



# Comparative study of the hemolymph microbiome between live and recently dead American lobsters *Homarus americanus*

Jibom Jung<sup>1</sup>, Patrick M. Gillevet<sup>2</sup>, Masoumeh Sikaroodi<sup>2</sup>, Jamal Andrews<sup>2</sup>,  
Bongkeun Song<sup>3</sup>, Jeffrey D. Shields<sup>3,\*</sup>

<sup>1</sup>School of Biological Sciences, Seoul National University, Gwanak-gu, Seoul 08826, Republic of Korea

<sup>2</sup>Microbiome Analysis Center, George Mason University, Fairfax, VA 22030, USA

<sup>3</sup>Virginia Institute of Marine Science, College of William & Mary, Gloucester Point, VA 23062, USA

**ABSTRACT:** Lobsters and other crustaceans do not have sterile hemolymph. Despite this, little is known about the microbiome in the hemolymph of the lobster *Homarus americanus*. The purpose of this study was to characterize the hemolymph microbiome in lobsters. The lobsters were part of a larger study on the effect of temperature on epizootic shell disease, and several died during the course of the study, providing an opportunity to examine differences in the microbiomes between live and recently dead (1–24 h) animals. The hemolymph microbiomes of live lobsters was different from those in dead animals and both were different from the tank microbiome in which the animals had been held. The microbiomes of live lobsters were more diverse and had a different suite of bacteria than those from dead animals. The dominant taxa in live lobsters belonged to *Flavobacteriaceae* and *Rhodobacteraceae*, whereas *Vibrionaceae* and *Enterobacteriaceae* were predominant in the dead lobsters. Although aquarium microbiomes overlapped with the hemolymph microbiomes, there was less overlap and lower abundance of taxa in comparison with hemolymph from live lobsters. Previous studies reporting bacteria in the digestive tract of lobsters suggested that *Vibrionaceae* and *Enterobacteriaceae* had invaded the hemolymph via the gut. Our study suggests that hemolymph bacteria abundant in live lobsters do not originate from the tank milieu and comprise a rich, natural, or native background of bacterial constituents.

**KEY WORDS:** Decapoda · Microorganism · Biodiversity · Bacteria · Succession · *Vibrio* · Gut microbiome · Comparative microbiomes

## 1. INTRODUCTION

The American lobster *Homarus americanus* is a highly valued commercial species and one of the main fishery products in the world. The ex-vessel landings are around 160 000 mt and represent approximately half of world-wide lobster catch (FAO 2018). Because of its abundance, relative ease of capture, maintenance, handling, and large size, the American lobster is also used as a model crus-

tacean for comparative studies (e.g. Romano et al. 2007, Ma et al. 2008, Waller et al. 2017). However, the microbiomes of the lobster have only recently been studied. The external, or shell microbiomes of the lobster have received the most attention, primarily because of the dysbiosis found in epizootic shell disease (ESD) (Meres et al. 2012, Quinn et al. 2013, Reardon et al. 2018). Bacterial taxa such as *Aquimarina*, *Tenacibaculum*, *Maribacter*, *Jannischia*, and others are more abundant on diseased

\*Corresponding author: jeff@vims.edu

animals than on healthy animals. Although there has been no work on the gut microbiome of the American lobster, a recent study has examined how the formation of gut microbiome changes in the European lobster *H. gammarus* (Holt et al. 2020). In addition, using ultrastructure and specialized staining, Martin et al. (2020) found that American lobsters and several other crustaceans do not have an active microbiome in the midgut region because the food bolus is encased in a peritrophic membrane that excludes bacteria from entering the lumen of the midgut.

As with many marine invertebrates, American lobsters often do not have sterile hemolymph. Previous studies used culture techniques to isolate and identify few culturable bacteria in the lobster hemolymph, including significant pathogens (Cornick & Stewart 1966, Bartlett et al. 2008), but these methods are highly selective and the results may not be representative of the taxa present in the hemolymph microbiome (Bent & Forney 2008). Moreover, culture-dependent methods often give negative results, leading to the notion that healthy hemolymph should be sterile. Quinn et al. (2013) examined bacteria in the lobster hemolymph using culture-independent methods (nested-PCR of 16S rRNA and denaturing gradient gel electrophoresis), but reported only a few taxa, and one sample did not have bacteria. In a study using next generation sequencing (NGS) of 16S amplicons, the whole hemolymph microbiome of the spiny lobster *Panulirus ornatus* was found to include a high diversity of potentially symbiotic bacteria (Ooi et al. 2019). Given that spiny lobsters (Infraorder Palinura: Palinuridae) are not closely related to clawed lobsters (Infraorder Astacidea: Nephropidae), and live in tropical and subtropical regions, they are likely to have very different microbiomes. Nonetheless, there have been no NGS studies to date on the microbiome in the hemolymph of *H. americanus* or other nephropid lobsters.

Our objective was to identify the hemolymph microbiome of the American lobster and to characterize its diversity using 16S amplicon NGS. Because the animals were part of a larger, long-term temperature experiment on ESD (see Barris et al. 2018), we had the opportunity to compare the microbiomes of live lobsters and those that had died of natural causes (within 24 h) during the experiment. In addition, we analyzed the microbiomes of the aquaria (tank microbiomes) to examine whether the hemolymph microbiomes originated from their external environment.

## 2. MATERIALS AND METHODS

### 2.1. Treatment of lobsters

The capture, care, feeding, and maintenance of *Homarus americanus* was described in Barris et al. (2018). In that study, we used 65 females and 14 males, 59 with ESD and 20 without shell disease (termed healthy) (total N = 79). The intensity of infection with ESD was categorized visually: 0: healthy, no scarring; light: shell disease <10 % of body surface with lesions; moderate: shell disease over 11–50 % of the body; and heavy: with shell disease >50 % of the body. The animals were held individually in 38 l aquaria at either 6° (n = 30), 12° (n = 25), or 18°C (n = 24) for 6 mo, and other variables such as salinity and water quality were controlled to match normal seawater conditions. Water quality was maintained with 30–50 % water changes weekly. Nine lobsters died during the course of the long-term temperature study; their hemolymph was sampled as described below. In addition, at the end of the experiment, hemolymph samples were taken from 9 haphazardly selected healthy lobsters and swab samples were taken from 39 aquaria in which many of these lobsters had been held.

### 2.2. Sampling

Hemolymph samples were drawn aseptically from the juncture of the basis and the ischium of the 5<sup>th</sup> walking leg from lobsters at the beginning and end of the experiment and on the day of death for those that died during the experiment. A 95 % ethanol swab was used to sterilize the region prior to bleeding. Several drops of hemolymph from every live animal were plated directly onto marine agar (Difco 2216) and assessed for colony growth after 24 and 48 h. For the microbiome analysis, approximately 100 µl hemolymph was taken from each of the 9 haphazardly selected animals, as well as from each of the 9 recently dead lobsters (Table 1), placed in 1.0 ml 95 % ethanol, placed on ice for a short period, and then frozen at –80°C. Because animals were monitored 1–2 times d<sup>–1</sup>, any dead lobsters had died within 1–24 h of their sampling. Hemolymph samples from live lobsters were taken again after 6–7 mo in captivity in November 2017. Hemolymph samples of dead lobsters were taken at the time of their death, with most mortalities in July and August 2017. In addition to the hemolymph samples, tank (aquarium) samples were taken from 39 aquaria in which lobsters were held at the end of

Table 1. Status of hemolymph samples from *Homarus americanus* used in this study. Dead animals were given a subjective 'smell test' to determine approximately when they died. If the animal smelled rank (dead smell), it was presumed to have died at least 12–24 h previous to the sample. If the animal smelled like a live animal, it was presumed to have died within the last 1–12 h. ESD: epizootic shell disease; ND: not determined; NA: animal did not die during experiment

ID	Internal reference number	Temperature treatment (°C)	Disease condition	Status	Time since death (h)
HM1	A1	6	ESD	Dead	12–24
HM2	A2	6	ESD	Dead	<12
HM3	A8	6	ESD	Dead	<12
HM4	AA22	6	ESD	Dead	<12
HM5	B10	12	Healthy	Dead	12–24
HM6	B17	12	ESD	Dead	ND
HM7	F1	18	ESD	Dead	12–24
HM8	C8	18	ESD	Dead	ND
HM9	F10	18	ESD	Dead	<12
HM10	AA5	6	Healthy	Alive	NA
HM11	AA15	6	ESD	Alive	NA
HM12	AA16	6	ESD	Alive	NA
HM13	AA17	6	ESD	Alive	NA
HM14	AA18	6	ESD	Alive	NA
HM15	AA19	6	ESD	Alive	NA
HM16	AA21	6	ESD	Alive	NA
HM17	AA23	6	ESD	Alive	NA
HM18	B1	12	ESD	Alive	NA

the experiment in November 2017. These samples consisted of sterile cotton swabs wiped along the inside of each aquarium and placed into sterile microfuge tubes. Hemolymph and tank samples were held on ice for short periods (20–30 min) until frozen at  $-80^{\circ}\text{C}$  and shipped to the Microbiome Analysis Center, George Mason University, for further processing. Due to cost constraints, only a subset of hemolymph samples were processed for microbiome analysis, those from the 9 recently dead lobsters and those haphazardly selected from 9 live lobsters at the end of the experiment.

### 2.3. Sequencing

Each sample was extracted using the FastDNA™ Spin Kit for Soil (MP Biomedicals) to obtain purified DNA samples. The DNA amplification protocol and sequencing methods followed those described by Reardon et al. (2018). Briefly, the primers 27F and 355R were used to amplify the first 2 variable regions of the bacterial 16s ribosomal RNA through PCR. Length heterogeneity (LH)-PCR fingerprinting was used as a quality control to assure linear amplification of the sample using an ABI 3130 XL fluorescent sequencer to check the quality and reproducibility of

the amplification (Suzuki et al. 1998, Sikaroodi & Gillevet 2012). Multi-tag pyrosequencing (MTPS) was then used to analyze the samples by creating fusion primers with 16S rRNA primers, a 7 base barcode, and emulsion PCR adaptors as in Sikaroodi & Gillevet (2012). Each DNA sample was amplified with both-directed and combined 16S rRNA primers; these were subjected to emulsion PCR and sequenced on an Ion Torrent PGM. The BioProject accession number of this study is PRJNA644743.

### 2.4. Analyses

The analyses of the microbiome sequences were conducted following a modified method of Semedo & Song (2020). Prior to analysis, the amplicon sequence variant (ASV) count data were filtered using the 'FilterAndTrim' command in DADA2 (Callahan et al. 2016). The 'fastq' files were collated and processed using DADA2 in R v.3.6.1 using a forward read only option. The sequencing results were examined as quality scores versus amplification cycles and were deemed acceptable for further analyses (Fig. S1 in Supplement 1 at [www.int-res.com/articles/suppl/d143p147\\_suppl1.pdf](http://www.int-res.com/articles/suppl/d143p147_suppl1.pdf)). ASVs were identified from each sample, resulting in 327 083 sequence reads identified with Silva Reference database v.132 (Quast et al. 2013). Mitochondrial and chloroplast sequences were removed for diversity analysis of microbiomes using Phyloseq (McMurdie & Holmes 2013). Rarefaction (Fig. S2), abundance-based coverage estimator (ACE), and Shannon indexes were used to estimate  $\alpha$ -diversity, and principal coordinate analysis (PCoA) with Bray distance was used to examine  $\beta$ -diversity of the microbiome. 'VennDiagram' (Chen & Boutros 2011) was used to draw a Venn diagram of the microbiomes, 'ggplot' (Wickham & Chang 2008) was used to make stacked bar plots of the microbiomes, and 'DESeq2' (Love et al. 2014) was used to compare microbiome from different conditions. PERMANOVA was performed on the Bray-Curtis resemblance matrix derived from Hellinger-transformed ASV counts to test for the effect of lobster status (live vs. dead), temperature treatment, and shell disease condition using the 'adonis2' and 'strata'

functions (strata=spawn). PERMDISP was conducted to determine whether multivariate dispersion had an effect on the 3 testing conditions. PERMANOVA and PERMDISP analyses were conducted in the 'vegan' R package. The ANOVA feature in 'DESeq2' (Love et al. 2014) was used to compare microbiomes from different conditions and distinguish differences in abundance among bacterial genera between treatment groups. For bar plots, ASVs with a relative abundance >1% were used to reduce the complexity of the plot and legend due to rare genera.

### 3. RESULTS

#### 3.1. General observation

Culturable bacteria were present in 52.2–86.7% of the hemolymph samples taken from lobsters (Table 2). There was a significant decrease in the prevalence of bacteremia over time ( $\chi^2 = 4.680$ ,  $p = 0.031$ ), but it was only observed in animals held at 6°C. Colonies growing on marine agar varied in their morphology, but no consistent patterns were observed. Of the hemolymph samples selected for high-throughput sequencing, all but one (HM18) had colony growth consistent with low level bacteremia.

The hemolymph samples from lobsters had diverse members in their microbiomes (Table 3). Collectively, 173 families, 316 genera, and 305 unclassified and 52 classified ASVs of bacteria were present in the hemolymph of live and dead lobsters (Table S1 in Supplement 2 at [www.int-res.com/articles/suppl/d143p147\\_suppl2.xlsx](http://www.int-res.com/articles/suppl/d143p147_suppl2.xlsx)). The diversity of

the microbiome was somewhat higher in the hemolymph of live than in dead lobsters (Fig. S2, Table 3). In addition, only 3 of 9 hemolymph samples from dead animals had diversity indices similar to those found in live lobsters. The lowest richness (ACE = 5 and 9) was observed in the hemolymph of 2 dead lobsters (HM2 and HM8), whereas the highest richness (ACE = 193) was from the hemolymph of a live lobster (HM17; Tables 2 & S1). Although some hemolymph samples from dead lobsters had a relatively high diversity, as indicated by their high ACE index (e.g. HM5, HM6, and HM9), they had relatively low Shannon diversity values and differed from those values in the live hemolymph samples. This tendency in the hemolymph samples from dead lobsters indicates that they had lower evenness despite higher richness. These differences were notable for the proportionally higher abundance of *Vibrio* (HM5, HM9) and other bacterial species (HM5, HM6, HM9) which were not common in hemolymph samples from live lobsters. In addition, PERMANOVA showed significant differences among microbiomes from live versus dead lobsters ( $F_{1,39} = 2.912$ ,  $p < 0.001$ ) and different temperatures ( $F_{1,39} = 1.338$ ,  $p < 0.05$ ) with homogenous dispersions. However, the sample sizes for animals at 12 and 18°C were too low to draw inferences. The presence of ESD did not have significant effect on microbiome compositions ( $F_{1,39} = 1.084$ ,  $p > 0.05$ ).

The microbiomes present in the hemolymph of live and dead lobsters exhibited communities different from those in the 39 tank (aquarium) microbiomes (Fig. 1). There were 186 families, 316 genera, and 339 ASVs present in the tank microbiomes (Table S2), and 43 families, 64 genera, and 68 ASVs overlapped with the hemolymph microbiomes (50 overlapped with hemolymph from live animals and 48 overlapped with hemolymph from dead animals) (Fig. 2).

Although tank microbiomes formed different groups depending on temperature (Fig. 1), there were too few hemolymph samples processed from animals held at different temperatures to make inferences about temperature. Nonetheless, there was no overlap in the PCoA values for microbiomes from both live and dead lobsters compared with those in tank microbiomes.

Several bacterial taxa in tank water overlapped with the taxa in lobster hemolymph (Fig. 2, Table S3). Among the 30 taxa shared between tank microbiomes and hemolymph microbiomes of live and dead lobsters, *Methylobacterium adhaesivum* and *Halioglobus* sp. were slightly more abundant in live lobsters, *Aquimarina* sp. was more abundant in dead

Table 2. Percentage of lobsters with culturable bacteria present in the hemolymph (bacteremia) at the start and end of a 6 mo temperature experiment. Details of the experimental conditions and temperature treatments are given in Barris et al. (2018)

Temperature treatment (°C)	% Positive at start (no. positive/total no.)	% Positive at end (no. positive/total no.)
6	86.7 (26/30)	60.9 (14/23) <sup>a</sup>
12	52.2 (12/23)	50.0 (9/18)
18	83.3 (20/24)	68.8 (11/16) <sup>b</sup>

<sup>a</sup>Significantly lower prevalence of bacteremia than at start ( $\chi^2 = 4.680$ ,  $p = 0.031$ )

<sup>b</sup>The lower sample size in this treatment reflects a contaminant present in the initial media. When possible, the animals were resampled using new media

Table 3. Condition, number of sequences, observed species richness, abundance-based coverage estimator (ACE, mean  $\pm$  SD) and Shannon statistics for the microbiomes from individual lobsters

Condition	Sample	No. of sequences	Observed richness	ACE	Shannon
Dead	HM1	3409	14.7	21 $\pm$ 2	1.03
	HM2	7870	3.4	5 $\pm$ 1	0.83
	HM3	7570	16.3	17 $\pm$ 1	2.40
	HM4	4815	19.5	24 $\pm$ 2	1.59
	HM5	4555	71.2	85 $\pm$ 5	3.20
	HM6	8446	107.4	160 $\pm$ 6	2.66
	HM7	6132	24.6	30 $\pm$ 3	1.88
	HM8	6242	8.6	9 $\pm$ 1	1.82
	HM9	6334	112.7	154 $\pm$ 6	3.72
Live	HM10	1416	112.7	120 $\pm$ 5	4.31
	HM11	1740	75.0	80 $\pm$ 4	3.60
	HM12	1175	76.5	80 $\pm$ 4	3.75
	HM13	1140	101.0	104 $\pm$ 5	4.33
	HM14	1771	101.8	106 $\pm$ 5	4.28
	HM15	801	66.3	67 $\pm$ 4	3.87
	HM16	1710	73.9	79 $\pm$ 4	2.99
	HM17	3395	154.9	188 $\pm$ 7	4.57
	HM18	2575	77.5	89 $\pm$ 5	3.01
Tank	TA1	4972	65.3	76 $\pm$ 4	3.36
	TA2	3769	56.7	64 $\pm$ 4	3.21
	TA3	11057	106.2	154 $\pm$ 6	3.69
	TA4	11175	91.9	133 $\pm$ 5	3.62
	TA5	8336	96.1	124 $\pm$ 5	3.76
	TA6	6749	97.4	123 $\pm$ 5	3.92
	TA7	9134	109.8	152 $\pm$ 6	4.09
	TA8	6612	87.2	108 $\pm$ 5	3.56
	TA9	10275	105.6	152 $\pm$ 6	3.68
	TA10	5934	79.1	102 $\pm$ 5	3.58
	TA11	10059	112.8	158 $\pm$ 6	3.96
	TA12	9019	90.8	130 $\pm$ 6	3.42
	TA13	5209	103.4	128 $\pm$ 6	3.99
	TA14	5216	93.0	118 $\pm$ 5	3.89
	TA15	5249	105.6	138 $\pm$ 6	3.99
	TA16	5431	94.1	123 $\pm$ 6	3.70
	TA17	5462	109.8	144 $\pm$ 6	4.00
	TA18	4816	117.4	141 $\pm$ 6	4.22
	TA19	6522	116.7	158 $\pm$ 6	3.93
	TA20	3089	98.0	115 $\pm$ 5	4.02
	TA21	5221	109.7	145 $\pm$ 6	3.97
	TA22	4692	72.8	91 $\pm$ 5	3.24
	TA23	3205	84.8	99 $\pm$ 5	3.57
	TA24	4707	91.8	112 $\pm$ 5	4.10
	TA25	4944	123.6	149 $\pm$ 6	3.97
	TA26	5211	75.7	98 $\pm$ 5	3.23
	TA27	2872	75.7	83 $\pm$ 4	3.73
	TA28	4409	65.3	78 $\pm$ 4	3.22
	TA29	3648	82.7	98 $\pm$ 5	3.52
	TA30	2893	109.2	125 $\pm$ 6	4.20
	TA31	4527	75.5	95 $\pm$ 5	2.99
	TA32	3489	114.4	132 $\pm$ 6	4.15
	TA33	2833	65.7	76 $\pm$ 4	2.96
	TA34	3964	105.2	123 $\pm$ 5	4.03
	TA35	3787	75.6	88 $\pm$ 5	3.50
	TA36	2877	91.6	103 $\pm$ 5	3.85
	TA37	3629	87.9	100 $\pm$ 5	3.75
	TA38	4525	111.1	136 $\pm$ 6	4.00
	TA39	5043	98.2	128 $\pm$ 6	3.28

lobsters, and *Andersenella* sp., *Marinicella* sp., *Paraglaciicola* sp., and *Chitinophagales* sp. were more abundant in tank samples. Among the 20 taxa shared between the tank microbiomes and those in the hemolymph of live lobsters, *Myxococcales* sp. was slightly more abundant in live lobsters. Among the 18 taxa shared between tank samples and hemolymph from dead lobsters, *Vibrio* sp., *Citrobacter* sp., *Photobacterium profundum*, and *P. indicum* were more abundant in dead lobsters.

### 3.2. Patterns of the hemolymph microbiome

In total, the hemolymph microbiome of live lobsters consisted of 242 distinct ASVs. The 3 most diverse families were *Flavobacteriaceae*, *Rhodobacteraceae*, and *Burkholderiaceae*, with many ASVs represented by unclassified genera and species. The 3 most diverse genera were *Bacteroides*, *Corynebacterium*, and *Methylobacterium* (Table S1). The 5 most abundant bacterial families in the hemolymph of live lobsters were *Flavobacteriaceae*, *Rhodobacteraceae*, *Beijerinckiacae*, *Saprospiraceae*, and *Pseudomonadaceae* (Fig. 3A). The abundance of genera was relatively evenly distributed with taxa in the *Flavobacteriaceae*, *Alkalimarinus*, *Ulvibacter*, *Methylbacterium*, *Gammaproteobacteriaceae*, and *Loktenella*, as well as *Halloglobus*, *Arenicella*, and *Saprospiraceae* (Figs. 3B & 4).

In total, the microbiome in the hemolymph of dead lobsters consisted of 232 ASVs, i.e. a similar diversity to those in the live animals, but as noted above, the relative abundances of several taxa were very high. The 3 most abundant families were *Flavobacteriaceae*, *Rhodobacteraceae*, and *Vibrionaceae*, as well as many ASVs representing unclassified genera and species. The 2 most diverse genera were *Vibrio* and *Colwellia* (Table S1). Six families—*Vibrionaceae*, *Enter-*



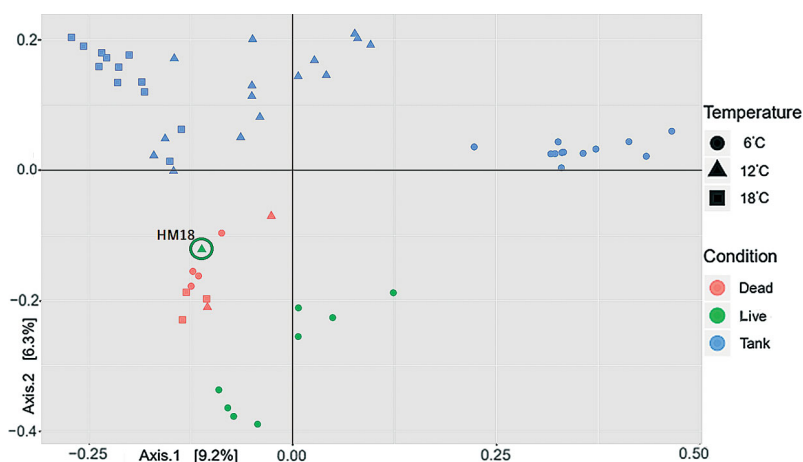


Fig. 1. Principal coordinate analysis of the microbiome of the hemolymph of *Homarus americanus* and tank, by condition and temperature. Differences between HM18 and the microbiome of dead animals are noted in Section 4

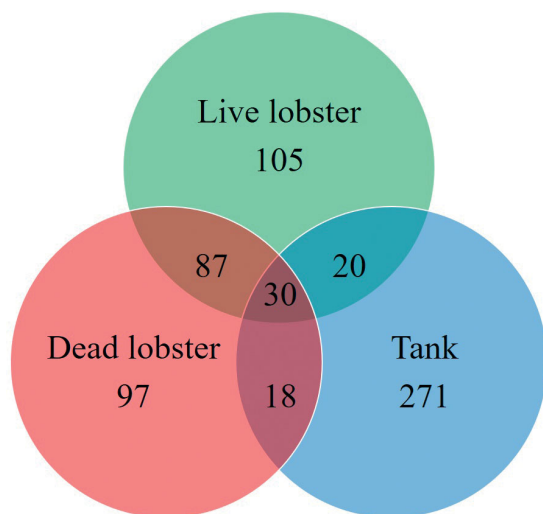


Fig. 2. Amplicon sequence variants in the hemolymph microbiomes of live and dead lobsters and tank samples. Those shared between and among groups are indicated

*bacteriaceae*, *Entomoplasmatales* incertae sedis, *Halomonadaceae*, *Colwelliaceae*, and *Arcobacteraceae*—were abundant in the hemolymph of dead animals but were not present in live animals. Two families in particular, *Entomoplasmatales* incertae sedis and *Halomonadaceae* were dominant in the hemolymph of 2 dead lobsters, HM5 and HM6. In addition, one family, *Pseudomonadaceae*, was present in the hemolymph from of 8 live and 4 dead lobsters (Fig. 3A).

Several genera were abundant in the hemolymph of dead lobsters, including *Vibrio*, *Photobacterium*, *Citrobacter*, *Candidatus* Hepatoplasma, *Aquimarina*,

*Oceanospirillum*, *Colwellia*, and *Arco-bacter* (Figs. 3B & 4). These genera were not present or were present at very low levels in the hemolymph of live animals. The microbiomes of 2 dead lobsters (HM4 and HM6) were especially dominated by *Citrobacter*, *Oceanospirillum*, and *Candidatus* Hepatoplasma.

The hemolymph microbiome of dead and live lobsters were significantly different at the genus level (Fig. 5; DESeq ANOVA). Many genera, such as *Flavobacteriaceae*, *Maribacter*, *Arenicella*, *Granulosicoccus*, and *Methylobacterium*, were much more abundant in the hemolymph of live animals than in that from dead animals, whereas the dominant genera in dead lobster such

as *Vibrio*, *Photobacterium*, *Oceanospirillum*, *Citrobacter*, and *Hepatoplasma* were significantly more abundant compared to hemolymph of live lobsters.

#### 4. DISCUSSION

Using next generation sequencing of 16S amplicons, we confirmed the presence of a diverse microbiome in all 18 *Homarus americanus* hemolymph samples. Live lobsters typically have culturable bacteria in their hemolymph (bacteremia) in a large proportion of their populations (Dove et al. 2005, Bartlett et al. 2008, Shields et al. 2012, Quinn et al. 2013), but here we show a much higher diversity than previously known. We have shown that the hemolymph of lobsters can have a richly diverse microbiome, with as many as 52 families present in one animal (Table S1). Moreover, the hemolymph microbiome appears to be quite different from that on the shell (cf. Meres et al. 2012) and in the milieu of the aquarium (this study). The potential effects of these hemolymph ‘infections’ remain largely unknown; they could be commensals, mutualists, sublethal parasites, or pathogens (such as *Aerococcus viridans* var. homari, causative agent of gaffkemia). The diversity of this community should be further characterized to improve our understanding of how they contribute to lobster health and disease resistance.

Early research on the bacterial flora of the shell and hemolymph of decapod crustaceans, including lobsters, postulated that the source of hemolymph infections was either via the ‘contaminated’ shell or through the gut (for review, see Shields et al. 2006,

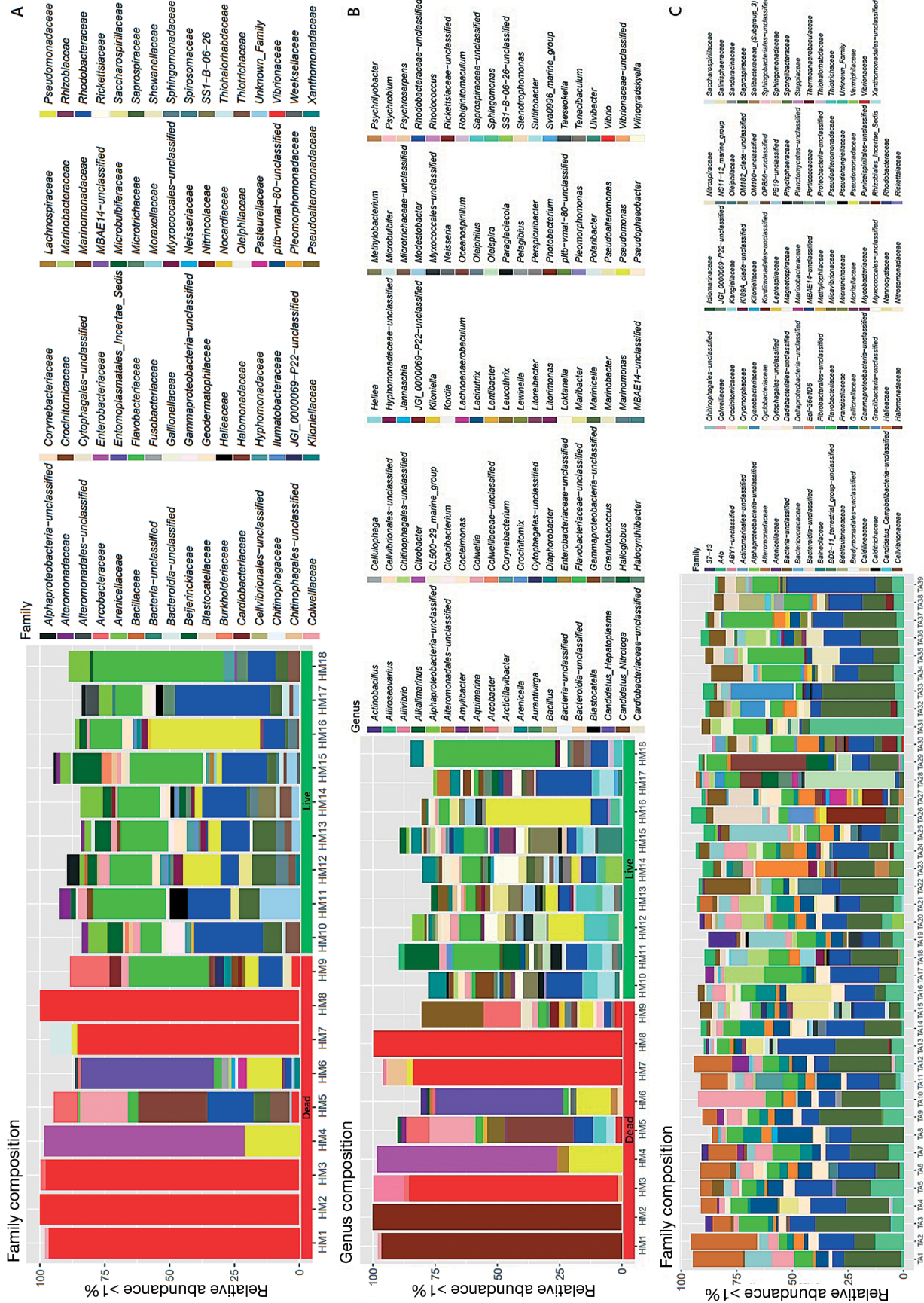


Fig. 3. (A) Family-level and (B) genus-level composition of the hemolymph microbiome of *Homarus americanus*. Dead lobsters are grouped in red and live lobsters in green on the x-axes. (C) Family-level composition of the tank microbiome based on amplicon sequence variants

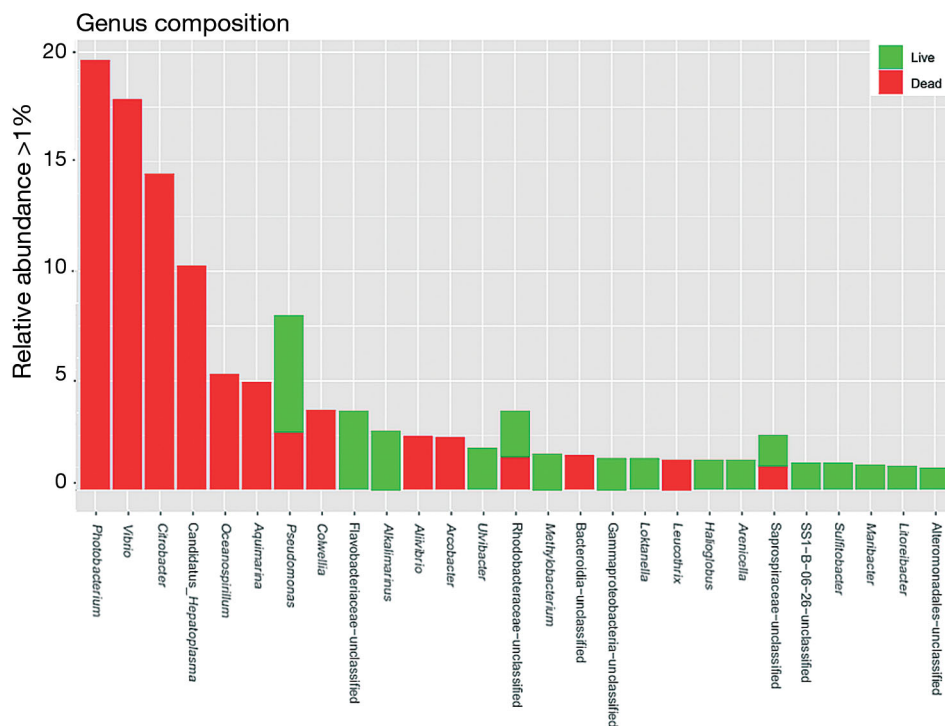


Fig. 4. Genus-level composition in hemolymph microbiome in relation to host status (dead or alive) of the lobster *Homarus americanus*

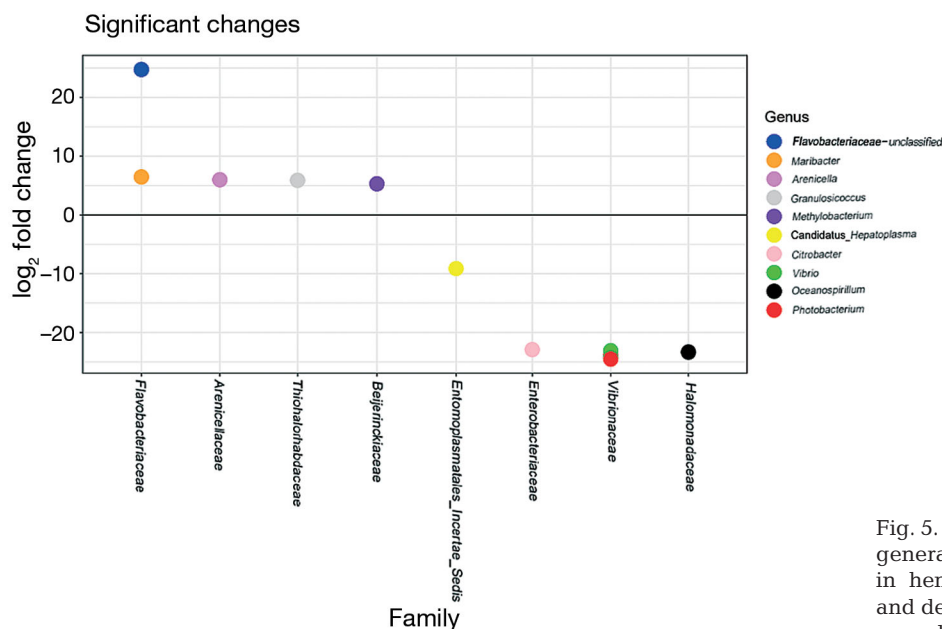


Fig. 5. DESeq ANOVA analysis showing genera that were significantly enriched in hemolymph microbiomes from live and dead lobsters. Above line: live samples, below line: dead samples

Shields & Overstreet 2007). Our data support that the microbiome in the hemolymph is distinctly different from that on the cuticle (cf. Meres et al. 2012). This is not surprising considering that the shell is exposed to the natural environment and in an external milieu different from that of the hemolymph, which is itself an intimate tissue bathing the internal organs of the host lobster. More importantly, our data indicate

that the microbiome in lobster hemolymph rapidly changes when the host lobster dies.

We conjecture that changes in the microbiome in the hemolymph of dead animals arise from invasion through the lobster gut. The gut microbiome of the American lobster *H. americanus* has received little attention. Nonetheless, in a recent study, juvenile sea-reared European lobsters *H. gammarus* had a



very different gut microbiome than those reared in aquaria (Holt et al. 2020). Early benthic stages reared at sea had more *Vibrio* spp. and *Photobacterium* spp. than those reared on land. However, the microbiome present on the feed given to lobsters was not assessed. This is important because Martin et al. (2020) showed that the lumen of the midgut of American lobsters, as well as 5 other crustaceans from disparate taxa, have no microbiome per se. The microbiome is solely present within the food bolus which is entirely encased by the peritrophic membrane. Indeed, the lack of bacteria in the lumen of the midgut has been well documented in other crustaceans (Boyle & Mitchell 1978, Sleeter et al. 1978, Johnson 1980). Collectively, these findings have implications for lobster health. For example, the hemolymph of dead lobsters appears more like that of the food bolus in that the pathogens are either entering through the food or arise from the food as a consequence of mortality. Another implication is that the gut microbiome may be comprised entirely of what is eaten by the lobsters and not by anaerobic fermenters that are normally present in vertebrate digestive systems and whose members are probiotic species providing essential nutrients.

In terrestrial studies of carcasses, changes in the microbial community are known to follow a rapid sequence of succession, loss of diversity, and increase in abundance of certain taxa within the *Proteobacteria* and *Firmicutes* (Pechal et al. 2013). In our study, the hemolymph microbiome in dead lobsters also showed a decline in diversity and shift in specific taxa (*Vibrionaceae* and *Enterobacteriaceae*) compared to the hemolymph of live lobsters. This change also appeared to follow a process of succession in relation to post-mortem change and decomposition. The succession appears to occur very rapidly and includes overgrowth by a few dominant taxa.

The hemolymph microbiome of live lobsters is different from that of the tank microbiome and is suggestive of that fact the lobsters have a native or natural microbiome that does not originate directly from the environment. Although there was some overlap in the taxa present in the microbiomes among groups (live, dead, tank), differences in the abundance levels and composition were evident. For example, the most abundant taxa present in the hemolymph of live lobsters such as *Flavobacteriaceae* and *Pseudomonas* were not present or were present at very low levels in tank microbiomes.

The correlation between hemolymph microbiomes in dead lobsters and tank microbiomes was less clear. Several abundant ASVs in the hemolymph of dead

lobsters were present in the tank samples; however, differences between these groups may have been due to the ability of different taxa to invade and grow as saprobes in the tissues of dead lobsters. Moreover, we did not find a shift in the dominant bacterial community in dead lobsters to be more like that in tank samples. There was, however, a notable flaw in our study as we did not match samples from dead lobsters taken at the same time as tank samples. Nonetheless, the dominant taxa in the tank samples were not the dominant taxa in hemolymph samples from live or dead lobsters.

The hemolymph microbiome of one live lobster (HM18) appeared similar to that of a dead lobster (Fig. 1). The family *Flavobacteriaceae* and its unclassified genera accounted for about half of the relative abundance of bacteria present in the hemolymph of this animal (Fig. 2B). This pattern was observed in the hemolymph from several dead lobsters, where few taxa were dominant. However, with one exception (HM9), the dominant taxa in dead lobster samples was not *Flavobacteriaceae*. Notably, HM18 was held at 12°C, whereas the other animals were held at 6°C. Although temperature is important in lobster homeostasis, it may not be modulating diversity in the hemolymph of lobsters. In the tropical ornate spiny lobster *Panulirus ornatus*, a 6 d increase to 34°C (+6°C above ambient) had no effect on hemolymph microbiomes (OTUs, richness, diversity indices) compared to those from animals held at ambient temperature (28°C), even the though those held at 34°C began to exhibit mortality after 4 d (Ooi et al. 2019). Given that lobsters and other crustaceans are often held at somewhat elevated temperatures for commercial shipment, additional studies are needed to determine how temperature affects hemolymph microbiome of these crustaceans.

In most cases, the hemolymph of dead lobsters had a lower microbial richness with the overgrowth or dominance of a few species of *Vibrionaceae*. *Vibrios* are common constituents of the marine environment and many are reported as pathogens of aquatic organisms, including lobsters (Tall et al. 2003). However, given the surprisingly low abundance and diversity of *vibrios* observed in the hemolymph of live lobsters, the role of these bacteria as pathogens or saprobes should be better established. The patterns in diversity in the hemolymph of dead lobsters are likely the result of sublethal stress leading to mortality with the subsequent decomposition of hemolymph through bacterial degradation.

Five families were highly represented in the hemolymph of the live lobsters. The presence of *Flavobac-*

teriaceae and *Rhodobacteraceae* in live hemolymph samples but not in dead samples suggests a possible commensal relationship between these families and the lobster host. Members of *Flavobacteriaceae* have been reported as insect symbionts (Bernardet & Nakagawa 2006). One genus of *Flavobacteriaceae*, *Aquimarina*, is regarded as a causal agent of ESD in the American lobster (Chistoserdov et al. 2012, Quinn et al. 2012, 2013). Although highly prevalent on the shell of diseased lobsters (data not shown), it was only found in one hemolymph sample (HM9) from a live lobster. However, several other members of the *Flavobacteriaceae* were present, although their roles in the hemolymph or lobster health remain to be determined. Members of the *Rhodobacteraceae* are aquatic photo- and chemoheterotrophs as well as symbionts in other organisms (Pujalte et al. 2014). *Rhodobacteraceae* has been reported as changing in richness in marine organisms in relation to temperature (Stratil et al. 2013, Ooi et al. 2019), but the role of this taxon in the lobster hemolymph is unknown. *Methylobacterium* spp. are members of the  *Beijerinckiaceae* that are facultative methyltrophs (Lidstrom & Chistoserdova 2002, Tamas et al. 2014); the genus was abundant in the hemolymph of live lobsters and may use methylated compounds derived from algae and seaweed, but their role in lobster health is unknown. *Pseudomonadaceae* is abundant in both live and dead lobsters. Members of this taxon have been reported in diseased and dead insects (Jurat-Fuentes & Jackson 2012), and this may be because they are rapid colonizers with the ability to use diverse energy sources (Palleroni 1981, 2008).

Six families were very abundant in the hemolymph of dead lobsters. *Vibrionaceae* are facultative anaerobes and infamous as pathogens of many marine organisms (Farmer 2006, Wendling et al. 2014, Sun et al. 2017, Williams et al. 2017). In the present study, several genera in the family (*Vibrio*, *Photobacterium*, and *Aliivibrio*) were present in the hemolymph of dead animals, but not in the hemolymph from live animals. As indicated previously, it is likely that they invaded the dead or dying animal through invasion of the gut. *Enterobacteriaceae* are also facultative anaerobes and pathogens (Donnenberg 2015). In the present study, *Citrobacter* was found in the hemolymph of dead lobsters, although their role in lobster health or disease resistance is unclear. *Arcobacter* spp., a genus in *Arcobacteraceae*, have been reported as pathogens of fish and mollusks (Fouz et al. 2000, Zhao et al. 2009, Pérez-Pascual et al. 2017, Burioli et al. 2018). They have been reported in biofilms associated with lobsters (Welsh et al. 2011) and as in-

dicators of fecal pollution (Collado et al. 2008); hence, they may be opportunistic invaders in this system.

Two studies have examined the bacterial community present in the hemolymph of the American lobster. In a study focused on cultivable bacteria, Bartlett et al. (2008) found several species that were absent in the present study, including *Staphylococcus epidermis*, *S. equorum*, *S. warnerii*, *S. xylosus*, *Delftia* (*Pseudomonas*) *acidovorans*, *Enterococcus faecalis*, *Hafnia alvei*, *Klebsiella pneumonia*, *Pseudomonas putida*, and *Vibrio fluvialis*. In a study using high-throughput sequencing, Quinn et al. (2013) also found several species that were absent in our study, including *Weissella paramesenteroides*, *D. acidovorans*, *Pelomonas aquatica*, *Geobacillus tepidamans*, *Marinosulfonomonas methylotropha*, and *Brochothrix thermosphacta*. At the genus level, *Weissella*, *Novosphingobium*, *Ralstonia*, *Pelomonas*, *Alphaproteobacterium*, *Hyphomicrobium*, *Sediminibacterium*, *Deltaproteobacterium*, *Geobacillus*, *Marinosulfonomonas*, and *Brochothrix* were not found in our samples. In both studies, the lobsters were from nearby locations around Rhode Island (Bartlett et al. 2008) and eastern Long Island Sound (Quinn et al. 2013); thus, spatial differences probably do not contribute to these differences. Other factors such as water temperature, time in captivity, handling stress, etc., likely contributed to the differences observed among these studies.

## 5. CONCLUSIONS

We found a high diversity in the hemolymph microbiomes of live American lobsters. The diversity and composition of microbiomes changed when lobsters died, apparently shifting to a more saprobic bacterial community comprised of potential facultative pathogens. The hemolymph microbiome from live lobsters was different from the tank microbiomes and consisted of several families with known symbiotic relationships in other host taxa. Hemolymph from dead lobsters had lower diversity than that from live animals and was mostly dominated by several bacteria rarely found in live lobsters. Tank microbiomes and hemolymph microbiomes of live and dead lobsters overlapped slightly. However, the most abundant ASVs present in the hemolymph of live lobsters were not found in tank samples, and this result suggests that these abundant ASVs did not originate from the external environment. In addition, the ASVs in the hemolymph of dead lobsters were found in tank samples, albeit at low abundance. We speculate that the

bacteria in the hemolymph of dead lobsters originates from the food in the gut, as there were similarities between the microbiome of the gut of European lobsters with that observed in the hemolymph of dead animals. To understand how the hemolymph microbiome is assembled, further research is needed into how lobsters control the hemolymph microbiome, how the succession of hemolymph microbiome proceeds when a lobster dies, and what role these microorganisms play in the hemolymph.

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