The effects of amoeba grazing on bacterial populations

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Introduction

Microbial communities are complex environments populated by bacteria, viruses, archaea, and single-celled eukaryotes. Within these communities, bacterial composition has become an important topic of interest as largescale projects such as the Human Microbiome Initiative and Earth Microbiome Initiative have surveyed the diversity of bacteria in a variety of habitats[1,2]. While we can easily survey the composition of bacterial communities with next generation sequencing, understanding the forces that influence bacterial compositions is still largely a mystery due to the complexity of biotic and abiotic interactions among microbes and their environments.

Bactiverous protozoans are recognized to be one of the forces that influences bacterial compositions[3,4]. Protozoans are single-celled Eukaryotes that include amoeba, flagellates, and ciliates. Some protozoans obtain nutrients by phagocytosing bacteria, and thus act as predators of bacteria. These kinds of amoeba are often found in ocean and soil environments, where bacterial food sources are plentiful. Protozoans grazing of bacteria has been shown to be selective, mainly based on bacterial morphology[3]. Efficiency of phagocytosis is dependent on bacterial size, with most protozoans preferring to feed on medium sized cells. While no lower size limit has been shown for protozoan grazing, phagocytosis efficiency decreases with both larger and smaller cells. Filamentous morphologies, increased aggregation, increased motility, and other strategies have also been observed to hinder protozoan grazing[5–8]. Preferential grazing on certain types and morphologies of bacterial cells will influence the overall composition of a bacterial community.

This project set out to determine the effect of two newly isolated amoebae from Cedar Swamp on populations of bacteria isolated from the same environment. Over the course of three days, bacteria and amoeba were grown together and the effect of the amoeba on the overall population morphology was measured by quantitative microscopy.

Methods

Culturing amoeba

Amoeba were isolated from freshwater samples taken from Cedar Swamp. The two species of amoeba were Achala amoeba and Lev amoeba. Amoeba were grown to high density in 25 cm^2 culture flasks containing Amoeba Isolation Media (17.1 mM NaCl, 1.95 mM $MgCl_2$, 0.68 mM $CaCl_2$, 7.17 mM KCl, 5 mM MES pH 6.15, 0.5 g/L yeast extract) with a low density mixed bacterial population at < 0.1 OD600.

Bacteria – amoeba microcosms

Four microcosms for each of the two amoebae were set up in 6 well plates. Amoeba were scraped from culture flasks and inoculated in each well containing 2 mL Amoeba Isolation Medium. One of four different bacterial compositions was added at a low concentration (< 0.1 OD600) to each well. The four bacterial compositions were as follows: a mixed bacterial community, a monoculture of long rods, a monoculture of cocci, and a monoculture of vibrio. A third six well plate was inoculated with only the bacteria. Since the amoeba had a background bacterial community, a droplet of this community from both amoeba flasks was added to the no amoeba wells. Plates were incubated in ambient conditions for three days.

Phase contrast microscopy

Three days after microcosms were inoculated, the bacterial composition of each was surveyed by spotting 2 ul of culture on a microscope slide observing at 630x magnification with a Zeiss AX10 microscope. Three viewing fields were randomly chosen from each microcosm and photographed with a Zeiss Axiocam 503 mono for image analysis. Amoeba were also imaged in the six well plate to ensure growth at 100x magnification using a Zeiss inverted microscope and Axiocam 503 mono camera.

Analysis of bacterial cell morphology

Images were analyzed using the software tools FIJI[9] (extension of ImageJ[10]), Celltool[11], and Matlab. In FIJI, raw images were background subtracted with a rolling ball radius of 50 pixels and manually thresholded. Segmentation was also done in FIJI using the "Analyze particles" tool with are between 0.5 – 5 um and circularity between 0.3 - 1.0. For each image, the area and circularity of each particle was recorded as well as the number of particles in each image. Images with fewer than 20 particles were excluded from further analysis. In Celltool, contours were extracted from bacterial populations grown under the three amoeba conditions (Achala, Lev, no amoeba) and aligned. For each bacterial population, a cell morphology model was constructed that explains 90% of the morphology variance. The distribution of cell morphologies was visualized by principal components analysis.

Statistics

Distributions of bacterial size and roundness as measured by FIJI were compared using a Wilcoxon rank sum test in R. Pairwise comparisons were done between the three amoeba conditions (Achala amoeba, Lev amoeba, no amoeba) for each of the four bacterial compositions. The distributions of cell size and circularity were plotted using the R package ggplot2[12].

Results and discussion

Four bacterial cultures were incubated for three days in the presence of Achala amoeba, Lev amoeba, or no amoeba (**Figure 1**). The first bacterial culture was a mixed community with a variety of cell morphologies ranging from short rods to longer rods. The second bacterial culture was a monoculture of longer rods. The third bacterial culture was a monoculture of *Vibrio*. The fourth bacterial community was a monoculture with a small vibrio shape. In the three days, bacterial cultures remained at a low density due to the replete media conditions and amoeba predation. Amoeba reached a high density in each of the cultures (**Figure 2,3**). Faster amoeba growth was observed in Achala amoeba compared to Lev amoeba.

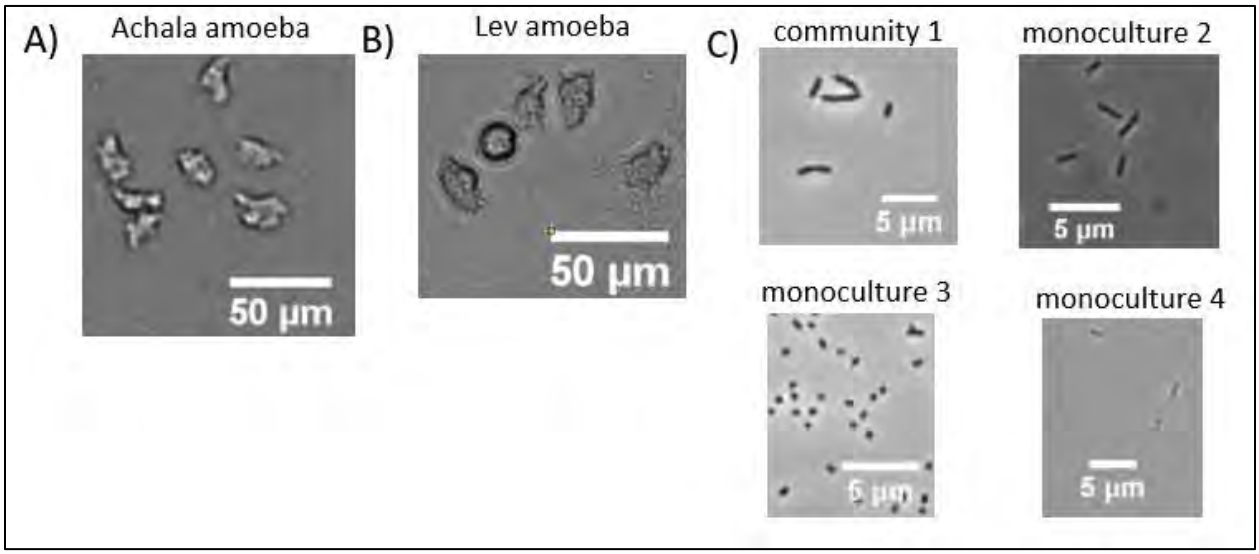

Figure 1. Amoeba and bacteria used in experiment. A) Achala amoeba viewed at 100x magnification. B) Lev amoeba viewed at 100x magnification. C) Bacteria used in experiment viewed at 630x magnification.

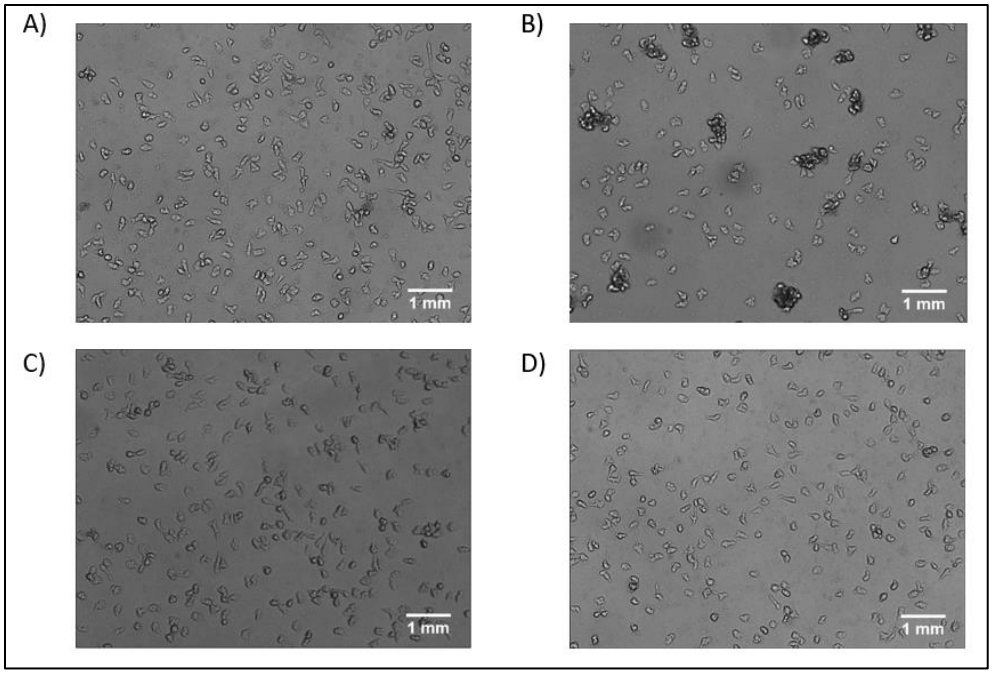

Figure 2. Achala amoeba growth after 3 days incubation with bacteria viewed at 100x magnification. A) Bacteria community 1, B) bacteria monoculture 2, C) bacteria monoculture 3, D) bacterial monoculture 4.

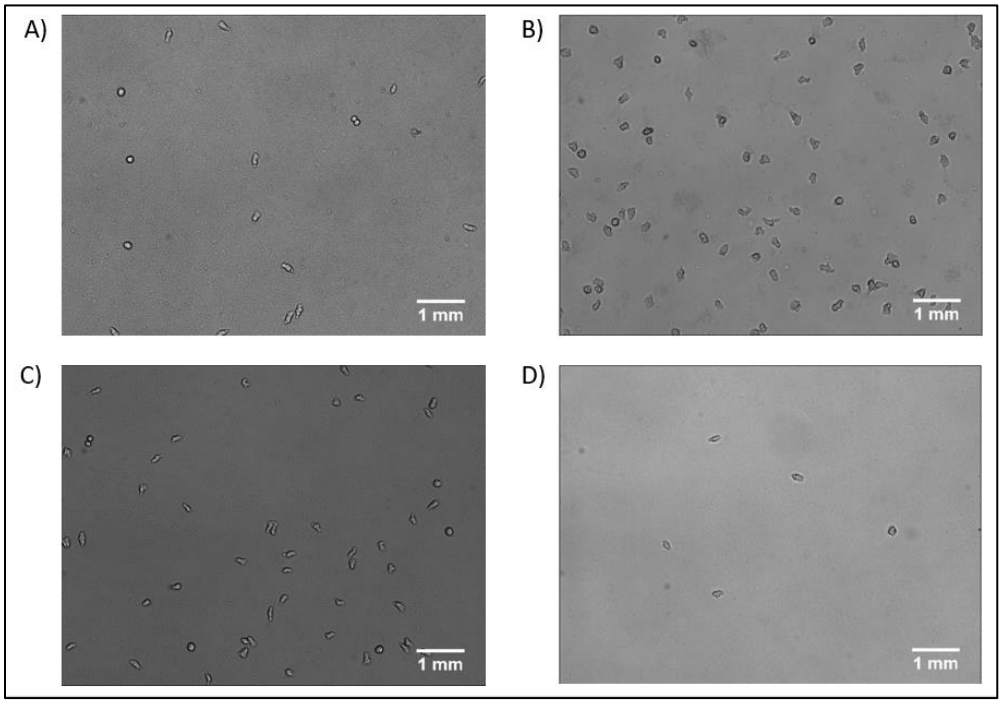

Figure 3. Lev amoeba growth after 3 days incubation with bacteria viewed at 100x magnification. A) Bacteria community 1, B) bacteria monoculture 2, C) bacteria monoculture 3, D) bacterial monoculture 4.

After three days of growth, bacterial communities in each culture were imaged at 630x magnification with phase contrast microscopy and cell morphologies were analyzed. Each bacterial condition was analyzed separately, comparing the bacterial community under the influence of predation by Achala amoeba, Lev amoeba, or no amoeba predation. Alignment of the cell contours indicated that bacterial populations varied mainly on area and roundness, together accounting for approximately 90% of morphology variation in all four bacterial conditions. Bacterial cultures varied most by cell area with the remaining variation due to cell roundness. In addition to these factors, culture 2 and 3 also varied by curvature of the rod shape between straight rods and vibrio shapes, although no major differences in this morphology were observed due to amoeba predation.

Predation by Achala amoeba resulted in a significant change in cell area in cultures 1, 2 and 4. In all three cases, bacterial cells were smaller after three days of amoeba predation than bacterial cells without amoeba predation. Roundness of the bacterial population was significantly affected in bacteria populations 1, 2 and 4. Roundness was increased in culture 1 and 2 and decreased in culture 4. Overall, the bacterial morphologies of culture 3 seemed to be unaffected by Achala amoeba predation.

Predation by Lev amoeba resulted in a significant change in cell area for cultures 1, 2, and 3. Bacterial cell size decreased in all three cultures. Roundness of the bacterial populations was significantly different in all four cultures under Lev amoeba predation. Roundness was increased in cultures 1, 2, and 3 and decreased in culture 4. The influence of Lev amoeba on bacterial population morphology is very similar to the changes caused by Achala amoeba. While these amoebae are currently unidentified, they look similar and have similar sizes and likely prey on similar types of bacterial cells.

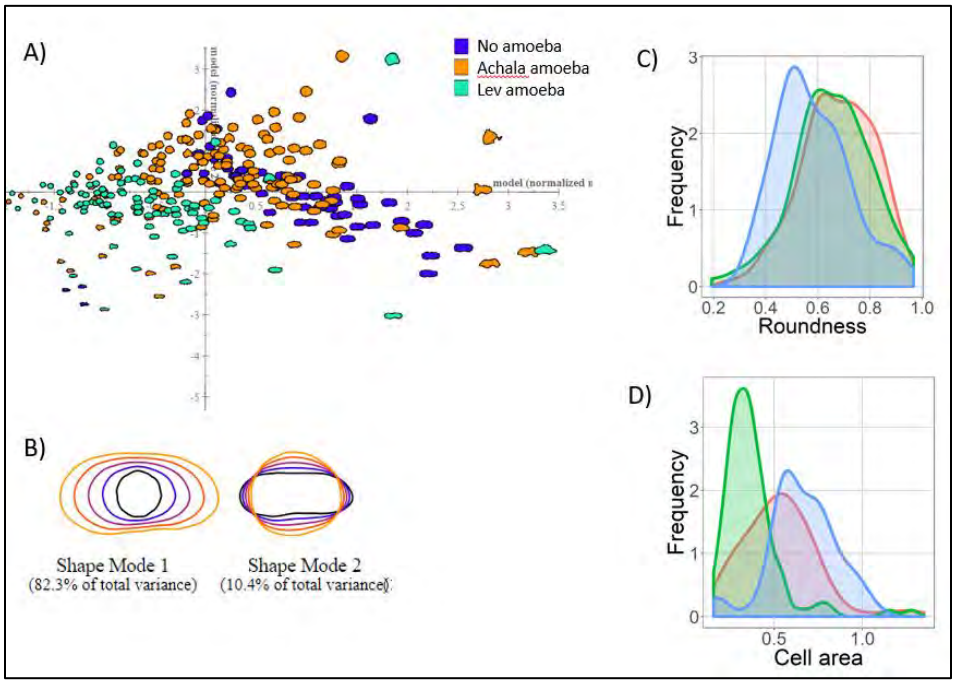

Figure 4. Cell shape analysis of the mixed bacterial community 1 after three days. A) PCA of all plotted bacteria colored by amoeba condition. B) Visualization of cell shape model showing the major contributors to cell shape variance. C) Frequency plot of the distribution of bacterial roundness. D) Frequency plot showing distribution of bacteria cell area.

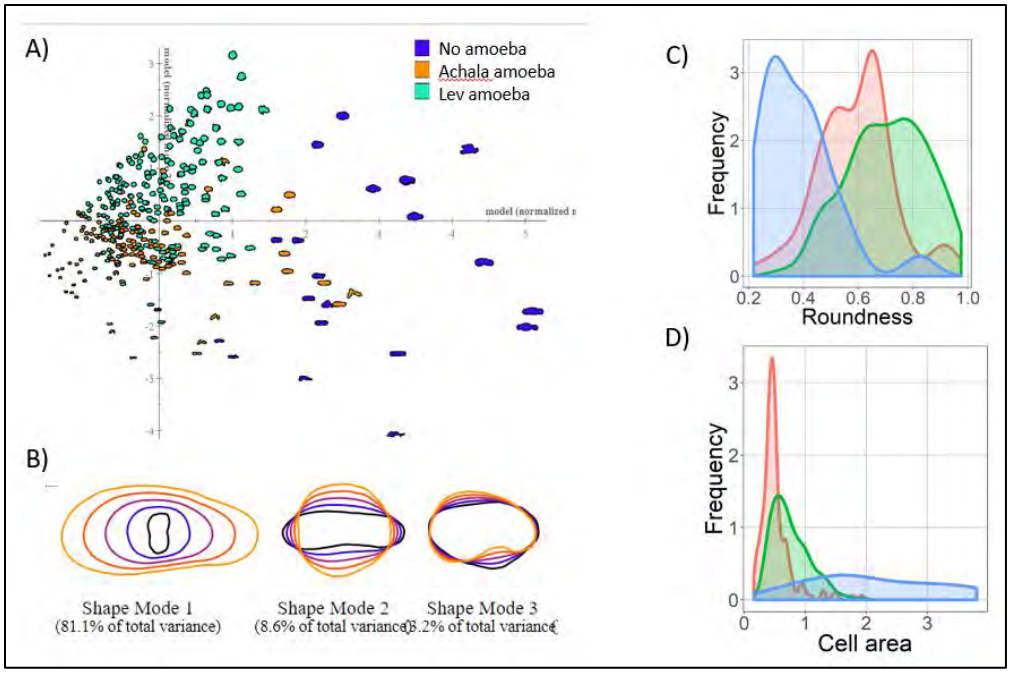

Figure 5. Cell shape analysis of bacterial monoculture 2 after three days. A) PCA of all plotted bacteria colored by amoeba condition. B) Visualization of cell shape model showing the major contributors to cell shape variance. C) Frequency plot of the distribution of bacterial roundness. D) Frequency plot showing distribution of bacteria cell area.

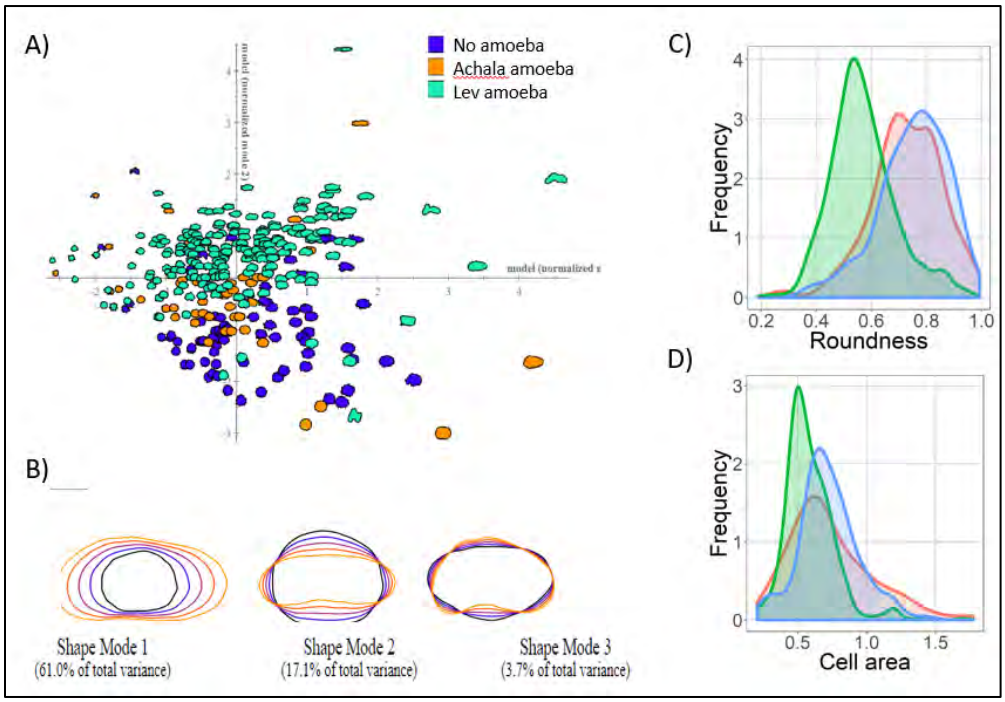

Figure 6. Cell shape analysis of bacterial monoculture 3 after three days. A) PCA of all plotted bacteria colored by amoeba condition. B) Visualization of cell shape model showing the major contributors to cell

shape variance. C) Frequency plot of the distribution of bacterial roundness. D) Frequency plot showing distribution of bacteria cell area.

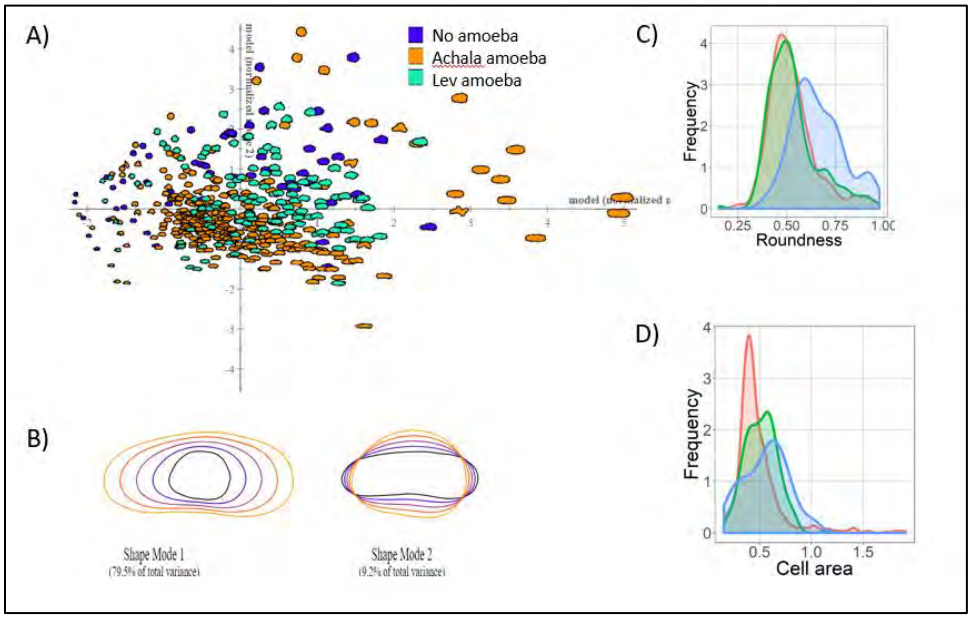

Figure 7. Cell shape analysis of bacterial monoculture 4 after three days. A) PCA of all plotted bacteria colored by amoeba condition. B) Visualization of cell shape model showing the major contributors to cell shape variance. C) Frequency plot of the distribution of bacterial roundness. D) Frequency plot showing distribution of bacteria cell area.

Morphological changes seen in these experiments are likely due to changes in population composition due to selective predation by amoeba. Amoeba are known preferentially feed on bacteria based on cell size among other factors[3]. The differences in cell morphology likely only provide modest protection from amoeba predation, not immunity. Nonetheless, bacteria with morphologies slightly further from the amoebas' preferred range will be more likely to persist. The modest changes seen after three days may become more dramatic if the experiment were continued, however the experiment was halted because many of the amoeba entered the cyst state after one week. In the cyst state, amoeba would no longer be preying on bacteria and would not exert any selective pressure. Addition of additional nutrients in the culture before amoeba form cysts would be advisable if the experiment were to be repeated for a longer time.

Achala amoeba were observed clumping together when grown with bacterial monoculture 2 (**Figure 8**). Clumping to this degree was not observed in Achala amoeba in any other condition or in Lev amoeba. Clumping began occurring after three days when all the amoeba were still in the amoeboid state. As the amoeba continued to grow and form cysts, clumping was observed to increase. The only difference in this condition was the presence of bacteria monoculture 2, thus the clumping may be a result of a direct or indirect interaction between the amoeba and bacteria.

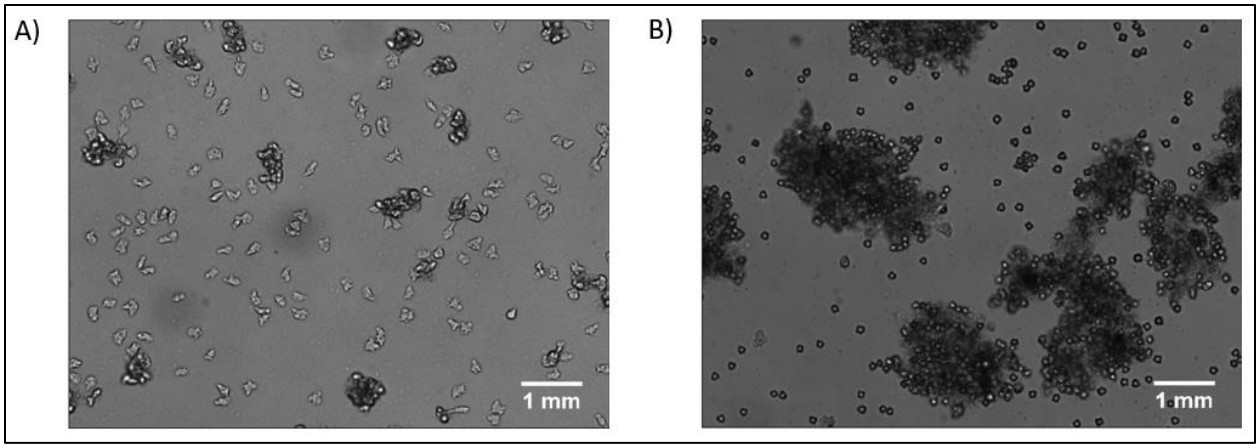

Figure 8. Achala amoeba growing with bacterial culture 2 after A) three days and B) 10 days. Amoeba are observed clumping only in this condition.

Bacterial community	Amoeba 1 area (Mean ± SD um^2)	Amoeba 2 area (Mean ± SD um^2)	No amoeba area (Mean ± SD um^2)
1	0.53 ± 0.21***	0.37 ± 0.17***	0.66 ± 0.19
2	0.52 ± 0.26***	0.76 ± 0.31***	2.11 ± 1.02
3	0.74 ± 0.30	0.59 ± 0.18***	0.73 ± 0.22
4	0.49 ± 0.23***	0.52 ± 0.16*	0.56 ± 0.22

Table 1. Average cell area of each bacterial population after 3 days growth with or without amoeba. Area measured by FIJI and displayed in microns^2. Distributions that are significantly different from the no amoeba condition as calculated by the Wilcoxon rank sum test are indicated. (* $p < .05$, ** $p < .001$, *** $p < .001$)

Bacterial community	Amoeba 1 roundness (Mean ± SD)	Amoeba 2 roundness (Mean ± SD)	No amoeba roundness (Mean ± SD)
1	0.68 ± 0.14***	0.65 ± 0.15***	0.58 ± 0.14
2	0.59 ± 0.14***	0.71 ± 0.15***	0.38 ± 0.14
3	0.73 ± 0.13	0.57 ± 0.12***	0.75 ± 0.13
4	0.53 ± 0.12***	0.53 ± 0.13***	0.66 ± 0.13

Table 2. Average cell roundness of each bacterial population after 3 days growth with or without amoeba. Roundness measured by FIJI and given on a scale between 0 and 1 with 1 being perfectly round. Distributions that are significantly different from the no amoeba condition as calculated by the Wilcoxon rank sum test are indicated. (* $p < .05$, ** $p < .001$, *** $p < .001$)

Conclusions

In three days, amoeba predation was observed to affect the population morphology of bacterial communities. These morphological changes are likely due to selective predation on certain species of bacteria, leaving more resistant morphologies to grow and dominate the culture. The main morphological change seen was in bacterial cell size, followed by cell roundness. Achala amoeba and Lev amoeba had similar effects on bacterial population morphologies when compared to bacteria growing without amoeba. These results suggest that bactiverous amoeba may affect the overall population structure and morphology of natural bacterial communities.

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References

1. Human Microbiome Project Consortium, T. Structure, function and diversity of the healthy human microbiome. *Nature* **486,** (2012).

2. Gilbert, J. A., Jansson, J. K. & Knight, R. The Earth Microbiome project: successes and aspirations. *BMC Biol.* **12,** 69 (2014).

3. Hahn, M. W. & Höfle, M. G. Grazing of protozoa and its effect on populations of aquatic bacteria. **35,** 113–121

4. Jürgens, K. & Matz, C. Predation as a shaping force for the phenotypic and genotypic composition of planktonic bacteria. **81,** 413–434

5. Matz, C. & Kjelleberg, S. Off the hook – how bacteria survive protozoan grazing. **13,** 302–307

6. Matz, C. & Jürgens, K. High Motility Reduces Grazing Mortality of Planktonic Bacteria. **71,** 921–929

7. Matz, C., Bergfeld, T., Rice, S. A. & Kjelleberg, S. Microcolonies, quorum sensing and cytotoxicity determine the survival of Pseudomonas aeruginosa biofilms exposed to protozoan grazing. **6,** 218–226

8. Simek, K. *et al.* Morphological and compositional shifts in an experimental bacterial community influenced by protists with contrasting feeding modes. **63,** 587–595

9. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9,** 676–682 (2012).

10. Schindelin, J., Rueden, C. T., Hiner, M. C. & Eliceiri, K. W. The ImageJ ecosystem: An open platform for biomedical image analysis. *Mol. Reprod. Dev.* **82,** 518–529 (2015).

11. PINCUS, Z. & THERIOT, J. A. Comparison of quantitative methods for cell-shape analysis. *J. Microsc.* **227,** 140–156 (2007).

12. Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*. (Springer-Verlag New York, 2009).