MICROCAPSULE CONTAINING LACTIC ACID BACTERIA FOR TREATMENT OF PEPTIC ULCERS

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ABSTRACT

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Probiotics are marketed throughout the world to promote the health of the consumer by improving the microorganisms that normally occur in the intestinal tract (Tannock, 1997). It has also been suggested that probiotics can prevent pathogen infections by adhering to the intestinal mucosa (Lee, Lim, Teng, Ouwehand, Tuomola, & Salminen, 2000). While probiotics can be delivered to the infected areas in multiple fashions, microencapsulation is a newer form of delivering probiotics straight to the infected area. A whey protein microcapsule is thought to protect the probiotics from stomach acid and delivers the treatment to the affected area. To ensure this microencapsulation treatment is effective, the microcapsules will be stained and imaged to see if the microcapsules are constructed in a way which is consistent with the theory: a whey protein microcapsule surrounding bacteria and fat droplets. Through these experiments, it was shown that the microcapsule was not constructed as previously thought. Instead of a thin layer of protein surrounding the bacteria, it more closely resembled a solid ball of protein with bacteria and fat trapped inside. The bacteria are able to survive stomach like conditions (0.1M HCl for 8 hours) due to other forms of microencapsulation.

Keywords: Lactic Acid Bacteria, probiotic, microcapsule, *H. pylori*, confocal, microscopy, ulcer, fluorescence staining.

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Introduction

Peptic ulcers are the fairly common sores on the lining of the stomach or duodenum which affect about half a million people in the United States annually (NDDIC, 2010). An ulcer is defined as a mucosal defect with a diameter of at least 0.5 cm penetrating through the muscularis mucosa (Kusters, van Vliet, & Kuipers, 2006). More extreme cases can lead to a perforation, which is a hole in the stomach or duodenum. Peptic ulcers are found in three areas along the gastrointestinal tract as shown in Figure 1. They form in the stomach, a gastric ulcer, or in the duodenum, a duodenal ulcer (PubMed Health, 2011). Less commonly they can be found just above the stomach in the esophagus (NDDIC, 2010).

Figure 1 - Locations of peptic ulcers along gastrointestinal tract (The 2005 Nobel Prize in Physiology or Medicine, 2005)

In a healthy person, the stomach and all intestines are protected against hydrochloric acid produced by the stomach (Saladin, 2010). If this protective layer stops working correctly, it can result in inflammation of the tissue or lead to an ulcer (PubMed Health, 2011). The epithelial cells of the stomach, the inner most lining which comes into contact with ingested food, provide this protection by producing bicarbonate. Bicarbonate is a basic substance that

neutralizes hydrochloric acid, preventing the acid from damaging the tissue (Saladin, 2010). This epithelial lining can be damaged in various ways, and bicarbonate production in that area will be reduced or stopped which allows hydrochloric acid to further damage the stomach or intestinal tissue (Saladin, 2010). While it may seem the hydrochloric acid is capable of more harm than good, it is necessary in the stomach as it has several functions. Hydrochloric acid in the stomach breaks up connective tissue and plant cell walls which help liquefy food. The acid also converts ferric ions (Fe^{3+}) into ferrous ions (Fe^{2+}) which can then be used for hemoglobin synthesis (Saladin, 2010). It also provides some disease resistance by eliminating pathogens before they can make it further into the digestive system (Saladin, 2010).

Causes of peptic ulcers

The most common cause of an ulcer is due to an infection of the bacterium *Helicobacter pylori* (*H. pylori*), not by stress or spicy foods as rumored (NDDIC, 2010). A majority of people with peptic ulcers have *H. pylori* living in their gastrointestinal tract. (PubMed Health, 2011). The prevalence of *H. pylori* in the gastrointestinal tract depends on geographic location and socioeconomic status. In western countries, the infection rate is under 40% while in developing countries the infection rate could be greater than 80% (Kusters, van Vliet, & Kuipers, 2006). After *H. pylori* was discovered, initial reports stated that approximately 95% of duodenal ulcers and 85% of gastric ulcers occurred in the presence of an *H. pylori* infection (Kusters, van Vliet, & Kuipers, 2006). About 20 years ago, researchers Barry Marshall and Robin Warren were able to isolate *H. pylori* from the human stomach. Experiments done by Marshall and Warren proved that *H. pylori* can colonize the stomach, causing inflammation of the gastric mucosa (Kusters, van Vliet, & Kuipers, 2006). Both researchers developed gastritis, or inflammation of the mucosa, after their self-ingestion experiment. Marshall and Warren's results were so convincing that it encouraged other researchers to further investigate. It was later found that *H. pylori* can

lead to a variety of upper gastrointestinal disorders, such as chronic gastritis, peptic ulcers, and even gastric cancer (Kusters, van Vliet, & Kuipers, 2006). *H. pylori* infection causes gastritis in all infected individuals, however very few develop any clinical signs of the infection (Kusters, van Vliet, & Kuipers, 2006). It is unknown why *H. pylori* can cause ulcers in some people and not others. Most likely, development of ulcers depends on the infected host, environmental factors, and strain of *H. pylori* may also play a role as shown in Figure 2. Figure 2 shows that an infection of *H. pylori* alone is not enough to cause gastric cancer. The bold arrows on the right are necessary for the next step down; such as *H. pylori* infection alone cannot cause gastric atrophy, immune response, diet, gastrin, and host genetics all play a role.

Figure 2 - *H. pylori* and other factors in gastric cancer (Kusters, van Vliet, & Kuipers, 2006)

There are several causes of peptic ulcers other than *H. pylori* infections. One common cause of peptic ulcers is from long term use of NSAIDs, or nonsteroidal anti-inflammatory drugs (NDDIC, 2010). A study in Hong Kong was done to test the effects of NSAIDs and *H. pylori* on

ulcer occurrence. Patients were randomized into two groups, one receiving treatment to eradicate the *H. pylori* infection, while the other was given a placebo. After six months, the patients were examined, and it was determined that 12.1% of the eradication group still was affected by ulcers, as compared to 34.4% in the placebo group (Kusters, van Vliet, & Kuipers, 2006). The 12.1% occurrence rate in the eradication group shows that removal of the *H. pylori* infection reduced the number of ulcers that treatment alone is not enough as NSAIDs play some role in the development of ulcers. Other possible causes of peptic ulcers include drinking too much alcohol, smoking cigarettes or using chewing tobacco, undergoing radiation therapy, or being very ill (Mayo Clinic, 2011). It is important to note that probiotics will not heal ulcers caused solely by the chronic use of NSAIDs, as the purpose of lactic acid bacteria is the eradication of *H. pylori*, and in this case there wouldn't be an *H. pylori* infection.

Symptoms

Most people do not realize they are infected with *H. pylori* until an ulcer develops because they are not sick from the infection (Mayo Clinic, 2011). An infection of *H. pylori* will cause chronic gastritis for as long as the bacteria are present in the stomach or duodenum as shown in Figure 3. However, this does not produce any clinical symptoms. It is not until an ulcer is present that symptoms show up. It is estimated that infected patients have a 10-20% chance of developing an ulcer over their lifetime, and a 1-2% risk of developing gastric cancer (Kusters, van Vliet, & Kuipers, 2006). The most common symptom is abdominal discomfort (NDDIC, 2010). The discomfort can range from a dull pain/feeling to a burning pain, most commonly occurring between meals or during the night. Other symptoms may include weight loss, a poor appetite, nausea, bloody stool, fatigue, or vomiting (PubMed Health, 2011). There are some severe symptoms which should be reported to a doctor immediately, such as bloody

stool which may show that a perforation is present, or that the ulcer has broken a blood vessel (NDDIC, 2010).

Figure 3 - Inflammation of gastric mucosa. (The 2005 Nobel Prize in Physiology or Medicine, 2005)

Helicobacter Pylori

H. Pylori was first observed in human stomachs in the early 1980s. The spiral bacteria were considered to be bacterial overgrowth or food contaminants. The organism was first named “*Campylobacter*-like organism”, “*Campylobacter pyloridis*”, and “*Campylobacter pylori*.” Its name was changed to *Helicobacter pylori* when it was discovered it was not a member of the *Campylobacter* genus (Kusters, van Vliet, & Kuipers, 2006). *H. pylori* is a gram-negative bacterium that measures 2-4 μ m in length and 0.5-1 μ m in width (Kusters, van Vliet, & Kuipers, 2006). Figure 4 shows a microscopy image of a single *H. pylori* bacterium. Even though most are usually spiral, *H. pylori* can appear as a rod, or coccoid shape after extended in vitro culture or after antibiotic treatment (Kusters, van Vliet, & Kuipers, 2006). The coccoids cannot be

cultured in vitro; it is thought these coccoids represent dead cells (Kusters, van Vliet, & Kuipers, 2006).

Figure 4 - Microscopy image of *H. pylori* (The 2005 Nobel Prize in Physiology or Medicine, 2005)

The composition of *H. pylori* is similar to that of other gram-negative bacteria. It has an inner membrane, a periplasm with peptidoglycan, and an outer membrane of phospholipids and lipopolysaccharide (Kusters, van Vliet, & Kuipers, 2006). All gram-negative bacteria have lipopolysaccharide (LPS) (Salton & Kim, 1996). This LPS is also called an endotoxin, which play an important role in the pathogenesis of many gram-negative bacterial infections (Salton & Kim, 1996). Because *H. pylori* is found in the stomach and duodenum of humans, its optimal growth requirements are unsurprising. Growth occurs at temperatures between 34-40°C but experiences optimal growth at 37°C, the average temperature of the human body (Kusters, van Vliet, & Kuipers, 2006). A key feature of *H. pylori* is its microaerophilicity with optimal growth at O₂ levels between 2-5%, and a need for 5-10% CO₂ as well as a high humidity (Kusters, van Vliet, & Kuipers, 2006). Oxygen levels in the stomach and intestines are extremely low. Given these requirements, it is easy to understand why *H. pylori* is able to flourish if introduced in the gastrointestinal tract.

Researchers are unsure how *H. pylori* is spread, however it may be spread through contaminated food or water. Like other pathogens, it is possible that *H. pylori* can be spread by food that has not been washed or cooked properly, and drinking water from an unclean source (NDDIC, 2010). The prevalence of *H. pylori* inversely correlates with socioeconomic status, especially during childhood (Kusters, van Vliet, & Kuipers, 2006). Infections are much more common in developing countries and among the poor because they are unable to prepare food in a clean environment, or may simply not have access to clean water (NDDIC, 2010). In some developing countries, more than 80% of the entire population is infected with *H. pylori*, including adults and children. This is significantly higher than the infection rate (less than 40%) in industrialized countries. This is most likely due to improved hygiene, sanitation, and elimination of the bacteria by antimicrobial treatments (Kusters, van Vliet, & Kuipers, 2006). Other research is being done to understand how the bacteria can spread from an infected person to an uninfected person (NDDIC, 2010). *H. pylori* may also be spread by person to person through direct contact with saliva, vomit or fecal matter (Mayo Clinic, 2011). There has been only one known instance of *H. pylori* being cultured from water, but the culture involved waste water which may have been contaminated with fecal matter. The bacteria can only survive brief periods of refrigeration (Kusters, van Vliet, & Kuipers, 2006). It is unknown if one mode of transmission is more common than another at this point. Because of *H. pylori*'s optimal growing conditions (37°C, 2-5% O₂), it's most likely that the infection is transmitted by person to person contact.

Diagnoses

There are multiple ways of effectively diagnosing a *H. pylori* infection. All these modes of diagnosing can be broken down into two categories: non-invasive or invasive techniques (Kusters, van Vliet, & Kuipers, 2006). Non-invasive techniques are a series of tests run by a

doctor, and could include less invasive techniques such as endoscopy. If a patient has the symptoms of peptic ulcers, the doctor may simply tell the patient to stop use of, reduce dose, or switch to a different NSAID. If symptoms are still present, then tests for an *H. pylori* infection will begin. Non-invasive diagnoses include blood sample tests, urea breath tests, and stool antigen tests (Mayo Clinic, 2011). In the blood sample tests, blood will be drawn from the patient to check for antibodies that attach to *H. pylori*. Presence of these antibodies will confirm an infection as they are not present otherwise. A stool antigen test will test the patients stool for *H. pylori* antigens (NDDIC, 2010). For a urea breath test, the patient will swallow a capsule that contains urea labeled with a special carbon atom (NDDIC, 2010). After a few minutes, the patient will blow into a container that checks for the ingested carbon atom. *H. pylori* contains urease, an enzyme that breaks down urea. If the carbon atom is detected, it signifies a presence of *H. pylori* (NDDIC, 2010). The invasive diagnostic technique used is endoscopy. The patient is sedated, and an endoscope will be passed into the patient to the stomach and duodenum. This allows the doctors to view the lining of stomach and duodenum. The doctors are also able to take pictures, remove pieces of the tissue for a biopsy, inject medicines, or even cauterize an actively bleeding ulcer (NDDIC, 2010). In hospital based care, the endoscopy procedure is most common. However, for children the stool antigen test is favored because it allows for accurate diagnoses without the need for an endoscopy or having blood drawn (Kusters, van Vliet, & Kuipers, 2006).

Treatment

There are many antibiotic treatments available to treat a patient with ulcers. Oddly, *H. pylori* is sensitive to a wide range of antibiotics in vitro, however if each therapy is used alone it will not work in vivo (Kusters, van Vliet, & Kuipers, 2006). The infections are usually treated with two varieties of antibiotics at once, such as clarithromycin, amoxicillin, tetracycline, or

metronidazole (PubMed Health, 2011). The most effective single drug is clarithromycin, when used twice a day for two weeks. Even with treatment of this antibiotic, the eradication rate is about 40% (Kusters, van Vliet, & Kuipers, 2006). If the ulcer is caused without being infected by *H. pylori*, such as one caused by extensive use of NSAIDs, the doctor may prescribe a histamine receptor blocker (H2 blocker) or a proton pump inhibitor (PPI) such as omeprazole (Prilosec), lansoprazole (Prevacid) or esomeprazole (Nexium) for up to eight weeks (PubMed Health, 2011).

PPIs work to suppress acid production by halting the mechanism that pumps acid into the stomach. PPIs cannot kill an *H. pylori* infection, but it has been shown that after 4 weeks PPI use can relieve pain and promotes healing (NDDIC, 2010). Common therapies today include a combination of antibiotics as well as PPIs to cover all potential causes of the ulcers. Triple therapies combine two antibiotics and a PPI as shown in Table 1. Tetracycline, amoxicillin, or imidazoles along with clarithromycin are probably the drugs most widely used to get rid of an *H. pylori* infection (Kusters, van Vliet, & Kuipers, 2006). After being taken for 14 days or longer, eradication rates can reach about 80%. The therapies cannot reach 100% due to the bacteria gaining antimicrobial resistance, patients failing to take their prescription after the ulcer is gone, or side effects caused by the treatment. In the case of a patient not responding to the triple therapy, a quadruple therapy will be prescribed which includes amoxicillin because *H. pylori* resistance is rare to that antibiotic (NDDIC, 2010). If the infection remains, ulcers could reoccur, and even progress into stomach cancer as shown in Figure 2.

Table 1- Triple and quadruple therapies prescribed to patients with *H. pylori* infection (US Pharm, 2010)

Antisecretory Agent (1)	Antibiotics (2)	Bismuth Salt (1)	Combination Product
TRIPLE THERAPY REGIMEN			
Esomeprazole 20-40 mg po daily <i>or</i> Lansoprazole 30 mg po bid <i>or</i> Omeprazole 20 mg po bid <i>or</i> Pantoprazole 40 mg po bid <i>or</i> Rabeprazole 20 mg po bid	Clarithromycin 500 mg bid + amoxicillin 1 g bid <i>or</i> metronidazole 500 mg bid	None	<i>Prevpac</i> : Lansoprazole 30 mg + clarithromycin 500 mg + amoxicillin 1 g po bid
QUADRUPLE THERAPY REGIMEN			
1 of 5 PPIs above <i>or</i> Cimetidine 300 mg po qid (800 mg qhs) <i>or</i> Famotidine 20 mg po bid (40 mg qhs) <i>or</i> Nizatidine 150 mg po bid (300 mg qhs) <i>or</i> Ranitidine 150 mg po bid (300 mg qhs)	Metronidazole 250 mg qid + tetracycline 500 mg po qid	Bismuth subsalcylate 525 mg po qid	<i>Helidac Therapy Kit</i> : Bismuth subsalcylate 262.4 mg (2 tab) + metronidazole 250 mg (1 tab) + tetracycline 500 mg (1 tab) po qid + a PPI or H ₂ RA <i>Pylera</i> : Bismuth subcitrate potassium 140 mg + metronidazole 125 mg + tetracycline 125 mg 3 cap po qid + a PPI or H ₂ RA
<i>bid: twice daily; cap: capsule; H₂RA: histamine-2 receptor antagonist; po: oral route; PPI: proton pump inhibitor; qhs: bedtime; qid: four times daily; tab: tablet; tid: three times daily.</i> <i>Source: References 3, 12, 13.</i>			

Use of probiotics

Another approach that may be more beneficial to the patient would be using probiotics in place of antibiotics, or as a supplement to previously mentioned (or antibiotic) therapies. A probiotic is a live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance (Tannock, 1997). Many of the probiotic bacteria are lactic acid bacteria and are useful in treatments of anything that may disturb intestinal microflora. (Lee, Lim, Teng, Ouwehand, Tuomola, & Salminen, 2000). Probiotics are marketed throughout the world, most commonly in yogurts, cheese, and other fermented food. Establishing scientific validity of probiotics will define and clarify the benefits of consuming probiotics, and it is apparent to the dairy-food industry (Tannock, 1997). There is strong evidence supporting that *H. pylori* is killed by lactic acid bacteria in vitro, and to some extent in vivo (Kusters, van Vliet, & Kuipers, 2006). While lactic acid bacteria alone will not eradicate an infection in the same way the triple therapy will, it will reduce the amount of bacteria which can prevent ulcers (Kusters,

van Vliet, & Kuipers, 2006). Aside from killing pathogens, the mere presence of probiotics is helpful. If the GI tract is missing some of its natural microflora, the probiotics are able to exclude the pathogens. Comparisons of lactic acid bacteria free mice and mice that had been intentionally colonized with the bacteria have demonstrated that the lactic acid bacteria have a major influence on intestinal biochemistry (Tannock, 1997).

Another advantage probiotics have is that pathogens cannot acquire resistance or immunity to probiotics like they can against antimicrobial medications. If the infection becomes resistant to a drug or therapy, it will cause an already resilient infection to become even more difficult to eradicate. Since probiotics do not work in the same manner as antibiotics, *H. pylori* cannot gain resistance or immunity to probiotics.

Lactic acid bacteria

Lactic acid bacteria (LAB) is the general name given to all of the Lactobacillus genus of bacteria that produce lactic acid. The Lactobacillus genus is all gram positive rod shaped bacteria which are resistant to acidic environments (Tannock, 1997). Even though they are resistant to acidic environments, they cannot survive the concentrated hydrochloric acid (HCl) produced by the stomach. They thrive in low oxygen environments, which make the GI tract an ideal location. It has been proposed that lactic acid production by these bacteria, unrelated to pH, is responsible for inhibition of *H. pylori*. The salts of lactic and acetic acids, namely sodium lactate and sodium acetate, vary in their ability to inhibit *H. pylori* at a neutral pH (Midolo, Lambert, Hull, Luo, & Grayson, 1995).

The Microcapsule

Lactic acid bacteria are incapable of surviving past the stomach acid unaided, so whey protein microcapsules have been developed to allow the lactic acid bacteria to reach areas affected by ulcers. The microcapsules can be stored long term after they have undergone a

spray drying process. Spray drying is simply drying the microcapsules with a hot gas and is one of the oldest and most commonly used methods of encapsulation in the food industry (Rodrigues, et al., 2011). Microcapsule synthesis starts with all the components mixed into solution as shown in Figure 5. The solution is then pumped to an atomizer, which is a centrifuge with small holes along the perimeter (Souza, Gebara, Ribeiro, Chaves, Gigante, & Grosso, 2012). Rodrigues, et al. centrifuged at 1789G to produce their cultures (Rodrigues, et al., 2011). This forces the solution out of the atomizer in a fine mist. The mist is exposed to 170 °C air at the inlet and 70 °C at the outlet which causes the water inside the microcapsule to evaporate, leaving behind the microcapsule and the lactic acid bacteria inside (Rodrigues, et al., 2011). The end produce is a white powder that collects at the bottom of the collecting funnel. Once the microcapsules have been rehydrated with cold water, they retain their normal function (Souza, Gebara, Ribeiro, Chaves, Gigante, & Grosso, 2012).

Figure 5 - Microcapsule synthesis procedure used by Souza et al. Diagram created by Dr. Jimenez of Cal Poly DPTC.

These microcapsules are spherical covers for the lactic acid bacteria. Figure 6 shows a spray dried microcapsule using a scanning electron microscope and a rehydrated microcapsule using a confocal microscope. During preparation for staining for an experiment done in 2011 by Hinkel and Nicolas, it was found that the microcapsules took much longer to stain than cells. While other cell staining protocols called for incubation times of 10 minutes, the microcapsules required incubation times of 48 hours. Even with the extended duration, it is unknown if even 48 hours is sufficient because incubation times longer than 48 hours were not examined. Though the microcapsules require a long incubation period, they are very fragile to mechanical forces. During the same experiment, the microcapsules were vortexed and sonicated in hopes to help in the rehydration process (Hinkel & Nicolas, 2011). When those initial microcapsules were imaged, all of the microcapsules had been destroyed. A sample image of the experiment is seen in Figure 7 (Hinkel & Nicolas, 2011).

Figure 6 – Two images of lactic acid bacteria microcapsules. Image on left of spray dried microcapsule using SEM. Image on right of rehydrated microcapsule using confocal microscope. (Souza, Gebara, Ribeiro, Chaves, Gigante, & Grosso, 2012)

Figure 7 – Confocal image of stained microcapsule. This microcapsule was not spherical as expected. Fluorescent particles were imaged throughout the cover well, suggesting that the microcapsule had ruptured. (Hinkel & Nicolas, 2011)

The microcapsules have three components: the lactic acid bacteria, whey protein, and fat. The lactic acid bacteria are for the treatment of peptic ulcers as described earlier. The whey protein microcapsule houses the lactic acid bacteria and fat (Rodrigues, et al., 2011) and allows the therapy to reach the affected area. Without the capsule, all components will be destroyed by the HCl in the stomach rendering the therapy useless. By the time the microcapsule reaches

the duodenum, it has degraded enough to release the lactic acid bacteria. The fat inside the microcapsule provide some unknown protection for the lactic acid bacteria during the spray drying process. When the fat is omitted during microcapsule synthesis, all of the lactic acid bacteria are killed during the spray drying method (Souza, Gebara, Ribeiro, Chaves, Gigante, & Grosso, 2012). When the fat is included, the bacteria are able to survive the process. It is hypothesized that the fat acts as a heat sink that absorb a majority of the heat from the hot gases experienced during spray drying. The 2011 experiment with microcapsules had attempted at proving or disproving the fat heat sink hypothesis but was unsuccessful due to lack of time and an inaccurate staining protocol (Hinkel & Nicolas, 2011).

Microscopy

This work utilizes extensive use of a confocal microscope. Confocal microscopy is used because it enables three dimensional visualization deep within both living and fixed cells and tissues (Olympus Corporation, 2009). Confocal microscopy also gives the ability to control depth of field, reduction of background information, and the capability to collect serial optical sections from thick specimens (Olympus Corporation, 2009). While the microcapsules might not be “thick”, they are three dimensional objects and need to be imaged at various depths to accurately view the bacteria and fat droplets inside of the microcapsule without destroying the outer protein capsule. Confocal microscopy also allows images to be taken with much further detail when compared to a widefield optical epi-fluorescence microscope (Olympus Corporation, 2009). Figure 8 shows the distinct increase in detail obtained from the result of confocal microscopy. The increased level of detail is especially important because it will allow for the imaging of all the components of the microcapsule. Using widefield microscopy to image the microcapsules might give results very similar to Figure 8c. This would be ineffective as it is impossible to gain any information about the structure of what is being imaged other than a

general shape. If it is desired, confocal microscopy is also capable of producing three dimensional images from z-series stacks, or consecutive images taken layer by layer in the specimen (Olympus Corporation, 2009). Three dimensional imaging might be useful for this work to determine if the whey protein capsule had been damaged during rehydration, which would allow HCl to kill the lactic acid bacteria.

Figure 8 - Comparison of widefield microscopy and confocal microscopy. Images (a-c) show widefield images while (d-f) show confocal images of human medulla (a,d), rabbit muscle (b,e), and sunflower pollen grain (c,e) (Olympus Corporation, 2009)

Figure 9 details the complexity of a confocal microscope in a very simplified manner. It is important to note the exclusion of the eyepiece on the figure. Confocal microscopes display the image directly to software installed on a computer; no light is reflected back to an eyepiece. A confocal microscope works differently than a common light microscope. Coherent light is emitted by the laser and reflected towards the specimen by a dichromatic mirror (Olympus Corporation, 2009). The laser is then scanned across the specimen in a defined focal plane; this

allows the entire specimen to be imaged rather than the single point where the laser hits the specimen. The laser will excite fluorescent markers on the specimen. The emitted fluorescence will pass back through the dichromatic mirror and is focused as a point on the photomultiplier detector (Olympus Corporation, 2009). The pinhole aperture filters out fluorescence that is not in the focal plane which results in a clearer image. That information is then passed on to a computer which reconstructs the image on a monitor.

Figure 9- Components of a confocal microscope (Olympus Corporation, 2009)

Along with the confocal microscope, Differential Interference Contrast (DIC) imaging will be used as well. Figure 10 shows a simplified view of the components that comprise a DIC microscope. DIC images look remarkably similar to images taken with white light, but DIC is far more complex. Light from under the sample is polarized and then split perpendicular to each other by a Wollaston prism. The light then passes through a condenser, which causes the two light rays to travel parallel to each other through the specimen being imaged (Olympus Corporation, 2009). When the light passes through the specimen, it can change the wave path of the light if the specimen has varying thickness, or refractive indices (Olympus Corporation,

2009). The two parallel light rays then pass through a second Wollaston prism which recombines the light. If the specimen being imaged had altered either of the light rays, the individual light rays will not be the same length. For the light to interfere, a second polarizer will bring the light rays into the same plane and axis (Olympus Corporation, 2009). This light is then viewed through the eye piece or on a monitor.

Figure 10 - Components of a DIC microscope (Olympus Corporation, 2009)

The DIC is used as a “base layer” for the confocal images. The confocal fluorescence images will be overlaid on top of the DIC images to show which parts of the microcapsule are fluorescing. Figure 24 D and H show two different filters being displayed over the DIC image of a microcapsule.

Work goals and aims

It is important to understand the causes and symptoms of ulcers to understand what patients go through and the risks they are exposed to when they are affected by ulcers.

Understanding *H. pylori* and how probiotics work with the body are critical in the development of novel treatments. Without this knowledge, the treatment would be composed of only antibiotics. The purpose of this work is to show that the microcapsule have the potential to be an effective treatment of ulcers by sufficiently shielding the lactic acid bacteria from exposure to hydrochloric acid. In order to study the integrity of the entire microcapsule individual stains of all three components ensure that all components are present and formed correctly. After imaging the microcapsules to determine if they are constructed as predicted, live/dead viability stains will be performed before and after exposure to HCl to identify the survival rate of the lactic acid bacteria. The results of this comparison will determine if microcapsules could potentially be a suitable therapy for the treatment of ulcers.

The Whey Protein Microcapsule

As stated previously in the Introduction, the microcapsule is composed of a whey protein capsule, filled with fat droplets and lactic acid bacteria (LAB). The first steps of the entire experiment were to simply find a way to clearly image the microcapsule with no stains used at all. This was done to determine how much incubation time, water, and dehydrated microcapsules are needed per sample so individual microcapsules could be imaged on the slide easily. All images shown in this section were either taken with the DIC, a camera put up to the eye piece to show the microcapsules under white light, or an SEM to show what the microcapsules look like dehydrated.

The DIC images are the control experiment for the rest of the fluorescence experiments being done. These images show what correctly rehydrated microcapsules look like, and that there is no auto fluorescence (AlexaFluor 488, AlexaFluor 633, and Texas Red filters were all activated one at a time along with DIC) when no stains are present. All samples were prepared by mixing a small amount (less than 0.001g) of dehydrated microcapsules with 50 μ L DI water, and letting the solution sit overnight so the microcapsules could absorb the water. The next day the contents were placed onto a microscope slide and imaged as shown in Figure 11.

Figure 11 – Microcapsules after 12 hours of rehydration. Image taken with DIC using 100x objective.

It was difficult to view bacteria in any great detail inside the microcapsule even at 100x magnification. The confocal microscope is capable of magnifying beyond 100x by means of a digital zoom. The digital zoom allowed small areas of the microcapsule to be enlarged slightly (1.5x) without much distortion while still using the 100x objective. Areas that were thought to include bacteria were further imaged 1.5x. These images can be seen along with the original 100x image as seen in Figures 12 and 13.

Figure 12 – Locations which are thought to contain bacteria. Images recorded with DIC. Top image uses 100x objective, side images at 150x. Cannot prove presence of bacteria without staining.

Figure 13 – More locations which are thought to contain bacteria. Images recorded with DIC. Top image uses 100x objective, side images at 150x.

Figures 12 and 13 only show what is thought to be bacteria. The contoured lines inside the microcapsule are shaped like bacteria, but could easily be fat droplets not completely in focus, a shadow, or imperfection in the microcapsule. It cannot be proven that bacteria are inside of the microcapsule until Live/Dead stainings are performed later in the experiment. Using the DIC it is impossible to differentiate the structures of the microcapsule because DIC is not a fluorescence microscopy method.

SEM images were also taken. While the SEM images were not crucial to the experiment at any stage, it was important to see what the whey protein microcapsules looked like compared to the SEM image shown in Figure 6. The images in Figure 6 were taken by the researchers, Souza et al, who constructed the microcapsules. If the SEM images in Figure 14 looked significantly different than what was shown in Figure 6, it could show that the microcapsules had been damaged during spray drying, storage, or even transportation, which could render the therapy useless.

Figure 14 – SEM images of dehydrated microcapsules. Images taken at Building 192 at California Polytechnic State University – San Luis Obispo. These images were used to compare samples worked on in San Luis Obispo with samples created in Brazil.

Figure 14 shows images of dehydrated microcapsules taken with an SEM. The images don't look exactly the same as Figure 6, but the images in Figure 14 show similar characteristics

such as jagged ridges, and a bumpy surface. Differences between Figures 6 and 14 can be attributed to sample preparation and familiarity with the SEM used. The samples in Figure 6 were prepared by experts, the researchers who constructed the microcapsules. In this case, the dehydrated microcapsule was prepared in a relatively crude fashion by rehydrating the microcapsules with ethanol, then allowing them to air dry on a microscope slide. The slide was covered to prevent as much contamination as possible. Rough, jagged edges are present everywhere on the dehydrated microcapsules as expected.

Once the structure of both dehydrated and rehydrated microcapsules was known, and that there was no auto fluorescence, staining of the microcapsule was started. Initially to ensure that the stains were specific to the intended component of the microcapsule, one stain was used at a time to control for interactions between stains and efficiency of the identifier itself. Once the procedures were demonstrated to work correctly, multiple stains were used at one time to illustrate an overview of the entire microcapsule.

Staining for Fat

The first step of testing the hypothesis of microcapsule structure was to determine if all components of the microcapsule are present and intact. Structurally, the microcapsule is thought to have a protein shell that surrounds the small fat droplets and LAB. During synthesis of the microcapsules, it was realized that fat was necessary for the survival of the lactic acid bacteria. Without the fat, the bacteria would not survive the spray drying process. It is thought that the fat acts as a heat sink protecting the bacteria when the bacteria is exposed to the high temperatures of the spray drying process, much like a CPU fan protects a processor in a computer from overheating. Since the fat droplets and protein microcapsule are thought to protect the bacteria in different manners, staining procedures for those two components were developed independently. The fat protocol was arbitrarily chosen first.

To image the fat inside of the microcapsules, the stain Nile red was chosen and provided by The Dairy Product Technology Center (DPTC). The DPTC located at California Polytechnic State University – San Luis Obispo has had great success using Nile red to stain fat in various dairy samples. The DPTC uses Nile Red, purchased from Fisher Scientific, dissolved in acetone at a concentration of 1mg/mL for staining of fat in dairy products. The microcapsules are not exactly similar to milk or cheese samples, but using Nile red for fat staining was a logical starting point.

Fat Staining Procedure

This procedure is an iteration of other procedures which are included in the appendix. Nile Red solution (1mg Nile Red/mL acetone) was placed in an open eppendorf tube which allowed the acetone to evaporate, leaving the Nile Red powder behind. 10µL of Nile Red solution was left to evaporate, leaving .01mg of Nile Red powder. Adding 25µL of water to the

Nile Red powder will result in a final Nile Red concentration of 0.4 mg/mL. This was a sufficient concentration to stain the fat while avoiding the dehydrating effects of the acetone.

Procedure

- Pipette 10 μ L Nile Red into 1.5mL eppendorf tube. Place in fume hood for 5 minutes or until pink film has formed at bottom, whichever comes first.
- Place small amount of dehydrated microcapsules(less than 0.001g) into same eppendorf tube.
- Add 25 μ L of DI water to eppendorf tube containing dehydrated microcapsules and Nile Red powder. Mix well by hand to ensure all microcapsules and Nile Red powder is in liquid.
- Pipette 25 μ L from tube onto microscope slide. Cover with cover slip.
- Let stain sit for 8 hours out of sunlight.
- Image on confocal microscope using Nile Red filter and 100x objective lens.

Results

Results were similar to the Nile Red powder dissolved in water; however the red fluorescence had a much more intense signal even at lower laser powers. Thus, the microcapsules were much easier to find when looking for them on the microscope slide. The red circles on the inside of the microcapsule are the fat droplets. There is some fat on the outside of the microcapsule, most likely there due to the way they are created.

Figure 15 – Representative images of Fat Stain. Image A shows rehydrated microcapsules using Nile Red filter. Red haze is visible on all microcapsules imaged on microscope slide. Image B shows DIC image of rehydrated microcapsules. Image C shows DIC with Nile Red overlay. Images should show individual red circles inside the microcapsule instead of the entire microcapsule fluorescing red.

The red haze that is viewable inside the microcapsule was a little concerning. The Nile Red looked like it could be staining components other than the fat, but since the fat droplets fluoresce so much brighter than the surrounding area the stain appears to be working as intended. Not knowing the specificity of the Nile Red led to the start of the next group of experiments: staining for protein.

Staining for Protein

Once the fat had been identified in the microcapsules, the protocol for staining the protein capsule was started. As mentioned previously, the capsule is made of whey protein and is meant to help shield the lactic acid bacteria from gastric acid produced by the stomach. If the capsule is damaged in any way the treatment will be ineffective because the probiotics will be killed by acids present in stomach.

The purpose of these experiments was to see if the protein is where it is expected to be on the outside of the capsule. Any gaps or breaks in the protein will expose the bacteria to acid or other substances which could render the microcapsules useless.

Protein Stain

The DPTC uses a stain called Fast Green to stain for protein. Like the Nile Red used previously, Fast Green can be obtained through Fisher Sciences. The Fast Green, which was once again provided by the DPTC, was mixed with nanopure water at a concentration of 1mg of Fast Green per 1mL of nanopure water. The results achieved with Nile Red were good, so the recommended Fast Green was used as well. For these experiments, Fast Green is simply combined with dehydrated microcapsules, and some rehydrating water. The samples can be imaged after approximately 8 hours of incubation.

Procedure

- Place small amount of dehydrated microcapsules(less than 0.001g) into 1.5mL eppendorf tube.
- Pipette 20 μ L Fast Green to same tube.
- Pipette 20 μ L DI water to same tube.

- Keep eppendorf tube out of sunlight for 8 hours.
- Pipette contents of eppendorf tube onto microscope slide and cover with cover slip
- Image on confocal microscope using AlexaFluor 488 and 100x objective lens.

Results

The staining procedure was quick and effective. The images taken show the inside of the microcapsule in much more detail than the Nile Red as expected. The green fluorescence in Figure 16 represents protein stained by Fast Green.

Figure 16 - Sample images taken from Protein Stain. Image A shows AlexaFluor 488 filter. Image B displays DIC. Image C displays DIC with AlexaFluor 488 filter overlay. Since image was taken near center of microcapsule's thickness, the fluorescence was expected to be along the perimeter of the microcapsule, not throughout. 10μm scale bar applies to images A-C.

Image 16a illustrates the structure of the protein capsule. The signal intensity is consistent over much of the microcapsules surface other than a few spots of very intense fluorescence. These bright spots look much like the fat droplets stained in other fat staining experiments, included in the appendix, which may indicate bleed through from other channels, autofluorescence, or that the fast green is not specific to the protein alone.

Figure 17 – Cropped sample image taken from Protein Stain using 100x objective. AlexaFluor 488 filter is used. Green fluorescence shows presence of protein on the microcapsule.

Figure 17 shows the microcapsules in more detail (original image was cropped and enlarged, no digital zoom used), which looks analogous to the Nile Red images, but with a green signal. It is unknown at this point if only one of or both of the stains is staining nonspecifically. Using both stains on a single sample make troubleshooting the specificity of each stain more effective. Being able to toggle the two filters on and off individually may show which staining protocol is not working correctly assuming that the fat structures and protein shapes are known.

Staining for Protein and Fat: The Combined Stain

Once protocols for staining fat and protein individually were completed, development of a protocol for staining both at the same time began. This experiment was not done first for a few reasons. The most important was for ease of determining what errors were made if the images did not turn out as expected. If an error was made in either the staining protocol or on the confocal, it would have been very difficult to pinpoint what exactly went wrong. But since both fat and protein staining protocols gave very similar images, it is important now to stain with both to identify which stain might be working incorrectly.

Combined Stain

Both Nile Red and Fast Green provided by the DPTC were used at the same time for the combined staining experiment. The combined stain experiment used AlexaFluor 488 for Fast Green and AlexaFluor 633 for Nile Red. Since the spectra for both filter sets are dissimilar enough, both filters could be activated at the same time.

Procedure

- Pipette 10 μ L Nile Red solution [1mg/mL acetone] into 1.5mL eppendorf tube. Place in fume hood for 5 minutes or until pink film has formed at bottom, whichever comes first.
- Place small amount of dehydrated microcapsules(less than 0.001g) into same eppendorf tube.
- Pipette 20 μ L Fast Green to same tube.
- Pipette 20 μ L DI water to same tube.
- Keep eppendorf tube out of sunlight for 8 hours.
- Pipette contents of eppendorf tube onto microscope slide and cover with cover slip

- Image on confocal microscope using AlexaFluor 488 and AlexaFluor 633 filters using 100x objective lens.

Results

Using AlexaFluor 488 and 633 at the same time resulted in dramatically different images than staining individually. The green and red signals are clearly distinguishable from each other illustrating what Dr. Jimenez had anticipated: a protein (green) capsule surrounding fat (red) droplets. The protein appears green and the fat is red. While the images looked good, the distribution of the protein wasn't exactly as expected. The images taken in Figure 18 were taken about midway through the microcapsule. At that level, the protein should appear only as a green ring around the perimeter of the microcapsule.

Figure 18 - Representative images from Combined Stain #2. Images A and D show AlexaFluor 633 Filter. Images B and E show AlexaFluor 488 filter. Images C and F show both AlexaFluor 488 and 633 filters. Fat appears as red while protein appears green. Protein appears more like a solid mass instead of thin capsule surrounding microcapsule. 10 μ m scale bar applies to images A-F.

In Figure 18 B and E, it looks like the protein is distributed throughout the thickness of the microcapsule. From these images it appears that the whey protein capsule might more closely be a solid whey protein ball when rehydrated. Images were taken at various depths of the microcapsule to record how the distribution of fat and protein changes. These images are included in the appendix in Figure 30.

Staining for Bacteria: Live/Dead

The purpose of this work is to prove or disprove the hypothesis that the protein microcapsule will protect LAB from HCl produced by the stomach. Using a Live/Dead stain will show if the bacteria are alive or not. The stain used in all Live/Dead staining is Live/Dead BacLight Bacterial Viability kit from Invitrogen.com, product number L7012.

The kit combines two different nucleic acid stains, SYTO-9 and Propidium iodide. SYTO-9 is a green fluorescent stain that targets nucleic acids in all bacteria in the sample, live or dead. It has an excitation/emission spectra of 480/500nm (Probes, Inc., 2004). Propidium iodide is a red fluorescent stain that will target only bacteria that have a damaged membrane. It has an excitation/emission spectra of 490/635nm. When both stains are present in bacteria with damaged membranes, the Propidium iodide will reduce the fluorescence of SYTO-9, causing the damaged membrane bacteria to fluoresce red, which means the bacteria is dead (Probes, Inc., 2004).

Bacteria Live/Dead

The purpose of this experiment is simply to develop a working staining procedure using the Live/Dead BacLight Bacterial Viability kit from Invitrogen. Staining bacteria alone is being done first to preserve microcapsule supply while a protocol is being worked out and to confirm that the kit is compatible with this particular strain bacteria. The bacteria used in this experiment was a culture of Lactic Acid bacteria (NCFM) prepared by the DPTC.

Procedure

- Dilute Lactic Acid bacteria in PBS at a 1:10 ratio. Pipette 10µL of suspended LAB and 90µL of PBS into 1.5mL eppendorf tube. Mix well by hand.

- Create Live/Dead staining solution by adding 1.5 μ L STYO-9, 1.5 μ L Propidium iodide, and 997 μ L DI water into 1.5mL eppendorf tube. Mix well.
- Pipette 100 μ L of suspended LAB and 10 μ L Staining solution into a 1.5mL eppendorf tube.
- Pipette 50 μ L of stained LAB on to microscope slide. Cover with cover slip. Let sit for 15minutes in dark at room temperature.
- Image using AlexaFluor 488 and Texas Red filters using 100x objective lens.

Results

Using the BacLight viability kit on a suspended culture of bacteria resulted in images that clearly differentiated live bacteria from those that were dead. The LAB bacteria from the DPTC are all easily distinguishable from one another with no haze seen in some of the protein and fat stains. Since the culture was newly made, most of the bacteria imaged were fluorescing green as anticipated.

Figure 19 - Representative images of Bacteria Live/Dead stain. Image A displays dead bacteria using Texas Red filter. Image B displays live bacteria using AlexaFluor 488 filter. Image C displays DIC image of bacteria on microscope slide. Image D displays DIC with Texas Red and AlexaFluor 488 overlays. Images show that Invitrogen kit is able to label both dead and live bacteria. 10 μ m scale bar applies to images A-D.

Image A of Figure 19 shows dead bacteria, while image B shows a higher quantity of bacteria, which are live as expected. Image C displays only the DIC image. Image D shows the

DIC image with confocal overlay. While it may appear that not all of the visible bacteria were stained, this may be because they are outside of the focus of the microscope when the image was taken.

The next step in Live/Dead staining was to use the same protocol on rehydrated microcapsules. If the stain is successful, the bacteria inside the microcapsule should fluoresce in the same way as Figure 19.

Microcapsule Live/Dead Stain

As stated previously, this experiment follows the same general steps as Bacteria Live/Dead, but using one eppendorf tube to make a staining solution, and a separate for rehydrating microcapsules which will help keep a higher concentration of microcapsules when the stain is added. This was done only to make locating the microcapsules easier when they were being imaged.

Procedure

- Create a 0.3% Live/Dead staining solution by adding 1.5 μ L STYO-9, 1.5 μ L Propidium iodide, and 997 μ L DI water into 1.5mL eppendorf tube. Mix well.
- Place small amount of dehydrated microcapsules(less than 0.001g) into a separate eppendorf tube.
- Pipette 50 μ L Live/Dead stain into eppendorf tube containing dehydrated microcapsules. Mix well.
- After 24 hours, pipette 50 μ L from eppendorf tube onto microscope slide, cover with cover slip.

- Image on confocal using AlexaFluor 488 and Texas Red filter sets using 100x objective lens.

Results

The microcapsules were much easier to locate and image using two eppendorf tubes to prepare the microscope slides compared to using a single tube as in failed experiments listed in the appendix. However, the change in preparing the slide doesn't explain for the change in appearance of the microcapsules. There is a distinct reduction of red fluorescence compared to the bacteria Live/Dead staining.

Figure 20 - Representative images of Microcapsule Live/Dead Stain. Number in images represents slice number out of 40. Image A display Texas Red. Image B display AlexaFluor 488. Image C display both Texas Red and AlexaFluor 488. Images recorded were expected to more closely resemble those of Figure 19.

Figure 21 - Representative images of Microcapsule Live/Dead Stain. Number in images represents slice number out of 40. Image A display Texas Red. Image B display AlexaFluor 488. Image C display both Texas Red and AlexaFluor 488. Images recorded were expected to more closely resemble those of Figure 19.

The microcapsule is shown as a green haze when imaged with the confocal microscope using AlexaFluor 488 and Texas Red filters. Inside the microcapsule are some lime green spots, but it cannot be said if the spots are bacteria or not. Figures 20 and 21 do show some bacteria outside the microcapsule, seen as a red fluorescence. The presence of bacteria outside of the microcapsule illustrates that there are in fact bacteria in the sample; it just can't be imaged inside the microcapsule using the Live/Dead stain kit from Invitrogen. The protein or fat could be interacting with the stains in an unknown manner. The kit was designed to work with both STYO-9 and Propidium iodide stains being used together, but with the inclusion of fat and protein, there may be some mechanistic changes in the way they work.

Alginate Microcapsules

From the lack of information that could be gathered from staining the protein microcapsules with the Live/Dead stains, it was obvious that a different approach was needed. Either the stains being used to target bacteria had to change or the microcapsule itself had to change. Dr. Jimenez found that “microcapsule-like” droplets could be created in the DPTC using alginate sprayed into a calcium chloride solution. To test the new alginate microcapsules, various protocols were devised. Each tested the components of the microcapsules. First, a control experiment was created to test microcapsules made only of alginate. The control experiment was done to ensure there wasn't any nonspecific staining of the alginate. The second experiment optimized the portions of the alginate solution mixed with LAB ranging from a 1:10 dilution to a 3:10 dilution. Lastly, fat was added to the solution varying from 1:10 to 3:10 of the total solution. Even though fat is not necessary for encapsulating the LAB within alginate, the inclusion of fat will make the alginate microcapsules more closely resemble the protein microcapsules.

Alginate Only Microcapsule

An alginate only microcapsule was tested to ensure that none of the problems that had occurred with the original microcapsules were present with the alginate microcapsules, namely, the interaction between the whey protein and Live/Dead bacteria stain. After synthesized and stained, there should be no fluorescence when imaged under any filter (AlexaFluor 488 and Texas Red) with the confocal microscope.

Procedure

- Mix 1.5g calcium chloride into 100mL DI water. Allow to dissolve completely at room temperature.

- Pour calcium chloride solution into petri dish until bottom is completely covered by solution. Place dish in sink.
- Pour 10ml of 1% alginate solution into 15mL container.
- Place spray bottle top tube into 15mL container.
- Spray all 10mL of alginate as a fine mist into petri dish containing calcium chloride solution
- Pour petri dish and all contents into another 15mL conical. Label "Alginate Stock Solution".
- Create Live/Dead staining solution by combining 1.5 μ L STYO-9 and 1.5 μ L Propidium Iodide with 1mL of DI water into 1.5mL eppendorf tube.
- Mix 40 μ L of Alginate Stock Solution and 10 μ L Live/Dead staining solution.
- Incubate for 30 minutes out of sunlight at room temperature.
- Pipette entire eppendorf tube onto microscope slide. Cover with cover slip.
- Image on confocal microscope using Texas Red, and AlexaFluor 488 filter sets with 100x objective lens, with DIC enabled.

Results

Images taken from this protocol showed nearly spherical droplets when imaged with DIC and no fluorescence in nearly all samples when imaged with either Texas Red or AlexaFluor 488 filter sets. Although some bacteria were identified in a few of the alginate microcapsules, it is important to note that the protocol was not carried out using sterile water, instruments, or tools. The species of bacteria in this sample is irrelevant.

Figure 22 - Images taken from Alginate Only experiment. Images A and E display Texas Red filter. Images B and F display AlexaFluor 488 filter. Images C and G display DIC only. Images D and H display DIC with both Texas Red and AlexaFluor filter sets overlaid. No fluorescence in either channel shows alginate does not autofluoresce.

The alginate only microcapsule protocol is the control for all experimentation done with alginate microcapsules. It demonstrates that alginate microcapsules can be constructed using the spray bottle method, and there is no sign of auto fluorescence.

Alginate and LAB Microcapsule

After it was determined there were no undesired results with the alginate microcapsule and the bacteria Live/Dead stain, LAB was added to the alginate solution. The goal of this experiment is to determine if the bacteria can be encapsulated within the alginate droplet, stained, and imaged. The same procedural steps were followed as in the alginate only experiment, but with a small portion of alginate replaced with bacteria. The protocol was repeated a second time, with HCl being added to the completed alginate microcapsule solution for 8 hours. The HCl application will show the protective qualities of alginate.

Procedure

- Mix 1.5g calcium chloride into 100mL DI water. Allow to dissolve completely at room temperature.
- Pour calcium chloride solution into petri dish until bottom is completely covered by solution. Place dish in sink.
- Pour 9mL of 1% alginate solution into 15mL container
- Pipette 1mL of LAB culture (prepared by DPTC) into same 15mL conical containing alginate. Mix well.
- Place spray bottle top tube into 15mL container
- Spray alginate-LAB mixture as a fine mist into petri dish containing calcium chloride solution
- Pour petri dish and all contents into another 15mL conical. Label "Alginate-LAB Stock Solution".
- Create Live/Dead staining solution by combining 1.5 μ L STYO-9 and 1.5 μ L Propidium Iodide with 1mL of DI water into 1.5mL eppendorf tube.
- Mix 40 μ L of Alginate-LAB Stock Solution and 10 μ L Live/Dead staining solution.
- Incubate for 30 minutes out of sunlight at room temperature.
- Pipette entire eppendorf tube onto microscope slide. Cover with cover slip.
- Image on confocal microscope using Texas Red, and AlexaFluor 488 filter sets with 100x objective lens, with DIC enabled.

Results

Images taken with LAB mixed into the alginate look significantly different than the original protein microcapsules after being stained with Live/Dead. Figure 23 shows the bacteria

inside the microcapsule are clearly distinguishable from the rest of the microcapsule. The images are proof that LAB can be encapsulated, and will survive the encapsulation process. While the bacteria are clearly visible, the amount of bacteria inside the microcapsule was less than expected. The alginate-LAB experiment involving HCl will add slightly more LAB into the protocol only so the bacteria will be easier to view.

Figure 23 – Representative images taken from Alginate + LAB microcapsules. Images A and E display Texas Red filter. Images B and F display AlexaFluor 488 filter. Images C and G display DIC only. Images D and H display DIC with both Texas Red and AlexaFluor filter sets overlaid. These images demonstrate that bacteria can be microencapsulated using the methods mentioned.

Since the bacteria are visible inside the microcapsule, the next step was to test the alginate microcapsule's protective qualities against 0.1M HCl. It was important to simulate as closely as possible to what a microcapsule might experience in the stomach; 0.1M HCl closely resembles the gastric acid produced by the stomach and 8 hours is the time it might take for some foods to pass through the stomach. The next experiment will follow the same steps, but will include the microcapsules being exposed to HCl for 8 hours and then neutralized by NaOH before viability assessment and staining occurs.

Procedure (HCl)

- Mix 1.5g calcium chloride into 100mL DI water. Allow to dissolve completely at room temperature.
- Pour calcium chloride solution into petri dish until bottom is completely covered by solution. Place dish in sink.
- Pour 8mL of 1% alginate solution into 15mL container
- Pipette 2mL of LAB culture (prepared by DPTC) into same 15mL container. Mix well.
- Place spray bottle top tube into 15mL container
- Spray alginate-LAB mixture as a fine mist into petri dish containing calcium chloride solution
- Pour petri dish and all contents into another 15mL conical. Label "Alginate-LAB Stock Solution".
- Create Live/Dead staining solution by combining 1.5 μ L STYO-9 and 1.5 μ L Propidium Iodide with 1mL of DI water into 1.5mL eppendorf tube.
- Mix 40 μ L of Alginate-LAB Stock Solution and 10 μ L 0.1M HCl for 8 hours.
- Neutralize solution by adding 10 μ L 0.1N NaOH to 1.5mL eppendorf tube.
- Add 10 μ L Live/Dead staining solution to 1.5mL eppendorf tube.
- Incubate for 30 minutes out of sunlight at room temperature.
- Pipette entire eppendorf tube onto microscope slide. Cover with coverslip.
- Image on confocal microscope using Texas Red, and AlexaFluor 488 filter sets with 100x objective lens, with DIC enabled.

Results (HCl)

There were no glaring differences from the control (no HCl) and experimental (HCl) groups. The shape of the microcapsule still spherical and the bacteria inside were still alive. Even though a fair portion of the bacteria were dead, it's impossible to determine at what stage they died at. Even using a fresh culture of bacteria, it is highly unlikely that 100% of the bacteria were alive. It was clear from these images in Figure 24 that alginate microcapsules are sufficient in protecting LAB for a minimum of 8 hours.

Figure 24 - Alginate + LAB microcapsules after exposure to 8 hours of 0.1M HCl. Images A and E display Texas Red filter. Images B and F display AlexaFluor 488 filter. Images C and G display DIC only. Images D and H display DIC with both Texas Red and AlexaFluor filter sets overlaid. These images demonstrate that bacteria can be protected from HCl exposure.

Inclusion of fat drastically changed the shape of the alginate microcapsules. It was thought that the fat being sprayed through the spray bottle nozzle affected the formation of the alginate droplets. Results of those experiments are included in the appendix.

Discussion and Conclusion

While these experiments were performed to discover if whey protein microcapsules could protect bacteria from stomach acid and proving their structure, other discoveries were made due to problems encountered during the experiments. Unfortunately from the experiments conducted, it is unknown if the main goal of proving if the whey protein microcapsules can protect bacteria because of problems encountered with the staining procedures. Without accurate staining of only the bacteria inside the microcapsule, it is impossible to compare before and after images of the microcapsules when they are exposed to HCl. It was shown that the Live/Dead staining procedure works on bacteria in suspension, but when the procedure is applied to microcapsules the images are inconclusive. The entire microcapsule is covered in a red or green haze depending on which filters are active. Live/Dead staining of the whey protein microcapsules resulted in little useful information.

The theorized structure of the microcapsule was mostly correct. It was thought that the microcapsule was composed of a whey protein capsule that surrounds fat droplets and bacteria inside the microcapsule. Based on images taken in the Combined Stains experiment, it appears that the whey protein more closely resembles a solid mass than a capsule. Green fluorescence in Figures 18 shows that the protein is present everywhere in the microcapsule. The fat droplets displayed in red were only on the inside of the microcapsule as assumed.

Unlike the whey protein microcapsules, it was discovered that the alginate microcapsules can protect bacteria from stomach acid. In the experiment “Alginate and LAB microcapsules”, the experimental group was exposed to 0.1M HCl for 8 hours. Samples showed that there were live bacteria inside the microcapsules after exposure. Some samples had more

live bacteria than others, but as a whole the alginate worked well. Survival rates weren't as high as the control group which was not exposed to the HCl which was unsurprising.

When fat was added to the alginate microcapsules, an entirely new set of problems arose. When slides were imaged, extremely large bubble like formations were present in each sample that included the emulsified fat or fat from milk cream. It was later proved these formations were entirely fat by staining the samples with Nile Red (as shown in Figure 46 in appendix). Some of the fat droplets were so large the 100x objective lens used in all imaging couldn't be used; instead the 10x objective was used to capture the entire globule. The inclusion of fat also made locating the alginate microcapsules extremely difficult. Some microscope slides had as few as a single microcapsule on it, but the problems weren't limited to only a drastic decrease in microcapsule number. Even though LAB was added to the initial solution, most microcapsules observed had no trace of bacteria at all (as shown by Figure 41 in appendix). It is unknown why the bacteria were unable to be encapsulated even though the bacteria were present in the solution. After imaging alginate microcapsules both with and without fat, it is clear that the inclusion of fat does not allow the staining to work properly using the methods described. Either using only fat and bacteria, or using a different method of spraying the alginate solution would be necessary if testing the efficacy of the microcapsules.

Future work with the microcapsules could include clearer differentiation of the fat and protein stains. In some of the images from the Combined Stain experiments, the same circular structures were present in both filter sets. A more clear differentiation of fat and protein could be accomplished by changing fat and protein stains as well as using filters that have a larger difference in their emission spectra. Other future work could include further attempts of Live/Dead staining using stains that target something in bacteria other than nucleic acids. Only

nucleic acid stains were used, other stains may not have the unwanted interactions with protein as the Invitrogen viability kit. If the bacteria are able to be imaged, the images could prove the effectiveness of the whey protein microcapsules. From this, there would be no need to create the alginate microcapsules which merely mimic the whey protein microcapsules. Since the alginate microcapsules can encapsulate bacteria, useful images were taken from those experiments. It was discovered that the alginate can protect the bacteria for at least 8 hours; however it doesn't result in a 100% survival rate. It is unknown how much the therapy would be affected by a drop in bacteria count; finding that information is outside the scope of this paper.

In conclusion, LAB can be protected from stomach acid by encapsulation in alginate. It doesn't protect all of the bacteria encapsulated, but enough bacteria could be alive to still be effective. Including fat in the alginate microcapsules should be avoided using the procedures used. Fat from either source essentially ruined the microcapsules; the fat greatly reduced the number of microcapsules formed and even prevented encapsulation of the bacteria. It is unknown if the whey protein microcapsules protect bacteria from HCl. The only way to prove the effectiveness of either type of microcapsule would be to test the microcapsules on test animals that have ulcers or are known to be infected with *H pylori*.

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Appendices

Fat Staining Procedure #1

The first attempt at fat staining was to verify Nile Red could be used to image dehydrated microcapsules.

Procedure

- Place small amount of dehydrated microcapsules from DPTC(less than 0.001g) into a 1.5mL eppendorf tube.
- Add 5 μ L DI water to same tube.
- Add 20 μ L Nile Red Solution (1mg/mL) to same tube. Shake tube held in fingers to mix well ensuring all microcapsules are evenly dispersed throughout the solution. Do not vortex to avoid compromising the microcapsule shell as seen in the preliminary experimentation (Figure 7).
- Pipette 25 μ L of the solution from tube onto microscope slide. Cover with cover slip.
- Let stain incubate for 8 hours out of sunlight.
- Image on confocal microscope using Nile Red filter set and 100x objective lens.

Results

Comparing the microcapsule structure seen in Fat Stain Procedure #1(Figure 25) to the rehydrated microcapsule seen under white light (Figure 6) and the dehydrated microcapsule seen in the SEM image(Figure 13), the results look more like the later. There are two possible explanations for the appearance of the microcapsules in this experiment. The first reason might be that the seal between the microscope slide and cover slip may have allowed evaporation to

occur. Using only 25 μ L of liquid, any evaporation that occurs could affect results. The second reason might be from the stain itself. The DPTC's stock solution of Nile Red comes from Nile Red in powder form dissolved in acetone. Acetone's mechanistic affects on the microcapsules are unknown at this time.

Figure 25 – First images of Fat Staining Procedure #1. 14a Shows Nile Red filter set only. 14b shows DIC of dehydrated microcapsule. 14c shows DIC with Nile Red filter set overlay.

Figure 26 - Second images of Fat Staining Procedure #1. 15a Shows Nile Red filter set only. 15b shows DIC of dehydrated microcapsule. 15c shows DIC with Nile Red filter set overlay.

All microcapsules imaged looked similar to those in the DIC images in Figures 10 and 11 were previously incubated in water for 12hrs. It is clear that the Nile Red is interacting with the microcapsule. Even though there is a signal, the Nile Red does not appear to bind to the fat as

expected. The images show a red haze instead of the spheres the fat droplets are thought to look like. The next experiment used Nile Red powder dissolved in water instead of acetone to control for the possible unknown adverse effects of acetone on the microcapsules. Given enough time, a small amount of water sufficiently rehydrated the microcapsules to look more similar to that shown in Figure 6.

Fat Staining Procedure #2

As stated previously, the second attempt at staining fat inside the microcapsules uses Nile Red powder dissolved in DI water at the same concentration the DPTC dissolves in acetone, 1 mg Nile Red powder for every 1mL of solvent. The Nile Red powder did not dissolve nearly as much in water as in acetone, since acetone is a much stronger solvent than water. The DI water did take on a slight tint of red, meaning some of the powder did dissolve. Not knowing if the Nile Red/Water solution would stain fat, the same steps used in Procedure #1 were followed.

Procedure

- Place small amount of dehydrated microcapsules(less than 0.001g) into 1.5mL eppendorf tube.
- Add 1mg of Nile Red powder and 1mL DI water into separate eppendorf tube. Label Nile Red Water.
- Add 25 μ L of Nile Red stain solution to eppendorf tube containing dehydrated microcapsules. Mix well by hand to ensure all microcapsules are in liquid.
- Pipette 25 μ L from tube onto microscope slide. Cover with cover slip.
- Let stain sit for 8 hours out of sunlight.
- Image on confocal microscope using Nile Red filter set and 100x objective lens.

Results

The shape of the microcapsules look significantly and structurally more intact using the above procedure. The microcapsule is much more circular with no jagged boundaries.

Figure 27 - Representative images taken using Nile Red dissolved in water for fat staining. Image A displays Nile Red filter. Image B shows only DIC with circular rings surrounding microcapsule that are distortions created by DIC and are not part of the microcapsule. Image C shows DIC with Nile Red overlay

Figure 28 - White light image of a correctly rehydrated microcapsule at 100x magnification taken with camera through eye piece.

While the shape of the microcapsule looks much more circular in Figure 27, the Nile Red still seems to be producing a red haze instead of circles, how fat would be shaped. In most of the microcapsules imaged, the signal was very faint. This is probably due to the Nile Red not completely dissolving into the water and thus resulting in a low concentration of Nile Red exposed to the microcapsules. The next experiment will use water alone to rehydrate the microcapsules, and additional Nile Red to stain the fat.

Combined Stain #1

Both Fast Green and Nile Red stains were giving signals, it was decided to stay with those two stains and try to alter the procedure or confocal microscope settings instead of trying new stains. The first attempt followed the same procedures as the working Fast Green and Nile Red experiments combined together.

Procedure

- Pipette 10 μ L Nile Red acetone solution [1mg/mL] into 1.5mL eppendorf tube. Place in fume hood for 5 minutes or until pink film has formed at bottom, whichever comes first.
- Place small amount of dehydrated microcapsules(less than 0.001g) into same eppendorf tube.
- Pipette 20 μ L Fast Green to same tube.
- Pipette 20 μ L DI water to same tube.
- Keep eppendorf tube out of sunlight for 8 hours.
- Pipette contents of eppendorf tube onto microscope slide and cover with cover slip.
- Image on confocal microscope using AlexaFluor 488 and Nile Red filters consecutively using 100x objective lens.

Results

Before imaging even began, there was a critical issue with the confocal microscope. AlexaFluor 488 and Nile Red cannot be active at the same time because the emission spectra of the two filters are too similar. The following images are taken by using only one filter at a time.

Figure 29 - Representative image of Combined Stain #1. Image A displays Nile Red only. Image B shows AlexaFluor 488 filter only. Image C was created by combining both A and B in ImageJ since both Nile Red and AlexaFluor 488 filters could not be used at the same time on the confocal. Image D shows a DIC image of same microcapsule.

Images A and B in Figure 29 look identical other than the channel color assigned. Since the two filters being used won't work at the same time, it's possible that the Nile Red and AlexaFluor 488 filters have emission spectra that are too similar. The same signal is being picked up by both filter sets. The next experiment dealt with changing the filters being used. If results looked similar to those of this experiment, different stains for both fat and protein would be investigated.

Combined Stain #3

Combined stain #3 followed the same protocol as #2, except that each microcapsule was imaged throughout its thickness. For each microcapsule imaged, 20 slices were recorded. This was done to observe any changes that might occur in protein or fat density with respect to location in the microcapsule.

Procedure

- Pipette 10 μ L Nile Red solution [1mg/mL acetone] into 1.5mL eppendorf tube. Place in fume hood for 5 minutes or until pink film has formed at bottom, whichever comes first.
- Place small amount of dehydrated microcapsules(less than 0.001g) into same eppendorf tube.
- Pipette 20 μ L Fast Green to same tube.
- Pipette 20 μ L DI water to same tube.
- Keep eppendorf tube out of sunlight for 8 hours.
- Pipette contents of eppendorf tube onto microscope slide and cover with cover slip.
- Image on confocal microscope using AlexaFluor 488 and AlexaFluor 633 filters using 100x objective lens.

Results

The images recorded looked slightly different than in protocol #2 included in the main paper. In protocol #2, the green haze in the image created by fluorescing protein appears to be more concentrated on the outside with very little on the center of the circle. This was expected, imaging near the center of the microcapsule should show protein only along the perimeter of the microcapsule. In protocol #3, most of the images display a much more uniform distribution

of protein throughout the microcapsule with only a faint haze around the edge, giving the appearance that the microcapsule could actually be completely filled with protein instead of the protein being a hollow sphere.

Based on the images taken from Combined Stain protocols #2 and #3, it appears that the protein capsule theory is not true. The protein is actually present throughout the entire microcapsule, making the microcapsule more like a solid protein “ball” than protein capsule. At this point it is unknown if this discovery will affect the survivability of the bacteria when it is exposed to HCl.

Figure 30 - Images of microcapsule taken at various depths. Number represents slice out of 20 slices image was taken at. Images A show AlexaFluor 633 filter. Images B show AlexaFluor 488. Images C show both AlexaFluor 488 and 633. Green signal is much more visible in each depth.

Microcapsule Live/Dead Stain #1

Since the protocol developed for staining bacteria in Bacteria Live/Dead was successful, the same protocol was used to stain the microcapsules. A successful image would show the microcapsule shell displaying no fluorescence; with bacteria clearly visible and fluorescing on the inside assuming the spray drying process didn't kill the LAB in the manufacturing process.

Procedure

- Create a 0.3% Live/Dead staining solution by adding 1.5 μ L STYO-9, 1.5 μ L Propidium iodide, and 997 μ L DI water into 1.5mL eppendorf tube. Mix well.
- Place small amount of dehydrated microcapsules (less than 0.001g) into same eppendorf tube.
- After 30 minutes, pipette 50 μ L from eppendorf tube onto microscope slide, cover with cover slip.
- Image on confocal using AlexaFluor 488 and Texas Red filters with 100x objective lens.

Results

Figure 31 was taken only 30 minutes after staining, the same length of time used to image the bacteria. However with the microcapsule, the entire microcapsule fluoresced solid green. There was no sense of structure, or any presence of bacteria.

Figure 31 - Representative image of Microcapsule Live/Dead Stain #1 after 30 minutes of incubation. Image was taken using both Texas Red and AlexaFluor 488 filter sets.

An incubation time of 30 minutes was significantly shorter than previous experiments. The same eppendorf tube had enough leftover to create another sample that was incubated for 36 hours before imaging. The results are displayed in Figure 32.

Figure 32 - Representative image of Microcapsule Live/Dead Stain #1 after 36 hours of incubation. Image A displays Texas Red filter. Image B displays AlexaFluor 488 filter. Image C displays both Texas Red and AlexaFluor 488 filters. No visible bacteria inside of microcapsules.

After 36 hours, the fluorescence was more defined but still provided no useful information. Structure of the microcapsule was visible, but there was no clear evidence of rod-shaped bacteria anywhere in the microcapsules. Images could show that too much stain was used, or that the microcapsules contain far more bacteria than initially expected. Knowing that the stain and filters work, the next experiments altered the concentrations of the stains and the amounts added to the dehydrated microcapsules. Invitrogen protocols recommended not using a stain concentration above 0.3% due to DMSO being included into the stain. In higher concentrations, DMSO might adversely affect staining (Probes, Inc., 2004). It is possible that with the inclusion of the microcapsule, a 0.3% stain might be too concentrated. The next experiment worked with a lower concentration to try and achieve clearer images.

Microcapsule Live/Dead Stain #2

Live/Dead Stain #1 used a 0.3% Live/Dead staining solution. The fluorescence on some of the images was rather intense compared to that seen in the bacteria stains. This experiment will follow the same steps as #1, but with a 0.1% Live/Dead staining solution. Incubation time was also changed to 24 hours. 24 hours was chosen mainly for convenience so images could be taken at the same time the next day. If images are still unclear, the same samples can be saved and imaged an additional 12 hours later.

Procedure

- Create a 0.1% Live/Dead staining solution by adding 0.5 μ L STYO-9, 0.5 μ L Propidium iodide, and 999 μ L DI water into 1.5mL eppendorf tube. Mix well.
- Place small amount of dehydrated microcapsules(less than 0.001g) into tube same eppendorf tube. Mix well by hand.
- After 24 hours, pipette 50 μ L from eppendorf tube onto microscope slide, cover with cover slip.
- Image on confocal using AlexaFluor 488 and Texas Red filter sets.

Results

The images taken after 24 hours with a 0.1% staining solution looked similar to those taken after 36 hours in Microcapsule Live/Dead stain #1. However, all of the microcapsules looked dehydrated; none had the spherical shape of a rehydrated microcapsule. It's unclear if the 0.1% concentration of Live/Dead stain dehydrated the microcapsules or if it never rehydrated in the first place. Even though the microcapsules aren't shaped correctly, there are no rod-shaped bacteria visible inside.

Figure 33 - Representative image of Microcapsule Live/Dead #2. Images A and D display Texas Red filter. Image B and E display AlexaFluor 488 filter. Images C and F display both Texas Red and AlexaFluor 488 filters. Microcapsules do not appear circular, may have dehydrated during incubation period. Colors look similar to Microcapsule Live/Dead #1.

It is unknown if the change in concentration or an error in procedure caused the dehydrated appearance of the microcapsules in this experiment. After reviewing procedure steps, the next experiment will go back to using a 0.3% concentration of stain, and using two separate eppendorf tubes instead of a single tube. One tube will be used for creating a staining solution at the desired concentration, and the second will be used to rehydrate and stain the microcapsules. Using two eppendorf tubes will allow the microcapsules to be stained at the correct concentration, but using less of the stain. This will make the microcapsules easier to image, resulting in more images being taken and hopefully some higher quality images due to imaging more.

Microcapsule Live Stain/ Dead Stain #4

The staining kit from Invitrogen is designed to be used with both stains at the same time. For this experiment it was decided only one stain would be used at a time. In one sample, only Propidium iodide was used, and in the other only STYO-9 was used. This procedure was done to ensure the individual stains weren't affecting each other with the inclusion of fat and protein in the solution.

Propidium Iodide

Propidium Iodide is one part in the Invitrogen Live/Dead kit that selectively targets bacteria in the sample that have disrupted membranes (Probes, Inc., 2004). If the microcapsules perform as theorized and the stain works correctly, there shouldn't be much to see when imaged with the Texas Red filter. If the microcapsule protects bacteria, therefore keeping the membranes intact, the Propidium iodide won't have any dead bacteria to bind to.

Procedure

- Pipette 1.5 μ L of Propidium iodide and 498.5 μ L DI water into an eppendorf tube. Mix well.
- Place small amount of dehydrated microcapsules(less than 0.001g) into a separate eppendorf tube.
- Pipette 50 μ L of Propidium iodide into eppendorf tube containing dehydrated microcapsules. Mix well.
- After 24 hours, pipette 50 μ L from eppendorf tube onto microscope slide, cover with cover slip.
- Image on confocal using DIC and Texas Red filter sets using 100x objective lens.

Results

Using Propidium iodide alone resulted in images that were mostly as expected. There was no haze present inside of the microcapsule as seen in the previous three Live/Dead staining experiments. Figures 34 and 35 show no signs of bacteria on the inside of the microcapsule. Having no dead bacteria was unexpected; even if the microcapsules do work, having some bacteria die during microcapsule synthesis would not be surprising.

Figure 34 - Representative image taken from Propidium Iodide stain. Image A displays Texas Red filter. Image B displays AlexaFluor 488 filter. Image C displays DIC only. Image D displays DIC with both Texas Red and AlexaFluor 488 filters overlaid. Even though no stain was included to be imaged with the AlexaFluor 488 filter, it was still used to ensure the signal didn't overlap.

Figure 35 - Second image from Propidium Iodide stain. Image A displays Texas Red filter. Image B displays AlexaFluor 488 filter. Image C displays DIC only. Image D displays DIC with both Texas Red and AlexaFluor 488 filters overlaid.

It is unknown what the red spheres in Figures 34 and 35 are. The fluorescence seen in these two figures are obviously not rod-shaped bacteria, but Propidium iodide should not be binding to anything else in the microcapsule since it is a nucleic acid stain.

STYO 9

STYO 9 is the second half of the live/dead staining kit. The same procedural steps were followed as in the Propidium iodide experiment, but Propidium iodide was replaced with an equal amount of STYO 9. The images taken using STYO-9 were expected to be a little more interesting than those taken with Propidium iodide. The microcapsules are thought to be filled with live bacteria, so there should be a lot of green fluorescent signal on the inside of the microcapsules. It is hoped that the fluorescence will be in the shape of bacteria, and not the haze as seen previously.

Procedure

- Pipette 1.5 μ L of STYO-9 and 498.5 μ L DI water into an eppendorf tube. Mix well.
- Place small amount of dehydrated microcapsules(less than 0.001g) into a separate eppendorf tube.
- Pipette 50 μ L of STYO-9 into eppendorf tube containing dehydrated microcapsules. Mix well.
- After 24 hours, pipette 50 μ L from eppendorf tube onto microscope slide, cover with cover slip.
- Image on confocal using DIC and AlexaFluor 488 filter sets with 100x objective lens.

Results

Unfortunately the STYO-9 stain didn't have the same specificity as the Propidium iodide appeared to have. The haze seen in other experiments is seen again. Using only STYO-9, it is evident that this stain is interacting with the fat or protein, and somehow binding to them causing them to fluoresce. Even though STYO-9 is used with the AlexaFluor 488 filter set, the Texas Red filter set was used to see if the signal would overlap. Unlike the Propidium iodide which only displayed a signal under one filter set; the STYO-9 stain was visible in both Texas Red and AlexaFluor 488. Although STYO-9 resulted in a significantly weaker signal through the Texas Red filter set, it was using almost double the laser power (9% for AlexaFluor 488 compared to 17% for Texas Red)

Figure 36 - Representative image from STYO-9 stain. Image A displays Texas Red filter. Image B displays AlexaFluor 488 filter. Image C displays DIC only. Image D displays DIC with both Texas Red and AlexaFluor 488 filters overlaid. Even though no stain was included to be imaged with the Texas Red filter, it was still used to ensure the signal didn't overlap.

Figure 37 - Second image from STYO-9 stain. Image A displays Texas Red filter. Image B displays AlexaFluor 488 filter. Image C displays DIC only. Image D displays DIC with both Texas Red and AlexaFluor 488 filters overlaid

HCl Application #1

Since the goal of this work is to determine if the microcapsules can deliver the lactic acid bacteria safely passed the gastric acids produced by the stomach, it was necessary to expose them to HCl. Only after a set amount of time being exposed to HCl will they be imaged.

This experiment followed the same Live/Dead staining protocol as the previous applications, but it included an experimental batch that was exposed to HCl. It was important to have both groups use the same microcapsules and live/dead staining solution to be certain nothing was being affected during sample preparation. Both samples were identical, but the experimental batch had 20µL of 0.1M HCl added to it for 24 hours. One-Tenth molar HCl is being used for the HCl experiments because it creates a pH of 1, the equivalent pH of acid produced by the stomach.

Procedure

- Create a 0.3% Live/Dead staining solution by adding 1.5µL STYO-9, 1.5µL Propidium iodide, and 997µL DI water into 1.5mL eppendorf tube. Mix well.

- Place small amount of dehydrated microcapsules(less than 0.001g) into each of two separate eppendorf tube. Label one as “Control” and the other “Experimental”
- Pipette 40μL Live/Dead stain into both eppendorf tube containing dehydrated microcapsules. Mix well.
- Pipette 20μL 0.1M HCl into eppendorf tube labeled “Experimental”.
- After 24 hours, pipette 50μL from Experimental eppendorf tube onto microscope slide, cover with cover slip. Repeat for “Control”.
- Image on confocal using AlexaFluor 488 and Texas Red filter sets with 100x objective lens.

Results

Figure 38 shows results from the control group of HCl Application #1. Sample was exposed to live/dead stain only. Figure 39 shows results from the experimental group of HCl Application #1. The microcapsules in the experimental group were exposed to 0.1M HCl for 24 hours.

Control Group (No HCl)

Figure 38 - Sample images control group. Number in image represents slice number out of 25 slices. Images A display Texas Red filter. Images B display AlexaFluor 488 filter. Images C display both Texas Red and AlexaFluor 488 filter sets.

The control group images look identical to images taken in Live/Dead Stain #3 because the protocol was intentionally similar. The control group's purpose was to see the difference between images taken with and without HCl.

Experimental Group (0.1M HCl)

Figure 39 - Sample images of experimental group displaying microcapsules after 24 hours exposure to 0.1M HCl(pH of 1). Image A displays Texas Red filter. Image B displays AlexaFluor 488 filter. Image C displays both Texas Red and AlexaFluor 488 filters.

The experimental group looks drastically different than the control. The HCl definitely had a major effect on the images taken. The haze in Figure 39 is a dark red color, rather than green normally seen like in Figure 38. The color change is most likely due to the HCl affecting the STYO-9 stain. If the STYO-9 worked as it did normally, there would be a green haze when only the AlexaFluor 488 filter was active. In this experiment, the confocal image had almost no fluorescence when AlexaFluor 488 filter was active.

There are a few things that could be changed during the HCl dosing to lessen the affect on STYO-9. Possible changes could be using different volumes of HCl and stain, a less concentrated HCl, or even using a buffer to neutralize the acid before adding the Live/Dead stain.

HCl Application #2

This experiment changes the volumes of HCl and staining solution added to the dehydrated microcapsules. More HCl was added, and staining solution was taken out of the microcapsule solution. The overwhelming red signal may be caused by an overabundance of Propidium iodide in the solution. While this doesn't explain the lack of green signal, altering the volumes is all that can be done at this point.

Procedure

- Create a 0.3% Live/Dead staining solution by adding 1.5 μ L STYO-9, 1.5 μ L Propidium iodide, and 997 μ L DI water into 1.5mL eppendorf tube. Mix well.
- Place small amount of dehydrated microcapsules(less than 0.001g) eppendorf tube
- Pipette 20 μ L Live/Dead stain into eppendorf tube containing dehydrated microcapsules
- Pipette 40 μ L 0.1M HCl into eppendorf tube
- After 24 hours, pipette 50 μ L from eppendorf tube onto microscope slide, cover with cover slip.
- Image on confocal using AlexaFluor 488 and Texas Red filter sets with 100x objective lens.

Results

After viewing the images taken, it is safe to say that the HCl does affect both the STYO-9 and Propidium iodide. The amount of fluorescence observed through both filters was significantly diminished with the additional HCl and decreased amount of stain.

Figure 40 - Representative images from HCl Application #2. Image A displays Texas Red filter. Image B displays AlexaFluor 488 filter. Image C displays DIC. Image D displays DIC with Texas Red and AlexaFluor 488 filter sets overlaid.

The red and green haze is entirely gone with an increased volume of HCl. After all the problems encountered with attempting to stain the bacteria in the microcapsule, with or without HCl, it is clear that no useful information is going to be gathered. The protein and/or fat are interacting in a way that is preventing the bacteria from being visible using the confocal microscope.

Alginate, LAB, and Fat Microcapsule #1

Fat was added to this final step of alginate experimentation so the alginate microcapsules resemble the whey protein microcapsules as closely as possible. A small portion of the alginate was replaced with emulsified fat prepared and provided by the DPTC. The amount of LAB added to the alginate remains the same. The alginate microcapsules were constructed to closely match the originals; however it is impossible to know the proportions of the protein, fat, and bacteria as they were made elsewhere. The chosen volumes of alginate, bacteria, and fat are estimates based on images taken and could be altered if deemed necessary.

Procedure

- Mix 1.5g calcium chloride into 100mL DI water. Allow to dissolve completely at room temperature.
- Pour calcium chloride solution into petri dish until bottom is completely covered by solution. Place dish in sink.
- Pour 7mL of 1% alginate solution into 15mL container.
- Pour 1mL of emulsified fat (prepared by DPTC) into same 15mL container.
- Pipette 2mL of LAB culture (prepared by DPTC) into same 15mL container. Mix well.
- Place spray bottle top tube into 15mL container.
- Spray alginate-LAB-fat mixture as a fine mist into petri dish containing calcium chloride solution.
- Pour petri dish and all contents into another 15mL conical. Label Alginate-LAB-fat Stock Solution.
- Create Live/Dead staining solution by combining 1.5 μ L STYO-9 and 1.5 μ L Propidium Iodide with 1mL of DI water into 1.5mL eppendorf tube.
- Mix 40 μ L of Alginate-LAB-fat Stock Solution and 10 μ L Live/Dead staining solution.
- Incubate for 30 minutes out of sunlight at room temperature.
- Pipette entire eppendorf tube onto microscope slide. Cover with cover slip.
- Image on confocal microscope using Texas Red, and AlexaFluor 488 filter sets with 100x objective lens, with DIC enabled.

Results

Results from the experiment were not expected. There is no evidence of fat anywhere inside of the microcapsule. The microcapsule shown in Figure 40 looks almost identical to the microcapsules with only alginate and LAB. Not only was fat missing from the microcapsule, the number of microcapsules was significantly lower; only one microcapsule was observed. In the one microcapsule, all of the bacteria were dead, which was also unlike the other experiments that didn't include HCl.

Figure 41 - Representative images of the alginate microcapsules with LAB and fat. Image A displays Texas Red filter set. Image B displays AlexaFluor 488 filter set. Image C displays DIC only. Image D displays DIC with Texas Red and AlexaFluor 488 filters over. No observable fat inside of alginate microcapsule.

Figure 41 depicts the lack of observable fat inside the alginate microcapsule. In other parts of the same microscope slide, there was bubble like structures seen in Figure 42. These objects were not seen in any previous experiments. The only stains present in this experiment were STYO-9 and Propidium iodide responsible for staining bacteria, so it was impossible to determine what the objects were without further staining. Based on substances added to the alginate solution, it was most likely fat.

Figure 42 - Most of solution contained large, unrecognizable bubble-like structures. It is impossible to definitively identify what they are made of without further staining

To determine if the objects were fat or not, a sample from the next experiment had only Nile Red instead of the Live/Dead staining solution. If the objects are still present and do in fact consist of fat, they will be easily observable using the AlexaFluor 633 filter set.

Alginate, LAB, and Fat Microcapsule #2

The fat containing microcapsule protocol #1 was unsuccessful because the fat was not visibly identified inside of the alginate microcapsules. It is thought that the emulsified fat separated from alginate solution before it was sprayed into the calcium chloride. The separation of fat and alginate may have resulted in clumps of only fat being sprayed through the nozzle, resulting in the droplets seen in Figure 42. This protocol followed the same general steps as #1, but used cream from centrifuged milk instead of the liquid emulsified fat. The fat from the cream could mix with the alginate more effectively, resulting in fat droplets inside the alginate capsules.

Procedure

- Using a hot water bath, warm 40mL of whole milk to 37°C.
- Centrifuge milk at 4000 RPM for 3 minutes, or until cream layer forms at top.
- Using a spatula, take cream layer from top of milk and place 1mL cream and put it into a 15mL conical.
- Add 2mL LAB solution to tube containing cream. Using hot bath warm to 37°C. Mix well by hand
- Mix 1.5g calcium chloride into 100mL DI water. Dissolve completely.
- Pour calcium chloride solution into petri dish until bottom is completely covered by solution. Place dish in sink.
- Add 7mL of alginate into conical containing LAB and cream. Mix well by hand.
- Place spray bottle top tube into 15mL container.
- Spray alginate-LAB-fat mixture as a fine mist into petri dish containing calcium chloride solution.
- Pour petri dish and all contents into another 15mL conical. Label "Alginate-LAB-Fat Stock Solution".
- Create Live/Dead staining solution by combining 1.5μL STYO-9 and 1.5μL Propidium Iodide with 997μL of DI water into 1.5mL eppendorf tube.
- Mix 40μL of Alginate-LAB-Fat Stock Solution and 10μL Live/Dead staining solution in a clean eppendorf tube.
- Incubate for 30 minutes out of sunlight at room temperature.
- Pipette entire eppendorf tube onto microscope slide. Cover with cover slip.

- Image on confocal microscope using Texas Red, and AlexaFluor 488 filter sets with 100x objective lens, with DIC enabled.

Results

While there were more a few more observable microcapsules in the second protocol, these microcapsules didn't contain the bacteria or the new fat made from the milk cream. Even though the cream appeared to mix more evenly with the alginate before spraying, the results were worse because nothing was inside the microcapsule. The large droplets outside the microcapsules that are thought to be fat were still present.

Figure 43 - Images taken from Alginate + LAB + Fat #2. Image A displays Texas Red filter. Image B displays AlexaFluor 488 filter. Image C displays DIC only. Image D displays DIC and both Texas Red and AlexaFluor 488 filter sets overlaid. No observable bacteria inside alginate microcapsules.

Figure 44 – Representative images of unidentified droplets present throughout microscope slide. Image A displays Texas Red filter. Image B displays AlexaFluor 488 filter. Image C displays both Texas Red and AlexaFluor 488 filters. Image D displays DIC only. Image E displays DIC with Texas Red and AlexaFluor 488 overlaid.

The droplets are present in all protocols that involved fat. To find out if the droplets are fat, the same protocol from Fat Staining Procedure #3 was used instead of adding the Live/Dead stain. It is important to determine if the droplets are fat or not. If they are fat, it could show that the microcapsules cannot be synthesized using the spray bottle technique with fat inside them.

Procedure (Nile Red)

- Using a hot water bath, warm 40mL of whole milk to 37°C
- Centrifuge milk at 4000 RPM for 3 minutes, or until cream layer forms at top.
- Using spatula, take cream layer from top of milk and place 1mL cream into 15mL conical.

- Add 2mL LAB to conical containing cream. Using hot bath warm to 37°C. Mix solution by gently shaking with hands.
- Mix 1.5g calcium chloride into 100mL DI water. Dissolve completely.
- Pour calcium chloride solution into petri dish until bottom is completely covered by solution. Place dish in sink.
- Add 7mL of alginate into conical containing LAB and cream. Mix well by vortexing.
- Place spray bottle top tube into 15mL conical.
- Spray Alginate-LAB-Fat mixture as a fine mist into petri dish containing calcium chloride solution.
- Pour petri dish and all contents into another 15mL conical. Label “Alginate-LAB-Fat Stock Solution”.
- Pipette 10μL Nile Red into 1.5mL eppendorf tube. Place in fume hood for 5 minutes or until pink film has formed at bottom, whichever comes first.
- Pipette 40μL of Alginate-LAB-fat Stock Solution into eppendorf tube containing Nile Red stain.
- Incubate for 30 minutes out of sunlight at room temperature.
- Pipette entire eppendorf tube onto microscope slide. Cover with cover slip.
- Image on confocal microscope using AlexaFluor 633 filters filter sets with 100x objective lens, with DIC enabled.

Results (Nile Red)

The DIC images looked similar to those taken previously that had milk cream. Fat was not observable under the DIC, but when the AlexaFluor 633 filter was toggled on, there was a faint red fluorescence that shows some fat is on the inside of the microcapsule.

Figure 45 - Representative images of microcapsules stained with Nile Red. Image A displays AlexaFluor 633 filter. Image B displays AlexaFluor 488 filter. Image C displays both AlexaFluor 633 and 488 filters. Image D displays DIC only. Image E displays DIC with AlexaFluor 633 and 488 overlaid.

After it was determined that fat from the milk cream was making it inside the microcapsules, the unknown droplets were imaged after. The hypothesis that not all the fat was making it inside the microcapsules was correct: Figure 46 shows the large droplets consist entirely of fat.

Figure 46 - Representative images of fat droplets stained with Nile Red. Images taken with 10x objective lens. Image A displays AlexaFluor 633 filter. Image B displays DIC only. Image C displays DIC with AlexaFluor 633 overlay.

Figure 46 demonstrates that the droplets seen in both alginate experiments using fat are fat from the milk cream or the emulsified fat. The droplets produced for the Nile Red experiment were far larger than previous observations; it is possible the fat from the cream was still separating from the alginate. Separation could have resulted in large clumps of fat going through the spray nozzle at once, which may have resulted in the large droplets seen on the microscope slide. To view the entire fat globule the images in Figure 46 were taken with the 10x objective instead of the 100x objective used in imaging all previous samples.