A simple microbiome in esophagus and gills of the European common cuttlefish, *Sepia officinalis*

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ABSTRACT

The European common cuttlefish, Sepia officinalis, is used extensively in biological and biomedical research yet its microbiome remains poorly characterized. We analyzed the microbiota of the digestive tract, gills, and skin in mariculture-raised S. officinalis using a combination of 16s rRNA amplicon sequencing and fluorescence spectral imaging. Sequencing revealed a highly simplified microbiota consisting largely of two single bacterial amplicon sequence variants (ASVs) of Vibrionaceae and Piscirickettsiaceae. The esophagus was dominated by a single ASV of the genus Vibrio. Imaging revealed a striking organization of bacteria distributed in a discrete layer that lines the esophagus. Imaging with specific probes confirmed the identity of these bacteria as Vibrionaceae. This Vibrio was also abundant in the microbiota of the stomach, cecum, and intestine, but occurred at lower density and in the lumen rather than in a discrete layer; it was present in only trace proportions in tank water and in the microbiome of shrimp that were used as feed for the cuttlefish. These Vibrio were resilient to treatment of animals with the commonly-used antibiotic, enrofloxacin. The gills were colonized by a single ASV in the family Piscirickettsiaceae, which imaging visualized as small clusters of cells. We conclude that bacteria belonging to the Gammaproteobacteria, especially Vibrionaceae, are the major symbionts of the cuttlefish Sepia officinalis cultured from eggs in captivity, and that the esophagus and gills are major colonization sites.
IMPORTANCE

Microbes can play critical roles in the physiology of their animal hosts, as evidenced in cephalopods by the role of *Vibrio (Aliivibrio) fischeri* in the light organ of the bobtail squid and the role of Alpha- and Gammaproteobacteria in the reproductive system and egg defense in a variety of cephalopods. We sampled the cuttlefish microbiome throughout the digestive tract, gills, and skin and found dense colonization of an unexpected site, the esophagus, by a microbe of the genus *Vibrio*, as well as colonization of gills by Piscirickettsiaceae. We found these associations to be resilient to the treatment of animals with a common antibiotic, enrofloxacin. This finding expands the range of organisms and body sites known to be associated with *Vibrio* and is of potential significance for understanding host-symbiont associations as well as for understanding and maintaining the health of cephalopods in mariculture.

KEYWORDS:

microbiome, fluorescence *in situ* hybridization, *Photobacterium*, Vibrionaceae, Piscirickettsiaceae

1. INTRODUCTION

Symbiotic associations between invertebrates and bacteria are common. Among cephalopods, the most intensely studied association is the colonization of the light organ of the bobtail squid *Euprymna scolopes* by the bioluminescent bacterium *Vibrio*
(*Aliivibrio*) *fischeri* in a highly specific symbiosis (1). A more diverse but still characteristic set of bacteria colonize the accessory nidamental gland from which they are secreted into the egg jelly coat and likely protect the eggs from fungal and bacterial attack (2). The accessory nidamental gland and egg cases of the squid *Doryteuthis (Loligo) pealeii* and the Chilean octopus (*Octopus mimus*) have also been reported to contain Alphaproteobacteria and Gammaproteobacteria (3, 4). These associations indicate that bacteria can play a critical role in the physiology of cephalopods.

*Sepia officinalis*, the European common cuttlefish (hereafter cuttlefish), is used extensively in biological and biomedical research (5-7) and is a model organism for the study of rapid adaptive camouflage (8-11). Cuttlefish are also widely represented among zoos and aquaria, and play an important role in educating the public about cephalopod biology and life history (12). Little is known about the association of bacterial symbionts with cuttlefish, and whether such associations may play a role in the health or behavior of these animals. Because cuttlefish behavior is well-studied and there exist standardized methods for documenting multiple behaviors (8), we hypothesized that these animals may provide a unique opportunity to study microbes and the gut-brain axis – the effect of gut microbiota on behavior (13) – in an invertebrate system. Understanding the importance, or lack thereof, of the cuttlefish microbiome will not only shed light on the basic biology of this model organism, but will also have practical implications for future husbandry practices and research design.

Using a combination of 16s rRNA amplicon sequencing and fluorescence *in situ* hybridization (FISH), we characterized the gastrointestinal, gill, and skin microbiota of the common cuttlefish in wild-bred, captive-raised animals (5) housed at the Marine...
Biological Laboratory (Woods Hole, MA). In addition to baseline microbiome characterization, we examined the extent to which cuttlefish microbiota were responsive to antibiotic treatment, and conducted behavioral experiments to assess possible effects of the microbiota on two well-studied behaviors: camouflage and feeding.

2. RESULTS

2.1 Two taxa dominate the *S. officinalis* microbiome.

This study comprised two time points – the first (20-21 June 2017) in which three healthy adult *S. officinalis* from the mariculture laboratory at Marine Biological Laboratory (Woods Hole, MA) were sampled, and the second (25 September -10 October 2017) in which antibiotic trials were conducted on 24 healthy adult *S. officinalis* (16 treatment individuals, 8 control individuals). 16s rRNA amplicon sequencing of the gastrointestinal tract, gills, and skin of *S. officinalis* revealed a highly simplified microbiota dominated by bacterial amplicon sequence variants (ASVs) in the *Vibrionaceae* and *Piscirickettsiaceae*. These results were consistent across both the pilot study and in the experimental study.

In particular, results showed a consistent and highly simplified microbiota in the esophagus (Fig. 1; Table 1). A single ASV in the genus *Vibrio* made up the majority of the sequence data from the esophagus of the three pilot investigation animals (mean 92.4% ± 9.8). This ASV was also detected in high abundance among experimental animals four months later, in control animals (mean 99.5% ± 1.2) and treatment animals.
(mean 94.4% ± 10.8). Thus, this ASV represents a dominant constituent of the esophagus microbiota stably over the two time points of this study. We note that another ASV of the related genus *Photobacterium* (Vibrionaceae) was present in the esophagus community in the pilot investigation animals (mean 6.8% ± 10). Combined, the two Vibrionaceae ASVs in these pilot animals constituted 99.2% of the esophagus community.

The major *Vibrio* ASV was also a major constituent of downstream sites in the digestive tract, and differed significantly between control and treatment groups for the most distal digestive organs (cecum and intestine) (Fig. 1; Table 1). The remainder of the sequence data from the digestive tract consisted of an assortment of taxa that varied between individuals or between the two time points of the study and thus suggest transient rather than stable microbial colonization. We thus consider the *Vibrio* ASV to be the dominant microbe of the lower digestive tract in these cuttlefish, although not in the same density and discrete layering as the esophagus (see 2.3).

Samples from gills were dominated by a single highly abundant ASV, classified as family Piscirickettsiaceae and making up an average 96.9% ± 2.5 in the gills of control animals, and 82.4% ± 1.9 in treated animals. In samples from other body sites this ASV was detected only sporadically and at low abundance (mean 0.2%, range 0 to 5.8%) (Fig. 1).

### 2.2 Effect of antibiotic treatment on *S. officinalis* microbiome composition and behavior

Analysis of behavioral data found no differences in the expression of disruptive camouflage behavior between cuttlefish before and after antibiotic treatment (p = 0.7,
paired t-test), or between treatment and control groups after antibiotic treatment ($p = 0.62$, t-test). Similarly, no differences in predation behavior (i.e. hunting live shrimp) were observed between cuttlefish before and after antibiotic treatment ($p = 0.65$, paired t-test), or between treatment and control groups after antibiotic treatment ($p = 0.58$, t-test).

Treatment of *S. officinalis* with the antibiotic enrofloxacin therefore had no discernable effects on camouflage or feeding behaviors, and resulted in only modest changes to the microbiome. Between organs (gills, esophagus, stomach, cecum, and intestine) of treatment and control groups, we observed a small but significant difference in Shannon diversity ($p = 1.55 \text{e}-7$, t-test) but saw no difference in observed ASV richness ($p = 0.46$, t-test). We also found experimental group (treatment vs control) to be a significant predictor of weighted UniFrac dissimilarity ($p < 0.01$, $R^2 = 0.042$, ADONIS), unweighted UniFrac dissimilarity ($p < 0.01$, $R^2 = 0.021$, ADONIS), and Bray-Curtis dissimilarity ($p < 0.01$, $R^2 = 0.040$, ADONIS).

The single *Vibrio* ASV maintained dominance in the digestive tract and did not differ significantly in relative abundance within the esophagus or stomach of treatment and control groups ($p > 0.09$, t-test). In the more distal organs of the digestive tract, however, the relative abundance of the major *Vibrio* ASV showed a dramatic and significant drop in mean relative abundance. In the cecum, this sequence made up an average $43.7\% \pm 23.4$ of the community in control individuals and only $6.4\% \pm 8.7$ in antibiotic-treated individuals ($p = 0.002$, t-test); in intestine the average from control animals was $56.8\% \pm 15.8$, and only $5.5\% \pm 5.9$ in antibiotic-treated animals ($p = 1.99 \text{e}-05$, t-test) (Table 1). A single Piscirickettsiaceae ASV maintained dominance in the gills.
of treated cuttlefish (Fig. 1; Table 1), although its relative abundance did differ
significantly between treatment and control groups ($p = 0.01$, t-test) (Table 1).

2.3 Imaging shows high microbial abundance and spatial structure in cuttlefish esophagus
and lower abundance and scattered distribution elsewhere in the digestive tract.

Fluorescence *in situ* hybridization (FISH) revealed a striking organization of
bacteria distributed in a layer lining the interior of the convoluted esophagus of cuttlefish
(Fig. 3). Hybridization with the near-universal Eub338 probe showed bacteria in high
density in a layer ~20-40 μm thick at the border between host tissue and lumen. Staining
with fluorophore-conjugated wheat germ agglutinin revealed a mucus layer that covered
the epithelium and generally enclosed the bacteria. To verify the identity of these bacteria
we employed a nested probe set including Eub338 as well as probes for
Alphaproteobacteria, Gammaproteobacteria, and one of two probes we designed
specifically for Vibrionaceae (Vib1749 and Vib2300, Table 2). Bacterial cells imaged in
the esophagus showed signal from all probes expected to hybridize with Vibrionaceae,
suggesting that the bacteria observed in this organ are a near-monoculture of this taxon
(Fig. 4B-D). The probe targeted to Alphaproteobacteria was included in the FISH as a
negative control and, as expected, did not hybridize with the cells (Fig. 4E). As an
additional control to detect non-specific binding of probes, we performed an independent
FISH with a set of probes labeled with the same fluorophores as the experimental probe
set, but conjugated to oligonucleotides not expected to hybridize with the cuttlefish
microbiota (Table 2). No signal from this non-target probe set was detected (Fig. 4F,G)
supporting the interpretation that the signal observed in the esophagus results from a specific interaction of the Vibrionaceae-targeted oligonucleotides with the visualized bacteria. In other parts of the digestive tract we observed a sparser distribution of bacteria without obvious spatial organization. Bacteria in the intestine were present not in a layer but scattered throughout the lumen and mixed with the luminal contents (Fig. 5). Similarly, in cecum bacteria were observed in low abundance in the lumen (Fig. 6). FISH was also applied to the stomach, posterior salivary gland (poison gland) and duct of the salivary gland, but no bacteria were detected (not shown). Fluorescence \textit{in situ} hybridization to cross-sections of the gills revealed clusters of bacteria (Figure 7). Gill samples were embedded in methacrylate resin and sectioned to 5 μm thickness, and FISH applied to the sections with universal probes (Fig. 7) and probes for Alpha- and Gammaproteobacteria (Fig. 7). Results showed hybridization of probes for gamma but not alpha proteobacteria, consistent with the identification of the clusters of gill bacteria as members of the gamma proteobacteria family Piscirickettsiaceae.

3. DISCUSSION

We sampled the cuttlefish microbiome of the digestive tract, gills, and skin and found dense colonization of an unexpected site, the esophagus, by a microbe of the genus \textit{Vibrio}. Both imaging and ribosomal RNA gene sequencing showed a near-monoculture of \textit{Vibrionaceae} in the esophagus, with imaging showing dense colonization of the
interior lining of the esophagus with a single morphotype that hybridized to probes targeting *Vibrionaceae*. In the remainder of the digestive tract, both imaging and sequencing indicated a less consistent microbiota. Sequencing showed lower relative abundance of the dominant *Vibrio* ASV. Imaging showed sparse and sporadic colonization in the stomach, intestine, and cecum, with scattered cells in the lumen and no clear colonization of the epithelium. In light of the imaging results, we interpret the sequencing results as consistent with a higher colonization density in the esophagus and a lower density in the distal gut. In the esophagus, the dominant *Vibrio* ASV appears to be resilient to antibiotic treatment, with no significant difference in relative abundance observed between groups. Moving along the digestive tract towards the distal end, however, we observed striking and significant differences in the *Vibrio* relative abundance between control and treatment groups. This may be due, in part, to the fact that antibiotics were administered via injection into the cuttlefish food source, grass shrimp, which would contain the antibiotic until broken down in the cecum and intestine of the cuttlefish, thereby shielding the esophagus and stomach from coming into contact with the full antibiotic dose. Alternatively, the esophagus microbiota may have formed a biofilm in which the microbes were shielded from the effects of the antibiotic.

Diverse associations with *Vibrio* and the *Vibrionaceae* are known from cephalopods. Among the most extensively investigated is the mutualistic association of the bioluminescent *Vibrio (Aliivibrio) fischeri* with the light organ of the bobtail squid *Eupryma scolopes* (1, 14). Other well-known symbioses include the colonization of the cephalopod accessory nidamental gland with Alpha- and Gammaproteobacteria, which enables the host to secrete a layer of bacteria into the protective coating of the egg.
capsule (3, 15-19). Thus, colonization by Gammaproteobacteria and specifically by
Vibrionaceae is common in cephalopods, yet dense colonization of the digestive tract,
and particularly the esophagus, was unexpected.

Bacteria from genus *Vibrio* and the related Vibrionaceae genus *Photobacterium* are frequent colonizers of the digestive tracts of marine fishes (20, 21) and are prominent in the microbiota of *Octopus vulgaris* paralarvae (22). Vibrionaceae have been reported to produce chitinases, proteases, amylase, and lipase (21), suggesting the possibility that colonization of the digestive tract by the Vibrionaceae serves to aid in host digestion (21). If the *Vibrio* and *Photobacterium* ASVs serve this function, their localization in high density in the esophagus, near the beginning of the digestive tract, may serve to seed the distal gut; colonization of the lining of the esophagus may provide a reservoir that permits the microbes to avoid washout from the gut by continually re-populating the lumen of downstream gut chambers.

An alternative hypothesis is that the colonization of the esophagus, and the rest of the gut, is pathogenic or opportunistic. Various *Vibrio* species are known pathogens of cephalopods, causing skin lesions and sometimes mortality in squids and octopuses (23-25). The genus *Vibrio* includes representatives that are pathogenic to corals (*V. coralliilyticus*), fish (*V. salmonicida*), diverse marine organisms (*V. harveyi*) and humans (*V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*) (26, 27). Likewise, the genus *Photobacterium* contains pathogenic as well as commensal representatives (28). A previous study of the microbiota of *Octopus vulgaris* paralarvae found that recently hatched paralarvae had a high-diversity microbiome that changed, in captivity, to a lower-diversity microbiome with abundant Vibrionaceae (22). Whether the
Vibrionaceae are an integral part of cuttlefish physiology or whether they represent opportunistic colonists of these laboratory-reared organisms is an interesting question for future research.

Our sequence data from gills was dominated by a single ASV classified as Piscirickettsiaceae that was in low abundance at other body sites. The Piscirickettsiaceae are a family within the Gammaproteobacteria (29) that includes the salmon pathogen *P. salmonis*. Rickettsia-like organisms have been described from the gills of clams and oysters (30, 31) as well as associated with the copepod *Calanus finmarchicus* (32). In recent years Piscirickettsiaceae have been identified in high-throughput sequencing datasets from seawater and sediment as a taxon that may be involved in biodegradation of oil and other compounds (33-39). Whether taxa in this family colonize the gills of cuttlefish and other organisms as symbionts or as opportunistic pathogens is, again, a subject for future investigation. Finally, studies of wild *S. officinalis* microbiota will be informative for understanding natural host-symbiont associations under natural conditions, as compared to the mariculture-reared animals in the present study. *S. officinalis* in the eastern Atlantic and Mediterranean are known to prey on small marine fishes and crabs, as compared to the grass shrimp (*Palaemonetes*) prey used in our study. It remains to be seen whether such differences in diet and natural variation in environmental conditions influence the association of microbial symbionts with *S. officinalis* in the wild.

4. MATERIALS AND METHODS
4.1 Behavioral experiment

We included 24 cuttlefish (16 test, 8 control) in an experiment designed to test the effect of the antibiotic enrofloxacin on cuttlefish behavior and the composition of the cuttlefish microbiome. Experimental animals were held in three separate water tables, all connected to the same open-filtration system fed by filtered seawater. Within each water table, animals were isolated into individual holding pens via plastic panels. Control and test animals were kept in separate water tables. The experimental period lasted for 21 days (25 Sep – 15 Oct 2017). The treatment consisted of administering antibiotic to treatment animals via injection into the food source (grass shrimp, *Palaemonetes* spp.), which were then fed to the animals. The shrimp were injected with enrofloxacin (Baytril®; 22.7 mg/mL, Bayer HealthCare LLC, Shawnee Mission, KS, USA) using a 0.5 cc, U-100 insulin syringe with an attached 28 g x 1/2” needle (Covidien LLC, Mansfield, MA, USA). The antibiotic dosage was 10 mg/kg rounded up to the nearest hundredth mL. The antibiotic was injected into the coelomic cavity of the shrimp which were then immediately fed to the cuttlefish once daily for 14 days. Behavioral assays were run for 7 days prior to antibiotic treatment, followed by a 7-day antibiotic treatment period, after which behavioral assays were repeated as antibiotics continued to be administered for 7 additional days.

We conducted two behavioral assays. The first assay was designed to elicit high contrast disruptive camouflage patterns in the cuttlefish, as described in Hanlon 1988 (8, 9, 11). Animals were placed in a holding pen (connected to the same filtered seawater system as the animals’ home water tables) comprising a base and a circular wall.
exhibiting high contrast black and white checkered squares (8, 9, 11, 40). The holding pen was covered in black fabric and contained a camera stationed directly above the animals, so that behavior could be documented without disturbing the animal. Each trial began once the animal had ceased swimming and had settled in a stationary position. Each animal was subjected to two trials per day for four days, for a total of 6 trials per animal, with at least 4 hours in between any individual’s trial period. This protocol was followed before antibiotic treatment (25-28 September 2017) and ten days later following antibiotic treatment (9-12 October). For disruptive camouflage experiments, animals were assigned discrete scores based on 11 chromatic elements (8, 41). Scores for individual elements were combined for one cumulative score per trial. These scores were then averaged for each individual across trials conducted before and after antibiotic treatment. Scores were compared between treatment and control groups after antibiotic administration, and within treatment and control groups before and after antibiotic administration (e.g. treatment before vs. treatment after antibiotic administration) using the Student’s t-test.

In the second experiment, referred to as the feeding experiment, the same holding pen and recording setup were used, but with the high-contrast background exchanged for a neutral background (as shown in Fig. 1). The trial began once the animal settled (stopped swimming), at which point a clear glass jar containing 5 healthy and active grass shrimp was placed directly across from the cuttlefish within the holding pen. Animals were scored based on three metrics: 1) time to attack, 2) number of attacks, and 3) duration of attack. These three metrics were combined into a single score. As with the camouflage experiment, scores were compared between treatment and control groups.
after antibiotic administration, and within treatment and control groups before and after antibiotic administration (e.g. treatment before vs. treatment after antibiotic administration) using the Student’s t-test.

4.2 Sampling

After the completion of all behavioral assays (pre- and post-antibiotic treatment), each animal was euthanized via immersion into a 10% dilution of ethanol in saltwater. The animals were then dissected under sterile conditions to obtain samples for microbial analyses. The gastrointestinal tract was dissected into four components: esophagus, stomach, cecum, and intestine. A portion of gill tissue was sampled as well. All tissues were stored in separate tubes and flash-frozen in liquid nitrogen. At the end of the experimental period, a 1L water sample was taken from each water table and filtered using a 0.22 micron Sterivex filter for DNA extraction. Lastly, grass shrimp used as the food source throughout the duration of the experiment were collected into 1.8ml sterile cryotubes and frozen for DNA extraction.

4.3 DNA extraction, sequencing, and 16S rRNA gene statistical analyses

DNA extractions were performed on gut, tongue, and skin samples using the MoBio PowerSoil 96 Well Soil DNA Isolation Kit (Catalog No. 12955-4, MoBio, Carlsbad, CA, USA). We used the standard 515f and 806r primers (49-51) to amplify the V4 region of the 16S rRNA gene, using mitochondrial blockers to reduce
amplification of host mitochondrial DNA. Sequencing was performed using paired-end 345
150 base reads on an Illumina HiSeq sequencing platform. Following standard 346
demultiplexing and quality filtering using the Quantitative Insights Into Microbial Ecology 347
pipeline (QIIME2) (52) and vsearch8.1 (53), ASVs were identified using the Deblur 348
method (19) and taxonomy was assigned using the Greengenes Database (May 2013 349
release; http://greengenes.lbl.gov). Libraries containing fewer than 1000 reads were 350
removed from further analyses.

Alpha diversity for each organ was measured using the Shannon index, as well as 352
by measuring species richness based on actual observed diversity. Significance of 353
differing mean values for each diversity calculation was determined using the Kruskal- 354
Wallis rank sum test, followed by a post-hoc Dunn test with bonferroni corrected $p$- 355
values. Three measures of beta diversity (unweighted UniFrac, weighted UniFrac, and 356
Bray-Curtis) were calculated using relative abundances of each ASV (calculated as ASV 357
read depth over total read depth per library). Significant drivers of community similarity 358
were identified using the ADONIS test with Bonferroni correction for multiple 359
comparisons using the R package Phyloseq (55). Code for microbiome analyses can be 360
found at http://github.com/hollylutz/CuttlefishMP. (Authors’ note: Sequences and 361
metadata for replication of analyses presented in this study will be uploaded to the Qiita 362
platform and to the European Nucleotide Database upon acceptance for publication.) 363

4.4 Sample collection, fixation and sectioning for imaging
Samples from esophagus, stomach, intestine and cecum of 9 cuttlefish (5 from antibiotic treatment and 4 controls) were dissected and divided in order to include the same individuals in both microscopy and sequencing analyses. Immediately after dividing, samples were fixed with 2% paraformaldehyde in 10 mM Tris pH 7.5 for 12 h at 4 ºC, washed in PBS, dehydrated through an ethanol series from 30 to 100%, and stored at -20 ºC. Samples were dehydrated with acetone for 1 h, infiltrated with Technovit 8100 glycol methacrylate (EMSdiasium.com) infiltration solution 3 x 1 hour or longer followed by a final infiltration overnight under vacuum, transferred to Technovit 8100 embedding solution and solidified for 12 h at 4 ºC. Blocks were sectioned to 5 um thickness and applied to Ultrastick slides (Thermo Scientific). Sections were stored at room temperature until FISH was performed.

4.5 Fluorescence *in situ* hybridization (FISH)

Hybridization solution [900 mM NaCl, 20 mM Tris, pH 7.5, 0.01% SDS, 20% (vol/vol) formamide, each probe at a final concentration of 2 μM] was applied to sections and incubated at 46 ºC for 2 h in a chamber humidified with 20% (vol/vol) formamide. Slides were washed in wash buffer (215 mM NaCl, 20 mM Tris, pH 7.5, 5mM EDTA) at 48 ºC for 15 min. Samples were incubated with wheat germ agglutinin (20 ug ml⁻¹) conjugated with Alexa Fluor 488 and DAPI (1 ug ml⁻¹) at room temperature for 30 minutes after FISH hybridization to label mucus and host nuclei, respectively. Slides were dipped in distilled cold water, air dried, mounted in ProLong Gold antifade reagent.
(Invitrogen) with a 1.5 coverslip, and cured overnight in the dark at room temperature before imaging. Probes used in this study are listed in table 2.

4.6 Image acquisition and linear unmixing

Spectral images were acquired using a Carl Zeiss LSM 780 confocal microscope with a Plan-Apochromat 40X, 1.4 N.A. objective. Images were captured using simultaneous excitation with 405, 488, 561, and 633 nm laser lines. Linear unmixing was performed using the Zeiss ZEN Black software (Carl Zeiss) using reference spectra acquired from cultured cells hybridized and imaged as above with Eub338 probe labeled with the appropriate fluorophore. Unmixed images were assembled and false-colored using FIJI software (Schindelin et al., 2012).

FIGURE LEGENDS

Figure 1. A single *Vibrio* taxon dominates the esophagus, and a single *Piscirickettsiaceae* taxon dominates the gills of the European common cuttlefish in captivity

Relative abundance of dominant ASVs (ASVs with >20% maximum relative abundance among samples), colored by ASV, arranged by experimental group (control or treatment), and faceted by organ. Penaid shrimp and sea water from experimental holding tanks are also shown. ASVs are labeled according to the finest level of taxonomic resolution provided by the GreenGenes database. Bars correspond to individual libraries, showing
Figure 2. Cuttlefish microbiota show negligible response to antibiotic treatment

(A) Shannon Index and (B) Observed ASV richness of organ microbiota, with and without enrofloxacin treatment. PCoA of (C) weighted UniFrac dissimilarity, (D) unweighted UniFrac dissimilarity, and (E) Bray-Curtis dissimilarity of organ microbiota, with and without enrofloxacin treatment. Points represent individual 16s sequence libraries, with colors corresponding to experimental group (control, treatment), and shapes corresponding to organ (esophagus, stomach, cecum, intestine, and gills).

Figure 3. Spatial organization of bacteria in the esophagus of *S. officinalis*. The image shown is a cross-section of esophagus that was embedded in methacrylate, sectioned, and subjected to fluorescence *in situ* hybridization. (A) Bacteria (magenta) lining the interior of the esophagus in association with the mucus layer (wheat germ agglutinin staining, green). (B) and (C) are enlarged images of the regions marked with arrowheads in (A) where bacteria extend past the edge of the mucus layer. Blue: Host nuclei. Scale bar =100 μm in (A) and 20 μm in (B) and (C).

Figure 4. Fluorescence *in situ* hybridization identifies bacteria in the esophagus of *S. officinalis* as Vibrionaceae. A methacrylate-embedded section was hybridized with a nested probe set containing probes for most bacteria, Gammaproteobacteria, Alphaproteobacteria, and Vibrionaceae. (A) near-universal probe showing a similar
bacterial distribution as in figure 3. (B, C, D) Enlarged images of the region marked by
the dashed square in (A) showing hybridization with near-universal, Vibrionaceae, and
Gammaproteobacteria probes, respectively. (E) Merged image of B, C, and D showing an
exact match of the signal from those three probes. (F) Alphaproteobacteria probe
showing no hybridization. (G) An independent hybridization with the non-target probe
set as a control; no signal is observed. (H) enlarged image of the dashed square in (G).
Scale bar=20 μm (A, G); 5 μm (B-F, H).

Figure 5. Fluorescence in situ hybridization in intestine of S. officinalis. A
methacrylate-embedded section was hybridized with the near-universal probe and stained
with fluorophore-labeled wheat germ agglutinin to visualize mucus. (A) Bacteria
(magenta) are sparsely distributed through the lumen. (B) enlarged image of the dashed
square in (A). (C) An independent FISH control with a non-target probe (Hhaem1007);
no signal was detected. Scale bar= 20 μm (A, C); 5 μm (B).

Figure 6. Fluorescence in situ hybridization in spiral cecum of S. officinalis. (A)
Bacteria are observed in low abundance in the lumen of spiral cecum. (B) Enlarged
image of dashed square in (A). Scale bar=20 μm (A); 5 μm (B).

Figure 7. Fluorescence in situ hybridization in gills of S. officinalis. Bacteria are
observed in small clusters. (A) Overview image. (B, C): enlarged images of the dashed
square in (A) showing hybridization with near-universal, and Gammaproteobacteria
probes, respectively. (D) Merged image of (B), and (C) showing an exact match of the
signal from those two probes. Display in (B, C, and D) was adjusted for clarity. Scale bar=20 μm (A); 5 μm (B-D).

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Table 1. Relative abundance dominant ASVs in the esophagus (ASV1, Vibrio sp.) and gills (ASV2, Piscirickettsiaceae), both in the class Gammaproteobacteria. T-test of change in relative abundance between treatment and control groups reveals varying degrees of microbial "knockdown" in the treatment groups. * indicates organs in which relative abundance differed significantly between treatment and control animals.

<table>
<thead>
<tr>
<th>Organ</th>
<th>ASV</th>
<th>Bacterial Family</th>
<th>Genus</th>
<th>Group</th>
<th>Relative Abundance</th>
<th>p-value</th>
<th>df</th>
<th>t</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gills*</td>
<td>ASV 2</td>
<td>Piscirickettsiaceae</td>
<td>Unknown</td>
<td>Control</td>
<td>0.969 ± 0.025</td>
<td>0.01</td>
<td>13.8</td>
<td>2.82</td>
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<td></td>
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<td></td>
<td></td>
<td>Treatment</td>
<td>0.824 ± 0.190</td>
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<tr>
<td>Esophagus</td>
<td>ASV 1</td>
<td>Vibrionaceae</td>
<td>Vibrio sp.</td>
<td>Control</td>
<td>0.995 ± 0.012</td>
<td>0.09</td>
<td>14.7</td>
<td>1.83</td>
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<td></td>
<td></td>
<td></td>
<td>Treatment</td>
<td>0.944 ± 0.108</td>
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<tr>
<td>Stomach</td>
<td>ASV 1</td>
<td>Vibrionaceae</td>
<td>Vibrio sp.</td>
<td>Control</td>
<td>0.431 ± 0.373</td>
<td>0.17</td>
<td>10</td>
<td>1.48</td>
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<tr>
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<td></td>
<td>Treatment</td>
<td>0.191 ± 0.309</td>
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</tr>
<tr>
<td>Cecum*</td>
<td>ASV 1</td>
<td>Vibrionaceae</td>
<td>Vibrio sp.</td>
<td>Control</td>
<td>0.437 ± 0.234</td>
<td>0.002</td>
<td>8.3</td>
<td>4.31</td>
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<td></td>
<td></td>
<td>Treatment</td>
<td>0.064 ± 0.087</td>
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<tr>
<td>Intestine*</td>
<td>ASV 1</td>
<td>Vibrionaceae</td>
<td>Vibrio sp.</td>
<td>Control</td>
<td>0.568 ± 0.158</td>
<td>1.99E-05</td>
<td>8.1</td>
<td>8.81</td>
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<td>Treatment</td>
<td>0.055 ± 0.059</td>
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<td>Probe Set</td>
<td>Probe name</td>
<td>Fluorophore(s)</td>
<td>Target organism</td>
<td>Sequence 5’ – 3’</td>
<td>Target position</td>
<td>Reference</td>
<td></td>
<td></td>
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<tr>
<td>Experimental</td>
<td>Eub338-I</td>
<td>Alexa 555 or Rhodamine Red X</td>
<td>Most Bacteria</td>
<td>GCTGCCTCCCGT AGGAGT</td>
<td>16S, 338-355</td>
<td>Amann et al. 1990 (42)</td>
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<td>Vib1749</td>
<td>Texas Red X</td>
<td>Vibrionaceae family</td>
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<td>23S, 1749-1769</td>
<td>Schlundt et al., in prep</td>
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<td>Vib2300</td>
<td>Texas Red X</td>
<td>Vibrionaceae family</td>
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<td>23S, 2299-2321</td>
<td>Schlundt et al., in prep</td>
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<td>Alf968</td>
<td>Atto 620 or Dy490</td>
<td>Alphaproteobacteria</td>
<td>GGTAAGGTTC GTGCGTT</td>
<td>16S, 968-985</td>
<td>Neef, A., 1997 (43)</td>
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<td>Gam42a</td>
<td>Atto 647N or Cy5</td>
<td>Gammaproteobacteria</td>
<td>GCCTCCCACAT CGTTT</td>
<td>23S, 1027-1043</td>
<td>Manz et al. 1992 (44)</td>
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<td>Alexa 555</td>
<td>Veillonella</td>
<td>CCGTGGCTTTCT ATTCCG</td>
<td>16S, 488-505</td>
<td>Chalmers et al. 2008 (45)</td>
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<td>Non-target control</td>
<td>PorTan34</td>
<td>Texas Red X</td>
<td>Porphyromonas and Tannerella</td>
<td>GTTAAGGCTATC GCTAGC</td>
<td>16S, 34-51</td>
<td>Mark Welch et al., in prep.</td>
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<tr>
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<td>Fus714</td>
<td>Atto 620</td>
<td>Fusobacterium</td>
<td>GGCTTCCCCATC GGCATT</td>
<td>16S, 714-731</td>
<td>Valm et al. 2011 (46)</td>
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<tr>
<td></td>
<td>Lept568</td>
<td>Atto 647N</td>
<td>Leptotrichia</td>
<td>GCCTAGATGCC TTTATG</td>
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<td>Hhaem1007</td>
<td>Rhodamine Red X</td>
<td>Haemophilus haemolyticus</td>
<td>AGGCACCTCCCAT ATCTCTACAG</td>
<td>16S, 1007-1028</td>
<td>Mark Welch et al., in prep.</td>
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</table>
Figure 1. A single Vibrio taxon dominates the esophagus, and a single Piscirickettsiaceae taxon dominates the gills of the European common cuttlefish in captivity. Relative abundance of dominant ASVs (ASVs with >20% maximum relative abundance among samples), colored by ASV, arranged by experimental group (control or treatment), and faceted by organ. Penaid shrimp and sea water from experimental holding tanks are also shown. ASVs are labeled according to the finest level of taxonomic resolution provided by the GreenGenes database. Bars correspond to individual 16s rRNA sequence libraries, showing only libraries with >1000 read depth. *S. officinalis* pictured in the lower right-hand corner.
Figure 2. Cuttlefish microbiota show negligible response to antibiotic treatment
(A) Shannon Index and (B) Observed ASV richness of organ microbiota, with and without enrofloxacin treatment. PCoA of (C) weighted UniFrac dissimilarity, (D) unweighted UniFrac dissimilarity, and (E) Bray-Curtis dissimilarity of organ microbiota, with and without enrofloxacin treatment. Points represent individual 16s sequence libraries, with colors corresponding to experimental group (control, treatment), and shapes corresponding to organ (esophagus, stomach, cecum, intestine, and gills).
Figure 3. Spatial organization of bacteria in the esophagus of *S. officinalis*. The image shown is a cross-section of esophagus that was embedded in methacrylate, sectioned, and subjected to fluorescence *in situ* hybridization. (A) Bacteria (magenta) lining the interior of the esophagus in association with the mucus layer (wheat germ agglutinin staining, green). (B) and (C) are enlarged images of the regions marked with arrowheads in (A) where bacteria extend past the edge of the mucus layer. Blue: Host nuclei. Scale bar =100 µm in (A) and 20 µm in (B) and (C).
Figure 4. Fluorescence in situ hybridization identifies bacteria in the esophagus of *S. officinalis* as *Vibrionaceae*. A methacrylate-embedded section was hybridized with a nested probe set containing probes for most bacteria, Gammaproteobacteria, Alphaproteobacteria, and *Vibrionaceae*. (A) Near-universal probe showing a similar bacterial distribution as in figure 3. (B, C, D) Enlarged images of the region marked by the dashed square in (A) showing hybridization with near-universal, *Vibrionaceae*, and Gammaproteobacteria probes, respectively. (E) Merged image of (B, C, and D) showing an exact match of the signal from those three probes. (F) Alphaproteobacteria probe showing no hybridization. (G) An independent hybridization with the non-target probe set as a control; no signal is observed. (H) Enlarged image of the region marked by the dashed square in (G). Scale bar=20 µm (A, G); 5 µm (B-F, H).

Specific probe set

![Specific probe set](image)

Non-target probe set

![Non-target probe set](image)
Figure 5. Fluorescence in situ hybridization in intestine of *S. officinalis*. A methacrylate-embedded section was hybridized with the near-universal probe and stained with fluorophore-labeled wheat germ agglutinin to visualize mucus (green). (A) Bacteria (magenta) are sparsely distributed through the lumen. (B) enlarged image of the region marked by the dashed square in (A). (C) An independent FISH control with a non-target probe (Hhaem1007); no signal was detected. Scale bar= 20 µm (A, C); 5 µm (B).
Figure 6. **Fluorescence in situ hybridization in cecum of S. officinalis.** A methacrylate-embedded section was hybridized with the near-universal probe and stained with fluorophore-labeled wheat germ agglutinin to visualize mucus (green). (A) Bacteria are observed in low abundance in the lumen of cecum. (B) Enlarged image of region marked by the dashed square in (A). Scale bar=20 µm (A); 5 µm (B).
Figure 7. Fluorescence in situ hybridization in gills of *S. officinalis*. A methacrylate-embedded section was hybridized with the near-universal probe and stained with fluorophore-labeled wheat germ agglutinin to visualize mucus (green). Bacteria are observed in small clusters. (A) Overview image. (B, C): enlarged images of the region marked by the dashed square in (A) showing hybridization with near-universal, and Gammaproteobacteria probes, respectively. (D) Merged image of (B), and (C) showing an exact match of the signal from those two probes. Display in (B, C, and D) was adjusted for clarity. Scale bar=20 µm (A); 5 µm (B-D).