Aerococcus varidans var. Homari in Phagocytic cells of *Homarus Americanus*

By

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Abstract

Aerococcus viridans var. homari causes Gaffkemia, a deadly disease in American lobsters (Homarus americanus) that spreads through wounds and overwhelms the immune system. The disease, now also seen in European lobsters (Homarus gammarus), is a major problem in both lobster pounds and wild populations. This study uses transmission electron microscopy (TEM) on previously collected samples to examine how A. viridans grow inside lobster immune cells (fixed phagocytes). TEM images show that after being engulfed, the bacteria proliferate and create intracellular voids filled with an unknown substance, which may help them survive and replicate. The formation of bacterial capsules likely compresses the phagocyte's contents, creating more space for bacterial growth.

Introduction

Aerococcus viridans var. *homari* is the bacterium responsible for Gaffkemia, a lethal and highly infectious disease that affects *Homarus americanus*, commonly known as the American

lobster. This pathogen gains entry into the host through external wounds or lesions in the lobster's hard exoskeleton and infiltrates the open circulatory system. Once inside, the bacterium specifically targets the arterioles, small branches of arteries, that contain fixed phagocytes—specialized immune cells that serve as the lobster's primary line of defense against invading pathogens. Despite the immune system's efforts to eliminate the bacterium through phagocytosis, *A. viridans* successfully evades destruction and ultimately overcomes these defenses, leading to a fatal systemic infection. Gaffkemia is a significant concern in both wild lobster populations and in captive environments such as lobster pounds. In recent years, the disease has also spread beyond North America, impacting *Homarus gammarus*, the European lobster, and posing a growing threat to lobster fisheries internationally.

The American lobster, *Homarus americanus*, is a decapod crustacean with an open circulatory system, meaning its blood, or hemolymph, is circulated not only through vessels but also directly into body cavities. Blood is pumped from the heart into major arteries, which then branch into smaller vessels called arterioles, supplying various organs—including the digestive gland—with oxygen and nutrients (Factor & Naar, 1990). In the American lobster, the hepatic artery, which further subdivides into smaller branches, is the main vessel responsible for delivering blood to the digestive gland, also known as the hepatopancreas (Lawton, 2008). This gland, closely associated with the midgut, plays a critical role in digestion and nutrient absorption. Structurally, the digestive gland consists of three lobes, each containing numerous digestive tubules where food is enzymatically broken down into nutrients for the lobster's use (Factor, 1995).

Within these digestive tubules lie the arterioles that house fixed phagocytes. These immune cells play a vital role in protecting the lobster from infection by engulfing and digesting

foreign particles—a process known as phagocytosis (Factor & Naar, 1995). Unlike circulating immune cells, fixed phagocytes remain stationary, attached along the walls of hemal sinuses and key organs such as the digestive gland and gills. In this research project, the fixed phagocytes studied are located within the digestive gland. These phagocytes function by engulfing pathogens or debris into intracellular vesicles, where they are broken down and neutralized to protect the lobster from disease. However, in the case of Gaffkemia, this defense mechanism appears to fail. Instead of being destroyed, *A. viridans* survives and replicates inside the very cells designed to eliminate it, compromising the lobster's immune system from within.

The infection process in lobster pounds is particularly concerning. Lobster pounds, used to store live lobsters before transport and sale, confine large numbers of lobsters in close quarters. This environment increases the likelihood of injury, as lobsters engage in aggressive interactions or territorial fights, resulting in wounds that serve as entry points for *A. viridans*. Once introduced into a population, the bacterium spreads rapidly, exacerbated by the crowded and stressful conditions of captivity. Consequently, Gaffkemia outbreaks in lobster pounds can lead to significant economic losses for the lobster industry.

The research presented here demonstrates that *A. viridans* is phagocytosed by the lobster's fixed phagocytes but instead of being destroyed, the bacteria proliferate within these immune cells. Transmission electron microscopy (TEM) images reveal the presence of intracellular voids forming around the bacteria, which appear to be filled with an unidentified material. Although the exact composition of this substance remains unknown, its consistent presence suggests it may be part of a bacterial survival strategy. It is hypothesized that *A. viridans* generates these intracellular spaces to create a protective environment that supports its persistence and replication inside the hostile setting of the host's immune cells. The formation of

bacterial capsules seems to displace the phagocyte's cytoplasmic contents outward, effectively compressing the cell's functional space while simultaneously expanding the area available for bacterial growth and capsule development.

To further investigate the interaction between *A. viridans* and the lobster's immune cells, this study utilizes transmission electron microscopy to visualize bacterial activity within infected phagocytic cells. Tissue samples containing fixed phagocytes, collected from prior senior research projects, were prepared using standard protocols of chemical fixation, dehydration, and resin embedding to preserve cellular structures. Ultrathin sections were then cut and examined under TEM. The resulting images, captured with an AMT digital imaging system, were carefully analyzed, with adjustments made to brightness and contrast to enhance the visualization of fine structural details. Through these observations, this study aims to deepen our understanding of the intracellular dynamics of *A. viridans* infection and its implications for lobster health and disease management.

Methods and Materials

Lobsters

To carry out the experiment, materials from prior projects were used. Commercially sourced American lobsters (*Homarus americanus*) were obtained and maintained in tanks at Purchase College. The experimental lobsters were housed in refrigerated seawater aquaria filled with Instant Ocean aquarium salts. Before injections, the lobsters were briefly kept in a

refrigerator at 4ºC. They were then injected with *A. viridans* into their bloodstream. Cold anesthetization methods were utilized, followed by dissection to obtain arteriole tissue samples.

Bacteria

An *Aerococcus viridans var. homari* culture was obtained by an isolated a sick lobster by Dr. Richard Robohm (NOAA, National Marine Fisheries Service, Milford Connecticut) and given to Dr. James Daly which was grown in the microbiology lab at Purchase College (LaRocca, 2004). The bacteria were frozen at -80ºC on site for experimental use. Previous senior projects injected the lobsters with various amounts of inoculant based on weight. The lobsters were then dissected a different time stamps- this was to monitor the bacterial growth in the fixed phagocytes.

Fixation and Embedding

The fixation process was then done to preserve and fix the dissected lobster samples. Fixation and preservation happen in multiple steps over multiple days, this ensures the sample is encased properly in the plastic moulds and can be thinly sliced with an ultramicrotome and observed with a transmission electron microscope.

The first day begins with primary fixation in 3% glutaraldehyde in 0.2 M NaCac buffer at 4ºC for a minimum of 6 hours. The second day is 4 hours of a minimum of 15 minutes for the buffer rinse followed by secondary fixation: 1% OsO4 in buffer #1 for a minimum of 6 hours and 1% OsO4 in buffer #2 for a minimum of 6 hours to overnight. The third day of fixation involves buffer rinse #1 and #2 for a minimum of 15 minutes each followed by 4 distilled water rinses for a minimum of 15 minutes, ending with En Bloc staining of 1% aqueous uranyl acetate at room temperature- if precipitate occurs, it means the buffer remains and additional water rinses are needed. The fourth day begins the EtOH dehydration series starting with 30% for a

minimum of 15 minutes followed by 50%, 70% and 90% EtOH also all for a minimum of 15 minutes. Then once completed immerse the sample in 100% EtOH #1 over CuSO4 for a minimum of 1 hour followed by another round of the same solution for a minimum of 2-3 hours. Once completed, the sample is placed in ½ EtOH and ½ Acetonitrile or propylene oxide for a minimum of 15 minutes then 2 rounds of 100% acetonitrile or propylene oxide for a minimum of 15 minutes. The infiltration process is then started by placing the sample in ½ Acetonitrile or propylene oxide and ½ embedding medium for a minimum of 2 hours, followed by 100% embedding medium for a minimum of 2-3 hours and 100% embedding medium for 6 hours-overnight. The fifth day is the final embedding in fresh medium in moulds to then be cured at room temperature for approximately 1 hour and then polymerized in a 60ºC-70ºC oven for 12-24 hours. In conclusion the fixation and preservation process should be a solid preserved mould of the specimen as close to its natural state as possible to allow for the best possible sectioning and viewing.

The fixation and embedding process was completed by following the procedures provided in the documentation from J.R Factor. 2014 Microscopy Laboratory Procedures. Purchase College Microscopy Laboratory.

Sectioning

Using a Leica ultramicrotome, the specimens preserved during the fixation process were sectioned with glass knives, which were carefully crafted by hand in the laboratory. Each glass knife was fitted with a "boat"—a reservoir created with tape along the knife edge and filled with deionized water to collect the ultrathin sections as they were cut. The ultramicrotome was set to a cutting thickness of 100 nanometers to obtain ultrathin sections suitable for transmission electron microscopy. The floating sections were then retrieved using fine metal loop tools and carefully transferred onto small copper grids specifically designed for imaging with a transmission

electron microscope. In this study, we used a Philips FEI Morgagni M268 microscope to view the sections at various magnifications, allowing detailed visualization of the cellular structures.

Results

Figure 1

Hemocyte cells found in lobster tissue sample

Seen in figure one is a hemocyte, a type of blood cell found specifically in invertebrates. The hemocyte plays a fundamental role in the invertebrate immune system. The cell in this image appears to have granules, which are one of the three major types of hemocytes found in invertebrates. The granules are the primary source in the hemocyte for encapsulation of an invading pathogen.

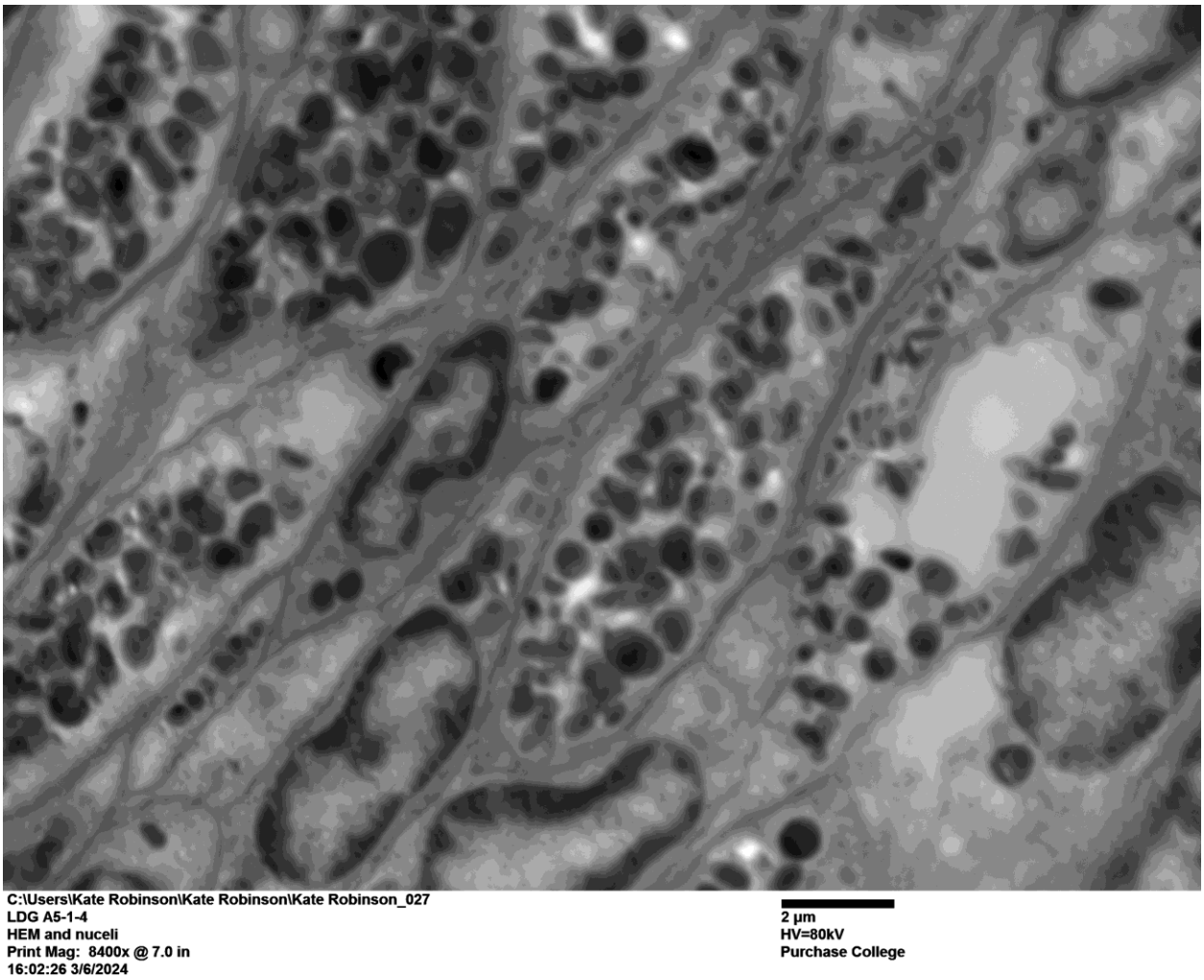

Figure 2

Lobster arteriole

The arteriole has a main job in the lobster's immune system by contributing to hemocyte and hemolymph flow. It also is where the phagocytes attach giving them the name fixed phagocytes. By regulating blood flow, arterioles serve to send hemocytes and hemolymph at the site of infection or wounds. This is a crucial part of the lobster's immune function. The image shows individual phagocytes that make up the arterioles forming a concentric circle-like pattern.

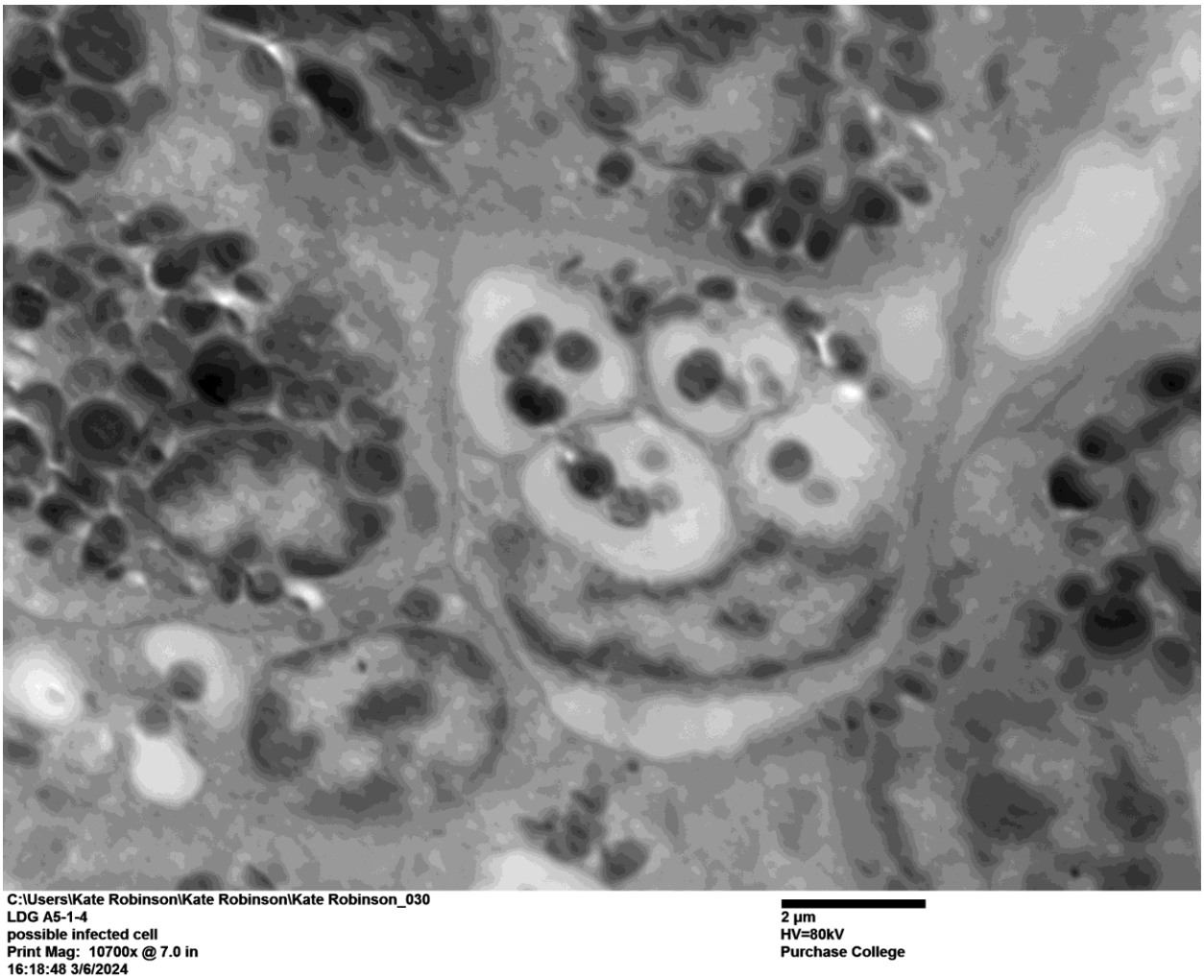

Figure 3

Phagocytes with *Aerococcus* encapsulated inside of it.

The individual phagocytes each being separated by a membrane. Inside of these cells appears to be clusters of *Aerococcus.*

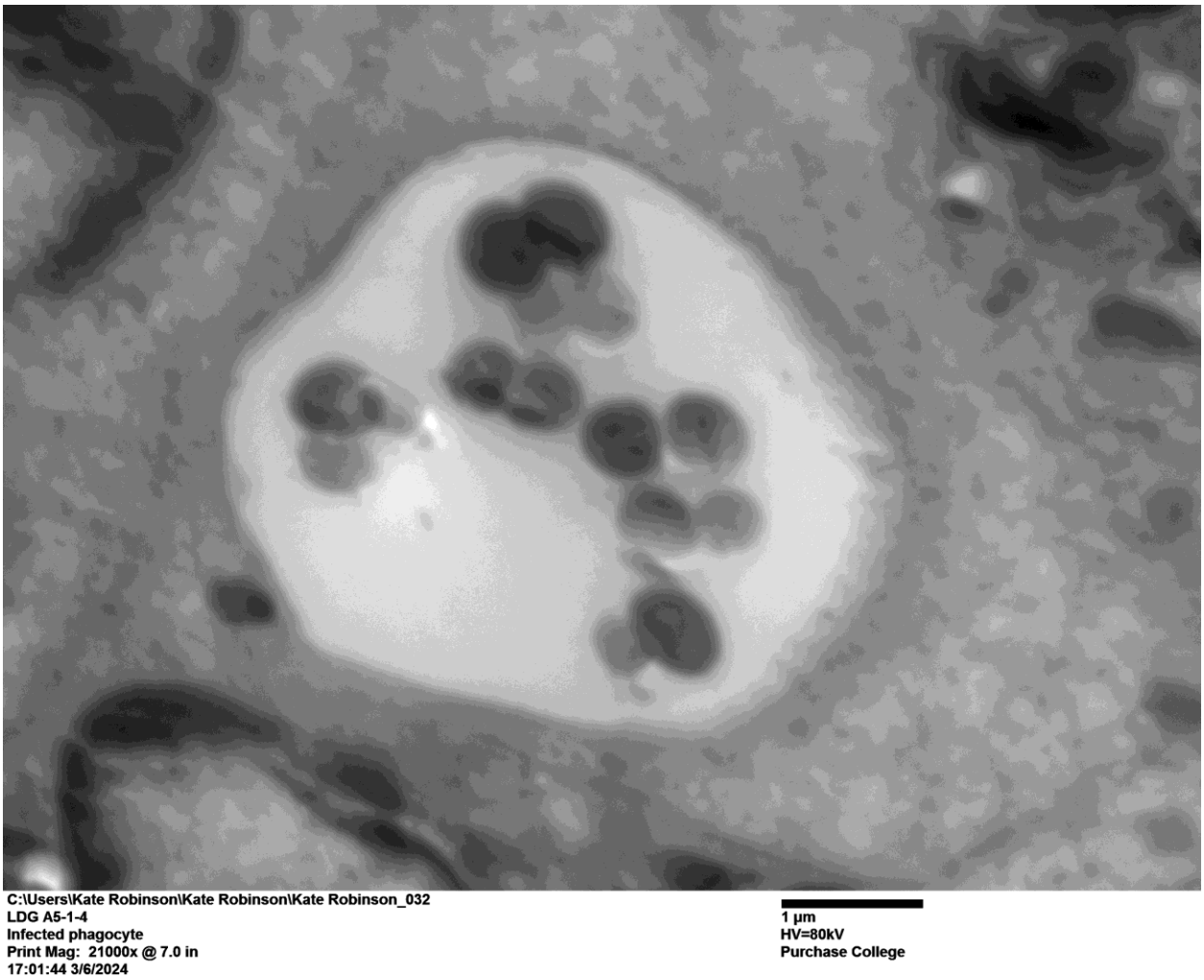

Figure 4

High magnification of an individual phagocyte with numerous Aerococcus bacteria inside.

This image uses a higher magnification on the previous image *Figure 3,* in order to show the growth feature of the bacteria. *Aerococcus* is known to grow into tetrads, meaning the bacteria divides into 4 separate bacteria shown using the arrow.

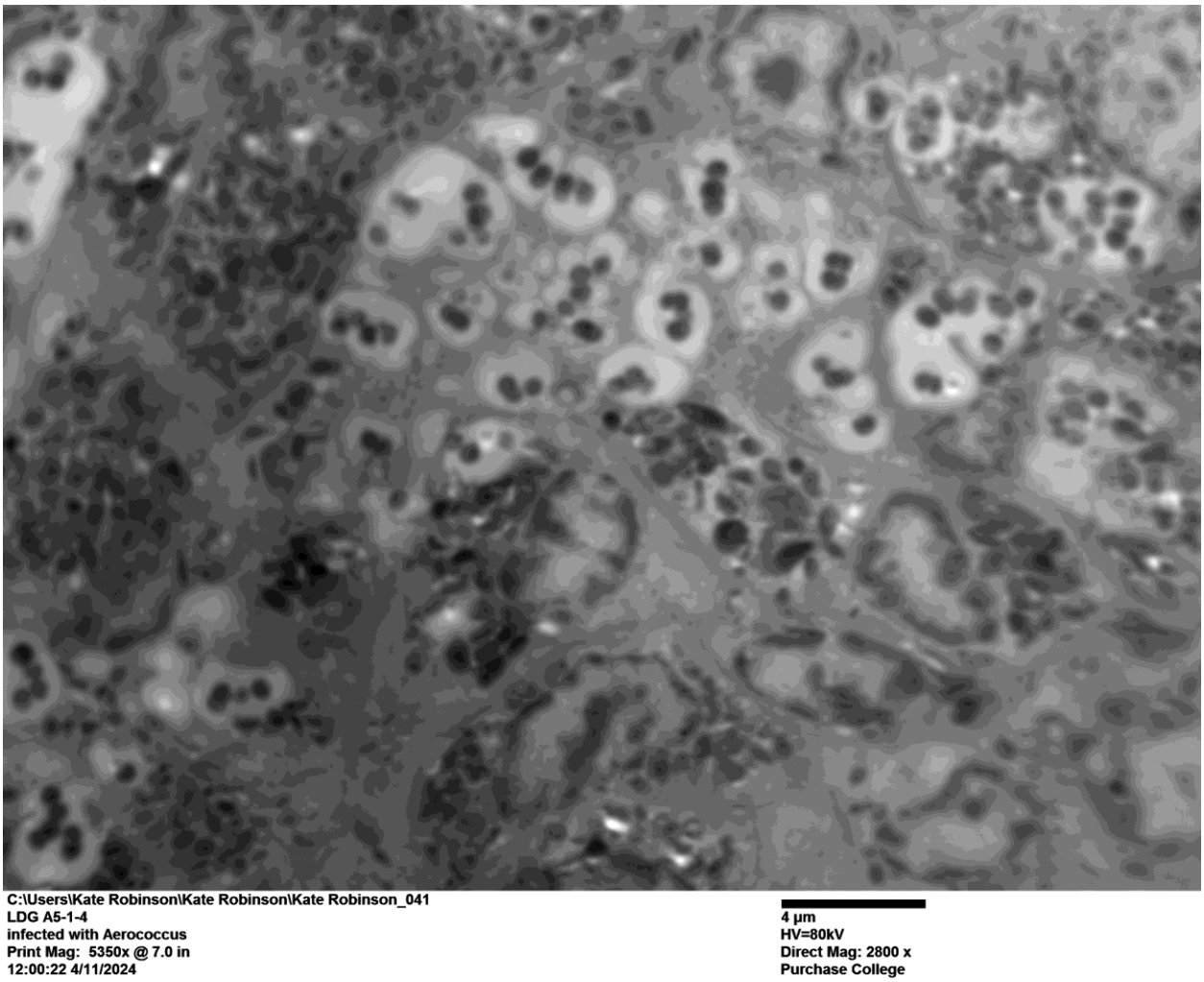

Figure 5

Lower magnification of a cluster of phagocytes, many infected with *Aerococcus*.

The Lower magnification of this sample allows for a better over all view of how rapidly the bacteria can infect the lobster. The bacteria in this image are in what appear to be translucent vacuoles, shown in this image-have begun to form the species-specific tetrads.

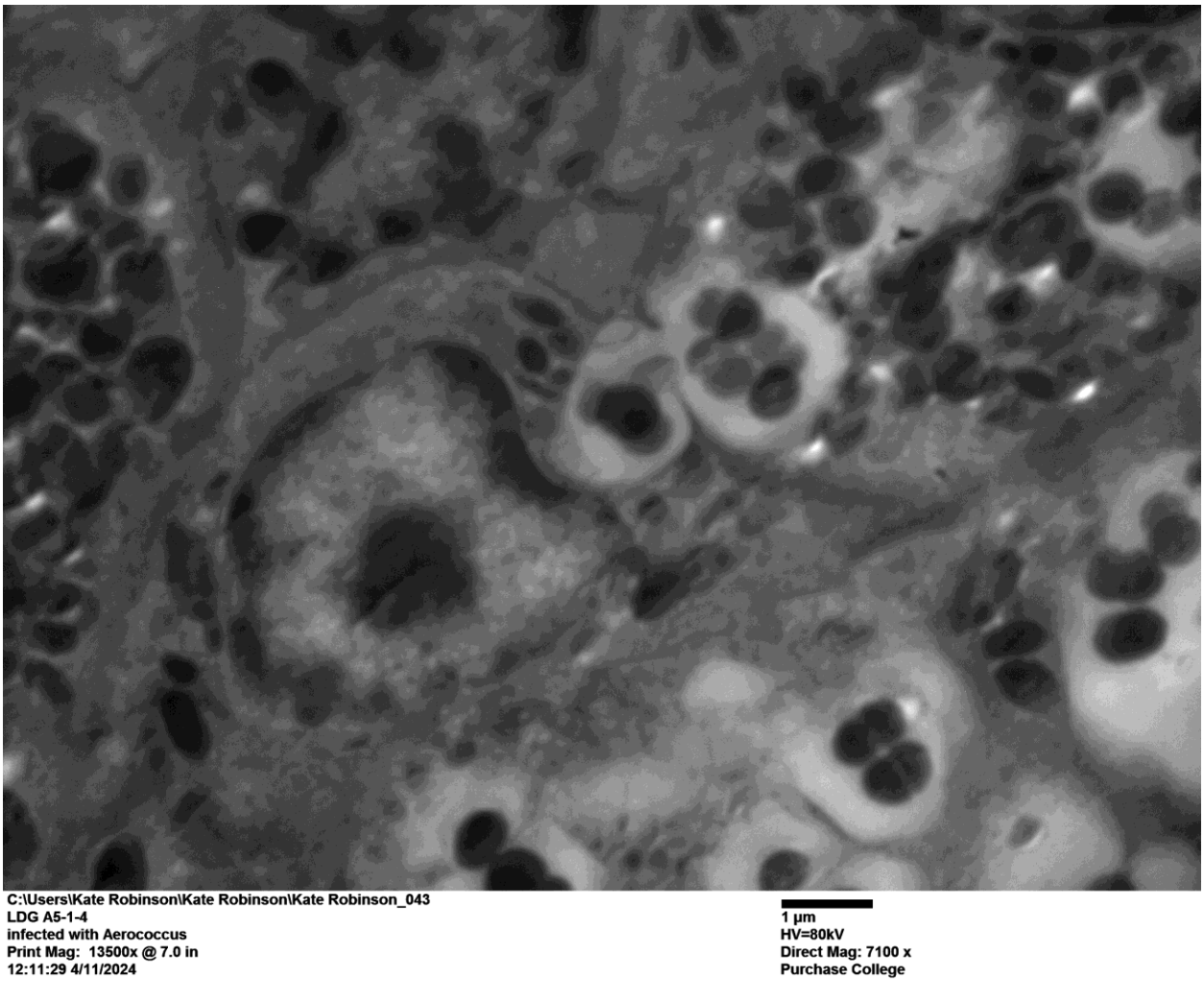

Figure 6

Higher magnification of phagocytes from *Figure 5.*

A tetrad the bacteria has formed very clearly in this image. Surrounding this translucent vacuole previously mentioned in *Figure 5,* are the phagocytes nucleus and granules.

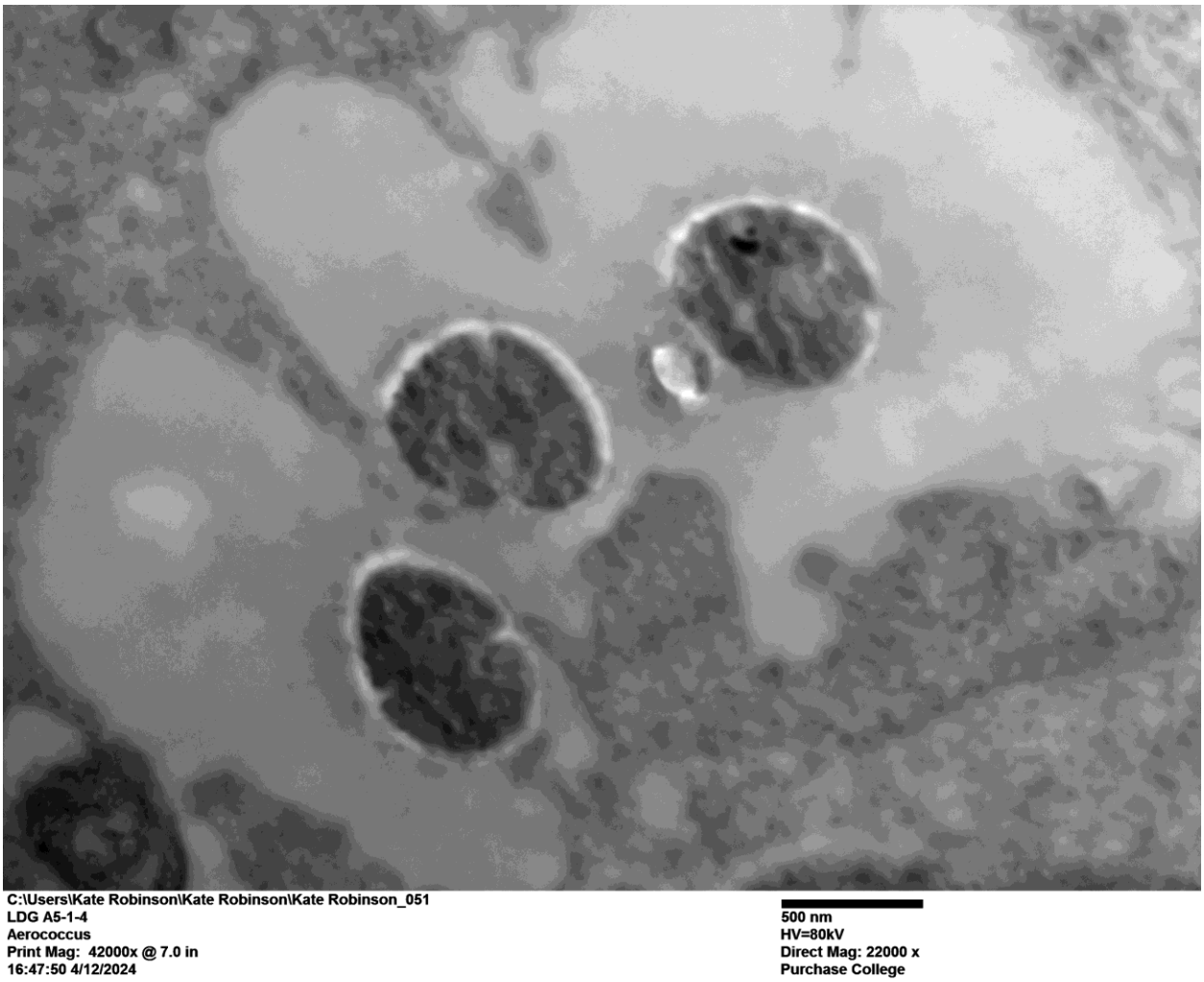

Figure 7

A high-magnification image of a cluster of bacteria is seen in *Figure 5*

In *Figure 5* is where this triplet of bacteria is found. Zooming in, a membrane becomes visible surrounding each bacterium. The bacterium shows ‘pinching’ on the left and right side, this is the beginning stages of dividing into two individual bacteria. Grey matter surrounding each of the bacteria. This can be seen to come off of the bacteria and spread outwards to the edges of the vacuole. This grey matter is what is hypothesized to me the bacterial capsule.

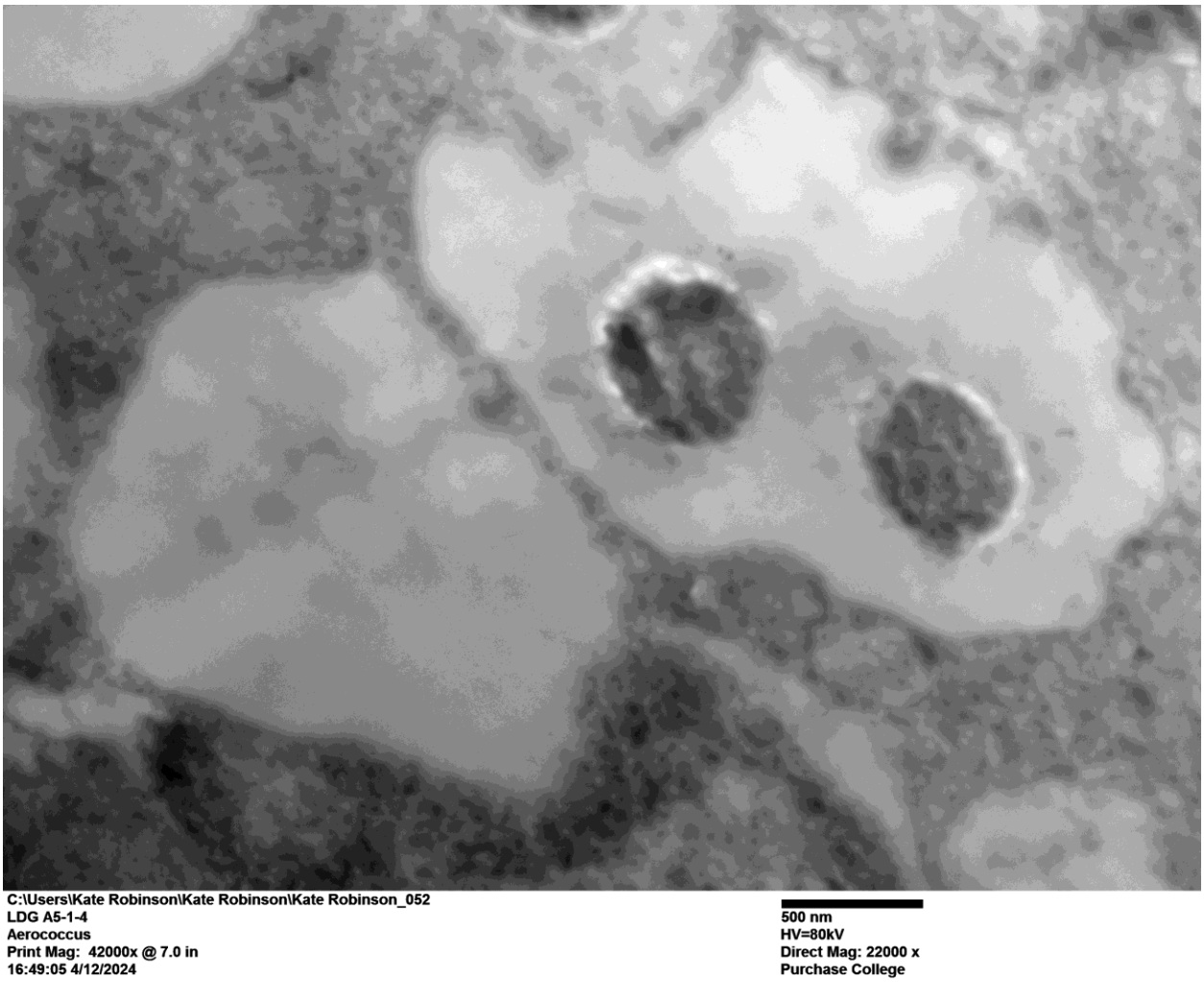

Figure 8

Two *aerococcus* bacteria inside of a vacuole.

In conjunction with *Figure 7*, the bacteria in this image are shown to be surrounded by this grey matter. Seen closely is the matter coming off of the bacteria resembling a stringy-like matter that spreads outwards to the edges of the vacuole.

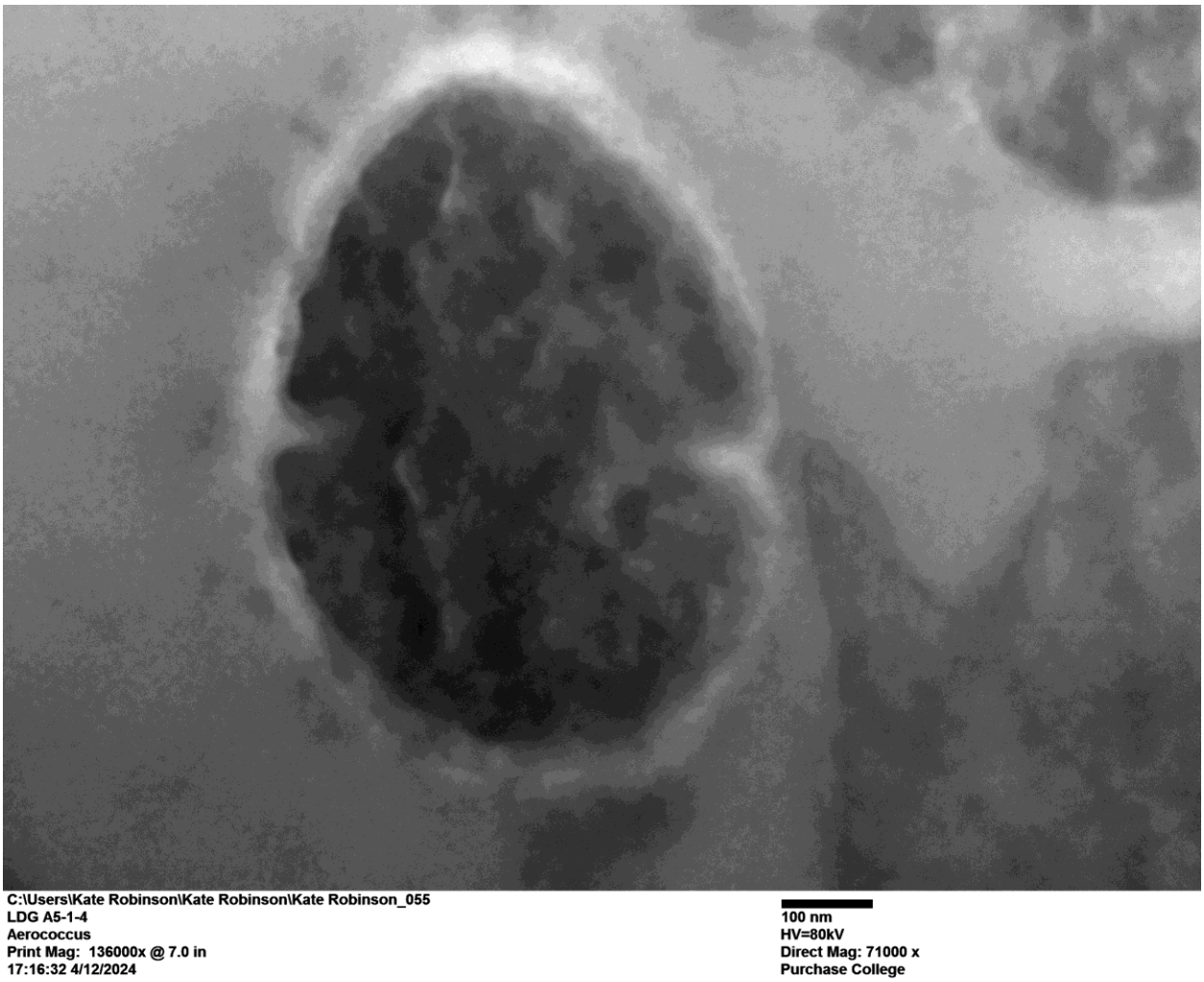

Figure 9

Aerococcus bacterium

A high magnification of an *Aerococcus* bacterium is shown to highlight the 'pinching' that occurs on the left and right side that begins the division. A membrane surrounding the entire bacterium is shown, and as previously mentioned in *figures 8* and *7,* is the grey matter around the membrane of the bacteria, specifically the string-like features spreading outwards.

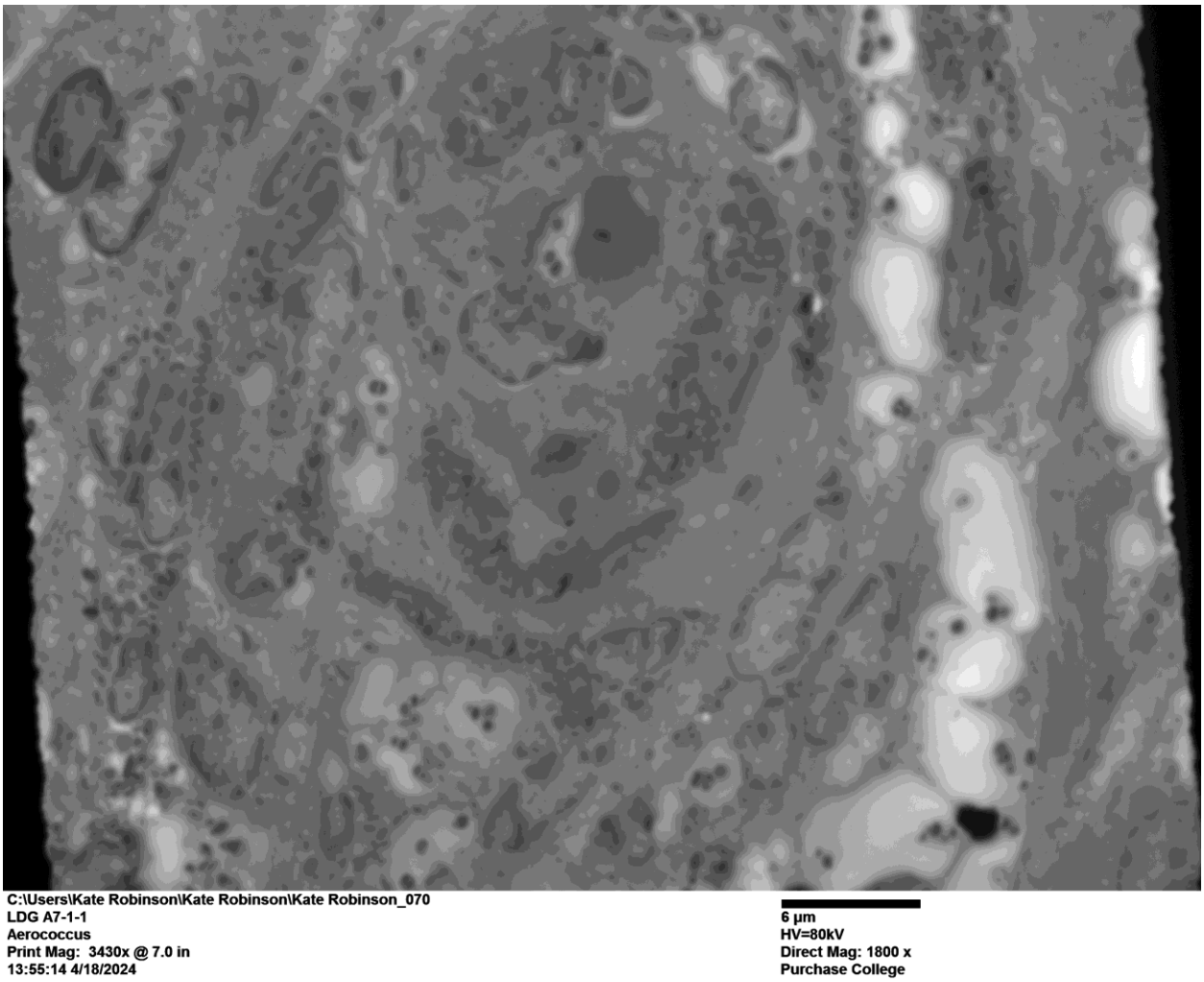

Figure 10

Low magnification of many phagocytes.

The low magnification of this image is to show the concentric circle like pattern that occurs in an arteriole, mentioned in *figure 2.* The specimen sample used for this image has a more progressed stage of gaffkemia. It can be seen by the large ‘white’ spaces around the edge of the image. This is thought to occur due to the rapid dividing of the bacteria pushing the vacuole outwards limiting space for necessary organelles in the phagocytes.

Figure 11

Cluster of vacuoles containing *Aerococcus*

Aerococcus can be seen inside the vacuoles to have divided into dyads with a clear split in between. This means that the bacteria are alive inside of the phagocytes which have the job of eliminating pathogens, unfortunately in this case, the phagocytes are not able to eliminate the pathogen. This will eventually allow *Aerococcus* to continue to divide leading to the death of the lobster.

Description

The findings of this study offer important insights into the intracellular behavior of *Aerococcus viridans* var. *homari* during infection of *Homarus americanus*. Transmission electron microscopy (TEM) revealed that while the bacteria are engulfed by the lobster's fixed phagocytes, they evade destruction and instead survive and proliferate within these immune cells. This bacterial replication leads to the formation of intracellular voids filled with an unidentified substance, suggesting that *A. viridans* has evolved a unique mechanism to persist within the hostile environment of the host's immune system. The expansion of bacterial capsules appears to displace the phagocyte's cytoplasmic contents outward, compromising its function and further weakening the lobster's immune defenses.

These results highlight a critical vulnerability in the immune response of *H. americanus* to *A. viridans* infection. The ability of the bacterium to survive and multiply within immune cells likely contributes to the rapid progression and high mortality associated with Gaffkemia, particularly in environments such as lobster pounds, where crowding and injury increase disease transmission. The presence of unidentified material within infected phagocytes raises further questions about its role in bacterial survival and pathogenesis, underscoring the need for additional research.

A deeper understanding of the interactions between *A. viridans* and fixed phagocytes is essential for developing effective disease management strategies. Future studies should aim to identify the composition and function of the intracellular material observed in TEM images, explore potential mechanisms of immune evasion, and investigate possible interventions to support immune function or inhibit bacterial proliferation in lobsters. Such research could contribute to the preservation of both wild and commercially harvested lobster populations impacted by Gaffkemia.

Literature Cited

https://www.frontiersin.org/journals/immunology/articles/10.3389/fimmu.2020.00268/full

Factor, J.R. 1995. The Digestive System. In: Factor, J.R. (Ed.), Biology of the Lobster Homarus americanus. Chapter 15, pp. 395-441. Academic Press, San Diego

Factor, J.R. 2008. Laboratory Manual. Purchase College Microscopy Laboratory.

Factor, J.R., and M. Naar. 1990. The digestive system of the lobster, Homarus americanus: II. Terminal arterioles of the digestive gland. Journal of Morphology, 206: 283-291.

LaRocca, T. May 2004. Phagocytic Removal of Bacterial Pathogens from the Blood of the Lobster, Homarus americanus. Purchase College, State University of New York.

Lawton, N.May 2008. Histology of Fixed Phagocytes in the American Lobster During *Aerococcus varidans* infection. Purchase College, State University of New York.

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