Predation enhances survival and growth of pathogenic and non-pathogenic isolates of *Vibrio cholerae*

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Jose M. Solis-Ruiz
Abstract:

*Vibrio cholerae* a major health problem in developing countries because of its ability to cause the disease cholera in humans. As *V. cholerae* bacteria are common inhabitants of temperate marine and brackish water environments worldwide, they are subject to intense top-down pressure by amoebae, which can affect their survival and replication in natural reservoirs. In these environments, interactions with amoebae, single celled eukaryotic predators of bacteria, are inevitable. This study examined the interactions between a model amoeboid predator, *Acanthamoeba castellanii*, and diverse strains of *V. cholerae* to better understand the adaptations of these bacteria to eukaryotic grazing pressure over 48 hours. Our analysis showed that *V. cholerae* TP (non-pathogenic GFP variant) and AR4100 (pathogenic GFP variant) increased growth (TP growth difference: 4.29-fold, AR4100 growth difference: 4.67-fold) when co-cultured with *A. castellanii* in comparison to *E. coli* co-culture, while SIO (non-pathogenic GFP variant) had no growth difference (SIO growth difference: -1.05-fold). In general, *V. cholerae* isolates induced cyst formation in amoebae within 48 hours and remained viable after ingestion. These findings suggest that complex predator-prey relations may ultimately impact the survival of strains of these potential pathogens in the environmental reservoirs in significantly different ways, changing the population structure of these organisms in their communities.

I. Introduction/background:

To date, *Vibrio cholerae*, a gram negative bacterium, a responsible for eight recorded pandemics of the severe diarrheal disease cholera since the 1800’s with an estimated 120,000 deaths annually [6]. The disease is caused by an acute intestinal infection characterized by
devastating watery diarrhea, which leads to rapid dehydration and death occurring in 50 to 70% of untreated patients [6,9]. Cholera is a major health issue in impoverished and developing countries with poor socioeconomic conditions and sanitation systems. Cholera epidemics are often spread after natural disasters (e.g. an earthquake in Haiti in 2010) or extreme weather events (e.g., flooding from monsoons in Bangladesh) when access to safe drinking water becomes restricted, forcing people to use water sources contaminated with pathogenic bacteria [8]. This situation is exacerbated in situations where fuel for boiling water is scare, such as in Bangladesh, India, Syria and East Africa [4,8]. In geographical regions such as Bay of Bengal, where V. cholerae are endemic, the prevailing patterns of disease frequencies along coastal areas follow distinct seasonal trends, suggesting that cholera infection cycles are driven by similar physical and environmental factors [13].

V. cholerae infections typically begins with the drinking of water contaminated with significant numbers of V. cholerae bacteria [28]. After ingestion, V. cholerae colonizes the small intestine of humans where it undergoes extensive growth and induces the development of disease symptoms [9]. Activation of the expression of pathogenic genes carried by the bacteria occur during colonization, as pathogenic V. cholerae strains begin to produce and secrete the main effector of the disease, Cholera Toxin (CTX), into the intestinal lumen. The CTX protein is an oligomeric complex comprised of one α subunit and five β subunits [28]. During the intoxication of the host, β subunits mediate receptor binding and entry into the vesicular pathway [7]. Upon binding, the enzymatic α1 domain of the α subunit is translocated into the target cell [7]. Here, covalent modification of intracellular targets leads to the activation of adenylate cyclase and a sequence of signaling events that results in the massive efflux of H₂O,
Cl\(^-\), Na\(^+\), K\(^+\), and HCO\(_3\)^\(-\) ions into the intestinal lumen [7 & 9]. This ionic imbalance causes diarrhea in infected individuals and completes the infection cycle by releasing copious amounts of newly replicated and highly infective *V. cholerae* microbes back into the environment [3].

In aquatic habitats, *Vibrios* are known to survive as free-living cells or as zooplankton- and phytoplankton-associated bacterial populations that grow in biofilms [13]. The ability to form biofilms on biological surfaces has been shown to promote the survival in aquatic environments for longer times than suspended free living cells [25]. The relevance of the interactions between *V. cholerae* and eukaryotes to disease outbreaks has been shown in endemic areas such as Bangladesh, where seasonal zooplankton blooms accompany have been linked to cholera outbreaks [25].

The ability to survive in biofilms provides one mechanism for this organism’s persistence in natural reservoirs. However, it has been shown that genetic variation between strains of pathogenic and non-pathogenic *V. cholerae* can lead to differences in the biofilm formation, suggesting that individual strains have developed alternative mechanisms of survival that may influence their respective fitness in different environmental niches [18]. This study attempts to further understand differences in individual population survival strategies by examining the interaction of strains of *V. cholerae* with protozoa grazers, and investigating whether these strains have differential sensitivity/resistance to grazing pressure. Bacterial ingestion by grazers was investigated to determine physiological changes in amoebae that may lead to enhanced bacterial persistence, as has been documented for other bacterial isolates [1, 2, 15, 17, & 26].

In a recent review by Thomas, et al. (2009) free-living amoebae were assessed for their ability to harbor intracellular pathogenic microorganisms as a potential risk to water quality
The authors noted that, despite serving a general role as prey for eukaryotic grazers like amoebae, some bacteria can actually benefit from interactions with these predators, specifically by their ability to escape predation by resisting intracellular digestion, for example, allowing growth in the presence of protozoan predators. Grazing pressure on bacteria from amoebae in natural environments is typically high, as amoebae are ubiquitous inhabitants of soils, freshwater and marine systems, and are found often in association with biofilms. In this review, it was also noted that protozoan grazing on bacteria is a key factor regulating community composition and population dynamics within biofilms. Antipredatory mechanisms, including microcolony formation, toxin production, and the presences of an intact capsule, all have a documented role in the ability of diverse types of bacteria to prevent protozoan predation [26]. This brings into question what the effects of amoebae grazing are on various strains of the water-borne pathogen, *Vibrio cholerae*.

A recent research study done by Shanan, et al. (2011) looked to determine if amoeba and *Vibrio cholerae* can be found in the same aquatic environment by collecting water samples from cholera-endemic areas in Sudan and quantifying the co-occurrence of these microbes [26]. Their results indicate *Vibrio cholerae* and amoebae are often found in the same environment, as both were present in 89 % of the water samples collected. In another study by Abd, et al. (2007), it was found that *Vibrio cholerae* can grow intracellularly within amoebae and that the intracellular bacteria were culturable even after amoebae underwent encystation [2]. Cyst formation by amoebae allows these cells to be highly resistant to various physical and chemical stresses, and can act as a protective environment allowing for the persistence of intracellular microorganisms [5,12, 13, & 26].
In another study done by Abd, et al. (2007), the intracellular behavior of *Vibrio cholerae* strain O139 and O1 after ingestion by *Acanthamoeba castellanii* was investigated with respect to the differences in the ability of these bacteria to express extracellular components [1]. For example, O139 and O1 both express the toxin-coregulated pilus (TCP) and the mannose-sensitive haemagglutinin (MSHA) pilus, which are needed for the colonization of the human intestine and zooplankton, respectively. Also, O139 has a capsular polysaccharide and different lipopolysaccharide from the O1 strain. Initially, these differences were hypothesized to contribute to differences in the grazing susceptibility of each strain in the presence of *A. castellanii*. Instead, both serotypes exhibited the same behavior [1]. Both *V. cholerae* serotypes co-cultured with *A. castellani* grew in the presence of the grazer, while when cultured alone *V. cholerae* abundances decreased to undetectable levels after two weeks. They were also able to show that amoebae supported bacterial viability, and viable after intracellular growth in the amoeba.

Evidence for strain-level variation in grazing resistance exists for the model organism of *Escherichia coli*. Here it has been documented that the K1 and K5 strains of *E. coli* respond significantly different to co-culture with *A. castellani* [15]. In their study, K5 showed low association, invasion, and intracellular survival within the amoebae [15]. While the opposite was true of the K5 strain. These results suggest that *A. castellanii* plays an important role in the dissemination of pathogenic *E. coli* in the environment [15]. These research findings also mirror those of Abd, et al. (2007) [1], by indicating distinct mechanisms of association, invasion, and intracellular survival for diverse groups of bacteria, which can act as prey for amoebae predators.
A feature of these studies that has gone unaddressed is how biofilm formation by prey bacteria, which can be highly variable between *Vibrio* strains, can influence grazing behavior. In a study by Matz, et al. (2005) [17] this question was investigated using mutant lines of *V. cholerae* that were deficient in some aspect of biofilm formation. Here, it was noted in that *V. cholerae* isolates tested were constrained in growth and survival by the predatory actions of bacterivorous protists. Further, results demonstrated that planktonic, free-living *V. cholerae* cells were eliminated by grazing forces, whereas cells associated with biofilms persisted. Interestingly, it was also seen that grazing on planktonic cells selects for variant cells that form “rugose” biofilms, which results in overproduction of hydrophobic exopolysaccharide material. Defensive strategies to survive grazing are an essential prerequisite for the environmental persistence of bacterial pathogens such as *V. cholerae*, and that biofilm production constitutes an environmental refuge for a number of bacterial pathogens providing an adaptive advantage promoting their persistence. This study demonstrates the advantage of biofilm-forming bacteria over planktonic cells for persistence in the presence of strong predation pressure [17].

It is clear from the above work that *V. cholerae* can persist in the presence of amoebae, and that these amoebae may act as an environmental incubator of sorts for this important water-borne pathogen (e.g., intracellular survival of *V. cholerae* throughout the encystment cycle of amoebae). It is also apparent that the ability to form biofilm by *V. cholerae* strains provides a protective niche against protozoa grazing. However, it is unclear whether or not *Vibrio cholerae* strain variants that differ in their ability to cause disease in humans (i.e., pathogenic strains that contain encode for the CTXαβ genes and other virulence determinants vs. nonpathogenic strains that typically replace pathogenicity islands in their genomes with
other traits) exhibit differences in grazing resistance, and what the effect is of ingestion of *V. cholerae* bacteria is on the physiological state and growth of amoebae cells.

The goals of this research project were to determine whether i.) *Vibrio cholerae* strain variants (pathogenic and nonpathogenic) are equally sensitive to grazing pressure by *A. castellanii*, ii.) ingestion of these bacteria affect amoebae cells causing a change in the growth of amoebae or its physiological state (i.e., a change from the predatory trophozoite for into a cyst), and iii.) if biofilm-formation ability confers resistance against amoebae grazing in these *V. cholerae* strain variants, as was shown when flagellates, which have different feeding mechanisms, were used against similar strains [17].

**II. Methodology:**

**Predator (*Acanthamoeba castellanii*):**

*Acanthamoeba castellanii* was chosen as our protozoan grazer because it is ubiquitous throughout the environment, specifically being found in the same aquatic environments as *Vibrio cholerae* isolates [14 & 22]. *A. castellanii* can be grown under axenic conditions and it’s genome is sequenced making it possible to perform downstream “omics” experiments. Table 1 lists the amoeba, bacterial strains and plasmids used for co-culture experiments. As a control for grazing in the presence of amoebae, *E. coli* S17-1 was used [15, 23, & 25].

<table>
<thead>
<tr>
<th>Strain Designation or Plasmid</th>
<th>Relevant Genotype or Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>A. castellanii</em> Neff</td>
<td>Wild-type</td>
<td>[20]</td>
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A.

E. coli
S17-1λπ  recA pro hsdR RP4-2-Tc::Mu-Km::Tn7, p519ngfp plasmid [23]

V. cholerae
S4100  SIO strain, gfp inserted downstream of ribosomal rRNA promoter, non-pathogenic to humans [19]
S100f1  SIO strain, p519ngfp plasmid, non-pathogenic to humans [19]
T100f1  TP strain, p519ngfp plasmid, non-pathogenic to humans [19]
AS4100  O1 El Tor, smooth variant, gfp downstream of ribosomal rRNA promoter, pathogenic to humans [27]
AR4100  O1 El Tor, rugose variant, gfp downstream of ribosomal rRNA promoter, pathogenic to humans [27]

Plasmids
p519ngfp  RSF1010 derivative containing pnptII promoter before gfp gene, mob, Km' [16]

A. castellanii axenic culture:

A. castellanii strain Neff obtained from the American Type Culture Collection was originally grown axenically prior to co-culturing with V. cholerae in PYG medium (Peptone 1.25 g, Yeast Extract 1.25 g, Dextrose 20.0 g, DI Water 1000 mL). After initial culturing for 5 days five parallel culture lines were created from this initial A. castellanii stock under aseptic conditions in sterile cell culture flasks with 10 mL PYG medium without shaking. After five days of culture, a new line of A. castellanii from each parallel stock was created using 500 μL of the most current line to inoculate a new flat flask containing fresh, sterile PYG medium. Periodic checks of axenic conditions of cultures were performed by plating on Luria-Burtani (LB)-agar medium (DI Water 1000 mL, Tryptone 10 g, NaCl 10 g, Yeast extract 5 g, Agar 15 g) and by fluorescence microscopy.

Co-culture Setup:

A. castellanii cells less than five days old were used for co-culture experiments. Cells were verified to be in the trophozoite (amoeboid, active feeding) stage of the growth cycle, with little evidence of cyst formation, under microscope analysis. All bacterial strains used in co-cultures
were first grown from glycerol stocks as streaks on LB agar with appropriate antibiotics at 37 °C. Overnight cultures from purified colonies were grown in LB broth with appropriate antibiotics at 37 °C with shaking. Prior to the start of each co-culture experiment bacteria were centrifuged at 15,000 rpm for 10 minutes to pellet cells. The supernatant was discarded and the cells were resuspended in sterile, fresh 354 Acanthamoeba medium (Proteose Peptone 15.0 g, Glucose 15.0 g, KH₂PO₄ 0.3 g, Vitamin B₁₂ 1.0 µg, Biotin 200.0 µg, Thiamine 1.0 mg, L-Methionine 14.9 mg, Salt Solution [CaCl₂ 2H₂O 0.15 g, FeCl₃ 0.02 g, MgSO₄ 7H₂O 2.46 g, DI water 100.0 ml] 1.0 ml, DI water 1000 mL; Autoclaved @121 °C for 15 minutes) at final concentrations of approximately 10⁴ cells/mL.

For each co-culture experiment two A. castellanii lines were mixed together aseptically into a sterile 15 mL centrifuge tube at a final volume of 10 mL. Cell counts were determined using a Cell Counting Bürker Chamber to set appropriate dilution volume needed. A. castellanii were then centrifuged at 500 rpm for 5 minutes to gently pellet cells. The supernatant was discarded and cells were resuspended in sterile, fresh 354 medium at approximately 10⁵ cells/mL. Cells were left without shaking at room temperature for 24 hours to acclimate to the culture conditions before setting up the co-culture experiment.

For the start of each co-culture experiment 10⁵ cells/mL of amoebae and 10⁴ cells/mL bacteria were added together in 354 medium at a final volume of three milliliters for each co-culture replicate. Experiments were carried out in 6-well microtiter dishes for up to five days at room temperature with no shaking. Two 6-well plates were used for the final setup. One plate contained A. castellanii and 354 media controls and replicates. Three replicate wells on this plate contained an inoculation of 10⁵ cells/mL of amoebae and brought up to 3 mL with 354
media. The other three wells on this plate contained 3 mL of 354 media alone to ensure sterile techniques. The other 6-well plate contained three replicates of *A. castellanii* and bacteria co-cultured together, the other three wells contained replicates of the bacterial strain tested alone in 3 mL of 354 media, as a no-grazing control.

Measurements of cell counts and growth status of amoebae and bacteria were made at t = 0, 24, and 48 hours. Amoebae were counted at each time point in control and co-cultured replicates using the cell counting Bürker Chamber to assess the numbers of trophozoite and cyst cells. Bacterial concentrations in co-cultures and no-grazing control replicates were determined by creating serial dilutions of $10^{-3}$, $10^{-4}$, and $10^{-5}$ and plating each on LB or TCBS agar plates (*Vibrio* selective media, Sigma-Aldrich, Inc) with appropriate antibiotics. The plates were then incubated at 37 °C. The colony forming units per plate were counted and were then multiplied by their dilution factor for the total amount of cells contained in each experimental replicate per mL at each time point.

**Cell Counting using Bürker Chamber:**

10 μL of resuspended *A. castellanii* in 354 media was pipetted onto Bürker chamber cell counting grid. The Bürker chamber has 9 large squares (1 mm² each), divided by double lines (0.05 mm apart) into 16 group squares. The double lines form small 0.0025 mm² squares, with the chamber depth being 0.1 mm. Cells are counted in 4 random large squares (identified by the triple line and shaded in the figure). Average cell counts are taken from all 4 squares. Counts of both trophozoite and cyst cell forms were recorded. Cells per mL were then calculated from the following equation:
Quantification of Surviving Bacteria Internalized by *A. castellanii*:

Co-culture experiments of *V. cholerae* and *A. castellanii* were performed as described above. After 48 hours co-cultures were centrifuged at 500 rpm for 5 minutes to separate amoebae from planktonic, external bacteria. Pelleted amoebae were resuspended in a phosphate-buffered saline solution containing the antibiotic Rifampicin (100 µg/mL) and Na-azide (0.01%). Centrifugation and lysis of external bacteria was repeated. Resuspended amoebae containing ingested *V. cholerae* were lysed with PBS and 0.5% Na-deoxycholate to release internal bacteria. Cell counts of internalized bacteria were made by plating dilutions of the amoebae lysate on TCBS *Vibrio*-selective media. Bacteria from amoebae that were not lysed in the presence Na-deoxycholate were counted and compared against as a negative control for background external bacteria in these co-culture experiments.

Statistical Analysis:

R version 3.3.1’s ANalysis Of VAriance (ANOVA) and Tukey’s Honest Significant Difference (TukeyHSD) was used for the all statistical analysis in this study. A two-way analysis of Variance was done to determine the differences between time points, bacterial isolates, and amoeba physiological state changes. A post hoc analysis was done using Tukey’s Honest Significant Difference (TukeyHSD) analysis, which allows the analysis for all pairwise comparisons [21].

III. Results:

Internalization dynamics of *Vibrio* in co-culture with *A. castellanii*: 

\[
\frac{\text{Cells}}{\text{mL}} = \frac{\sum \text{cells counted in 4 squares}}{4} \times \text{dilution factor} \times 10^2
\]
An investigation into the internalization dynamics of *V. cholerae* S4100 bacteria by *A. castellanii* was performed in collaboration with Drs. Malcom Lowry and Juan Giarrizzo at Oregon State University using flow cytometry to track and count amoebae cells containing internalized *gfp*-labelled bacteria at different time points. After 24 hours of co-culture with bacteria, approximately 50% of amoebae in the sampled co-culture (n = 5,000) demonstrated increased relative fluorescence of almost an order of magnitude over background levels (Figure 1a; background ≈ 10^2), indicative of rapid ingestion of *gfp*-labelled bacteria. After 48 hours of co-culture incubation with S4100 bacteria, nearly 100% of the amoebae sampled demonstrated increased fluorescence over background levels of fluorescence evident in control cultures of *A. castellanii* without bacteria (Figure 1b). These results indicate that almost all amoebae internalize *V. cholerae* bacteria within 48 hours under the defined co-culture conditions.

**Figure 1:**
A. Internalized *V. cholerae* (S4100 strain) in *A. castellanii*. Relative fluorescence (a.u. = arbitrary units) of amoebae in co-culture with *V. cholerae* S4100 for 24 hours (purple histogram). Green lines represent the histogram for fluorescence of control cultures of *A. castellanii* with no bacteria present (i.e., background fluorescence of amoebae).
B. *A. castellanii* fluorescence after 48 hours of co-culture with S4100 (purple histogram) and with no bacteria (green line).

**Survival of Internalized bacteria in co-culture with *A. castellanii*:**
Experiments quantifying the viability of internalized bacteria by amoebae were performed in order to address the question of whether *V. cholerae* bacteria are able to survive intracellularly after ingestion by *A. castellanii*. After 48 hours of co-culture with amoebae to ensure sufficient time for ingestion, external bacteria that were not internalized were killed with antibiotics and Na-azide. Figure 2 depicts the number of ingested and culturable *V. cholerae* that were released from amoebae cells lysed with Na-deoxycholate and grown on TCBS selective agar plates. In two replicate experiments more than two orders of magnitude greater numbers of culturable cells were obtained from amoebae lysate than control samples of non-lysed amoebae, indicating an increased viability of *V. cholerae* cells that have been ingested by *A. castellanii*.

![Internalized SIO Viability](image)

**Figure 2:** Culturability of ingested *V. cholerae* S4100 variant in *A. castellanii* after amoebae lysis using PBS and 0.5% sodium-deoxycholate.
Differential Bacterial Growth Under Grazing Pressure:

Pathogenic and nonpathogenic isolates *V. cholerae* (Table 1) were co-cultured with *A. castellanii* to better understand prey sensitivity between strains over time. As a control, *E. coli* S17-1 was grown in co-culture with *A. castellanii* and alone. Figure 3 provides an overview of the differential bacterial growth observed under predatory grazing pressure.

![Bacterial Isolate Growth Under A. castellanii](image)

**Figure 3:** Change in colony forming units (CFUs) of bacterial prey over two days when grown in co-culture *A. castellanii*.

Under these conditions all bacterial isolates demonstrated a trend of increased colony-forming units throughout the course of the experiment (p-value < 0.05). Here, the colony-forming units of the S100f1 strain, which is non-pathogenic to humans, increased significantly by 4.443-fold over the 2-day experiment (p-value < 0.05). A two-way analysis of variance yielded significant difference between bacterial isolate colony forming units at 24 and 48 hours. TukeysHSD post hoc pairwise comparison analysis (see Statistical Analysis) indicated no
significant difference between 24 and 48-hour samples of E. coli, and T100f1. Although, the colony-forming units of AR4100 at 24 and 48-hours showed a significant difference (AR4100 difference = 2.926-fold, p-value < 0.05) as well.

In order to take into consideration growth in the absence of predation, the values of CFUs recorded for each experiment were normalized by the CFUs recorded for the bacteria when grown in isolation. Here the patterns of growth in the presence of the predator relative to absence are more clearly illustrated (Figure 4).

Using R’s two-way ANOVA and TukeyHSD’s function determined a significant difference between bacterial isolates (p-value < 0.05), and bacterial isolates at different time points (p-value < 0.05) was seen. Under the first model, bacterial isolate CFUs showed a significant pairwise differences. Both AR4100 and T100f1 had increased CFUs in comparison to E. coli (p-value < 0.05). AR4100 showed a 2.260-fold CFU increase in comparison to E. coli, while T100f1 showed a 2.524-fold increase. S100f1 in comparison to E. coli had no significant difference (p-value = 0.865).

Under the secondary model, bacterial top down pressure relative to absence of a predator can be assessed at 48 hours. Isolates T100f1 and AR4100 showed increased bacterial CFUs in comparison to E. coli (p-value < 0.05) at 48 hours. T100f1 showed a 4.289-fold CFU increase and AR4100 showed a 4.669-fold CFU increase relative to E. coli at 48 hours. Only S4100 showed no difference in CFUs at 48 hours in comparison to E. coli. Figure 4 illustrates bacterial top down pressure relative to bacteria grown in the absence of a predator. Figure 5 shows that similar phenotypic characteristics were exhibited by all the Vibrio strains with
respect to locomotion, fluorescence, and individual *Vibrio* morphology after 24 and 48 hours of culturing with and without amoebae predators.

Figure 4: Relative bacterial growth of co-cultured bacteria with *A. castellanii* vs. cultured bacteria alone. Reported values are the averages of three independent experiments and error bars represent standard deviations of these values.

Figure 5: *Vibrio* isolate activity in co-culture and in cultured in isolation for AR4100 and S100f1 after 48-hours. Other strains exhibited similar characteristics of locomotion, fluorescence, and individual morphology (data not shown).

**Physiological State Change of *A. castellanii* Grown in Co-culture with Bacteria:**

To understand if pathogenic and nonpathogenic *V. cholerae* strains induce physiological changes in *A. castellanii*, the morphology of *A. castellanii* was assessed when grown in culture
with and without prey *V. cholerae* or *E. coli*. Here, amoebae cultures were assessed for the percentage of cells observed to be in either the trophozoite or cyst developmental state, the latter being indicative of a general stress response in the predators.

Figure 6 shows the ratio of free-living amoebae to cyst in various co-cultures when normalized to control cultures of amoebae alone. Here, *E. coli* strain S17-1, which was used as a grazing sensitive control bacterial culture, did not significantly induce cyst formation relative to the negative controls, indicating that this bacterium did not have a negative effect on the morphology of the amoebae under co-culture conditions. Similarly, co-culture of *A. castellanii* with *V. cholerae* strain AR4100 appeared to not significantly induce cyst formation in the predators, although a small, but insignificant, increase in the proportion of cysts in the co-culture was observed after 48 hours. Interestingly, the AS4100 strain, which is almost genetically identical to AR4100 with the exception of a small mutation that switches its biofilm forming phenotype from rugose to smooth, also demonstrates no significant effect on cyst formation after 24 hours. However, after 48 hours there is a dramatic increase in the amount of cysts counted in the co-culture.

In contrast to co-culture with these pathogenic isolates, co-cultures of amoebae with the non-pathogenic *V. cholerae* strains of S100f1 and T100f1 demonstrated significant increases in the cysts present after only 24 hours (p-values = 0.0167 & 0.0033, respectively). This trend continued at the 48 hours with further increases in the proportions of cysts formed under co-culture conditions with S100f1 and T100f1 (p-values = 0.0029 & 0.0005, respectively). Notably, after 48 hours nearly all amoebae co-cultured with T100f1 had converted into the cyst developmental state. The ratio of free-living trophozoites to cysts in all negative control
experiments through all time points was 94.36% ± 0.03%, demonstrating that growth alone does not significantly trigger a switch to the cyst form.

Figure 6: Cyst formation of A. castellanii co-cultured with E. coli, AR4100, AS4100, T100f1, and S100f1 relative to the negative controls.
In order to qualitatively assess the culturability of bacteria after more prolonged incubations, *Vibrio* bacteria from cultures with and without amoebae were plated on selective TCBS medium for up to six days after initial inoculations. Figure 7 shows the results of these experiments. Under these conditions, *V. cholerae* co-cultured with amoebae remained active and viable after 6 days, while the negative control *V. cholerae* cultures were not.

**IV. Discussion:**

*V. cholerae* is a bacterium that persists in aquatic environments, and is the etiological agent of the disease cholera [13 & 22]. It is a major issue in developing countries with poor socioeconomic conditions, sanitary systems and public hygiene are rudimentary, no safe
drinking water is available, and during floods [15]. In order to cause cholera, \textit{V. cholerae} must colonize the small intestine with extensive bacterial multiplication [9]. Even then, \textit{V. cholerae} cells must persist in the environment at high concentrations, as infectious doses in humans have been shown to require upwards of $10^6$ cells per mL [1 & 6].

The goals of research project were to investigate the interaction between predatory amoebae and potential prey bacteria, in order to better understand the population dynamics of \textit{V. cholerae} in natural reservoirs. Specifically, this work investigated: i) whether \textit{Vibrio cholerae} strain variants (pathogenic and nonpathogenic; rugose and smooth biofilm forming variants) are sensitive to amoeba grazing, and ii) whether different strains affect amoebae cells causing a physiological change (trophozoite or cyst formation).

Flow cytometry results of \textit{A. castellanii} cells grown in co-culture with S4100 bacteria indicate that \textit{V. cholerae} are susceptible to rapid phagocytosis by predatory amoebae under the conditions tested. These experiments demonstrated that near 100 \% of amoebae cells were fluorescent after 48 hrs of co-culture, indicating that GFP-labeled bacteria were being actively and rapidly consumed. Importantly, these flow cytometry results do not reveal whether the predators digested the internalized bacteria. Figure 2 provides a clear answer to this question by showing that bacteria released from lysed amoebae cells were viable upon cultivation. These results support the conclusion that \textit{Vibrio cholerae} bacteria, despite being susceptible to phagocytosis, are able to survive predation by \textit{A. castellanii}, and that they perhaps use the intracellular environment as a replicative niche, as has been demonstrated for other bacteria [24]. This conclusion is further supported by the qualitative growth results reported in Figure 8,
where bacteria grown in alone completely lost viability, whereas those inoculated in the presence of amoebae retained viability after six days.

A primary goal of this research was to test whether certain phenotypic traits of different *V. cholerae* strains determined the sensitivity of the bacteria to predation pressure. Figure 4 indicates that no clear pattern was observed between grazing sensitivity and the phenotypes of these bacterial strains. Variance among T100f1 and AR4100 and S100f1 *V. cholerae* biofilm morphology survival and viability under co-culture conditions suggest a mechanism other than biofilm alone in survival and proliferation of *V. cholerae* strains under grazing pressure. That is, the rugose biofilm isolates of S100f1 and AR4100 showed opposite patterns of grazing sensitivity with S100f1 demonstrating an overall decrease in cell counts relative to no grazing controls, whereas AR4100 demonstrated an overall increase in cell counts after 48 hours when in co-culture with amoebae. Additionally, there was no consistent pattern observed when pathogenicity of the isolates was considered. For example, the non-pathogenic isolate T100f1 increased in abundance during co-culture experiments, whereas the non-pathogenic S100f1 strain decreased. Overall these results suggest that trait of grazing sensitivity may not be linked to the broader phenotypes of pathogenicity or biofilm formation, as has been observed in other contexts [17].

A second goal of these experiments was to determine the overall effect of prey interactions on the predators themselves. Namely, it was tested whether different strains of bacteria induce cyst formation in *A. castellanii* cells. Here it was found that *A. castellanii* co-cultured with S100f1 or T100f1 showed strong evidence of induced cyst formation relative to the negative controls of amoebae alone. Importantly, this phenotypic switch in the amoebae
was not observed in all co-culture experiments. For example, *E. coli* cells, which were used as a grazing sensitive control, did induce a switch to the cyst developmental state. Similarly, *V. cholerae* strain AR4100 did not induce a significant change in the morphology of the amoebae. Additionally, AS4100 did significantly induce cyst formation in amoebae, but only after 48 hours. These results indicate that ingestion of the pathogenic isolates of *V. cholerae* may be less stressful to the predators than non-pathogenic isolates. It should be noted that isolates of *V. cholerae* that are non-pathogenic to humans, and which do not encode the genes for the CTX toxin in their genome, often carry alternative toxins, of which the target is not generally known [9]. The mechanism of this morphological change in the amoebae might be due to a chemical signal or direct interaction with the bacteria after ingestion, although further tests will need to be performed to determine this.

This work suggests that amoebae interactions can proliferate growth in *V. cholerae* strains (selective), possibly acting as protective reservoir and vector for *V. cholerae*. Certain individual *V. cholerae* strains will proliferate and survive under top down pressure as seen with pathogenic and nonpathogenic *V. cholerae* strain isolates; AR4100, S100f1 and T100f1 potentially influencing their respective fitness [26]. In addition, strains whom are able to survive the amoebal intracellular environment, as seen with S4100, are able to persist in their respective environment [24]. This predatory-prey interaction suggests a mechanism of persistence and proliferation in endemic areas where *V. cholerae* are known to persist.

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