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Exploring the protist microbiome: The diversity of bacterial communities associated with *Arcella* spp. (Tubulina: Amoebozoa)

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Abstract

Research on protist-bacteria interactions is increasingly relevant as these associations are now known to play important roles in ecosystem and human health. Free-living amoebae are abundant in all environments and are frequent hosts for bacterial endosymbionts including pathogenic bacteria. However, to date, only a small fraction of these symbionts have been identified, while the structure and composition of the total symbiotic bacterial communities still remains largely unknown. Here, we use the testate amoeba *Arcella* spp. as model organisms to investigate the specificity and diversity of *Arcella*-associated microbial communities. High-throughput amplicon sequencing from the V4 region of the 16S rRNA gene revealed high diversity in the bacterial communities associated with the wild *Arcella* spp. To investigate the specificity of the associated bacterial community with greater precision, we investigated the bacterial communities of two lab-cultured *Arcella* species, *A. hemispherica* and *A. intermedia*, grown in two different media types. Our results suggest that *Arcella*-bacteria associations are species-specific, and that the associated bacterial community of lab-cultured *Arcella* spp. remains distinct from that of the surrounding media. Further, each host *Arcella* species could be distinguished based on its bacterial composition. Our findings provide insight into the understanding of eukaryotic-bacterial symbiosis.

Keywords: *Arcella*- associated microbiome; Intracellular bacterial diversity; Heterotrophic amoebae; Amoeba-resistant bacteria

Introduction

Heterotrophic protists are among the most abundant and diverse groups in the eukaryotic tree of life. They play critical roles in the microbial food web by grazing on bacteria and other microbial communities, thus contributing to organic matter recycling and nutrient remineralization (Jumars et al., 1989; Sherr and Sherr, 2002). Existing research has shown that the diversity and abundance of heterotrophic protists in a given habitat are influenced and con-

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trolled by the richness, composition and the stability of the bacterial communities that serve as food source (Saleem et al., 2013). Evidence of preferential/selective feeding behavior on distinct bacterial communities has been demonstrated in various groups of protists, both in laboratory and field studies (Martinez-Garcia et al., 2012; Montagnes et al., 2008; Saleem et al., 2013). However, the interactions between heterotrophic protists and bacteria are not limited only to grazing activity; protists and bacteria are involved in complex symbioses ranging from mutualism to parasitism.

Protist-bacteria associations are widespread in nature and have been found to play fundamental biological processes for their protist hosts, e.g., nutrition, development, defense and other functions (Douglas, 2014; Gast et al., 2009; Husnik et al., 2021). Protists can host bacterial endo- and ecto-symbionts, and these associations can vary temporally from transient to stable and from facultative to obligate (Gast et al., 2009; Nowack and Melkonian, 2010). Several molecular studies have addressed protist-bacteria associations (Delafont et al., 2013; Gast et al., 2009; Gong et al., 2016), however, most focus on specific bacterial species or clades, while much remains to be discovered about the diversity and specificity of the total bacterial communities associated with protists. Of note, a study investigated the taxonomic composition of bacterial communities associated with different marine heterotrophic protist species isolated from environmental samples using flow cytometric cell sorting and single-cell genome amplification (Martinez-Garcia et al., 2012). Correlation analyses showed evidence of species-specific prey interactions and suggested novel putative symbionts.

Free-living amoebae and ciliates are known to host diverse assortments of bacterial symbionts, often shaped by host species (Balczun and Scheid, 2017; Delafont et al., 2013; Lanzoni et al., 2019; Plotnikov et al., 2019; Tsao et al., 2017). Surveys of bacterial symbiont diversity in amoebae and ciliates have shown species differ in their ability to harbor intracellular bacteria and the identity of the hosted bacteria, which for some groups includes human pathogens (Balczun and Scheid, 2017; Gong et al., 2016). For example, one study that isolated free-living amoebae and subjected them to 16S rRNA pyrosequencing analyses found more than 50 genera of digestion-resisting intra-amoebal bacteria, highlighting the power of high throughput, next-generation sequencing analysis in revealing the composition, diversity and abundance of the protist- associated microbiota (Delafont et al., 2013).

Furthermore, diatoms and dinoflagellates can select and maintain specific bacterial epibionts, fostering a community distinct from that of the surrounding media (Amin et al., 2012; Crenn et al., 2018). Shifts in the bacterial community in response to the culture conditions were also observed by others, suggesting that such associations may be influenced by other factors beyond the host (Jasti et al., 2005; Sapp et al., 2007).

We were particularly interested in exploring the protist-bacteria interactions in Arcellinida testate amoebae, a group of diverse and abundant protists with a growing body of literature documenting their importance in nutrient cycling and contribution at different trophic levels within the microbial food web (Jassey et al., 2013). Most Arcellinida species (e.g., genus *Arcella*) are bacterial predators (bacterivory) often found in sphagnum peatlands and freshwater ecosystems (Anderson, 2012; Beyens and Meisterfeld, 2002). Stable isotope analysis demonstrated that species belongs to genus *Arcella* and other testate amoebae species are top bacterial predators with a direct influence on the prey abundance and microbial food web structure in wetlands (Jassey et al., 2013). However, some members of the genus *Arcella* are also known to establish symbiotic association with diverse bacteria including pathogens as well (Gomaa et al., 2018; Katalin et al., 2008). The genus *Arcella* has many closely related species that can co-exist in the same habitats, making them appealing model organisms to experimentally test host-bacteria specificity. Further, Arcellinid testate amoebae are abundant, diverse and widely used as environmental bioindicators in aquatic to terrestrial ecosystems. *Arcella* in particular are used as environmental bioindicators, as they can tolerate hostile environmental conditions including low pH (<5), low oxygen levels, and high concentrations of heavy metals, and even some amount of salt (Nasser et al., 2016; Patterson et al., 2018; Roe and Patterson, 2014).

In this study we use *Arcella* as model organisms to investigate the protist microbiome, focusing on disentangling the roles of the environment and host species on the microbiome. We first performed a pilot study to sequence *Arcella*-associated bacterial communities collected from environmental samples. The aim of this initial sampling was to preliminarily assess whether *Arcella* in the wild naturally associate many or few bacterial groups. Then, to assess whether the *Arcella*-associated community was influenced more by the identity of their *Arcella* host or their environment, we conducted relatively controlled laboratory experiments where we cultured two *Arcella* species (*A. hemispherica* and *A. intermedia*; Supplementary Fig. 1) under different conditions varying the media type (wheat and rice), and for one species, *A. intermedia*, the pH (pH 7 and pH 5). Using high-throughput amplicon sequencing of the bacterial 16S rRNA gene V4 hypervariable region, we found that indeed, each species associates with a particular group of bacteria distinct from media, but that media type and pH do affect the community. These findings suggest a tightly knit association between host and associated bacterial community and underscores the importance of co-evolutionary processes in shaping the microbiome.

Material and methods

Specimens

The diversity of the intact core microbiome in *Arcella* species was investigated using freshly collected *Arcella* cells from the environment as well as isolates from culture collections (Supplementary Table 1, Supplementary Fig. 1). Environmental isolates of *A. vulgaris* ($N = 1$), *Arcella* sp. ($N = 2$), two different morphospecies, were collected from Thoreau's Bog in Concord, Massachusetts and from Mystic River, Medford, Massachusetts, USA. The environmentally-obtained *Arcella* cells were isolated through a series of filtrations following a previously established protocol (Gomaa et al., 2018; Gomaa et al., 2014) for isolating testate amoebae from environmental samples. *Arcella* cells were examined under a microscope, and live individuals, with cytoplasm enclosed in the shell, were identified based on the morphological characteristics of the shell shape and size (Lahr, 2009).

Cultured *A. hemispherica* were obtained from Carolina Biological Supply (item number #131310) and cultures of *A. intermedia* are maintained at the Laboratory of Evolutionary Protistology, University of Sao Paulo, Brazil – this particular lineage is thoroughly described both molecular and morphologically (Porfírio-Sousa et al., 2017). Both obtained cultures were subsequently maintained for 8 months in the laboratory (sub-cultured every week) with two different media treatments per species: rice and wheat, with pH 7, and pH 5. However, samples of *A. hemispherica* in pH 5 did not survive. Therefore, only *A. intermedia* are represented by four treatments (wheat and rice media at pH 7 and pH 5) while *A. hemispherica* has two treatments (wheat and rice media at pH 7). pH was adjusted with 1 M HCl and 1 M NaOH. All media was prepared using 50 ml (0.2 μm filtered) spring water from Carolina Biological supplies, and two grains of wheat or rice, which then were autoclaved. *Arcella* cells were inoculated in T-25 tissue culture flasks with vented cap (Thermo Fisher Scientific, USA), with slightly opened cap, containing the rice or wheat media, and incubated at approximately 20–22 °C on the lab bench top. Every week 1 ml of the cultured cells were spun, washed in ddH_2O and subcultured in fresh media. To harvest cells for PCR, cells were collected on 40 μm nylon-mesh Falcon Cell Strainers (Fisher Scientific, USA). The strainers were then placed upside down into clean Petri dishes, and a clean syringe filled with sterile ddH_20 was used to flush the cells out of the filter into the Petri dish. Specimens from both cultures (*A. hemispherica* and *A. intermedia*) were sorted and amplified within a one-week period. Cells were sorted and processed as the environmental samples above.

As controls, flasks filled with sterile media (rice and wheat, at both pH 5 and 7) but lacking *Arcella,* were incubated for 2 weeks along with the cultures to capture the bacterial diversity from media alone. The bacterial diversity from these culture media was also sampled by filtering 5 ml of media with a UV sterilized syringe, 0.2 μm Whatman filter, and 25 mm filter holder. The DNA was then extracted from the whole filter with the Power-Water DNA isolation kit (MOBIO laboratories) according to the manufacturer's instructions, including homogenization by bead-beating. The DNA was stored at −20 °C until processed for PCR amplification.

16S rRNA gene PCR amplification

Cells from both environmental samples and cultures were sorted using an inverted microscope and narrow pointed Pasteur pipettes. Single cells were placed into 0.2 ml PCR tubes and re-suspended in 25 μl of PCR amplification reaction containing the Q5 High-Fidelity 2X Master Mix (NEB) and 16S rRNA gene barcode-tagged primers (Supplementary Table 1). The 16S rRNA gene primers are dual index primers designed by (Kozich et al., 2013) for amplifying the V4 region. Each primer consists of the appropriate Illumina adapter, an 8-nt index sequence, a 10-nt pad sequence, a 2-nt linker. Each of the *Arcella* species from each culture medium (i.e., wheat or rice, pH 7 or pH5), environmental *Arcella* isolates, and media-only controls were amplified with treatment-specific dual-index barcoded forward and reverse primers (Supplementary Table 2). Twenty to thirty individual *Arcella* cells from each of the cultured *Arcella* species from the two media and pH levels (e.g., *A. hemispherica* in rice at pH 7) were isolated and amplified independently, i.e., twenty PCR reactions for twenty cells. Individual cells from the same culture were amplified with the same dual-index forward and reverse primer combinations and then pooled together for sequencing (Supplementary Table 1). For the environmental *Arcella* spp., single cells from each of the samples were amplified individually with a unique dual-index barcode (Supplementary Table 2). For the media controls, each was amplified independently with unique barcoded dual-index primers per culture medium type (Supplementary Table 2). Negative controls of PCR master mix with sterile water instead of DNA template or *Arcella* cell were also run and sequenced as technical controls to evaluate the degree of bacterial contamination in the PCR step and the workflow. The 16S rRNA gene amplification profile consisted of 10 min hold at 95 °C, followed by 30 cycles consisting of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. The amplified products were screened by gel electrophoresis and positive results at the expected size of approximately 400 bp were excised and then purified with the EZNA® Cycle Pure kit (Omega Bio-tek, USA). Purified DNA was quantified using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA).

The purified amplicons were then pooled in equimolar concentrations to a final concentration of 2 ng/μl. Libraries were mixed with Illumina-generated PhiX control libraries and denatured with fresh NaOH. Samples were sequenced on an Illumina MiSeq with the v3 kit for 2x300bp reads.

Sequence data processing

Upon sequencing, 7,907,067 reads were generated. Raw sequence data in fastq format were analyzed using the dada2 pipeline (Callahan et al., 2016) based off the standard workflow provided at https://benjjneb.github.io/dada2/tutorial.html. Reads were prefiltered to remove sequences containing N's (ambiguous nucleotides) using the dada2 function filterAndTrim and subsequently trimmed to remove any primer sequences using cutadapt (Martin, 2011). Trimmed reads were then filtered with the filterAndTrim function with default parameters except for setting the minimum length to 50 nt (minLen = 50) and the maximum expected errors, based on the quality score, to 2 (maxEE = c(2,2)). From this filtered set, the learnErrors function estimated the error rate from a subset of reads, and this model was then applied to the dada2 function to denoise the reads into amplicon sequence variants (ASVs). ASVs were merged with the mergePairs function and filtered for chimeras with the removeBimeraDenovo function, both with default parameters, and the processed dataset retained 4,612,508 reads. Rarefaction curves were obtained and plotted with the rarecurve function in vegan (Oksanen et al. 2018) and revealed the number of ASVs per sample plateaued before reaching the maximum sequencing depth (Supplementary Fig. 2)

Reference sequences for each ASV were reported and mapped to SILVA for closest taxonomic affiliation using the online SILVA Alignment, Classification and Tree (ACT) service which employs the SINA algorithm (Pruesse et al., 2012). These classifications were used to aggregate ASVs by taxonomic group as needed. The majority of ASV reference sequences differed by >1 nucleotide (Supplemental Fig. 3) based on pairwise alignments generated by MUSCLE (Edgar, 2004).

The table of ASV abundances by sample and the taxonomic association table were read into R for analyses and presented here as Supplementary Table 3.

We strictly treated the ASVs that appeared in technical controls by removing any ASV with non-zero reads in any technical control (PCR negative control, DNA extraction kit) from all other samples, i.e., environmental, laboratory cultures, and media controls. Similarly, to produce Fig. 3 which focuses exclusively on the *Arcella*-specific bacteria, we also dropped the ASVs with non-zero abundance in any media-only control.

Diversity *analyses*

All stacked bar charts were created in R using the ggplot function in the ggplot2 package on normalized relative abundances, subsetting and aggregating the data to different taxonomic levels (e.g., phylum, class, etc.) for the various figures with the reshape2 and plyr packages. α-diversity was calculated by counting the number of distinct ASVs (species richness) and the Shannon and Simpson indices (based on read count data) to assess diversity values within each sample using the vegan package in R (Oksanen et al., 2018). Similarities in community composition between media treatments (the different culture medium and pH levels) were investigated using the venn.diagram function from the VennDiagram R package (Chen and Boutros, 2011) and non-metric multidimensional scaling (NMDS). All beta diversity analyses were performed at the ASV level using ASV relative abundances. Overall sample similarity was investigated with a dendrogram generated with R's hclust function (method="complete") based on the binomial dissimilarity matrix computed from ASV relative abundances using vegan's vegdist function (method= "binomial").

Results

The diversity of bacterial communities associated with *Arcella* spp. cells was characterized by sequencing 16S rRNA gene amplicons from environmentally isolated *Arcella* spp., clonal *Arcella* laboratory cultures from two species, and culture-media-only samples. The processed dataset resulted in a total of 3056 amplicon sequence variants (ASVs) after removing all ASVs detected in the technical controls. While we detected 74 different bacterial classes, the majority of them were rare (Supplementary Fig. 4), so in almost all of our figures we are presenting the top 15 classes, which comprised over 95% of each sample. The majority of these ASVs belong to Proteobacteria, Bacteroidetes, Firmicutes, Verrucomicrobia and Acidobacteria, Actinobacteria and a few other phyla (Fig. 1A)

Arcella species harbor a *diverse* microbial community

Sequencing a few representatives of environmental *Arcella* isolates elucidated that *Arcella* in their natural environments host diverse bacterial communities. These environmental isolates were identified as *Arcella* spp. and *A. vulgaris* based on morphological characteristics. Cells of an environmental *Arcella* sp. isolated from Mystic River and Thoreau's Bog harbored a great diversity of associated bacteria, with Shannon diversities of 5.78 and 5.59, respec-

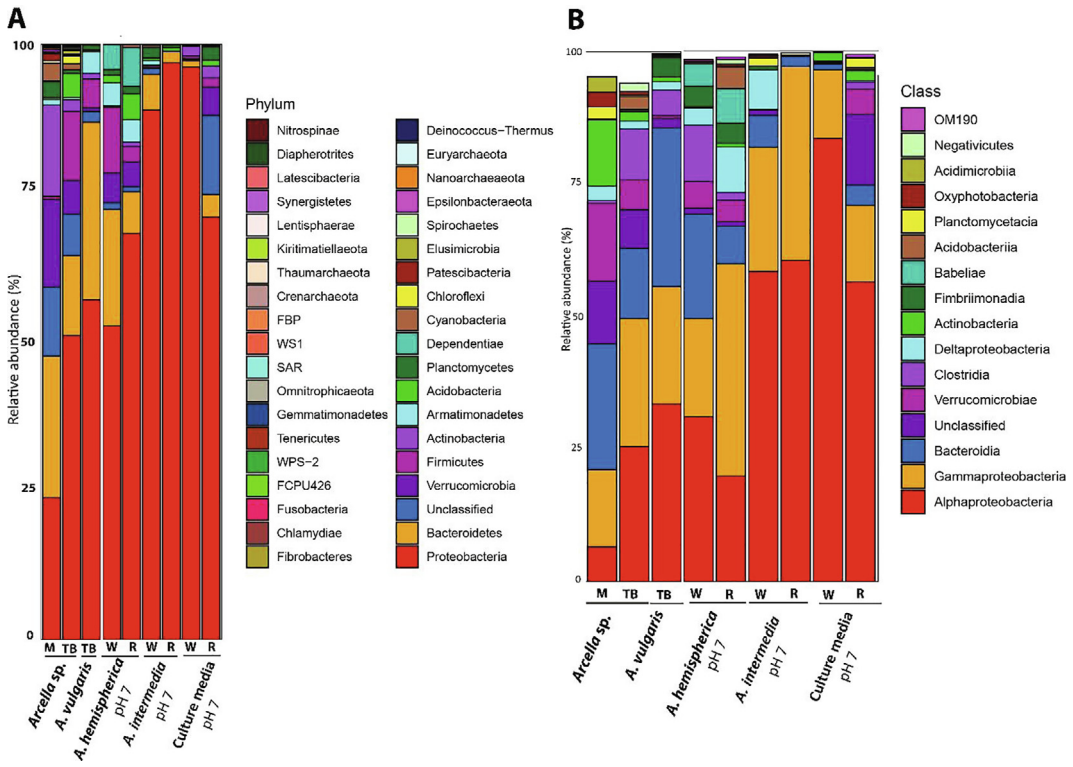

Fig. 1. Bar charts showing the relative abundance of bacterial phyla (left) and classes (right), comprising at least 95% of the bacterial community, characterized from *A. hemispherica* (cultured in wheat (W) and rice (R) media, pH 7), *A. intermedia* (cultured in wheat and rice media, pH 7), the control groups (the natural bacterial community that grew in wheat and rice media only both at pH 7), and the environmental *Arcella* isolates, including the *A. vulgaris* and *Arcella* sp. from Thoreau's Bog (TB) and *Arcella* sp. from Mystic river (M). Each bar in *A. hemispherica* and *A. intermedia* refers to groups of around 20–30 amoeba that were amplified independently and then pooled together for the 16S rRNA sequencing. Within each bar, box color denotes phylum-level or class-level taxonomic association (based on ASVs representative sequences) and box height gives the relative abundance (based on total reads per sample).

tively, and hundreds to thousands of ASVs detected (Table 1). However, *A. vulgaris* from Thoreau's Bog was an exception to this high diversity, having a Shannon diversity of 3.08 with only 90 ASVs detected (Table 1). The composition of the microbiota associated with these environmental *Arcella* was broadly similar at the phylum level, with each dominated by Proteobacteria, Bacteriodetes, and Verrucomicrobia (Fig. 1A). However, it appears that environmental *Arcella* associated with a rich diversity of microbes.

We then conducted a controlled laboratory experiment using cultures of two *Arcella* spp., *A. hemispherica* and *A. intermedia,* to investigate whether the associated bacterial communities are characteristic for each species or are largely influenced by the surrounding environments (i.e., the culture media). Both *Arcella* species were cultured in autoclaved and filtered media, with the culture flask caps were kept slightly open to allow oxygen into the cell cultures. Contaminant bacteria from the air may colonize the media as well. Thus, media controls without *Arcella* to account for the non-*Arcella*-associated microbiota were included in this study and also maintained in similar conditions to the cultured *Arcella* spp.

Between the cultured *Arcella* spp., *A. hemispherica* hosted more diverse bacterial communities whether in wheat or rice media, ranging from 120 to 155 ASVs compared to *A. intermedia* in wheat and rice with only 47–48 ASVs found (Table 1). Despite the difference in ASVs richness, the Shannon diversity indices for *A. hemispherica* and *A. intermedia* communities were similar, ranging from 2.7 to 3.4. On the other hand, the media controls had ASV richness between that of *A. hemispherica* and *A. intermedia*, with 102–129 ASVs and Shannon diversities of 2.32–3.71. Relative to the environmental *Arcella*, Simpson's diversity index was lower in the cultured *Arcella* spp., although the diversity of *A. vulgaris*, the least diverse of the sampled environmental *Arcella*, was on par with that of the cultured *Arcella* spp. Thus, the microbial communities associated with *Arcella* spp. in cultivation appeared to be overall less diverse than most environmental *Arcella* spp. but potentially different than their media, requiring investigation into the taxonomic composition of each community.

Table 1. The total bacterial ASVs number with non-zero abundance detected in the studied *Arcella* species and media control samples. ASV-level abundances were used to calculate the Shannon and Simpson indices of alpha diversity.

	A. hemispherica		*A. intermedia*				Media control		*Arcella* spp.		*A. vulgaris*
Media type/Source	Wheat, pH7	Rice, pH7	Wheat, pH7	Rice, pH7	Wheat, pH5	Rice, pH5	Wheat, pH7	Rice, pH7	Medford River	Thoreau's Bog, pH5	Thoreau's Bog, pH5
Number of ASVs	120	155	47	48	74	42	102	129	1990	729	90
Shannon Diversity	3.4	3.32	2.91	2.7	2.59	1.98	2.32	3.71	5.78	5.59	3.08
Simpson Diversity	0.93	0.89	0.91	0.87	0.78	0.75	0.82	0.95	0.99	0.99	0.91

Arcella-associated microbiota are shared among wild and cultured cells at broad taxonomic levels

Broad similarities in the taxonomic composition were found among *Arcella*-associated bacterial communities at the phylum and class levels (Fig. 1A and B, respectively). The bacterial communities are dominated by Proteobacteria, Bacteroidetes, and unclassified Bacteria, followed by appreciable amounts of Verrucomicrobia, Firmicutes, Actinobacteria, Planctomycetes and Dependentiae (TM6). Interestingly, the cultured isolates contain fewer phyla at high abundance than the wild *Arcella* spp.; notably the phyla, Cyanobacteria, Chloroflexi, Chlamydiae, and some archaeal phyla (Fig. 1A) are missing or much less abundant in the cultured *Arcella* species.

Differences in the bacterial communitY composition and relative abundance INDICATE species-specific associated microbiomes (Fig. 1B) For example, in *A. intermedia,* Alphaproteobacteria are dominated by over 50% of reads, while *A. hemispherica* and environmental *Arcella* spp. contained less Alphaproteobacteria (below 30%). Other associations include an abundance of Babeliae (TM6; teal), Fimbriimonadia (dark green), and Clostridia (light purple) in *A. hemispherica* over *A. intermedia* (Fig. 1B). Thus, each *Arcella* species appears to harbor a characteristic microbiota distinct from, but possibly influenced by, that of their growth media (Supplementary Fig. 5A and 3B).

We calculated the number of bacterial ASVs that are shared between host species and culture medium using Venn diagrams analysis as shown in (Fig. 2A–C), *A. hemispherica* grown in wheat has 46 unique ASVs (ASVs unique to that host/media combination), *A. hemispherica* grown in rice has 80 unique ASVs, while both wheat- and rice-grown *A. hemispherica* share 35 ASVs (out of total 120–155 ASVs). These 35 ASVs does not include any ASVs shared between the two *Arcella* spp., or shared between the *A. hemispherica* and the two media controls, rice and wheat. Wheat and rice grown *A. hemispherica* share 49ASVs with wheat and rice media (Fig. 2A). On the other hand, wheat grown *A. intermedia* has 14 specific ASVs and rice-grown *A. intermedia* has 16 specific ASVs, both wheat and rice grown *A. intermedia* share 14 ASVs out of total 47–48 ASVs, while sharing 23 ASVs with wheat and rice media (Fig. 2B). In addition, a Venn diagram of both *A. hemispherica* and *A. intermedia* species in wheat and rice medium, confirms that more ASVs are shared between the same species regardless of medium type. For example, *A. hemispherica* grown in wheat and rice share 54 ASVs, some of these ASVs possibly occur in either medium, but not in *A. intermedia*, while *A. intermedia* grown in wheat and rice share 23 ASVs, and also some of these ASVs possibly occur in either medium, but not in *A. hemispherica*. On the contrary, the different species of the same growth medium share 0 to 2 ASVs (Fig. 2C). Indeed, a beta diversity analysis (quantifying differences in community

composition between samples based on abundances of all ASVs) revealed that the bacterial communities associated with the cultured *Arcella* species (*A. hemispherica* and *A. intermedia*) is species-specific regardless the media type. In contrast, the media controls and environmental *Arcella* each formed distinct groups (Fig. 2D). Thus, the identity and relative abundance of ASVs can discriminate each host despite different environmental conditions.

To investigate this result more closely, we performed a non-metric multidimensional scaling (NMDS) ordination based on ASV relative abundances to visualize the differences and similarities between each sample (Fig. 2E). The NMDS differentiated between the cultured *Arcella* and the media controls, as expected, but also revealed consistent distinctions in ASV composition between both cultured *Arcella* species (color) and by media type (shape). Further, the environmental *A. vulgaris* sample appears to be more similar to the samples of cultured *A. hemispherica* than to the other environmental samples. However, because there are only few environmental samples represented in our study it is not possible to draw any conclusion with regard to distinguishing between the environmental and cultured *Arcella* species.

A. intermedia and *A. hemispherica* host distinct communities at and below the family level

The ASVs detected in the wheat and rice control media (Fig. 2) were then removed to eliminate the possibility that our analyses included media-associated ASVs. This strategy maximizes our confidence in the specificity of the bacterial community, if any, associated to *A. intermedia* and *A. hemispherica*, although the overall community composition will be biased by the removal of potentially shared bacteria between the media environment and the *Arcella* microbiome. The number of the ASVs declined from 3056 to 2854 ASVs (93.4%) not associated with culture media, wheat and rice. After removing potential media contaminants, cultivated *Arcella* spp. are clearly distinguished at the class level based on the remaining taxa present and their relative abundances (Fig. 3A). The patterns mentioned above are recapitulated but more strongly. Further, the distinct community hosted by each *Arcella* sp. was markedly similar regardless of media type. For instance, *A. hemispherica* is characterized by ASVs belonging to Bacteriodia, Clostridia, Deltaproteobacteria, Verrucomicrobia and BD7-11 that were conserved whether *A. hemispherica* was grown in wheat or rice culture media (Fig. 3A). On the other hand, *A. intermedia* from wheat and rice cultures shared similar bacterial communities at the family level that were clearly distinct from those of similarly grown *A. hemispherica* (Fig. 3B).

Additional investigation of the Alphaproteobacterial families found in the *Arcella*-associated communities revealed a nearly complete lack of overlap between the two *Arcella* spp. (Fig. 3C). For instance, Caulobacteriaceae, Parvibaculaceae, Devosiaceae, and Rhodospirillaceae among others dominated the *A. intermedia* community but were low to vanishingly rare in *A. hemispherica*, while the opposite pattern was observed for Sphingomonadaceae and Reyranellaceae (Fig. 3C). This pattern suggests a species specificity at fine taxonomic / phylogenetic levels.

The *A. intermedia*- associated bacterial community is consistent across different pH levels

We then characterized the diversity of the bacterial community associated with *A. intermedia* grown in wheat and rice medium at different pH levels, natural (pH 7) and acidic (pH 5), as a proxy to assess the stability of *Arcella*-bacteria association (Fig. 4). Of note, the comparison between *A. intermedia* from wheat and rice media pH 7 and pH 5 were done without the subtraction of the media controls as all pH 5 media controls did not meet sequence quality thresholds and so could not be analyzed, likely due to problems during libraries preparation or sequencing. The pH does appear to influence the taxonomic makeup of the bacterial community, although the differences in taxonomic composition between *A. intermedia* grown in wheat and rice media at pH 7 or pH 5 remain minimal relative to their differences from *A. hemispherica* or the media controls (Fig. 2D, E; Fig. 4A). This result suggests that the bacterial community membership is relatively stable across pH levels compared to inter-host differences. As between the two cultured *Arcella* species, the differences in community composition across pH levels were more apparent at the family level than at the class level, with the most abundant family Pseudomonadaceae going from approximately 20% of the microbiome at pH 7 to approximately 40% at pH 5 (Fig. 4B). Interestingly, the Pseudomonadaceae were dominated by a single ASV accounting for over 80% of the Pseudomonadaceae reads in each sample regardless of pH (Fig. 4C). This dominance of a single ASV across different pHs fits with the previous results underscoring the distinct consistency of the host species' characteristic microbiomes, while the ASV's dramatic change in abundance highlights the responsiveness of the species-characteristic microbiome to its environmental conditions.

Discussion

Expanding the host-microbiome field to protists

This is a preliminary study aims to explore the diversity of the bacterial communities associated with free-living amoeboid protists such as *Arcella*. As proof of concept that members of the genus *Arcella* harbor diverse bacterial communities in natural environments, we included few representatives of environmental *Arcella* isolates in our study. Further, we used laboratory cultured *A. hemispherica* and

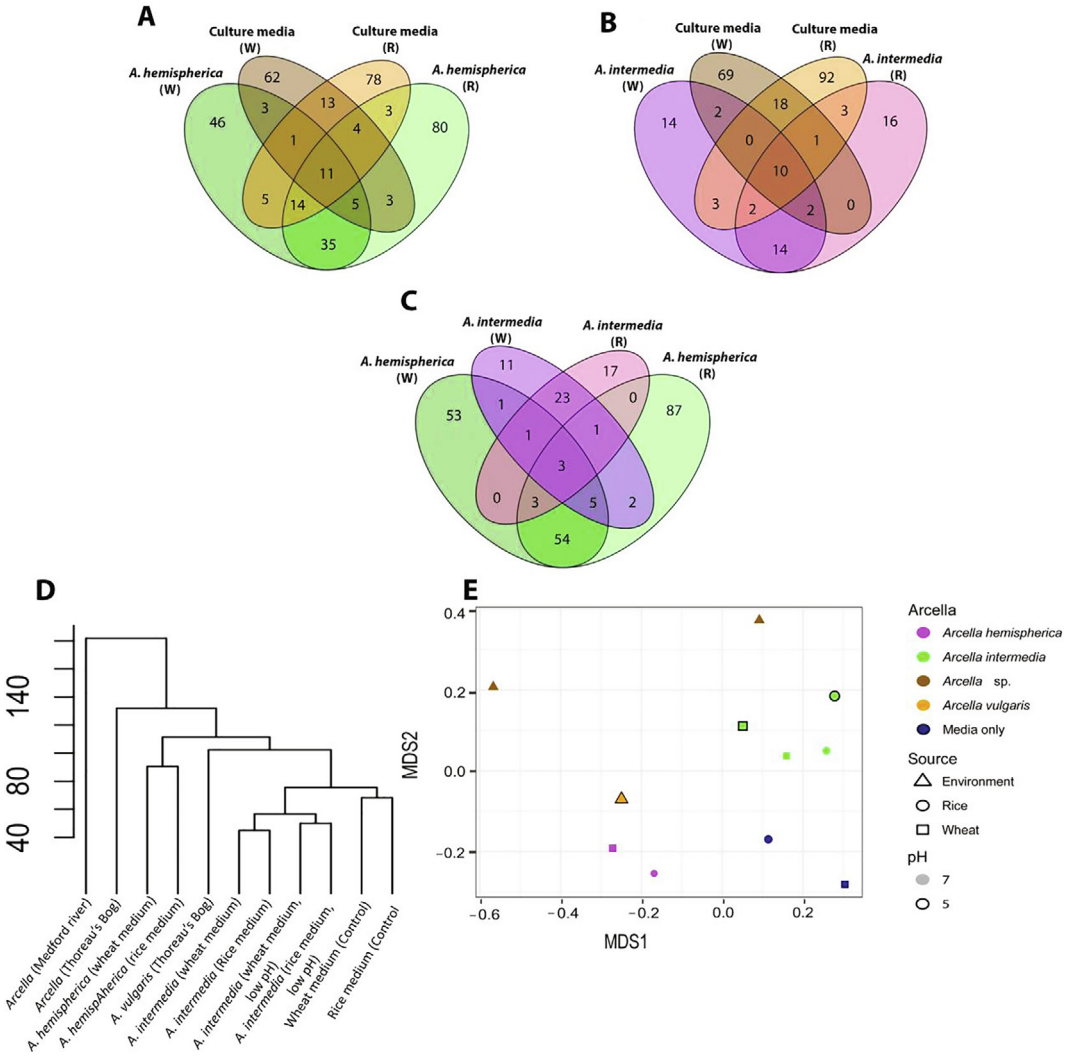

Fig. 2. Venn diagrams of specific and shared ASVs between. (A) *A. hemispherica* from wheat and rice cultures and the culture media controls; (B) *A. intermedia* from wheat and rice cultures and the culture media controls; (C) *A. hemispherica* and *A. intermedia* from both wheat and rice cultures; (D) Dendrogram based on binomial distance matrix calculated from ASV relative abundances illustrating that *Arcella*- associated bacterial communities from the same host species cluster together into subclade; (E) NMDS ordination based on the proportional abundance of all ASVs detected in each sample. Each point represents a different sample. Colors mark the different *Arcella* spp., while shape displays the source of the sample, whether environment or the specific culture media. Points with borders mark samples with pH = 5; all other points originate from pH 7.

A. intermedia to elucidate that the *Arcella*-bacteria association appears to be species-specific and distinct from the microbes of their surrounding environment. There are several emerging studies exploring the diversity and specificity of host-microbiota association in many host organisms, addressing questions related to whether host identity controls microbiome diversity by selecting specific bacterial lineages / phylotypes or whether host-microbiome association follows a stochastic colonization pattern (Foster et al., 2017). So far most microbiome research has been focused on metazoans, particularly humans, non-human animal models, and animals of social or economic value (Kostic et al., 2013). In many cases, it was found that microbiome composition is species-specific and the correlations between host phylogenetic relationships and microbial community composition illustrated a pattern of phylosymbiosis, co-evolution or at least co-divergence of host and symbiont communities (Loo et al., 2019a; Loo et al., 2019b). However, in other cases the microbiome is strongly, if not more, correlated with host geography, diet or other environmental factors (Schnorr et al., 2014; Youngblut et al., 2019). Yet, the microbiome of protists, the largest and most diverse eukaryotic group, remains largely unexplored. We know very little about whether the associated bacterial communi-

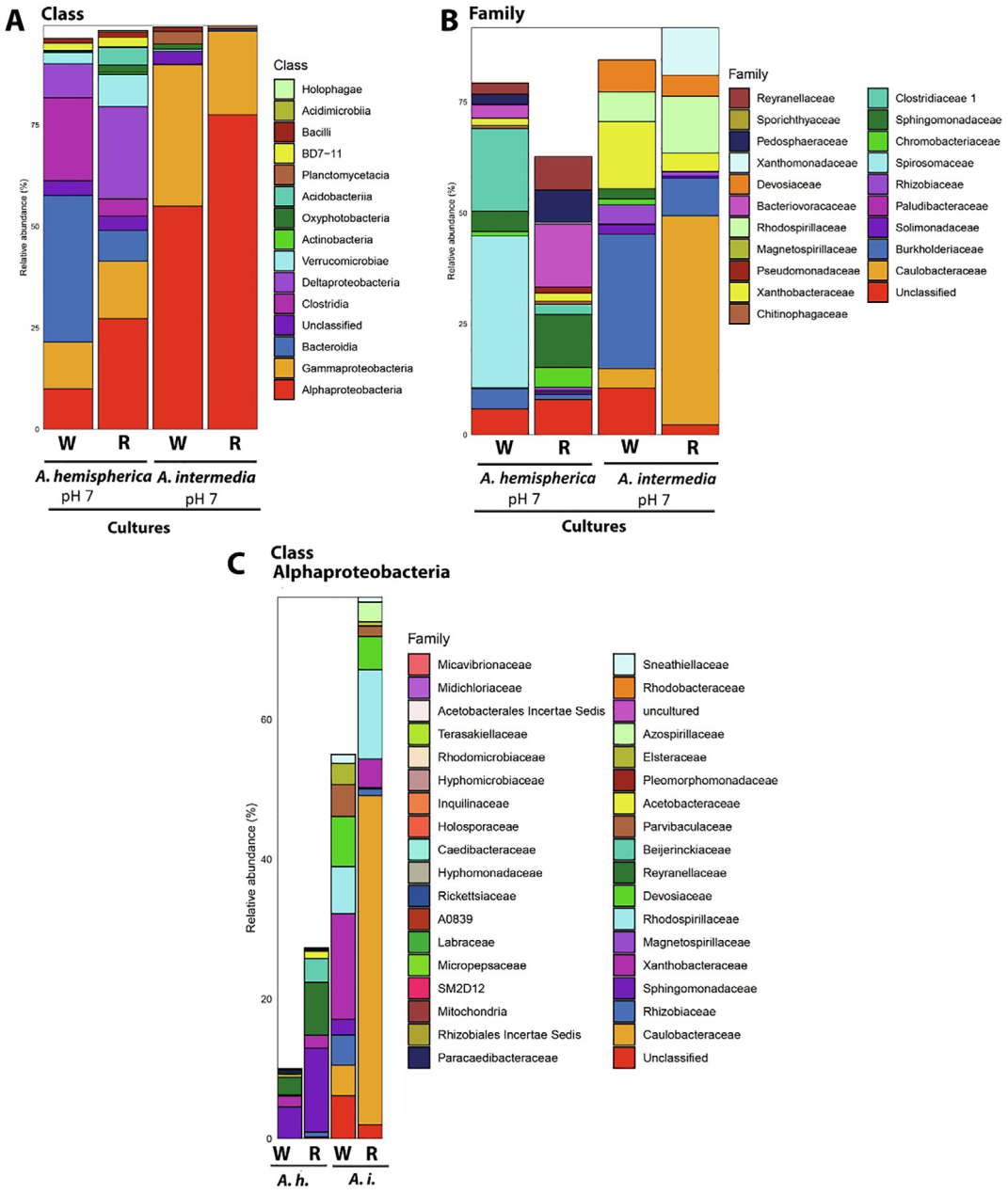

Fig. 3. Bar charts showing the relative abundance of bacterial classes (A), and families (B), Alphaproteobacterial families (C) characterized from *A. hemispherica* (A. h.) (cultured in neutral pH wheat and rice media), and *A. intermedia* (A. i.) (cultured in neutral pH wheat and rice acidic media), after removing the shared ASVs from the media controls. Each bar in *A. hemispherica* and *A. intermedia* refers to groups of 20–30 amoeba that were amplified independently and then pooled together for the 16S rRNA gene sequencing. Within each bar, box color denotes class and family-level taxonomic association (based on ASV representative sequences) and box height gives the relative abundance (based on total reads per sample).

ties with a given species of protist are random or specific, and if specific, whether related to host species or to the environment (Lanzoni et al., 2019; Rossi et al., 2019).

Our approach of using media controls for comparison allowed us to distinguish between contaminant or recently consumed bacteria and the stable, potentially symbiotic communities of bacteria habitually associated with *Arcella* spp. For both of the cultured *Arcella* spp., *A. hemispherica* and *A. intermedia* from wheat and rice media, each of the sequenced libraries combined the relative abundances of pooled PCRs from 20 to 30 amoebae individuals from the same treatment that were amplified independently with the same bacorde sequence. Thus, the observed abundances reflect the sum of several individuals' communities, which increases our confidence that taxa measured at low abundance are either truly low abundance or infrequent commu-

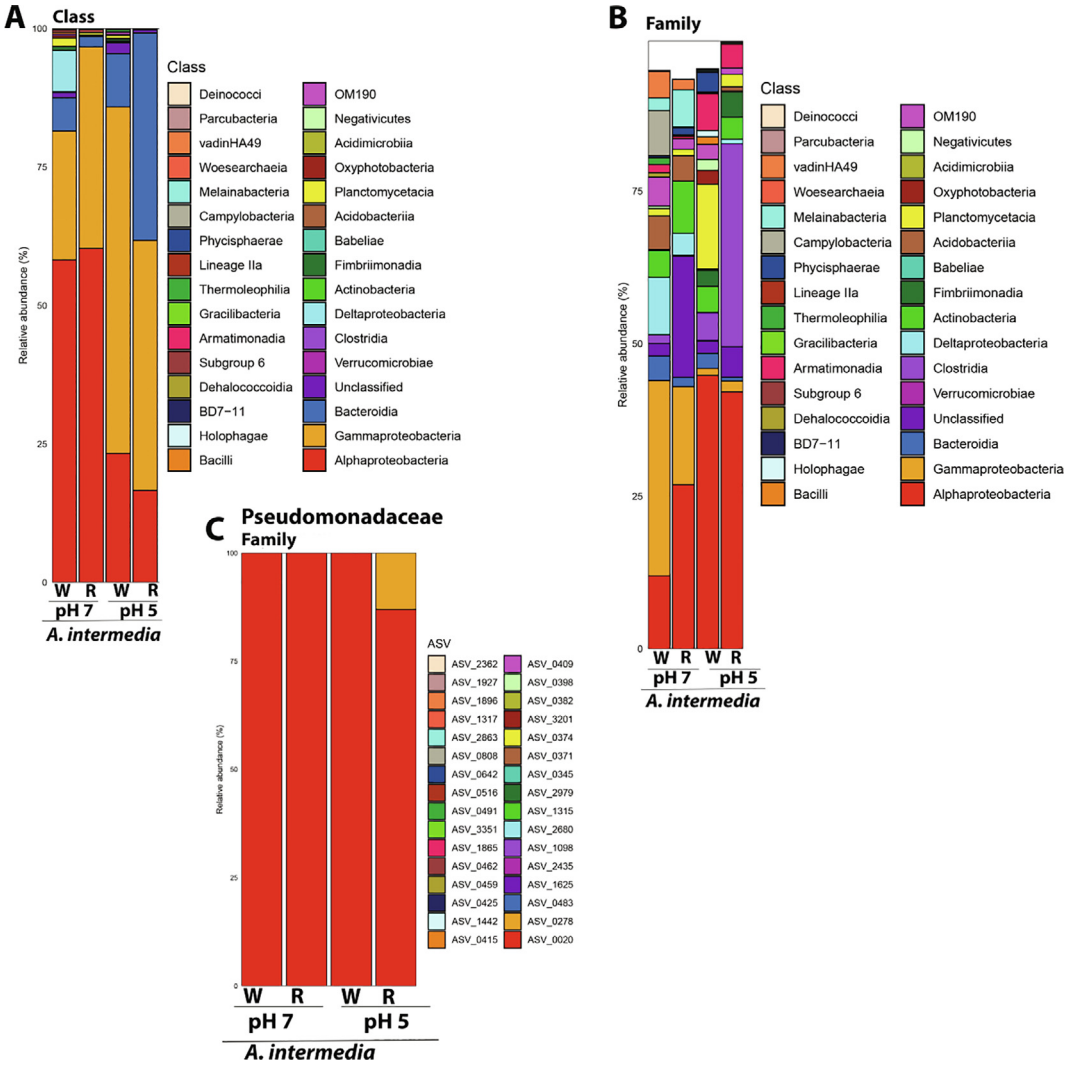

Fig. 4. Bar charts showing the relative abundance of bacterial classes (A), Families (B) and Pseudomonadaceae ASVs (C) characterized from *A. intermedia* (cultured in natural pH wheat and rice media), and *A. intermedia* (cultured in wheat and rice acidic media, pH 5). Each bar in *A. intermedia* refers to groups of 20–30 amoeba that were amplified independently but then pooled together for 16S rRNA gene sequencing. Within each bar, box color denotes class-level taxonomic association (based on ASV representative sequences) and box height gives the relative abundance (based on total reads per sample).

nity members. However, our data cannot address prevalence, i.e., the fraction of *Arcella* cells hosting a given taxon; this is beyond the scope of this study.

Composition, richness and assemblage variation

By plotting the *Arcella*–bacterial community assemblage of different habitats (Fig. 1A and B), our results showed that Proteobacteria and Bacteroidetes are the most abundant bacterial phyla associated with both cultured and environmental *Arcella* species. Our results recapitulate the findings of studies on other protists that have documented the predominance of Gammaprotebacteria, Alphaprotebacteria, and Betaprotebacteria recovered from ciliates and flagellates from marine and freshwater habitats (Gong et al., 2014; Gong et al., 2016; Martinez-Garcia et al., 2012; Serra et al., 2020).

While we could match ASVs to recognized taxa for many bacteria and some archaea, a portion of the community from environmental isolates of *Arcella* remained unclassified, encompassing almost 10–15% of the total associated bacterial community. This result underscores the need for further identification of environmental and non-pathogenic bacteria and subsequent improvement of reference databases (Lynch and Neufeld, 2016). Free-living amoebae and other protists have been recently recognized as hosts for unique and novel groups of bacteria, including the so-called microbial 'dark matter' (Gong et al., 2014; Tsao et al., 2017; Yeoh et al., 2016). These 'dark matter' bacterial lineages are often characterized by

small genome sizes, predicted auxotrophic relationships, and a total lack of cultured representatives while they represent important evolutionary lineages that holds answer to questions about symbiosis and symbiogenesis (Castelle and Banfield, 2018).

Arcella spp. host unique microbiota not shared with the surrounding media

Venn diagram analysis (Fig. 2A–C) revealed that both *A. hemispherica* and *A. intermedia* growing in rice or wheat culture each host unique ASVs, and most of these ASVs were not shared with ASVs occurring in wheat or rice media alone (Fig. 2A–B). This result suggests two scenarios: Potentially, each *Arcella* host species preferentially selects certain bacteria, represented by ASVs, from vanishingly rare abundance in the cultured medium or the environments which are then cultivated by the host to measureable abundances. Another possibility is that certain bacterial ASVs already existed as symbionts in each *Arcella* species prior to domestication in the lab. In either cases, whether the selection of bacterial communities is controlled by the host, or with flexibility to ensure environmental transmission, our results show a clear variability in the bacterial communities associated with each of the *Arcella* species.

Furthermore, the distinction in the composition of microbiome between the two species and the relative stability of their microbiome composition regardless of the culture media type suggests a strong species-specific association. For instance, *A. hemispherica* host diverse bacterial communities, but maintain taxonomically similar bacterial assemblages whether the *A. hemispherica* was cultured in wheat or rice media, which is different from *A. intermedia* that also appear to host similar taxonomic profile of bacterial communities whether it was cultured in wheat or rice media, natural or low pH. This distinction in the composition of microbiomes between closely related species suggests that the host species affects its bacterial composition, but it also points out that these colonized bacteria are resistant to host predation and successful competitors (Chen et al., 2019).

On the other hand, there were common bacterial groups showed between both *Arcella* spp. An interesting alphaproteobacterial family is Sphingomonadaceae, which are strictly aerobic chemoheterotrophs with the ability to degrade and tolerate xenobiotic compounds including many antibiotics (Suhadolnik et al., 2017; Vaz-Moreira et al., 2011). Additionally, many sphingomonads are recognized opportunistic pathogens (Vaz-Moreira et al., 2011), prompting further study of this group and the role of *Arcella* as potential environmental reservoirs. While Sphingomonadaceae were shared between both *Arcella* spp., they were noticeably more abundant in *A. hemispherica*, regardless of media condition, than *A. intermedia*. Thus, while broad taxonomic levels were sometimes present in both *Arcella* spp., their abundances were often different, suggesting possible different environmental circumstances associated with each host. Recent findings demonstrated that Alpha- and Gamma-proteobacteria, Bacteroidia, and Planctomycetacia are the most abundant classes of bacteria associated with FLA (free-living amoebae) in wastewater treatment plant (Moreno-Mesonero et al., 2020). Altogether, the similarity of each species' community composition was found to vary based on taxonomic level, as has been reported for other communities (e.g., sponge microbiome, oral microbiome) (Cuvelier et al., 2014; Utter et al., 2016). While the differences in community composition between *Arcella* spp. were less noticeable at the phylum or class levels (Fig. 1A and B), the communities were strikingly dissimilar at the family level (Fig. 3B) and even more so when fully maximizing the resolution (ASVs) (Supplementary Fig. 6). Although ASVs allow for maximum resolution from a 16S rRNA gene amplicon and can often distinguish ecologically relevant subgroups, the 16S rRNA gene sequence is ultimately a proxy for an organism. As such, genomic and physiological diversity can occur without being marked by a corresponding SNP in the 16S rRNA gene, which renders that diversity invisible to any 16S-amplicon-based method. Thus, our reports likely underestimate the true diversity present in *Arcella*.

In conclusion, we demonstrated that *Arcella* spp. host diverse bacterial communities mostly from Proteobacteria and Bacteroidetes. Further, environmental *Arcella* spp. host similar communities to cultivated *Arcella*, while cultivated *Arcella* are distinct from the bacteria in their surrounding media. Comparisons among two closely related cultured species, *A. hemispherica* and *A. intermedia,* indicated that while some community members are shared between both species, each species hosts a specific community regardless of its growth media type (rice or wheat). Our results suggest that each *Arcella* spp. tends to conserve a relatively large fraction of their microbiome, potentially through vertical transmission from a mother to daughter cell or by selection from environmental bacterial populations in the media below the detection limit. This study opens the door for future research investigating the interactions and associations between protists and bacteria that maintain these specific community compositions, which ultimately contributes to our understanding of the eukaryotic-bacterial co-evolution, speciation, and symbiosis.

Data availability

All raw sequence data generated during this study and the metadata are available under BioProject accession number PRJNA747988. The raw MiSeq reads are deposited in the NCBI SRA under accession number SRX11726850-SRX11726863.

Contributions

F.G and C.M.C. conceived of the study. F.G collected and cultured the samples. F.G. performed experiment. D. R.U. and F. G. analyzed the 16S rRNA gene sequences data. F.G. composed manuscript. All authors gave feedback on the manuscript.

Ethics declarations

Competing interests: The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejop.2021.125861.

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