pestle. Homogenized gut samples were then stored at − 80 °C until DNA extraction.

DNA Extraction, PCR, and Sequencing

Mussel gut and fecal samples were processed in the same manner. The ZymoBiomics DNA Microprep Kit (Zymo Research Corp., Orange County, CA) was employed to extract total genomic DNA from all samples following the manufacturer's instructions, with the inclusion of a proteinase K (20 mg/mL) digestion at 55 °C for 15 min. After DNA extraction, bacterial 16S rRNA gene fragments (V4 hypervariable region) were amplified with PCR using modified primers from the Earth Microbiome Project [19], supplied with indices by the University of Connecticut's Microbial Analysis, Resources, and Services center (MARS). Fragments were amplified with a GoTaq DNA polymerase kit and dNTPs from Promega (Promega Corporation, Madison, WI). Reactions were incubated at 94 °C for 3 min for initial denaturation. Reactions were then subjected to 31 cycles of 94 °C for 45 s, 55 °C for 60 s, and 72 °C for 90 s, followed by a final elongation step of 72 °C for 10 min. For each sample, amplification was confirmed visually by gel electrophoresis and imaged under UV following staining with GelRed (Biotium, Fremont, CA). Amplicons were submitted to MARS and sequenced on an Illumina MiSeq platform producing 2 × 250-bp paired-end read libraries. Raw reads were uploaded to the NCBI Short Read Archive (SRA) under submission ID SUB7223904 (BioProject ID: PRJNA622268).

Amplicon Sequence Analysis

All bioinformatic amplicon read analysis was conducted in R. Amplicon sequence variants (ASVs) were generated with the DADA2 pipeline [20]. Paired reads were quality filtered and merged, and chimeric sequences were removed. Taxonomic assignments down to the species level, when possible, were conducted via the SILVA rRNA gene database (version 132) [21, 22]. A generalized, time-reversible maximum likelihood phylogenetic tree, with gamma rate variation, was generated de novo using the phangorn package and a multiple alignment produced with the DECIPHER package [23, 24].

Further data analysis, manipulation, and figure generation were conducted with the phyloseq package [25]. ASVs with taxonomic assignments associated with eukaryotes, chloroplasts, and mitochondria were considered erroneous and removed from analysis. Additional classification efforts for selected ASVs were conducted manually with MEGA X and reference sequences from the SILVA database [21, 26]. A phylogenetic tree was constructed using the neighbor-joining method [27] with evolutionary distances calculated via the maximum composite likelihood method [28] and confidence

evaluated with a bootstrap test [29]. Data were never rarefied. Prior to analysis of beta diversity, a variance stabilizing transformation was conducted with DESeq2 to correct for differences in library size [30, 31]. Bray-Curtis dissimilarity and UniFrac distance metrics were both computed and compared [32]. DESeq2 was also employed to examine differential representation of ASVs between substrate types [30].

Categorical differences in community composition were tested statistically with permutational analysis of variance (PERMANOVA) [33]. Homogeneity of variance was evaluated with the betadisper function which utilizes a multivariate analogue of Levene's test to compare category dispersions. Although the data violated the homogeneity assumption of the PERMANOVA test, results were treated as valid considering that PERMANOVA is robust to departures from homogeneity in balanced designs [34]. Paired comparisons of alpha diversity between fecal and gut samples were conducted with paired Student's *t* tests. Normality of pair differences was confirmed with Shapiro-Wilk tests.

Results

16S rRNA Gene Amplicon Sequencing

A total of 393,250 raw reads from the 22 samples (median 18,256 reads) were loaded into the DADA2 pipeline. After rigorous quality control, i.e., read filtering and removal of erroneous taxonomic assignments, 1664 ASVs were observed. Rarefaction analysis revealed that, despite variability in filtered read depth, this sequencing effort sufficiently captured the ASV richness in all samples (Fig. 2).

Beta Diversity Analysis

There were obvious distinctions between the microbial communities of mussel feces and gut. Principal coordinates analysis on weighted UniFrac distances showed that fecal and gut communities from the same mussel never clustered together (Fig. 3a). However, all fecal samples and all gut samples, regardless of mussel identity, clustered together reasonably well (Fig. 3b). PERMANOVA on the weighted UniFrac distances confirmed the significant difference in multivariate ASV composition between sample types (pseudo-$F_{(1,20)} = 17.80$, $p < 0.001$). Fecal samples clustered more tightly with each other than gut samples did with each other; there was less distance between individual fecal samples and more distance between individual gut samples (Fig. 3). The nonhomogeneous dispersions were confirmed with a multivariate homogeneity of groups dispersions (betadisper) test ($F_{(1,20)} = 118.81$, permutations = 999, $p < 0.001$), and can also be visualized via a compositional dissimilarity network plot (Fig. 4).

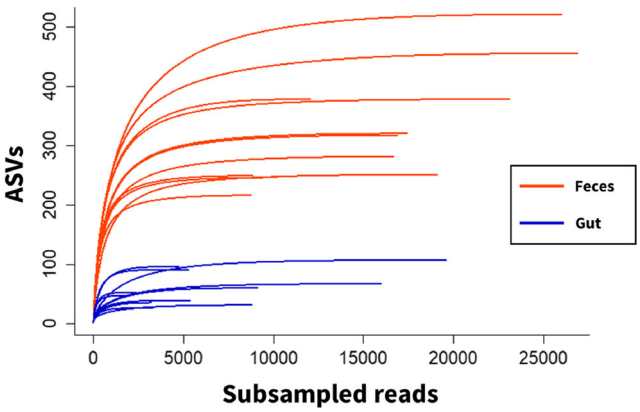

Fig. 2 Rarefaction curves for all 22 samples produced by the rarecurve command in phyloseq with a step size of five. Each of the curves for fecal samples (red) and gut samples (blue) saturated prior to termination

Alpha Diversity Analysis

Gut and fecal microbial communities had considerable differences in alpha diversity. Gut communities had a mean ASV richness of 59 (SD = 27.9), whereas fecal communities had a mean ASV richness of 328 (SD = 96.0), over five times greater. Pairwise comparisons of gut and fecal ASV richness values from individual mussels revealed a significantly greater ASV richness among fecal communities (Fig. 5a, paired Student's *t* test, $t_{(10)} = -8.36$, $p < 0.001$). Similarly, fecal communities were more diverse according to the Shannon index. Gut communities had a mean Shannon index of 2.3 (SD = 0.44), whereas fecal communities had a mean Shannon index of 4.6 (SD = 0.27), twice as great. Pairwise comparisons of Shannon index values between gut and fecal samples from individual mussels revealed a significantly greater Shannon index among fecal communities (Fig. 5b, paired Student's *t* test, $t_{(10)} = -14.82$, $p < 0.001$). These differences in diversity were also evident at higher taxonomic levels (e.g., phylum-level, Fig. 6).

Taxonomic Composition

Seven bacterial ASVs that could not be assigned taxonomic classification at the phylum level by the DADA2 pipeline were putatively assigned to the phylum Tenericutes after phylogenetic analysis (Fig. S1). Phylum-level taxonomic composition was relatively uniform across all fecal communities with major representation from the Proteobacteria, Bacteroidetes, and Tenericutes (Fig. 6). In contrast, phylum-level taxonomic composition was more variable among gut communities, with the Tenericutes usually representing 50–80% of the total relative abundance (Fig. 6). ASVs that could be assigned taxonomic classification to the genus level were utilized for in-depth taxonomic characterization. Fecal communities were not dominated by singular genera, but typically consisted of the following genera ranging from ca. 2.5 to 30% in relative

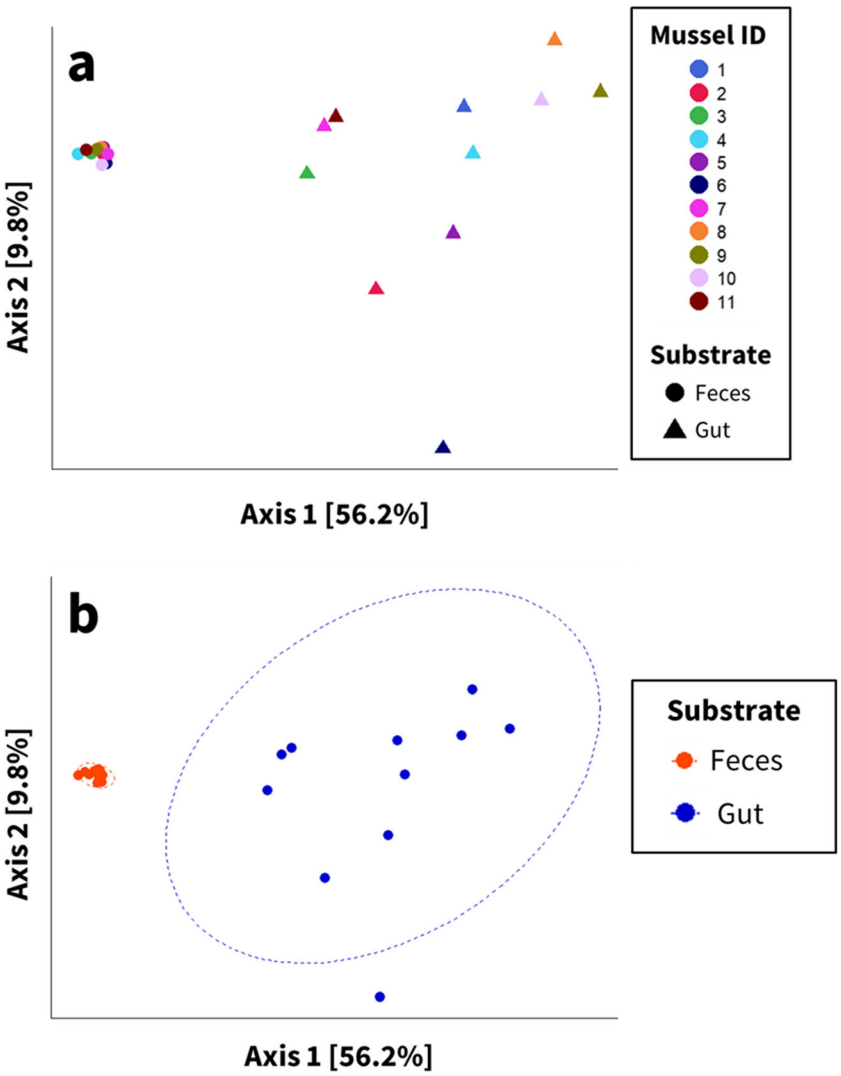

Fig. 3 Ordination plots displaying principal coordinates analysis on weighted UniFrac distances. **a** Microbial communities colored by mussel identity and shaped according to substrate type. **b** Microbial communities colored by substrate type and drawn with 95% data ellipses (PERMANOVA, pseudo-$F_{(1,20)} = 17.80$, $p < 0.001$)

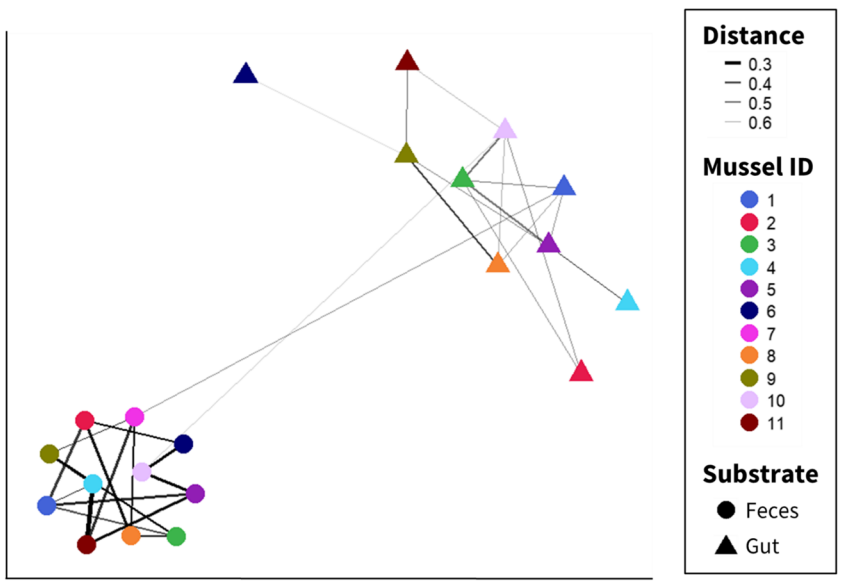

Fig. 4 Compositional dissimilarity network based on the Bray-Curtis dissimilarity metric created with the plot_net command in phyloseq using the Fruchterman Reingold network layout algorithm. Nodes represent microbial communities with color denoting mussel identity and shape denoting substrate type. Edges between nodes represent dissimilarity values less than 0.7 with thickness scaled according to the dissimilarity between nodes

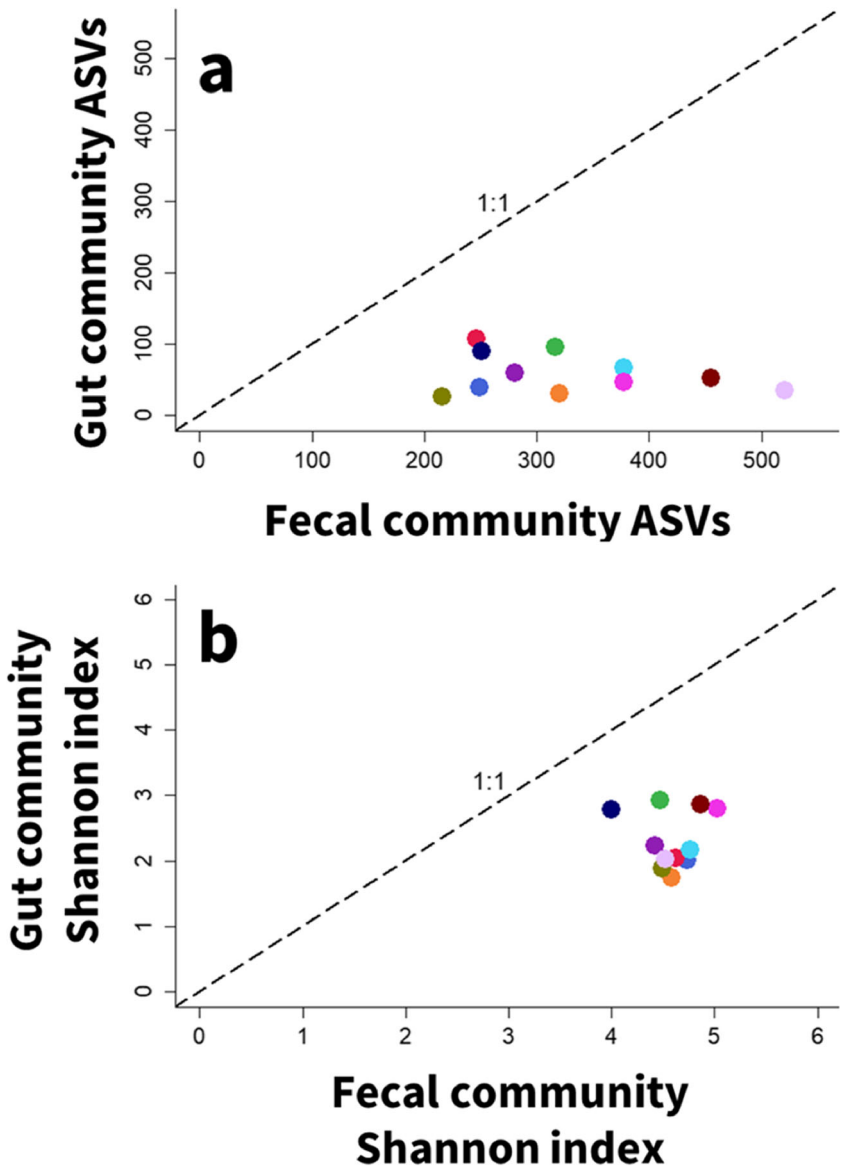

Fig. 5 Comparison of **a** ASV richness (paired Student's *t* test, $t_{(10)} = -8.36$, $p < 0.001$), and **b** Shannon diversity index (paired Student's *t* test, $t_{(10)} = -14.82$, $p < 0.001$), between gut and fecal microbial communities from individual mussels. Coloration distinguishes individual mussels and the broken line represents a 1:1 relationship

abundance (high to low): *Mycoplasma*, *Aliivibrio*, *Vibrio*, *Halioglobus*, *Psychromonas*, *Haloferula*, *Blastopirellula*, and *Algibacter* (Table 1). Gut communities were always dominated by the genus *Mycoplasma*, ranging from roughly 50 to 95% in relative abundance, and some also had other prominent genera ranging from roughly 10 to 35% in relative abundance belonging to the following genera: *Psychrilyobacter*, *Brochothrix*, *Alteromonas*, or *Lutibacter* (Table 1).

Differential abundance analysis with DESeq2 revealed that 200 ASVs (12.0% of all ASVs observed) were significantly more abundant in fecal communities (Fig. 7, $p_{adjusted} < 0.01$). Of the ASVs significantly more abundant in fecal communities, 79 (39.5%) belong to the class Gammaproteobacteria, 51 (25.5%) belong to the Bacteroidia, 16 (8.0%) belong to the Deltaproteobacteria, 15 (7.5%) belong to the Alphaproteobacteria, and 14 (7.0%) belong to the Verrucomicrobiales (Table S1). There were two ASVs with notable overrepresentation in fecal communities, one belonging to the clade Sva0081_sediment_group [35] and the other belonging to the genus *Ulvibacter*, with $\log_2$-fold differences of 24.2 and 23.8, respectively (Fig. 7). All other ASVs that were significantly more abundant in fecal communities had $\log_2$-fold differences between 4.4 and 10.3 (Table S1). Concurrently, there was only one ASV significantly more abundant in gut communities than fecal communities. This ASV belonged to the phylum Proteobacteria but could not be assigned classification any more specifically (Fig. 7, Table S1).

Discussion

The design of this study allowed for an informative comparison of resident and transient microbiota of *M. edulis*. Unlike

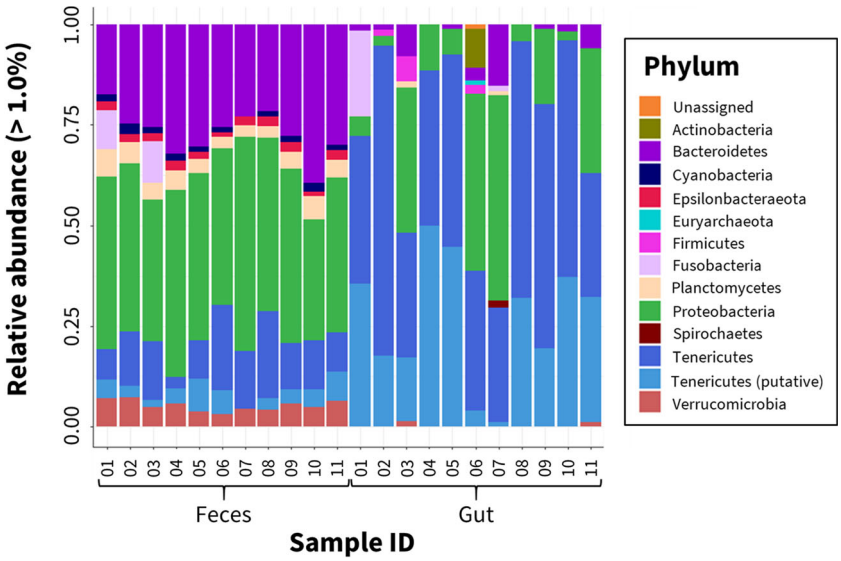

Fig. 6 Relative abundance of ASVs grouped at the phylum level for each sample. ASVs comprising < 1.0% of total composition of a sample were excluded for simplicity of viewing

many vertebrate digestive systems that can be divided into mucosal and luminal compartments as habitats for microbes to occupy, the complexity of the bivalve gut does not allow for such a binary distinction. Instead, microbes associated with the gut are better classified simply as resident or transient.

Under normal physiological conditions, mussels generate and void feces from two compartments [36]. Intestinal feces are composed of undigested material subjected to extracellular digestion in the stomach, and are voided on short timescales (e.g., 0.2–2 h). Glandular feces are composed of undigested material subjected to intracellular digestion by phagocytic cells that line the sacs of the digestive diverticula, and are voided on longer timescales (e.g., 2–9 h or longer) [37–40]. Because the microcosms were sterile and mussels were not fed after collection from the natural environment, microbial signatures recovered in feces voided in 3 h represent a valid approximation of transient microbiota of primarily intestinal origin. Concurrently, microbial signatures recovered in gut tissues 6 h after collection from the natural environment represent an approximation of resident microbiota that inhabit the gut and avoid routine clearance by digestive processes. It should be recognized that because feces were collected at only a single time point, it is possible that some fluctuations in microbial composition took place within fecal material during the period after egestion but before sampling. Additionally, as a result of the time course of glandular feces egestion, it is likely that some glandular feces destined for egestion remained in the gut at time of sampling. Therefore, results from gut samples likely contain mostly resident but also some transient microbes.

The microbial communities sampled from mussel feces and gut tissue were markedly different. For fecal microbiome sampling to be a reliable proxy for resident gut microbiome sampling, fecal and gut communities from individuals would need to resemble more closely each other than any communities from different individuals. The data presented here do not satisfy that requirement. Fecal communities from individual mussels resembled each other and did not resemble gut communities regardless of mussel identity (Fig. 3). Based on these results, sampling the fecal microbiome in place of direct gut sampling would provide little information about the resident gut community. Thus, the use of fecal sampling as a noninvasive and nondestructive substitute for direct gut sampling is strongly discouraged for experiments concerned with the resident microbiota of the mussel gut. Lack of interchangeability between fecal and invasive gut sampling has also been observed in bats and cane toads [10, 11], and likely applies to other groups of animals.

Considering that fecal communities of mussels formed a cohesive group distinct from gut communities, it is pertinent to evaluate specific ways that fecal and gut microbiomes differed. The evidence supports the notion that fecal microbiomes represent largely transient groups of microbes that become ingested by mussels and voided shortly afterwards (i.e., within hours). Fecal communities were significantly more diverse, in terms of ASV richness and evenness, than their gut community counterparts (Fig. 5). This disparity in alpha diversity would be expected if fecal communities represent the diverse community of environmental microbes that normally reside suspended in coastal seawater, and incidentally become ingested and voided by suspension-feeding mussels. Conversely, diversity would expectedly be low for residents of the mussel gut, a complex environment characterized by low pH, abundant digestive enzymes, and mucociliary flux of material [12]. Fecal communities also formed a more homogenous grouping than gut communities; dissimilarity was larger among gut communities than it was among fecal communities (Fig. 4). This homogeneity among fecal samples

Table 1 Relative composition of the fecal and gut communities from each individual mussel at the genus level. Taxonomic classification according to the SILVA database are listed; all genera listed are bacterial. ASVs that could not be assigned to a genus and genera comprising < 2.5% of a community's total composition were excluded for simplicity of viewing

	Feces						Gut					
	Relative abundance	Phylum	Class	Order	Family	Genus	Relative abundance	Phylum	Class	Order	Family	Genus
Mussel 1	0.128	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Psychrilyobacter	0.593	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma
	0.103	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma	0.348	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Psychrilyobacter
	0.081	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio						
	0.050	Proteobacteria	Gammaproteobacteria	Cellvibrionales	Halieaceae	Halioglobus						
	0.038	Planctomycetes	Planctomycetacia	Pirellulales	Pirellulaceae	Blastopirellula						
	0.038	Proteobacteria	Gammaproteobacteria	Alteromonadales	Psychromonadaceae	Psychromonas						
	0.029	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Rubritaleaceae	Haloferula						
	0.027	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio						
Mussel 2	0.119	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio	0.842	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma
	0.104	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma	0.036	Firmicutes	Bacilli	Bacillales	Listeriaceae	Brochothrix
	0.045	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Rubritaleaceae	Haloferula						
	0.042	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio						
	0.040	Proteobacteria	Gammaproteobacteria	Cellvibrionales	Halieaceae	Halioglobus						
	0.039	Proteobacteria	Gammaproteobacteria	Alteromonadales	Psychromonadaceae	Psychromonas						
	0.036	Cyanobacteria	Oxyphotobacteria	Synechococcales	Cyanobiaceae	Synechococcus_CC9902						
	0.036	Planctomycetes	Planctomycetacia	Pirellulales	Pirellulaceae	Blastopirellula						
	0.034	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Algibacter						
Mussel 3	0.205	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma	0.543	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma
	0.143	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Psychrilyobacter	0.097	Firmicutes	Bacilli	Bacillales	Listeriaceae	Brochothrix
	0.066	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio	0.050	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Tenacibaculum
	0.041	Proteobacteria	Gammaproteobacteria	Cellvibrionales	Halieaceae	Halioglobus	0.038	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Endozoicomonadaceae	Endozoicomonas
	0.029	Proteobacteria	Gammaproteobacteria	Alteromonadales	Psychromonadaceae	Psychromonas						
	0.027	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio						
Mussel 4	0.130	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio	0.917	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma
	0.061	Proteobacteria	Gammaproteobacteria	Cellvibrionales	Halieaceae	Halioglobus						
	0.057	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio						
	0.052	Proteobacteria	Gammaproteobacteria	Alteromonadales	Psychromonadaceae	Psychromonas						
	0.043	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma						
	0.032	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Algibacter						
	0.031	Planctomycetes	Planctomycetacia	Pirellulales	Pirellulaceae	Blastopirellula						
	0.029	Epsilonbacteraeota	Campylobacteria	Campylobacterales	Sulfurovaceae	Sulfurovum						
	0.027	Proteobacteria	Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas						
	0.025	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Rubritaleaceae	Haloferula						
Mussel 5	0.158	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio	0.862	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma
	0.069	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma	0.045	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio
	0.066	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio						
	0.054	Proteobacteria	Gammaproteobacteria	Alteromonadales	Psychromonadaceae	Psychromonas						
	0.047	Proteobacteria	Gammaproteobacteria	Cellvibrionales	Halieaceae	Halioglobus						
	0.029	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Algibacter						
	0.028	Planctomycetes	Planctomycetacia	Pirellulales	Pirellulaceae	Blastopirellula						
	0.027	Proteobacteria	Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas						
	0.026	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Rubritaleaceae	Haloferula						

Table 1 (continued)

	Feces						Gut					
	Relative abundance	Phylum	Class	Order	Family	Genus	Relative abundance	Phylum	Class	Order	Family	Genus
Mussel 6	0.316	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma	0.673	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma
	0.155	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio	0.052	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	Streptomyces
	0.077	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio						
	0.059	Proteobacteria	Gammaproteobacteria	Alteromonadales	Psychromonadaceae	Psychromonas						
	0.031	Proteobacteria	Gammaproteobacteria	Cellvibrionales	Halieaceae	Halioglobus						
Mussel 7	0.185	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma	0.493	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma
	0.074	Proteobacteria	Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas	0.156	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Lutibacter
	0.073	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio	0.040	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	Candidatus_ Megaira
	0.060	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Polaribacter_4	0.039	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Winogradskyella
	0.040	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	0.036	Proteobacteria	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella
	0.034	Proteobacteria	Gammaproteobacteria	Alteromonadales	Psychromonadaceae	Psychromonas	0.036	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Donghicola
	0.028	Proteobacteria	Gammaproteobacteria	Cellvibrionales	Halieaceae	Halioglobus	0.031	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Spirochaeta_2
Mussel 8	0.256	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma	0.932	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma
	0.095	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio	0.050	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio
	0.071	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio						
	0.052	Proteobacteria	Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas						
	0.039	Proteobacteria	Gammaproteobacteria	Cellvibrionales	Halieaceae	Halioglobus						
	0.032	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Polaribacter_4						
Mussel 9	0.166	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma	0.938	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma
	0.104	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio						
	0.057	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio						
	0.038	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Algibacter						
	0.038	Proteobacteria	Gammaproteobacteria	Cellvibrionales	Halieaceae	Halioglobus						
	0.038	Proteobacteria	Gammaproteobacteria	Alteromonadales	Psychromonadaceae	Psychromonas						
	0.030	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Rubritaleaceae	Haloferula						
	0.027	Proteobacteria	Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas						
	0.027	Planctomycetes	Planctomycetacia	Pirellulales	Pirellulaceae	Blastopirellula						
Mussel 10	0.080	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio	0.882	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma
	0.077	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma	0.035	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Endozoicomonadaceae	Endozoicomonas
	0.055	Proteobacteria	Gammaproteobacteria	Alteromonadales	Psychromonadaceae	Psychromonas						
	0.052	Proteobacteria	Gammaproteobacteria	Cellvibrionales	Halieaceae	Halioglobus						
	0.048	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Algibacter						
	0.042	Planctomycetes	Planctomycetacia	Pirellulales	Pirellulaceae	Blastopirellula						
	0.036	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio						
	0.035	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Rubritaleaceae	Haloferula						
	0.031	Cyanobacteria	Oxyphotobacteria	Synechococcales	Cyanobiaceae	Synechococcus_ CC9902						

Table 1 (continued)

	Feces						Gut					
	Relative abundance	Phylum	Class	Order	Family	Genus	Relative abundance	Phylum	Class	Order	Family	Genus
Mussel 11	0.140	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma	0.576	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma
	0.097	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio	0.125	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Alteromonas
	0.056	Proteobacteria	Gammaproteobacteria	Cellvibrionales	Halieaceae	Halioglobus	0.047	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Donghicola
	0.042	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	0.032	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio
	0.039	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Rubritaleaceae	Haloferula	0.025	Proteobacteria	Gammaproteobacteria	Alteromonadales	Psychromonadaceae	Psychromonas
	0.030	Proteobacteria	Gammaproteobacteria	Alteromonadales	Psychromonadaceae	Psychromonas						
	0.030	Planctomycetes	Planctomycetacia	Pirellulales	Pirellulaceae	Blastopirellula						
	0.028	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	Polaribacter_4						
	0.026	Epsilonbacteraeota	Campylobacteria	Campylobacterales	Sulfurovaceae	Sulfurovum						

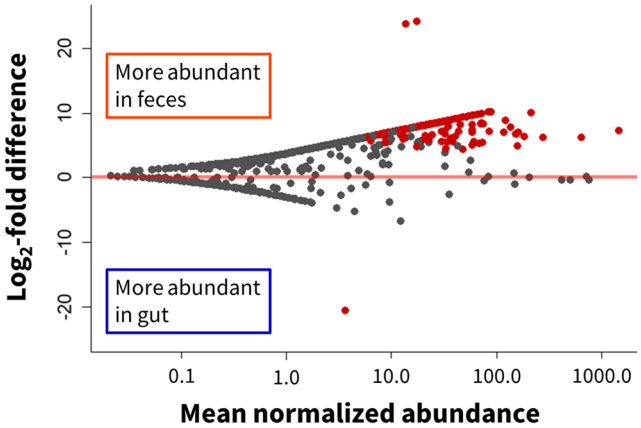

Fig. 7 Ratio intensity plot comparing mean normalized ASV abundance in fecal and gut microbial communities created from the plotMA command in DESeq2. Points on the plot represent all ASVs in the dataset. Points colored red are the 201 ASVs with abundances that were statistically different ($p_{adjusted} < 0.01$). Positive values on the *y*-axis indicate increased abundance in fecal communities relative to gut communities. Negative values indicate increased abundance in gut communities relative to fecal communities

likely reflects the common location of collection for all mussels in this study. Prior to collection, the mussels were presumably filtering and ingesting the same ambient seawater- and particle-associated microbiota. Similar findings were obtained by Ingala et al. [10] through a direct comparison of guano and intestinal microbiota in bats; fecal samples were more indicative of environmental factors such as host diet.

Analysis of taxonomic composition also supports the characterization of fecal communities of mussels as largely transient. Abundant classified genera in fecal communities included many that are known to be associated with temperate coastal seawater and sediments (Table 1), such as *Vibrio* [41], *Halioglobus* [42, 43], *Psychromonas* [44, 45], and *Haloferula* [46]. *Algibacter*, a genus known to be associated with macroalgae [47], the detritus of which is ingested by mussels [48], was also relatively abundant in feces. The genus *Aliivibrio*, which includes the well-known symbiont of the Hawaiian bobtail squid, *Euprymna scolopes*, was also a notable member of fecal communities [49, 50]. The identities of the differentially abundant ASVs in fecal communities also reinforce their inferred roles as transients (Table S1). The two most differentially abundant ASVs were of the Sva0081_sediment_group and *Ulvibacter*, known as sulfate-reducing sediment bacteria and associates with green macroalgal detritus, respectively [35, 51]. Additionally, significantly more abundant in fecal communities were some known and putative photosynthetic ASVs belonging to the Cyanobacteria and Chromatiaceae [52], which cannot be resident gut microbes considering the lack of light inside the mussel gut.

Harris [53] provided an effective conceptual framework for the differences between resident and transient gut microbiota

in aquatic invertebrates. Resident microbes are those that form permanent, relatively stable populations and may live attached to epithelia or in gut pouches or crevices. Transient microbes are those that become ingested and traverse the length of the gut; they may colonize gut contents or proliferate within the gut habitat. Importantly, members of both resident and transient communities are expected to be voided with feces. Although informative, the data synthesized by Harris [53] lacked any of the high-throughput sequence analysis available currently. Hammer et al. [54] provided a compelling argument that caterpillars do not retain, or minimally so, resident gut communities by demonstrating that caterpillar fecal microbiota were primarily leaf-associated taxa and host-specific gut taxa were largely absent. Caterpillars may represent an extreme case, and the data presented here do not suggest that resident microbiota are absent from the mussel gut.

Of the 201 ASVs with abundances that were statistically different, only one was more abundant in resident gut communities (Fig. 7). These data suggest that the resident gut community is mostly a subset of the fecal microbiome, which is not unexpected for a community that must undergo regular turnover and voidance in the feces. With that in mind, and considering the dominance of the *Mycoplasma* in gut communities, it is hypothesized that the relatively large abundance of *Mycoplasma* in the feces is a consequence of routine residential voidance. Although Rubiolo et al. [14] also observed increased relative abundance of the Tenericutes in mussel guts following a depuration period, other phyla (e.g., Cyanobacteria, Proteobacteria, and Planctomycetes) were still prominent, likely reflecting the non-sterile nature of the depuration period in their experiment. Members of the *Mycoplasma* are often dominantly abundant in 16S rRNA gene sequencing surveys of gut microbial communities of bivalves [17, 55–57], and have recently been proposed to be members of a "core" gut microbiome [58]. Although the functional role of *Mycoplasma* in the mussel gut is unconfirmed, metagenomic data from an analysis of oyster gut communities suggest that these bacteria might utilize chitin and arginine supplied by the host as carbon sources (Pimentel, personal communication). These results, coupled with the sheer numerical dominance of *Mycoplasma* in the resident community observed in the present study, hint at an important symbiosis between these bacteria and host mussels.

Although the use of fecal sampling as a substitute for direct sampling of the mussel gut is discouraged for studies concerned with the resident gut microbiota, fecal sampling may have other utility. For example, sampling the microbial communities of bivalve feces may lead to significant insight into the biogeochemical processes associated with biodeposits [59], which have profound ecological implications (e.g., carbon/nitrogen cycling, benthic-pelagic coupling) [3, 60]. Additionally, results of this study have implications for the interpretation of results of numerous gut microbiome studies that sampled bivalves directly from the natural environment or laboratory tanks, without allowing for a period of fecal egestion (see Pierce and Ward [58] and references therein). When no egestion period is allowed for the host to void the gut, subsequent results mainly identify robust transient microbial communities. These studies still have merit, especially considering that transient microbes may be metabolically active and important for host physiology. Results of the current study, however, highlight the importance of tailoring experimental and sampling design schemes to match the experimental questions being addressed. Any work that aims to study resident microbes of the bivalve gut should employ sterile microcosms and a fecal egestion period, similar to the design described here, prior to lethal gut sampling to allow time for transient microbes to be voided.

Conclusion

Fecal and gut microbiota of blue mussels were compared. The microbial communities sampled from feces voided within 3 h and gut tissue after 6 h of egestion were markedly different. Fecal communities of different mussels resembled each other but did not resemble gut communities. Furthermore, fecal communities were significantly more diverse, in terms of ASV richness and evenness, than their gut community counterparts. Thus, the use of fecal sampling as a noninvasive and nondestructive substitute for direct sampling of the gut is strongly discouraged for studies concerned with the resident microbiota of the mussel gut. The results suggested a mostly transient nature for the majority of fecal microbiota. Mussels retained a distinct resident microbial community in their gut after fecal egestion that was dominated by ASVs belonging to the *Mycoplasma*, which suggests an important symbiosis between these bacteria and host mussels. Prior studies of the gut microbiome of bivalves that lack an egestion period for the voidance of transient microbes likely include results biased toward transient communities. Any work that aims to study the resident microbes of the bivalve gut should employ sterile microcosms and a fecal egestion period, similar to the design described here, prior to lethal gut sampling to allow time for transient microbes to be voided.

Author's Contributions All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Julia Baer and Tyler W. Griffin. The first draft of the manuscript was written by Tyler W. Griffin and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Availability of Data and Material The data from this work are available upon email request from Tyler W. Griffin. Sequence data were uploaded to the NCBI Short Read Archive (SRA) under submission ID SUB7223904 (BioProject ID: PRJNA622268).

Compliance with Ethical Standards

Competing Interests The authors declare that they have no conflict of interest.

Ethics Approval NA

Consent to Participate NA

Consent for Publication NA

References

1. Dame R (1993) Bivalve filter feeders and estuarine and coastal ecosystem processes: conclusions. Bivalve Filter Feeders. Springer, pp 565–569
2. Prins TC, Smaal AC, Dame RF (1998) A review of the feedbacks between bivalve grazing and ecosystem processes. Aquat Ecol 31: 349–359. https://doi.org/10.1023/A:1009924624259
3. Newell RIE (2004) Ecosystem influences of natural and cultivated populations of suspension-feeding bivalve molluscs: a review. J Shellfish Res 23:51–61
4. Cranford PJ, Ward JE, Shumway SE (2011) Bivalve filter feeding: variability and limits of the aquaculture biofilter. In: Shumway SE (ed) Shellfish aquaculture and the environment 1st edn. Wiley, pp 81–124
5. Hammer TJ, Sanders JG, Fierer N (2019) Not all animals need a microbiome. FEMS Microbiol Lett 366:366. https://doi.org/10.1093/femsle/fnz117
6. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, Magris M, Hidalgo G, Baldassano RN, Anokhin AP, Heath AC, Warner B, Reeder J, Kuczynski J, Caporaso JG, Lozupone CA, Lauber C, Clemente JC, Knights D, Knight R, Gordon JI (2012) Human gut microbiome viewed across age and geography. Nature 486:222–227. https://doi.org/10.1038/nature11053
7. Aagaard K, Petrosino J, Keitel W, Watson M, Katancik J, Garcia N, Patel S, Cutting M, Madden T, Hamilton H, Harris E, Gevers D, Simone G, McInnes P, Versalovic J (2013) The human microbiome project strategy for comprehensive sampling of the human microbiome and why it matters. FASEB J 27:1012–1022. https://doi.org/10.1096/fj.12-220806
8. Smits LP, Bouter KEC, de Vos WM, Borody TJ, Nieuwdorp M (2013) Therapeutic potential of fecal microbiota transplantation. Gastroenterology 145:946–953. https://doi.org/10.1053/j.gastro.2013.08.058
9. Durbán A, Abellán JJ, Jiménez-Hernández N, Ponce M, Ponce J, Sala T, D'Auria G, Latorre A, Moya A (2011) Assessing gut microbial diversity from feces and rectal mucosa. Microb Ecol 61: 123–133. https://doi.org/10.1007/s00248-010-9738-y
10. Ingala MR, Simmons NB, Wultsch C, Krampis K, Speer KA, Perkins SL (2018) Comparing microbiome sampling methods in a wild mammal: fecal and intestinal samples record different signals of host ecology, Evolution. Front Microbiol 9. https://doi.org/10.3389/fmicb.2018.00803
11. Zhou J, Nelson TM, Rodriguez Lopez C, Sarma RR, Zhou SJ, Rollins LA (2020) A comparison of non-lethal sampling methods for amphibian gut microbiome analyses. Mol Ecol Resour n/a. https://doi.org/10.1111/1755-0998.13139
12. Owen G (1966) Digestion. In: Wilbur KM, Yonge CM (eds) Physiology of mollusca. Academic Press, New York, pp 53–88
13. Simons AL, Churches N, Nuzhdin S (2018) High turnover of faecal microbiome from algal feedstock experimental manipulations in the Pacific oyster (*Crassostrea gigas*). Microb Biotechnol 11:848–858. https://doi.org/10.1111/1751-7915.13277
14. Rubiolo JA, Lozano-Leon A, Rodriguez-Souto R, Fol Rodriguez N, Vieytes MR, Botana LM (2018) The impact of depuration on mussel hepatopancreas bacteriome composition and predicted metagenome. Antonie Van Leeuwenhoek 111:1117–1129. https://doi.org/10.1007/s10482-018-1015-y
15. Li Y-F, Yang N, Liang X, Yoshida A, Osatomi K, Power D, Batista FM, Yang J-L (2018) Elevated seawater temperatures decrease microbial diversity in the gut of *Mytilus coruscus*. Front Physiol 9:839. https://doi.org/10.3389/fphys.2018.00839
16. Vezzulli L, Stagnaro L, Grande C, Tassistro G, Canesi L, Pruzzo C (2018) Comparative 16SrDNA gene-based microbiota profiles of the Pacific oyster (*Crassostrea gigas*) and the Mediterranean mussel (*Mytilus galloprovincialis*) from a shellfish farm (Ligurian Sea, Italy). Microb Ecol 75:495–504. https://doi.org/10.1007/s00248-017-1051-6
17. Pierce ML, Ward JE (2019) Gut microbiomes of the eastern oyster (*Crassostrea virginica*) and the blue mussel (*Mytilus edulis*): temporal variation and the influence of marine aggregate-associated microbial communities. mSphere 4:e00730–e00719. https://doi.org/10.1128/mSphere.00730-19
18. Greenberg AE, Hunt DA (1985) Laboratory procedures for the examination of seawater and shellfish. American Public Health Association, Washington DC
19. Apprill A, McNally S, Parsons R, Weber L (2015) Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. Aquat Microb Ecol 75:129–137
20. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP (2016) DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods 13:581–583. https://doi.org/10.1038/nmeth.3869 https://www.nature.com/articles/nmeth.3869#supplementary-information
21. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2012) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41:D590–D596. https://doi.org/10.1093/nar/gks1219
22. Callahan B (2018) Silva taxonomic training data formatted for DADA2 (Silva version 132). https://doi.org/10.5281/zenodo.1172783
23. Schliep KP (2010) Phangorn: phylogenetic analysis in R. Bioinformatics 27:592–593. https://doi.org/10.1093/bioinformatics/btq706
24. Wright ES (2015) DECIPHER: harnessing local sequence context to improve protein multiple sequence alignment. BMC Bioinformatics 16:322. https://doi.org/10.1186/s12859-015-0749-z
25. McMurdie PJ, Holmes S (2013) Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8:e61217. https://doi.org/10.1371/journal.pone.0061217
26. Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35:1547–1549. https://doi.org/10.1093/molbev/msy096
27. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454
28. Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc

Natl Acad Sci U S A 101:11030–11035. https://doi.org/10.1073/pnas.0404206101
29. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x
30. Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15. https://doi.org/10.1186/s13059-014-0550-8
31. McMurdie PJ, Holmes S (2014) Waste not, want not: why rarefying microbiome data is inadmissible. PLoS Comput Biol 10:e1003531. https://doi.org/10.1371/journal.pcbi.1003531
32. Lozupone C, Lladser ME, Knights D, Stombaugh J, Knight R (2011) UniFrac: an effective distance metric for microbial community comparison. ISME J 5:169–172. https://doi.org/10.1038/ismej.2010.133
33. Anderson MJ (2014) Permutational multivariate analysis of variance (PERMANOVA)Wiley StatsRef: statistics reference online. Wiley
34. Anderson MJ, Walsh DCI (2013) PERMANOVA, ANOSIM, and the mantel test in the face of heterogeneous dispersions: what null hypothesis are you testing? Ecol Monogr 83:557–574. https://doi.org/10.1890/12-2010.1
35. Dyksma S, Pjevac P, Ovanesov K, Mussmann M (2018) Evidence for H2 consumption by uncultured Desulfobacterales in coastal sediments. Environ Microbiol 20:450–461. https://doi.org/10.1111/1462-2920.13880
36. Weel PBV (1961) The comparative physiology of digestion in Molluscs. Am Zool 1:245–252
37. Menzel RW (1955) Some phases of the biology of *Ostrea equestris* (Say) and comparison with *Crassostrea virginica* (Gmelin). Inst Mar Sci 4:69–153
38. Owen G (1974) Feeding and digestion in the Bivalvia. In: Lowenstein O (ed) Advances in Comparative Physiology and Biochemistry. Elsevier, pp 1–35
39. Foster-Smith RL (1975) The effect of concentration of suspension and inert material on the assimilation of algae by three bivalves. J Mar Biol Assoc U K 55:411–418. https://doi.org/10.1017/S0025315400016027
40. Gagnon C, Fisher NS (1997) The bioavailability of sediment-bound Cd, Co, and Ag to the mussel *Mytilus edulis*. Can J Fish Aquat Sci 54:147–156. https://doi.org/10.1139/f96-256
41. Oliver JD, Pruzzo C, Vezzulli L, Kaper JB (2013) *Vibrio* species. In: Doyle M, Buchanan R (eds) Food microbiology. ASM Press, Washington, DC, pp 401–439
42. Park S, Yoshizawa S, Inomata K, Kogure K, Yokota A (2012) *Halioglobus japonicus* gen. Nov., sp. nov. and *Halioglobus pacificus* sp. nov., members of the class Gammaproteobacteria isolated from seawater. Int J Syst Evol Microbiol 62:1784–1789. https://doi.org/10.1099/ijs.0.031443-0
43. Li S-H, Song J, Lim Y, Joung Y, Kang I, Cho J-C (2020) *Halioglobus maricola* sp. nov., isolated from coastal seawater. Int J Syst Evol Microbiol. https://doi.org/10.1099/ijsem.0.003985
44. Kawasaki K, Nogi Y, Hishinuma M, Nodasaka Y, Matsuyama H, Yumoto I (2002) *Psychromonas marina* sp. nov., a novel halophilic, facultatively psychrophilic bacterium isolated from the coast of the Okhotsk Sea. Int J Syst Evol Microbiol 52:1455–1459. https://doi.org/10.1099/00207713-52-5-1455
45. Hosoya S, Jang J-H, Yasumoto-Hirose M, Matsuda S, Kasai H (2009) *Psychromonas agarivorans* sp. nov., a novel agarolytic bacterium. Int J Syst Evol Microbiol 59:1262–1266. https://doi.org/10.1099/ijs.0.003244-0
46. Yoon J, Matsuo Y, Katsuta A, Jang J-H, Matsuda S, Adachi K, Kasai H, Yokota A (2008) *Haloferula rosea* gen. nov., sp. nov., *Haloferula harenae* sp. nov., *Haloferula phyci* sp. nov., *Haloferula helveola* sp. nov. and *Haloferula sargassicola* sp. nov., five marine representatives of the family Verrucomicrobiaceae within the phylum 'Verrucomicrobia'. Int J Syst Evol Microbiol 58:2491–2500. https://doi.org/10.1099/ijs.0.2008/000711-0
47. Nedashkovskaya OI, Kim SB, Han SK, Rhee M-S, Lysenko AM, Rohde M, Zhukova NV, Frolova GM, Mikhailov VV, Bae KS (2004) *Algibacter lectus* gen. nov., sp. nov., a novel member of the family Flavobacteriaceae isolated from green algae. Int J Syst Evol Microbiol 54:1257–1261. https://doi.org/10.1099/ijs.0.02949-0
48. Duggins DO, Eckman JE (1997) Is kelp detritus a good food for suspension feeders? Effects of kelp species, age and secondary metabolites. Mar Biol 128:489–495. https://doi.org/10.1007/s002270050115
49. Urbanczyk H, Ast JC, Higgins MJ, Carson J, Dunlap PV (2007) Reclassification of *Vibrio fischeri*, *Vibrio logei*, *Vibrio salmonicida* and *Vibrio wodanis* as *Aliivibrio fischeri* gen. nov., comb. nov., *Aliivibrio logei* comb. nov., *Aliivibrio salmonicida* comb. nov. and *Aliivibrio wodanis* comb. nov. Int J Syst Evol Microbiol 57:2823–2829. https://doi.org/10.1099/ijs.0.65081-0
50. McFall-Ngai M (2014) Divining the essence of symbiosis: insights from the squid-vibrio model. PLoS Biol 12:e1001783. https://doi.org/10.1371/journal.pbio.1001783
51. Nedashkovskaya OI, Kim SB, Han SK, Rhee MS, Lysenko AM, Falsen E, Frolova GM, Mikhailov VV, Bae KS (2004) *Ulvibacter litoralis* gen. nov., sp. nov., a novel member of the family Flavobacteriaceae isolated from the green alga Ulva fenestrata. Int J Syst Evol Microbiol 54:119–123. https://doi.org/10.1099/ijs.0.02757-0
52. Imhoff JF (2014) The family Chromatiaceae. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (eds) The prokaryotes: Gammaproteobacteria. Springer, Berlin Heidelberg, pp 151–178
53. Harris JM (1993) The presence, nature, and role of gut microflora in aquatic invertebrates: a synthesis. Microb Ecol 25:195–231
54. Hammer TJ, Janzen DH, Hallwachs W, Jaffe SP, Fierer N (2017) Caterpillars lack a resident gut microbiome. Proc Natl Acad Sci 114:9641–9646. https://doi.org/10.1073/pnas.1707186114
55. King GM, Judd C, Kuske CR, Smith C (2012) Analysis of stomach and gut microbiomes of the eastern oyster (*Crassostrea virginica*) from coastal Louisiana, USA. PLoS One 7:e51475. https://doi.org/10.1371/journal.pone.0051475
56. Milan M, Carraro L, Fariselli P, Martino ME, Cavalieri D, Vitali F, Boffo L, Patarnello T, Bargelloni L, Cardazzo B (2018) Microbiota and environmental stress: how pollution affects microbial communities in Manila clams. Aquat Toxicol 194:195–207. https://doi.org/10.1016/j.aquatox.2017.11.019
57. King WL, Siboni N, Williams NLR, Kahlke T, Nguyen KV, Jenkins C, Dove M, O'Connor W, Seymour JR, Labbate M (2019) Variability in the composition of Pacific oyster microbiomes across oyster families exhibiting different levels of susceptibility to OsHV-1 μvar disease. Front Microbiol 10. https://doi.org/10.3389/fmicb.2019.00473
58. Pierce ML, Ward JE (2018) Microbial ecology of the Bivalvia, with an emphasis on the family Ostreidae. J Shellfish Res 37:1–14. https://doi.org/10.2983/035.037.0300
59. Murphy AE, Kolkmeyer R, Song B, Anderson IC, Bowen J (2019) Bioreactivity and microbiome of biodeposits from filter-feeding bivalves. Microb Ecol 77:343–357. https://doi.org/10.1007/s00248-018-01312-4
60. Kautsky N, Evans S (1987) Role of biodeposition by *Mytilus edulis* in the circulation of matter and nutrients in a Baltic coastal ecosystem. Mar Ecol Prog Ser 38:201–212