Optimizing Hyperswarming Bacterial Plate Assay Serving As a Protective Host Response to Intestinal Inflammation

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Abstract

Approximately seventy-four percent of Americans are living with digestive symptoms like diarrhea, gas, bloating, and abdominal pain. A distinct property of intestinal inflammation is characterized by bacterial swarming - rapid movement across a nutritious environment through flagellar propulsion. Early research indicates that bacterial swarming could act as a protective host response to intestinal inflammation. In order to further study the protective properties of bacterial swarming on intestinal inflammation, an effective replicable assay for swarming accommodating complex material was created. Using diseased and unafflicted human fecal samples, an optimized plate assay was created to ensure diseased samples to swarm while simultaneously suppressing the unafflicted sample from growing. Four imperative variables were discovered and tested for reproducibility: The method by which the frozen samples were thawed, the concentration of agar used in the LB-agar plate, the volume of the sample included, and the volume of the petri dish. While the method of thaw and the volume of the plate showed no significant effect on the ability to swarm, the volume of the inoculant and the agar percentage both showed distinct correlations. The optimized plate consisted of 0.5% agar LB plates, 25 mL volume, and 7.5 µl sample inoculants. Using this assay, clinics can diagnose intestinal issues while avoiding complex, invasive procedures like colonoscopies.

1. Introduction

Approximately seventy-four percent of Americans are living with digestive symptoms like diarrhea, gas, bloating, and abdominal pain. Intestinal issues can indicate the possibility of more serious conditions in the future [7]. In patients with Inflammatory Bowel Disease (IBD) such as Crohn's Colitis, Gastrointestinal (GI) tract cancers, or Ulcerative Colitis (UC), chronic inflammation puts the individual at a major risk for gastrointestinal malignancies most commonly, the terminal ileum or the perianal region. Crohn's disease (CD) and ulcerative colitis (UC) are conditions that cause inflammation in the GI tract. Inflammatory Bowel Diseases including Crohn's Disease and Ulcerative Colitis are both characterized by chronic relapsing intestinal inflammation. CD and UC are usually characterized by damage created to the epithelial barrier separating the intestinal lumen and the mucosal immune system.

At a normal state, the intestinal epithelium exists in a state of physiological hypoxia. When the intestine is under any type of stress, which refers to inflammation, the body will reach a hyperoxic state in which there are increased levels of oxygen in the body. During this state, there is an abundance of swarming of the bacteria in the intestine [5]. The swarming of bacteria in the intestine can therefore act as an indicator for IBD or predict a possible flare up in the near future. The significance of this assay plays a role in the medical field for patients who either suffer from IBD or are concerned due to the presence of related symptoms. As opposed to a colonoscopy which is a highly invasive and costly procedure, this method would involve the patient in giving their doctor a stool sample while the clinic proceeds to perform the rest of the tests.

When the intestines are under stress and in a hyperoxic state, the swarming bacteria undergoes a tremendous amount of respiration in order to produce large amounts of ATP. The ATP acts as its fuel in order for the bacteria to swarm or move at a very rapid speed. In order for the bacteria to go through the electron transport chain and oxidative phosphorylation, it consumes the oxygen very rapidly. The process of cellular respiration in bacteria is somewhat similar to that in humans. Aerobic respiration in bacteria involves four stages: glycolysis, citric acid (Krebs) cycle, and an electron transport chain (chemiosmosis). During the electron transport chain, oxygen acts as the final electron acceptor. This part of respiration also produces the most ATP which is why swarming bacteria uses a lot of oxygen [1]. The paradoxical nature of swarming bacteria can theoretically help a patient diagnosed with IBD, Colitis, or Crohn's. While bacterial swarming acts as an indicator for the possibility of IBD, bacterial swarming is a protective host response during intestinal inflammation.

Those individuals that are afflicted with these diseases are at a higher risk of developing cancers associated with the GI tract [2]. Since there is currently no cure for Crohn's disease, its poses a great burden on those that suffer from the disease. Considering that the disease is usually diagnosed at a young age, IBD can put a significant financial burden on the individual as well as their family

The hypothesis presented in this paper is that if an assay for bacterial swarming can be optimized, then patients can be diagnosed with IBD using a quicker, cheaper, and non-invasive method. Furthermore, if the swarming bacteria could be injected out of a patient and preserved in its state, it can be given to a patient in large quantities to possibly treat IBD, Crohn's, and Ulcerative Colitis.

The aim of this research is to provide clinics with a reproducible assay in which they can avoid extensive surgeries as well diagnose many intestinal diseases with a simple stool sample. As the conditions are optimized, resources, time, and money are saved changing medicine for the better.

2. The Human Gut Microbiota

The gut microbiota refers to the collection of bacteria, archaea, and eukarya colonizing the gastrointestinal tract. The microbiota offers many benefits to its hosts by serving as a barrier to the external environment. It helps strengthen the gut and shape the intestinal epithelium. This protects the body against pathogens and helps build a strong immune system. All multicellular organisms harbor a vast population of bacteria as well as other microorganisms and form symbiotic partnerships with their microbial inhabitants. The gastrointestinal tract is germ free at birth and is then colonized sequentially by disparate microorganisms. The mode of delivery also seems to pose an effect on the microbiota composition. Research shows that vaginally delivered infants' microbiota contains a high abundance of lactobacilli while infants delivered by c-section have a delayed response in the proper colonization of the gut. The initial step at infancy involves the facultative anaerobes such as *Escherichia coli* and *Enterococcus.* A facultative anaerobe is an organism that makes ATP by aerobic respiration when oxygen is present but also capable of switching to fermentation, also known as anaerobic respiration, if oxygen is absent from the environment. The gastrointestinal tract is colonized by facultative anaerobes immediately following birth. The facultative anaerobes consume a large portion of the oxygen in the intestines allowing for the creation of a suitable environment in which obligate anaerobes can reside in [15,16].

A human microbial community can contain up to 5,000 species and 100 trillion individual organisms. The gut microbiota in patients with IBD greatly differs from that in a healthy individual. Dysbiosis (altered gut bacterial composition) is an imperative issue that all IBD patients live with. Patients with Crohn's Disease and Ulcerative Colitis have a large imbalance in their gut microbiota. The diversity of the species residing in the gut significantly decreases in patients diagnosed with IBD. These patients also

have 25% fewer genes than the microbiota of healthy individuals [15]. Many of the functional and protein pathways are depleted. The microbiota composition is subject to change depending on the host as well as externally selective pressures.

Gut microbes are adapted to certain lifestyles due to the fewer number of biochemical niches available in the gut. Bacteria that reside in the gut normally are energized through aerobic respiration or fermentation when oxygen is limited. Current research shows strong evidence towards diet playing a key role on the gut microbiota. The forming and shaping of the colonies is highly dependent on the availability of microbiota-accessible carbohydrates (MAC's) that are present in high doses in dietary fiber. Any diet that follows an extreme, whether it is animal-based, plant-based, etc, has shown correlation to dysbiosis. High fiber diets resulted in the formation of an enriched environment in which different bacterial species were able to coexist.

3. Pathology of the Disease

A) Genetics

The past few decades have played a significant role in enhancing our knowledge and understanding on the genetic contributions to IBD. Studies of gene loci have been the most valuable way to conduct a DNA analysis. Studies have brought the number of IBD associated gene loci to 163. From this number, 110 are associated with both Ulcerative Colitis and Crohn's Disease, leaving only 30 CD specific and 23 UC specific. Fifteen percent of patients with CD have an affected family member with IBD. Population studies have yielded compelling evidence that genetics play an imperative role in the pathogenesis of IBD. The studies showed that individuals who have an afflicted family member are 8-10 times more likely to develop UC or CD themselves. Most of all, twin studies have shown the strongest evidence for genetic correlation. Twin studies have reported that a child is at a 26 fold increased risk for developing CD when its twin has it. Unlike CD, UC only shows a 9 fold increased risk.

B) Environmental

It is no surprise that environmental factors play a role in the pathogenesis of IBD. Many factors are included in the long list of environmental factors such as diet, smoking, drugs, geography, psychological state, and social stress. Smoking has been studied the most among all of these factors. Studies have shown that smoking and UC have an inverse relationship while there is a direct relationship with CD. Heavy smoking has shown to have a protective effect on UC as well as a lower rate of relapse. On the other hand, with CD, smoking increased the risk of relapse and a higher rate of postoperative disease [18].

Nonsteroidal Anti-Inflammatory drugs (NSAIDs) such as aspirin and others play a well-recognized effect in the gastrointestinal tract. NSAIDs have shown to trigger onset or relapses of IBD. They exert their anti-inflammatory effects by inhibiting the enzyme cyclooxygenase (COX) and have many toxicities that cause the limited use of these medications. COX is used to catalyze the first step in the biotransformation of local hormones. Furthermore, NSAIDs, in many cases, have led to macroscopic gastrointestinal complications such as ulcerations and erosions. These complications can be life threatening if left untreated for a long period of time [14].

Patients who have any type of disease, especially intestinal diseases, are prone to be more stressed. Their immune systems are already overactive to begin with. Reducing stress inducing factors in their environments can greatly help their immune systems relax. Recent studies have begun to show that stress induced alterations in gastrointestinal inflammation may be mediated through changes in bacterial-mucosal interactions. (Psychological stress in IBD) Bitton's research suggested that individuals with lower levels of stress were at a lower risk level of disease onset [18].

Vitamin D deficiency is a common thread among patients with IBD as well as many other diseases. Low vitamin D levels also put individuals at a higher risk for developing IBD. Vitamin D functions in repairing the intestinal mucosal barrier. This puts IBD patients at the risk of bone loss and fractures [13].

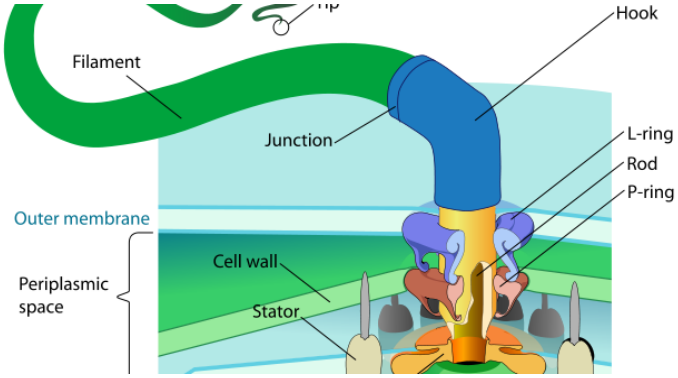
4. Bacterial Motility

The process of swarming is a fundamental characteristic possessed by bacteria in order to rapidly move across a surface. This process is primarily driven by the flagella. The flagella is a threadlike structure that allows distinct forms of bacteria to swim. Bacteria usually swims through liquid filled pores but in order to distinguish swimming from swarming, the concentration of the agar is varied. For bacteria movement to be considered swarming, the agar percentage has to be higher than 0.3% while agar concentrations higher than 1% can inhibit the swarming of many bacterial species [10]. Research shows that the swarming of bacteria can be drastically affected by a multitude of factors. Some of these can possibly include the freshness of the bacterial culture, agar concentration (%), thickness of the plate, dryness of the plate, pH, incubation temperature, and even the location of the petri dish inside the incubator [12].

A) Structure and Function of Flagellum

The flagellin gene (fliC) codes for a major component of the flagellum. Flagellin is a globular protein that is shaped like a hollow cylinder to form the flagellum. A bacterial flagellum has 3 basic parts: a filament, hook, and basal body. The filament is the rigid, helical structure that extends from the cell surface. It is

made of the protein flagellin. The shape is arranged like helical chains to create a hollow core. While the flagellar filament is being synthesized, flagellin molecules that come of the ribosomes are transported through the hollow core. They continue to all attach to the growing tip of the filament. The hook is a flexible structure connecting the filament to the basal body. The basal body is made up of a rod and a series or rings that anchor the flagellum to the cell wall and cytoplasmic membrane. It acts as a rotary molecular motor which enables the flagellum to rotate and propel the bacterium through the surrounding fluid [8].



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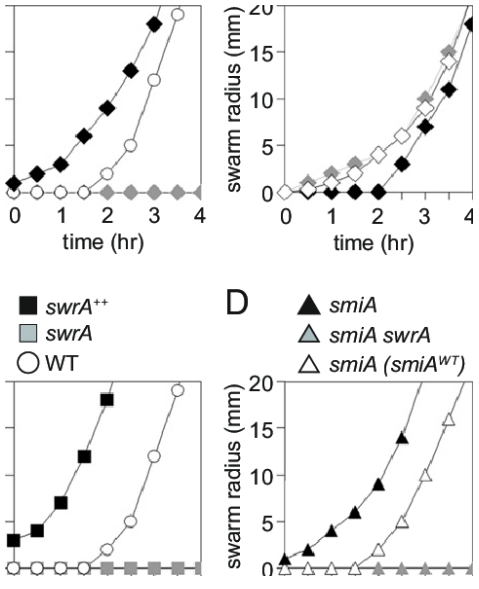
B) Swimming Versus Swarming

When flagellated bacteria are grown over solid surfaces, they can adapt to their environment to present a specialized form of flagellum-dirven motility called swarming. Swimming is characterized by individual cells independently perceiving chemical signals to trigger a response in a liquid environment. On the other hand, swarming is characterized by multicellular movement and communication in which bacteria migrate above solid mediums tightly bound to one another. Swarming is highly dependent on the ability of bacteria to adhere to its surface. Bacteria that possess the ability to swarm undergo a differentiation process which provide them with longer flagella. These cells have the option to revert back into their short flagellated form when the swarming has saturated the environment [3].

5. Phenotype

A) Lag Time

With bacterial swarming, there is a period of lag time in which non motile behavior precedes the commencement of swarming motility. The lag period can be shortened or elongated by increasing the volume of the inoculation spot or by using mutants. The lag time shows evidence that swimming bacteria change to swarming bacteria when existing a liquid medium and entering a semi-solid or solid medium. The lad time produces an exponential graph in which the swarming radius is relatively low for the first few hours. After the completion of the lag time, the swarming



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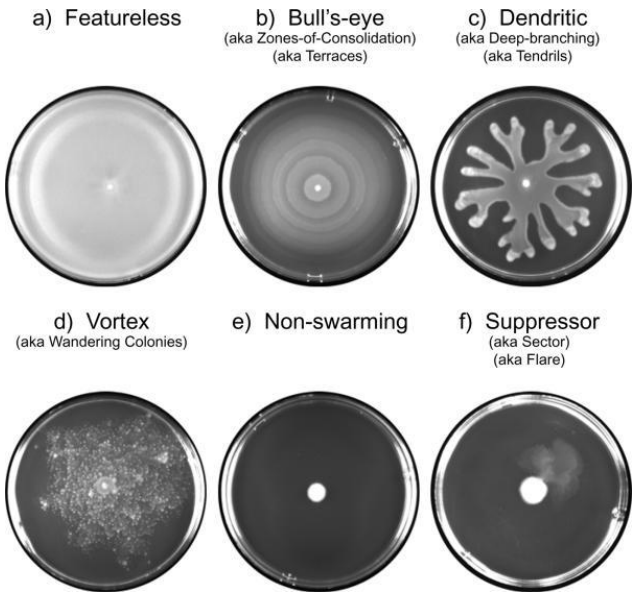
abruptly begins and grows exponentially by the hour until it reaches saturation in the environment [10].

B) Colony Pattern Formation

Swarming bacteria can form a multitude of different colony patterns. The patterns are highly dependent on environmental conditions. Featureless swarms are those that do not have distinct patterns to distinguish them from others. They are usually made when cells spread evenly and continuously outwards from the inoculation point. Cell density in this type of formation is high and uniform throughout the plate. When the plate is saturated, the cells cause the plate to become matte and opaque.



[10]

The most common form of swarming is the bull's-eye formation. This pattern results from cyclic bursts of swarming and rest. The phenotype that is forms represents that of concentric circles. The "bull's-eye" refers to the initial inoculation in the middle. Dendrites are long and thin regions that are colonized all connected to the center inoculation. This formation is usually associated with the secretion of multiple surfactants [10].

6. Cell-Surface and Cell-Cell Interactions

Swarming is initiated by the introduction of one or more cells to a nutrient rich environment. When a small drop of liquid with suspended cells is inoculated onto a petri dish, the additional liquid is absorbed by the hydrogel (medium in the petri dish). This allows the remaining cells that were suspended in the liquid to come into contact with the surface. At this time, swarming cells differentiate from the others and elongate into multinucleate filaments.

A) Cell-Surface Interaction

In order for surface motility to occur, there needs to be a wet substrate. The presence of water is one of the most critical components of swarming. In most labs, researchers use agar in order to study swarming. Agar gels contain water that is bound within all layers and very little free water on the surface. Agar gels

used to represent swarming have agar percentages higher or equal to 0.4%. This is because if the percentage dips under this value, the pore size of the gel starts to increase. This makes it easy for the bacterial cells to penetrate and start swimming. Swarming only refers to surface migration unlike swimming which can refer to swimming inside the agar gel [4].

B) **Cell-Cell Interactions**

In swarming bacteria, cells interact in intricate ways when placed in small confined areas. The way the cells interact can determine a lot of their phenotypes. In order for the cells to migrate across a surface, they need to be interacting with one another. If cells get isolated from a colony, they don't move unless the agar has a surfactant or a layer of liquid on the surface. Swarming is driven by the opposite movement of members in the population of the swarm [4].

7. Methodology

A) Variables Tested

Many swarming assay factors were tested in this experiment in order to optimize the plate assay. The tested factors included: Vortexing, centrifuging, pipetting, PBS Dilutions, dry time, varied volumes of the plate, varied agar concentrations, and varied volumes of the inoculation. Human fecal samples were obtained in 2015 (under protocol IRB# 2009-446 and 2015-4465) and since have been preserved in -80°C freezers. The standard assay has consisted of: preparation of a 0.5% agar Lysogenic Broth 20 mL plate (100ml H20, 1g Tryptone, 0.5 g Yeast Extract, 0.5g NaCl, 0.5g agar), removal of frozen samples from -80 degree Celsius freezer, thawing on ice, inoculating 5 microliter spot on 0.5% agar LB plates, drying of plates under biological hood, and placement in standard incubator.

I. The vortexing of the samples was done through a standard vortex shaker. The samples were taken out of the freezer, thawed, and vortexed for varied amounts of time: 5 seconds, 30 seconds, and 60 seconds. After vortexing the samples, they were inoculated in 5 µl spots.

II. When centrifuging the samples, they were placed in a table top microcentrifuge for one minute. When they were removed, plates were inoculated with either the supernatant or the pellet.

III. The pipetted samples were pumped with a micropipette for various values. The number of pumps included 5 pumps, 25 pumps, and 50 pumps. After pumping the sample, the plates were inoculated with 5 microliter spots.

IV. PBS Dilutions were conducted using a phosphate buffered saline solution. The values included ½, ¼, and ⅛ dilutions. The dilutions were created by placing 5 µl of PBS into an aloquote with 5 µl of the samples. 5 µl were taken from this mixture and placed into another 5 µl of the sample to

create the ¼ dilution. Lastly, 5 µl were taken from this mixture and placed into 5 µl of the sample to create a ⅛ dilution.

V. The two different dry times used were either before or after the plates were inoculated. In one experiment, the plates were dried under a standard biological hood before the plates were inoculated while in another experiment, the plates were dried after they were inoculated under a biological hood.

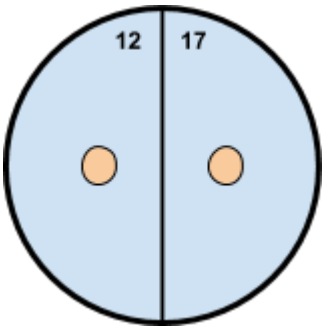
VI. Varied volumes of the plate included 15 mL, 20 mL, 25 mL, and 30 mL. The plates were poured with varied volumes of LB Agar and dried.

VII. The different agar concentrations used in this experiment were 0.4%, 0.5%, 0.6%, and 0.7%. The LB Agar was made with different percentages of agar following the standard directions of preparation, only altering the grams of agar.

VIII. There were 3 different volumes of inoculation used including 2.5 µl, 5 µl, and 7.5 µl. Plates were spotted with one of these values using a micropipette.

B) Optimized Plate Procedure

The methodology of creating the optimized plate was conducted as follows: A standard 100 mL PYREX media storage bottle was used. A weighing boat was used to measure 1 gram of tryptone, 0.5 grams of yeast extract, 0.5 grams of NaCl, and 0.5 grams of agar were placed into the bottle. 100 mL of distilled water was placed into the bottle along with a stir rod and placed on the hot plate. The heat was set to 8 and the stir speed was set to 2. After a homogenous mixture was created, the bottle was placed in the autoclave at 121 degrees Celsius for one hour. Upon retrieving the bottle, it was cooled while stirring to maintain a homogenous solution. After the lysogenic agar broth had cooled, a pipette gun was used to pour 20 mL into each petri dish. 37.5 µl of both sample 12 (non-swarmer) and 17 (swarmer) were obtained from the -80 degree Celsius freezer and put on ice. The five petri dishes were left to cool along with the samples thawing on ice for one hour. A line was drawn down the middle of each petri dish labelling the left side “12” and the right side “17.” A 10 µl micropipette was used to inoculate each petri dish with 5 µl of sample 12 on the left side and 5 µl of sample 17 on the right side as shown. After the plates were spotted, the lids were removed and the petri dishes were dried for 10 minutes under a biological hood. The lids were replaced onto the petri dishes and each dish was scanned. After scanning them, they were placed in a standard incubator at 37 degrees celsius and 80% humidity. The petri dishes were placed upright and stacked with 4 in each stack. They were left in the incubator for 24 hours at which time they were scanned



again and discarded following standard biological material procedure. The procedure was repeated for a total of 20 petri dishes yielding 20 trials.

8. Data/Results

Four variables were investigated prior to the finding of replicable data. These variables consisted of: 1) Vortexing the fecal samples prior to inoculation for varied time constraints: 5 seconds, 10 seconds, and 30 seconds. 2) Centrifuging the fecal samples and inoculating either the supernatant (surface liquid) or the pellet (bottom solid substance). 3) Phosphate buffered saline dilutions at different ratios: ½ dilution, ¼ dilution, and a ⅛ dilution. 4) Pipette pumps of the sample prior to inoculation at varied values: 5 pumps, 25 pumps, 50 pumps. These tests revealed that the conditions did not show a significant or replicable swarming change when compared against the control in each experiment. **Figure 1** summarizes the data of all unaffecting variables in the experiment. The lines on the top of each set of bars shows the percent change between the sub alterations to each variable. For example: The percentage of swarmers when the sample was vortexed was subtracted by the percentage of swarmers when the sample was not vortexed. The absolute value yielded a percentage of 7.43%. This percentage was relatively low as are all the other values presented in **Figure 1**.

Figure 1

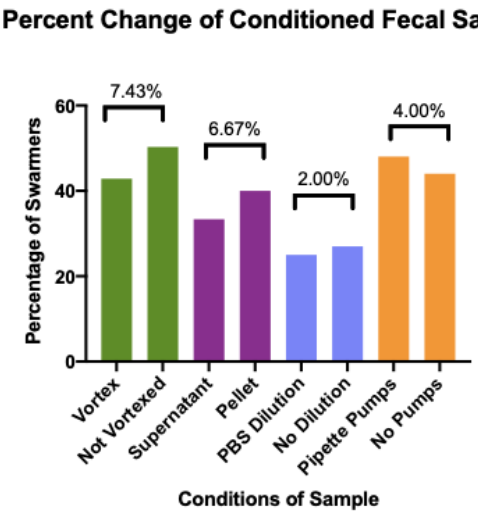


Figure 1 | The effect of varied conditions on fecal sample swarming expansion rate. Fecal samples were thawed on ice before inoculating on LB agar. The graph indicates the fold of expansion of the swarm colony after different conditions were placed on the sample. Each set of bars show comparisons between placing a condition and not placing the condition. The relatively low percent differences indicate that applying these conditions on the samples do not significantly affect the way the samples are able to swarm. The following variables did not show to have a significant effect on the swarming ability of the fecal sample respective to the graph: vortexing, centrifuging, PBS Dilution, and pipette pumps. Data are represented as median and interquartile range, significance tested using Mann-Whitney test. LB, Lysogenic Broth. PBS, Phosphate Buffered Saline.

Agar concentration showed to play a significant role in being inversely correlated with the propensity to swarm. After the completion of 24 hours, 100% of pre-ordained swarmers exhibited swarming on the 0.4% agar plates (n = 10). The 0.5% agar plates had 85% swarm (n = 20). Notably, as the agar concentration in the plate increased, there was a compelling drop in the percentage of swarmers. At 0.6% agar, only 20% of the plates swarmed (n = 10) and at 0.7% agar, only 25% of the plates swarmed (n = 20)

[see Figure 2A]. Furthermore, the magnitude of swarming, as measured by the folds of expansion in area [((final area - initial area)/initial area]) was significantly lower for the plates with the highest percentage of agar **[see Figure 2B]**. A scoring system was used with three possible scores: 1, 2, or 3. All scores of 1 contained expansion fold values between 0-5. All scores of 2 contained expansion fold values between 5-25. All scores of 3 contained expansion fold values greater than 25. The violin plot allows for a clear representation of the inverse correlation between increasing agar percentage and decreasing in swarming. A Mann Whitney test was conducted to show the significance in inverse correlation presented in the violin plot. The test yielded a p-value less than 0.0001. **[See Figure 2C]**.

Figure 2

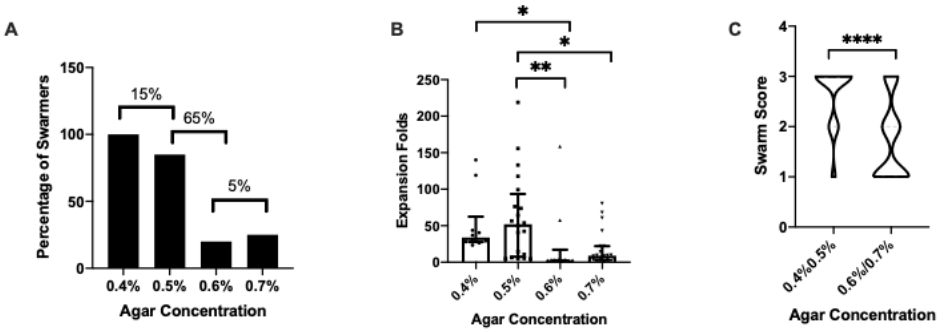


Figure 2 | The effect of agar concentration on fecal sample swarming expansion rate. LB Agar was prepared with varying agar percentages of: 0.4%, 0.5%, 0.6%, and 0.7%. 5 µl spots were inoculated on 20 mL petri dishes (n = 10, in singlets and duplicates). **A.** indicates the percentage of swarmers displaying phenotypical swarming. "N zig-zag" lines on top of the bars resembles the percent change when comparing different agar percentages. **B.** indicates the expansion folds of the swarm colony in varied agar concentrations. Data are represented as median and interquartile range, significance tested using the Kruskal-Wallis ANOVA test. Significance results are indicated on the graph with 1 "*" indicating a p-value less than 0.05 and 2 " ** " indicating a p-value less than 0.01. **C.** indicates the swarm score given to each swarmer with a value of: 1, 2, or 3. All scores of 1 encompass expansion folds of 0-5. Scores of 2 encompass expansion folds of 5-25. Scores of 3 encompass expansion folds of any value greater than 25. A Mann Whitney test was conducted to show the statistical significance of the inverse correlation between the increased concentration of agar and decreased expansion folds. " **** " resembles a p-value of less than 0.0001. LB, Lysogenic Broth.

The volume of fecal sample inoculated on the plate was positively associated with swarming. Plates were inoculated with varied volumes including 2.5 µl, 5.0 µl, and 7.5 µl (n=10, duplicates).

Figure 3

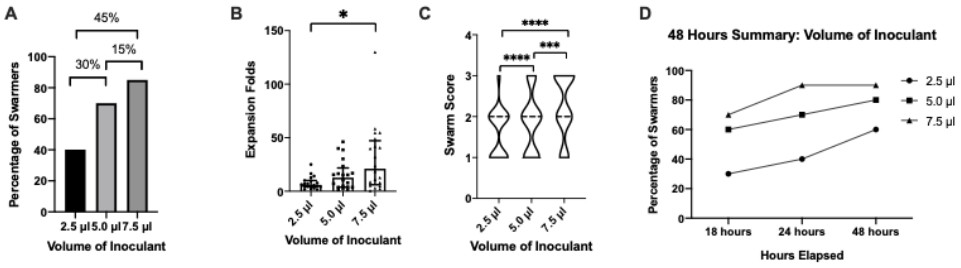


Figure 3 | The effect of the volume of the inoculant on fecal sample swarming expansion rate. 20 mL LB Agar plates were poured and spotted with varying volumes of inoculations: 2.5 µl, 5.0 µl, and 7.5 µl (n = 10, duplicates). **A.** indicates the percentage of swarmers displaying phenotypical swarming. "N zig-zag" lines on top of the bars represent the percent change when comparing different volumes of inoculants. **B.** indicates the expansion folds of the swarm colony in varied volumes of inoculation. Data are represented as median and interquartile range, significance tested using the Kruskal-Wallis ANOVA test. Significance results are indicated on the graph with 1 " * " indicating a p-value less than 0.05 and " ns " indicating no significance. **C.** indicates the swarm score given to each swarmer with a value of: 1, 2, or 3. All scores of 1 encompass expansion folds of 0-5. Scores of 2 encompass expansion folds of 5-25. Scores of 3 encompass expansion folds of any value greater than 25. A one sample t and wilcoxon test was conducted to show the statistical significance of the direct correlation between increased volume of inoculation and increased expansion folds. " **** " resembles a p-value of less than 0.0001. **D.** indicates a 48 hour summary of phenotypic swarmers percentages. Visible swarming was recorded at 16 hours, 24 hours, and 48 hours. LB, Lysogenic Broth.

As the volume of the inoculated spot increased the percentage of plates with swarming increased from 40% to 70% to 85%, respectively (8 out of 20; 7 out of 10; 17 out of 20, respectively). **[see Figure 3A]** Notably, the average expansion folds in the size of the colony over the 24-hour period was also significantly higher for the plates inoculated with 7.5 µl of fecal sample. **[see Figure 3B]** The violin plot using the same scoring system as mentioned above clearly shows the direct correlation between increased volume of inoculation and increased swarming **[see Figure 3C].** Furthermore, concerning the parameters of the volume of inoculation, a phenomenon was observed between all three inoculations. The 48 hour summary shows that by 48 hours, each volume of inoculant is close to leveling off showing that if left in the incubator for a longer period of time, most inoculants will show phenotypical swarming **[see Figure 3D].**

The method of thawing the samples from -80 degrees to room temperature liquid was found to have minimal to no dramatic impact (defined as a response that is less than half-log when comparing methods) on swarming. When samples were removed from the freezer and thawed at room temperature, 73.68% of plates showed swarming (28 out of n = 38); when samples were thawed on ice the percentage of swarming plates was similar at 68.57% (24 out of n = 35). **[see Figure 4A]** The magnitude of swarming did not significantly differ between room temperature thaw and ice thaw [**see Figure 4B].** The violin plot shows an image close to that of a normal distribution clearly representing the lack of impact that thaw type had on the percentage of swarmers as well as expansion folds **[see Figure 4C].**

Figure 4

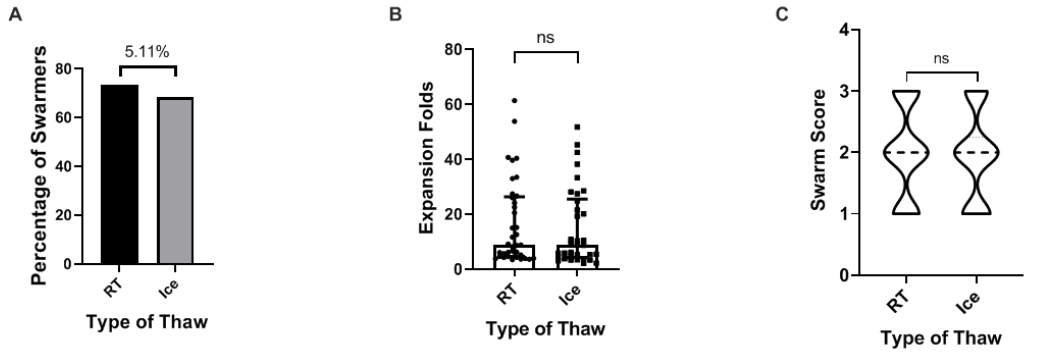


Figure 4 | The effect of type of thaw on fecal sample swarming expansion rate. Fecal samples were thawed at room temperature or on ice before inoculating on LB agar (n = 10, at least in triplicates). A. indicates the percentage of swarmers displaying phenotypical swarming. "N zig-zag" lines on top of the bars represent the percent change when comparing visible RT and visible Ice swarmers. B. indicates the fold of expansion of the swarm colony after room temperature thaw and after ice thaw. Data are represented as median and interquartile range, significance tested using Mann-Whitney test. Significance results are indicated on the graph; "ns" = no significance. C. indicates the swarm score given to each swarmer with a value of: 1, 2, or 3. All scores of 1 encompass expansion folds of 0-5. Scores of 2 encompass expansion folds of 5-25. Scores of 3 encompass expansion folds of any value greater than 25. A normality and lognormality test shows that the occurrence of each swarm score compared in both types of thaw show no significant difference. LB, Lysogenic Broth. RT, Room Temperature.

The volume of the plate showed little to no effect on the propensity to swarm. After the completion of 24 hours, 62.5% of the 15 mL plates swarmed (n = 16), 50% of the 25 mL plates swarmed (n = 16) and 75% of the 30 mL plates warmed (n = 16). There was no notable pattern observed in the occurrence of phenotypical swarming **[see Figure 5A].** The magnitude of swarming measured by the folds of expansion showed no significant change as the volume of the plate increased or decreased **[see Figure 5B].** The violin plot shows no significant difference between each of the volumes. All three figures are close representations of one another and exhibit no significance in occurrences **[see Figure 5C].**

Figure 5

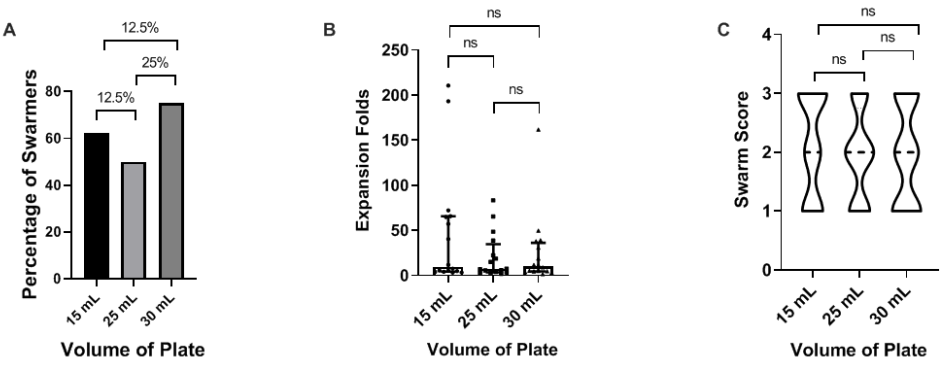


Figure 5 | The effect of the volume of the plate on fecal sample swarming expansion rate. LB Agar was poured with varying volumes of: 15 mL, 25 mL, and 30 mL. 5 µl spots were inoculated on 20 mL petri dishes (n = 8, duplicates). A. indicates the percentage of swarmers displaying phenotypical swarming. "N zig-zag" lines on top of the bars represent the percent change when comparing visible swarming in 15 mL, 25 mL, and 30 mL plates. B. indicates the expansion folds of the swarm colony in varied volumes of plate. Data are represented as median and interquartile range, significance tested using the Kruskal-Wallis ANOVA test. Significance results are indicated on the graph with " ns " resembling no significance. C. indicates the swarm score given to each swarmer with a value of: 1,2, or 3. All scores of 1 encompass expansion folds of 0-5. Scores of 2 encompass expansion folds of 5-25. Scores of 3 encompass expansion folds of any value greater than 25. A Mann Whitney test was conducted to show that the difference in expansion fold presented between each volume of plates shows no statistical significance. LB, Lysogenic Broth.

Discussion/Conclusion

The findings both concur and contradict the suggestions for assay optimization posited by those who have studied swarming in cultured bacteria, implying that covariates for the optimal assay of swarming in complex human material, like stool, may vary considerably.

First, agar concentration was inversely linked to swarming aligns with what previous researchers had identified. It appeared that increasing amounts of agar slowed down the rate of swarming although it did show that if incubated for more time, the higher agar concentration plates could possibly have eventually swarmed. Nevertheless, for researchers with time constraints, the optimized plate from this research was shown to be of 0.5% concentration. The reason that the 0.4% with a 100% swarming incident was not considered to be the most optimal plate was due to the concentration possibly being to

low. Meaning, a standard clinical procedure would require the sample to be incubated for 24 hours. 0.4% agar showed to be hazardously low in that it allowed for the non swarmer (sample 12 strand) to grow at exceeding amounts. The swarming was so extensive that it took over the entire plate as well as overtaking the sample 12 non swarmer inoculation. This made it problematic in identifying which inoculation sample had swarmed and if the petri dish presented swarming or simply colony growth. When swarming reaches the carrying capacity (such as in a petri dish where it does not have unlimited area to expand), the longer it is incubated, the more layers it will form on top of one another. Once the antecedent layer is covered, the is difficult to distinguish swarming. Regardless, using the 0.5% agar plates showed to yield the most reliable results while aiming to minimize the usage of reagents. Studies would caution against lowering the agar concentration any further as it may allow characteristically non-swarmers to exhibit plate-covering morphology, making the distinction between swarmers and non-swarmers difficult - and functionally damage the sensitivity and specificity of the assay.

The second robust finding was that greater volumes of sample inoculated on the plate produced greater expansion folds of swarming. This refuted what was predicted being that some research had shown a complex sample such as stool could have made it possible for different bacteria strains to compete and kill each other off. On the other hand, the experiment showed adverse results. The 7.5 µl inoculants tended to produce the greatest swarming expansion folds. However, an important observation taken with further experimentation was that 2.5 µl inoculants had the internal capability to reach a swarming area close to that of the 5 µl sample as well as the 7.5 µl sample if incubated for a longer period of time. Figure 3D showed a 48 hour summary of the experiment in which each volume sample leveled out close to the others. While the experiment supports that any of these volumes can be used to identify swarmers, the 7.5 µl inoculants were able to peak at 24 hours, therefore making this volume a part of the optimal and time efficient assay for clinical purposes. Furthermore, the 2.5 µl inoculants contained some trials of petri dishes in which there was questionable results when separating swarming and simple colony growth. For researchers considering replicating this assay, this presented a trade-off between sparing sample and sparing time. However, most samples captured from human feces should provide more than 7.5µl, even after multiple aliquots have been made. Therefore, the higher volume of inoculant was added to the optimized assay.

Third, the finding that the type of thaw did not play a significant role in swarming was a robust discovery. Assuming that most fecal samples would be frozen prior to transport from one location to another, the method of thaw would have two viable options. In this experiment, samples were thawed at room temperature and on ice. At the outset, it was speculated that the modality of thaw would play a role in the occurrence of swarming. However, multiple technical and biological replicates showed that there was no significant difference in the results. If anything, thawing at room temperature had a slightly higher

swarming yield than thawing on ice did (73.68% vs. 68.57%). The relative inconsequentiality of the type of thaw would allow researchers and technicians to save valuable time in heir ascertainment of swarming from human stool by thawing at room temperature (seven minutes) rather than on ice (one hour).

Fourth, there was no compelling difference in the ability to observe swarming based on the volume or thickness of the plate. While the experiment showed that 30 mL plates produced the highest percentage of swarmers, the data did not show a clear trend in correlation. As the volumes of the plates increased, the percentage of swarmers did not specifically increase or decrease. Moreover, the differences in swarming yield between each of the volumes of plates was insignificant as shown in Figure 5A. With this information, the optimal volume was marked as 25 mL to follow standard protocol allowing for the judicious use of lab resources.

Finally, considering the cardinal variables tested in the beginning of the experiments shown in Figure 1, there were many possible explanations for their lack of significant data produced. Concerning all of these conditions places on the samples, it was predicted that the samples could have possible been disturbed or harmed due to the stress they were put under.

Further research could allow for the extraction of swarming bacteria and multiplication of it to be used in order to treat intestinal distress. This could aid in reducing flare ups when diseases like CD or UC are active. This procedure would be similar to that of a fecal transplant but would require more treatment to the feces before they are introduced into the patient's body. Regardless, while other covariates may in the future be considered in order to further enhance this assay and application, this current assay is reproducible while keeping reagents at a minimal use and with efficient use of time. The implementation of this technique into clinics and hospitals could revolutionize medicine by avoiding major, invasive procedures as well as save time and money.

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