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CERVIS: Cervical Cancer Early Response Visual Identification System

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CERVIS: CERVICAL CANCER EARLY RESPONSE VISUAL IDENTIFICATION SYSTEM

BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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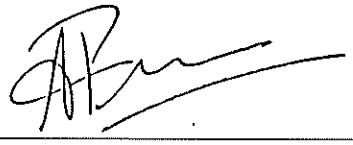

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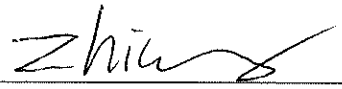

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CERVIS: CERVICAL CANCER EARLY RESPONSE VISUAL IDENTIFICATION SYSTEM

By

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ABSTRACT

The goal of CERVIS is to make a substantial, positive impact in the cervical cancer screening space through the development of a minimally invasive, cost effective solution that enables women in low-resource settings to test for cervical cancer on a frugal and effective platform. In the developed world, there are a variety of options that can aid in early detection, including Pap smears. However, due to the high cost and laboratory requirements that accompany this procedure, women in low-resource settings rarely have access to this preventative care or regular screenings for cervical cancer. Using new research about the changes in the vaginal microbiome, CERVIS aims to create a frugal, visual diagnostic screening tool for early stage cervical cancer as an alternative to the existing expensive, invasive, and clinic-dependent methods. Outcomes will be measured by partnering with a Kenyan NGO to collect data from several clinics.

CHAPTER 1. INTRODUCTION

1.1 Background

Cervical cancer accounts for 6.6% of all female cancers worldwide. The WHO states that 90% cervical cancer-related deaths occur in low and middle income countries, most of which could be prevented by screening and early diagnosis (WHO, 2018). Additionally, the link between Human Papillomavirus (HPV) and cervical cancer has been thoroughly established. Around one-half of individuals diagnosed with HPV infections have high-risk infections. HPV 16 and HPV 18, two of the most common strains of high risk HPV cause 70% of cervical cancers.

Current HPV infection and cervical cancer screening identify DNA and protein biomarkers from cervical samples using expensive, high tech devices (see 1.2 Review of Field). These factors limit test feasibility in low-resource areas, creating a need for new screening technologies.

Team CERVIS[1], a group of undergraduate students from Santa Clara University departments of Bioengineering and Public Health, are building on promising research related to changes in the vaginal flora to meet this need. Emerging research demonstrates the link between vaginal flora and cervical cancer, which has informed the device design. Specifically, *Fusobacteria* is undetectable in swabs taken from a healthy vagina but constitutes approximately 17% of bacteria in cervical cancer vaginal samples (Audirac-Chalifour et al., 2016). Despite a more robust library of scientific journal articles linking protein biomarkers to cervical cancer, the possibility of bacteria as a biomarker was explored as a cheaper and easier method for cervical cancer screening. Our goal is to use this research to design an early-stage cervical cancer screening test using microbial cultivation techniques to consistently and accurately detect the presence of *Fusobacteria.*

[1] Cervical Cancer Early Response Visual Identification System

1.2 Review of Field & Critiques of Current Technology

The key to preventing and addressing cervical cancer development is preventative medicine and routine screenings. The HPV vaccination and Pap smear are most common in developed nations while Visual Inspection with Acetic acid (VIA) is used more often in poorly resourced areas (Gaffikin, 1999).

Pap smears and colposcopies are the most common cervical cancer screening methods used in developed nations to accurately detect both precancerous and cancerous cellular processes within a swab sample of the cervix (Markovic, 1998). Analysis of Pap smears requires a high degree of technical and clinical knowledge to identify and swab the cervix correctly to interpret sample results for appropriate diagnosis . Additionally, expensive equipment to analyze the sample and laboratory access is required for proper and effective analysis. These procedures have a high number of false positives. Finally, from an individual patient standpoint, Pap smears and colposcopy are invasive, painful, and costly (Markovic, 1998).

HPV vaccinations, in combination with cancer screenings, are the greatest preventative measure against cervical cancer (WHO, 2018). FDA approved vaccinations including Gardasil and Cervarix have been found to be nearly 100% effective at preventing cervical infections of HPV 16/18, the two strains most commonly associated with the development of cervical cancer. The Center for Disease Control (CDC) recommends that all women through the age of 26 and all men through the age of 21 start the vaccination series between the ages of 11-15. While the HPV vaccine provides protection from HPV infection, it does not offer any therapeutic treatment of the virus or cervical cancer. Additionally, social stigmas prevent many from getting vaccinated. The vaccine, delivered in a two or three-part series, must be delivered by a health care professional in a clinical setting, making it difficult for those with limited access to clinics and hospitals to receive the full dose of the vaccine. Finally, the vaccine is expensive, costing up to $330 for an complete dosage.

Visual Inspection with Acetic Acid (VIA) of the cervix is an inexpensive screening method in poorly resourced areas (Gaffikin, 1999). Trained health workers and nurses work in mobile screening camps in low income areas to perform this screening test. The test is invasive and can

cause some discomfort similar to the Pap smear method because of the insertion of a self-retaining vaginal speculum. After insertion, acetic acid is applied to the cervix and watched for a reaction between the suspected lesion and the acetic acid (Poli, 2015). Similar to the limitations of the previous devices, VIA also requires a high degree of technical knowledge to properly administer the screening, must be done in a clinical environment, and is highly invasive. If the test is not administered properly, bubbles may still form, resulting in false positives.

Nurx HPV Test tests for molecular markers in cervical tumorigenesis, however, this test remains out of reach financially for clinics in many LMICs. NurX uses nucleic acid based HPV screening to test for the presence of high risk strains of the virus. While this method is accurate and highly specific, it relies on expensive pieces of laboratory equipment that require technical expertise to operate and understand machine output. (Nurx™).

Roche CINtec PLUS Cytology is a qualitative immunohistochemistry (IHC) test using the p16 biomarker, which is a cancer biomarker specifically linked to cervical cancer. The test is conducted using microscopic assessment of $p16^{INK4a}$ protein in formalin-fixed, paraffin-embedded (FFPE) cervical punch biopsy tissues. The test confirms cervical abnormalities based on hematoxylin and eosin (H&E) stained slides. This test is a more specific alternative to Pap smear, but is invasive, expensive, requires a clinician, and results in high rates of false positives (CINtec® PLUS Cytology).

The table below summarizes the benefits and limitations of each of the current innovations to combat cervical cancer that were mentioned previously.

Table 1: Current Technologies in CC/HPV Screening

Device	Benefits	Limitations
Pap smear & Colposcopy	Accuracy	Invasive Expensive lab equipment required for testing Clinical and technical

		expertise required for analysis
Visual Inspection with Acetic Acid (VIA)	Inexpensive Primary screening technique in low resource nations	Invasive Moderate technical expertise required Low sensitivity if administered incorrectly
Roche CINtec PLUS Cytology	Specific for Ki-67 and p16 markers of HPV infection	Invasive Multiple clinic visits Specific for ≥CIN-2 Technical expertise required
Nurx HPV test	Ease of use No clinical training required to administer the test	Tests only for HPV, not directly for cervical cancer Expensive lab equipment required for testing Technical expertise required for analysis

The limitations of these current innovations highlight the need for a cost effective medical screening device that enables women in low resource settings, without access to high-grade medical care, to accurately test for cervical cancer. The device must be non-invasive, making it easier to implement on a global scale. Additionally, the results of the screening should be simply interpreted and analyzed by individuals with little to no technical medical background.

1.3 Microbiome Screening Potential

The human microbiome contains a vast diversity of microorganisms that play critical roles in maintaining health. Each individual has a unique microbiome, and the differences in microbial populations can make people more resistant or susceptible to illness (Huang, 2018). By understanding the microbial communities that make up a healthy microbiome and the factors that affect microbial composition, new ways to diagnose, treat, and prevent a variety of human diseases are being considered.

The vagina is a complex, dynamic microbial niche within the human microbiome that is influenced by age, sexual activity, and other lifestyle factors (Huang, 2018). In general, during female maturation, the ratio of aerobic to anaerobic bacteria increases due to changes in vaginal pH, hormones, and sexual activity. In women of reproductive age (15-49), the average ratio of aerobic to anaerobic vaginal bacteria is approximately 1:10 (Gorbach et. al). Below is a table highlighting some of the principle microbiota within a healthy female genital tract.

Table 2. Select Normal Vaginal Flora

Species	**O_2 preference**	**Gram stain (+/-)**	**Morphology**	**Catalase test (+/-)**	**Prevalence in vaginal flora (%)[1]**
Lactobacillus	Aerotolerant anaerobe	+	Rods	-	60
Gardnerella vaginalis	Facultative anaerobe	Gram variable	Short Rods	-	7.2
Streptococcus Agalactiae	Microaerophile	+	Cocci	-	34
Diphtheroids	Facultative anaerobe	+	Rod	+	40

Notes:

1) Values reported are mean values for analyzed vaginal swabs. Source: (Hutt et. al, 2001)

Prior understanding of vaginal health focused on cell turnover, mucus production, and local immune defenses as important players in the maintenance of a normal vagina (Reid, 2018). Recently, it has become more clear that bacteria within the vagina also play protective roles and can contribute to a variety of diseases.

1.4 Significance of Project

Cervical cancer is a women's health issue on a global scale with an estimated 530,000 women diagnosed each year, according to the World Health Organization (WHO 2018). HPV vaccinations are one of the strongest available measures against cervical cancer especially when used in combination with accurate and routine screening methods such as a Pap smear. These methods, though commonly used in the developing world, are not widely available to women in poorly resourced areas for barriers previously discussed.

Additionally, HPV is a viral infection with more than 30-40 subtypes (Dixit, 2011). The most common oncogenic subtypes of HPV, HPV16 and HPV18, produce E6 and E7 oncoproteins. Currently, DNA and RNA based tests that require the use of polymerase chain reaction (PCR) are most commonly used to diagnose HPV (Engstrom-MeInyx, 2014). To eliminate the need for PCR, the proposed device uses a bacterial biomarker to produce a binary output based on the work of Audirac-Chalifour et al., thus contributing to the field of microbiology (2016).

In 2008, the National Institutes of Health (NIH) launched the human microbiome project to understand the microbial microenvironments in the human body and how these microbial flora contribute to human health and disease (Gevers, 2012). The vaginal microbiome was explored to gain insight into the means by which vaginal microbiomes fight against urogenital diseases. Using pyrosequencing of barcoded 16s rRNA genes, the researchers identified communities that were clustered into five groups. Although this project produced a baseline for understanding the

healthy vaginal microbial community, questions remain as to how to further quantify and define "healthy."

Researchers under the guidance of Dr. Ami Bhatt, Stanford University's Director of Global Oncology, are looking beyond the results from the human microbiome project to understand variations in the microbiome resulting from factors including location and lifestyle. Bhatt's research aims to draw conclusions on the microbiome that can be broadly applied, focusing on populations living in areas that are in the process of industrialization gaining access to new, processed foods to get a holistic look on the microbiome and its relation to health. Once published, research from this study will influence how CERVIS is used in the field.

The CERVIS project is novel because it contributes to the growing field of microbiology, looking not at traditional protein biomarkers that require expensive analytical techniques but at a bacterial biomarker that is upregulated in the presence of precancer and cervical cancer. By designing a test capable of screening for the presence of *Fusobacterium* spp., a screening method for implementation in low- and middle-income countries around the world will improve access to care and empower women, reducing the burden of disease.

1.5 Proposed Goals, Objectives, and Expected Results

During the Fall of 2018, specific goals, objectives, and expected results were created and agreed upon by all members of the team. These goals dynamically shifted throughout the year, as decisions were made about the biomarker of interest and the intended target community. Table A outlines the initial goals, objectives, and expected results as they were understood during the early stages of the project.

Chapter 2: PROJECT OVERVIEW

2.1 Target Population

Cervical cancer ranks as the first most common female cancer in Kenyan women ages 15 to 44 years old with over 5,000 cases diagnosed annually (De Sanjosé et al., 2007). The current target population for CERVIS pilot testing and implementation is Kenya, due to the high incidence and mortality rate of cervical cancer. Kenya also has an extremely high incidence rate of high risk strains, HPV 16/18, as it is more than double that of the United States and other developed countries. Currently, 68% of Kenyan women diagnosed with cervical cancer will die from the disease, making cervical cancer the first leading cause of cancer death in women (De Sanjosé et al., 2007). Many of these deaths could be prevented by HPV screening and early diagnosis of cervical cancer; however there is a lack of a low cost, non-invasive screening for low resource settings (Coleman et al., 2016).

2.1.1 Customer Needs & Cultural Relevance

Cervical cancer is the leading cause of cancer deaths in sub-Saharan Africa (WHO, 2018), and the incidence is likely underreported due to poor cancer registration (Kimani, Sherif, & Bashir, 2012). Cervical cancer screening methods including Pap smear and VIA have significantly reduced the burden of disease in several sub-Saharan African countries, and the "screen-and treat" approach allows for women who test positive to receive cryotherapy immediately following diagnosis (Coleman et al., 2016).

Although these methods are available in Kenya as part of the Ministry of Health's National Cervical Cancer Prevention Strategic Plan from 2002-2006, adoption remains low because these methods are invasive, they have difficulty navigating cultural barriers, and most women do not seek out medical treatment until they suspect a health issue (WHO, 2018). Additionally, the negative stigma about cervical cancer and fear of screening results prevent screening methods from being more widely employed. If screening and treatment for cervical cancer does not increase, the number of cervical cancer deaths is estimated to double in Kenya by 2025, demonstrating the need to educate women about cervical cancer screening and treatment options (Sudenga, 2013). Increasing smartphone usage rates in Kenya provides a technological platform

that can reduce prevailing barriers and thus the burden of disease (Poushter, 2016). Additionally, eliminating the need for women to travel to the clinic by focusing efforts on designing in-the-field screening methods has the potential to address cervical cancer deaths in low and middle income countries.

2.2 Project Components

A list of device criteria were defined based on the culturing conditions of the selected bacterial biomarker and the development of a screening device (Figure 1). First, in order to create a binary output, meaning the test detects the absence or presence of this species of bacteria, the device must be capable of selectively growing the bacteria of interest from a patient sample. Secondly, the device must maintain an anaerobic environment during the growth period because *Fusobacterium* is unable to grow in the presence of oxygen. And finally, the device must seamlessly incorporate the patients sample to allow for ease of use and result interpretation. These criteria were organized depending of the form of the device or the function of the device. Experiments were designed to address each of these criteria, and the results of these experiments ultimately informed the design of the proposed screening device.

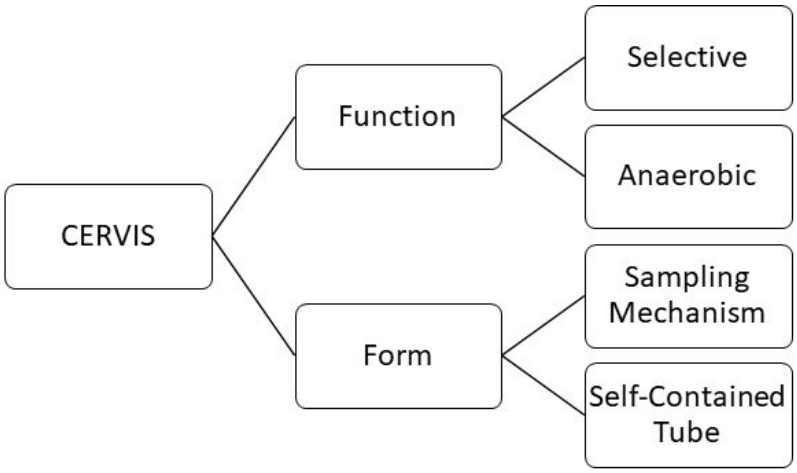


Figure 1: Subsystems Overview

2.2.1 Bacterial Biomarkers of Interest

Proteins were originally explored as biomarkers indicative of cervical cancer for this project. DNA amplification arose as an issue when dealing with these biomarkers because DNA

amplification is done by polymerase chain reaction (PCR) and subsequent analysis by sequencing or gel electrophoresis. PCR uses a strand of template DNA, the enzyme taq polymerase, primers, and exposure to repeated cycles of heating and cooling, which is done using a PCR machine that typically costs between $800 and $20,000. The primers, template DNA, and enzyme must be preserved by refrigeration.

Because CERVIS is intended for use in low- and middle-income countries, this route proved ineffective based on the need for expensive laboratory equipment and refrigeration. The link between vaginal microbiome diversity or concentrations and their association with HPV, precancerous lesions, and cervical cancer was explored as a promising alternative to protein biomarkers (Audirac-Chalifour et al., 2016). Additionally, using bacterial biomarkers showed potential for cheaper and easier culturing methods. *Lactobacillus* and *Fusobacterium* were explored as potential target biomarkers for CERVIS.

2.2.1.1 Lactobacillus

Lactobacillus is often the predominant species in the normal vaginal microbiome (Audirac-Chalifour et al., 2016). Given the abundance of *Lactobacillus* in a healthy vaginal microbiome, *Lactobacillus* was chosen to test the efficacy of using bacterial biomarkers. Initial experiments were designed to isolate lactobacillus from the vaginal microbiome. The purpose of these experiments was to both learn microbiology laboratory techniques and understand the difficulty in selecting against related species for the isolation of one particular bacteria.

This species plays a critical role in regulating the presence of other vaginal flora as well as protecting the host from pathogens (Larsen and Monif, 2001). Mechanisms for pathogen and microbial inhibition have been attributed to the ability of *Lactobacillus* to produce hydrogen-peroxide, an oxidizing agent that disrupts the plasma membrane of certain microbes, as well as lactic acid which can alter the pH within the vagina (Larsen and Monif, 2001). Previous studies show a significant correlation between absence of hydrogen-peroxide producing Lactobacilli and colonization by bacteria associated with vaginal disease as well as an increased abundance of Lactobacilli in in young adolescents (Hiller et. al). Both of these findings point to the importance of *Lactobacillus* in defending the host from potential pathogens.

Previous research has identified Lactobacilli at the genus level by gram stain, colony morphology, and catalase test (Hütt et. al, 2016). Specifically, *Lactobacilli* are gram positive, rod shaped, and produce a negative catalase test (Hütt et. al, 2016).

2.2.1.2 Fusobacteria

Studies show that *Fusobacterium* spp. is undetectable in vaginal swabs from a healthy vagina but constitutes approximately 17% of bacteria in a vaginal sample from a cervical cancer patient (Audirac-Chalifour et al., 2016). Based on this information, this bacteria species was chosen as the final bacterial biomarker used in CERVIS.

Fusobacterium spp. is an anaerobic, gram-negative bacteria usually absent in healthy tissue but upregulated in instances of disease. Fusobacterium nucleatum is associated with inflammatory diseases including appendicitis and ulcerative colitis, as well as multiple cancers, including colorectal, oropharyngeal, and cervical cancers (Audirac-Chalifour et al., 2016).

Our team selected a strain of *F. nucleatum* and *F. necrophorum* to investigate practical field-based cultivation methods for obligate anaerobes. By conducting a series of experiments, a set of criteria was developed for the optimum culturing of fusobacterium nucleatum and necrophorum without an anaerobic hood, which is unavailable in frugal contexts.

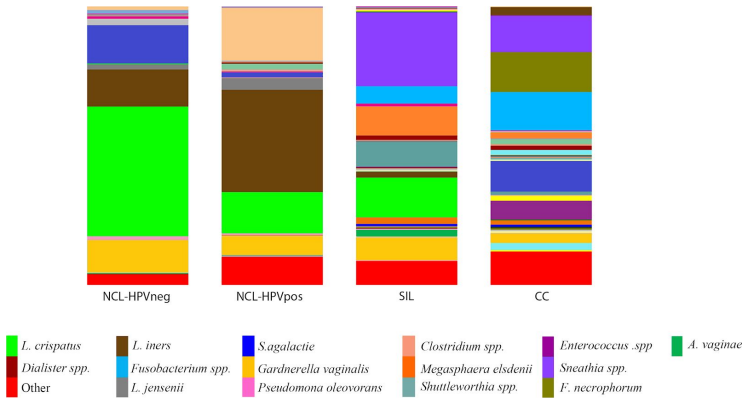


Figure 2: The Link between *Fusobacterium* and Cervical Cancer (Astride Audirac-Chalifour, 2016).

2.2.3 Physical Manifestation of the Assay

2.2.3.1 Device Format

The assay development was an iterative process. As initial laboratory results were collected, the anaerobic requirement for the future prototype was realized. In creating initial sketches, the designs took two forms, a plate and tube design (Figure 3). The tube design with semi-solid media was determined the best option for maintaining an anaerobic environment for *Fusobacterium* growth and detection. The tube design with the swab inserted and twist helped maintain an anaerobic environment capable of growing fusobacteria. To justify the final device format, questions were answered about the density of the media and the shape of the device for anaerobic maintenance and optimal growth conditions.

The plate design reduced the possibility of creating a truly anaerobic environment. Creating a sealed enclosure that made the entire chamber remain anaerobic posed a challenge because a larger surface area would be exposed to oxygen upon sample introduction. Furthermore, the application of the plate design required a more in depth understanding of microbiology and of microbiology techniques to properly streak the plate.

Plate Design	**Tube Design**
	

Figure 3: Initial Prototype Ideations

2.2.3.2 Swab Collection

To determine the optimal collection method, five cytology applicators- the Cervex brush, the cervical spatula, the cotton swab, the polyester swab, and the bristle brush- were chosen to measure the amount of oxygen introduced into a semi-solid media in a closed glass tube. In

addition to oxygen exposure, the swabs were each evaluated based on criteria of size, toxicity, and sample collection.

2.2.4 Growth Media

To create the media for the growth of *Fusobacterium*, several conditions were required. The media had to be nutrient-rich to support the growth of bacteria and had to be selective for the growth of *Fusobacterium*. Additionally, the media had to be transparent in order to easily observe growth. Finally, the media, in combination with the device form, was required to maintain anaerobic conditions to support the growth of fusobacterium, an obligate anaerobe. To meet these criteria, several ingredients were included in the media: Brain Heart Infusion broth, antibiotics, and Gelrite.

The media recipe was tested for functionality and altered to improve results in two different iterations. The results of each iteration and the criteria for altering the recipe are discussed in 4.2.

2.2.4.1 Anaerobic Conditions

Since *Fusobacterium* is an obligate anaerobe, the media also needed to be able to maintain anaerobic conditions within the tube. Currently, it is challenging to keep liquid media tubes free of oxygen because it is more easily mixed throughout the media even if only the surface becomes exposed to oxygen (See 2.2.3.2). To achieve a semi-solid consistency, Gelrite was added to the media. Gelrite was used instead of agar because it allows the same consistency as agar but prevents cloudiness when the media is poured. Oxygen inevitably enters the tube when it is opened for sample penetration. While the semisolid media prevents the oxygen from spreading throughout the media, it does not actively reduce the amount of oxygen in the media. To address this concern, an oxygen scavenger was incorporated into the CERVIS 1.0 formula. Additionally, an oxygen indicator was added to the media to identify oxygen exposure. When oxygen enters the system, the resazurin reacts with the oxygen to turn blue, showing exactly where oxygen has entered the media. This allowed close monitoring of the anaerobicity of the media.

2.2.6 Educational Component

An educational component teaching women about their anatomy, the risks of cervical cancer, and the importance of screening will be developed with the help of local community members for ensured cultural relevance and incorporated into the device implementation strategy. This program will extend to both patients and providers to increase screening uptake by the women living in the community and minimize the risk of false positive results. This is an important step in navigating cultural barriers and ensuring that the device is implemented in the most effective and ethical way. This component was outside the scope of the project for this year, but a preliminary outline of next steps is discussed in Chapter 6.

2.3 Team and Project Management

The project team is composed of four Santa Clara University (SCU) faculty members, four bioengineering students, and three public health students from the Engineering World Health (EWH) partnership. The faculty advisors are Dr. Prashanth Asuri, Dr. Michele Parker, Dr. Craig Stephens, and Dr. Tracy Ruscetti. Dr. Prashanth Asuri has served as a Bioengineering and Bioinnovation advisor. Dr. Michele Parker has served as a public Health advisor and a mentor to the Engineering World Health aspect of the project. Dr. Craig Stephens has served as a Microbiology advisor and mentored the students in common microbiology techniques. Additionally, Dr. Stephens provided the students with lab space and supplies. Finally, Dr. Tracy Ruscetti has also served as a Microbiology advisor, training the students in the anaerobic chamber and assisting with experimental design. The public health students that partnered with the bioengineering students through EWH are Dave Heil, Nicola Gerbino, and Mason Seeley. The project originally stemmed from the work of a 2017-2018 senior design group. Within the bioengineering student group, all experimental design, presentations, team member communication, company visits, and lab work were completed as a team. In terms of individual roles, Claire Hultquist managed supplies orders, Julia Lanoha planned School of Engineering showcasing dates, Rosie McDonagh organized meeting notes, and Hallie McNamara was the lead contact with EWH.

2.3.1 Budget

The funds for necessary supplies for experimentation and development of the frugal screening biodevice were requested from the Santa Clara University Undergraduate Programs. The awarded funding for the Frugal Cervical Cancer screening is summarized in Table B found in the appendix. Additionally, a detailed outline of the expenditures of the project totalling $1,230.27 can be found in Table C in the appendix.

2.3.2 Timeline

Reference appendix A for a complete overview of completed action items for the CERVIS project between Fall 2018 and Summer 2019.

CHAPTER 3: Bacteria Biomarkers of Interest (Subsystem A)

3.1 Introduction

Up until the 1970s, there was poor characterization of the vaginal microbiome due to the narrow range of media and oxygen levels that allow for cultivation of these bacteria (Larsen and Monif, 2001). Today, over 150 different bacterial species have been characterized as populates of the vagina that participate in many interactions within this ecosystem (Larsen and Monif, 2001).

Being able to rapidly recognize changes in vaginal flora and how this corresponds with specific disease states has a number of implications for screenings and the future of personalized medicine. Similar to the way a patient's blood pressure and weight is monitored over time, tracking changes in an individual's vaginal flora at different stages of their lifespan (i.e. childhood, adolescence, reproductive age, later adulthood) can provide healthcare providers with a more comprehensive understanding of a patient's health (Larsen and Monif, 2001). Experiments which examine the bacteria present in a patient's vaginal microbiome can be scaled up and translated to a comprehensive library with bacterial concentrations, species presence, and associated patient factors that can be used to monitor an individual's health and how it is related to the vaginal microbiome.

To reduce the risk of producing false positive results, bacterial biomarkers were considered with the potential of producing a binary outcome. Relying on reading a bacterial gradient with an array of microbes present in the vaginal microbiome requires further quantification and analysis beyond the scope of our device. *Lactobacillus* and *fusobacteria* species were selected for further investigation for possible device integration. *Lactobacillus* was selected based on its high prevalence in the healthy vaginal microbiome and involvement in regulating the vaginal pH. The first steps in this experiment were to determine whether vaginal microbes could be cultivated from vaginal swabs on rich media as well as the optimal aerobic environment for growth. *Fusobacteria* was selected due to its association with different disease states present in the vagina. Experiments were designed to inform the final device design.

3.2 Methods

An in depth literature review was conducted, evaluating the current literature on the vaginal microbiome and the present microbes association with disease state, specifically cervical cancer. Peer-reviewed articles were selected using the SCU One Search feature on the Santa Clara University library website. The findings from this review are summarized in the Table E in Appendix A.

Lactobacillus

Culturing Vaginal Samples

Four female students from the Fall 2018 Santa Clara University microbiology class took three sterile cotton swabs to procure three vaginal samples. Each vaginal sample was used to inoculate a separate tryptic soy agar (TSA) plate. The plates were cultivated in the 37°Celsius incubator for 48 hours in different aerobic conditions: aerobic, anaerobic, and microaerophilic. To achieve the anaerobic growth condition, a "GasPak" culture system was enlisted. In the GasPak jar, the gas generator envelope uses a catalyst to combine Oxygen gas with Hydrogen gas, which removes O_2 from the sealed container. To create the microaerophilic system, a candle was placed on top of the inoculated plates, lit, and then sealed in a jar. The flame from the candle consumes oxygen in the sealed chamber and produces carbon dioxide until it is finally extinguished due to insufficient oxygen quantity creating a microaerophilic environment. Oxygen test strips were placed in both the anaerobic chamber and candle jar system to assess the effectiveness of these chambers in producing the desired oxygenation levels.

After 48 hours, each plate was examined for growth. Colonies that appeared to be distinct species based on color and morphology were each analyzed via Gram stain and catalase test. The procedures enlisted for both of these tests were taken from Dr. Craig Stephens' *Biology 113-Microbiology Lab Manual*. To perform the Gram stain, a distinct colony from each separate identified strain was picked using a sterile loop and then smeared on a clean glass, microscope slide. The colonies were then heat fixed to the slide by passing the slide through the fire three times. Following this, the smears were flooded with the primary, crystal violet, stain and were left to sit for a minute. Afterwards, the slides were rinsed with deionized water. After the rinse, the slides were flooded with the mordant (Gram's Iodine) and were left to sit for a minute. The

slides were then rinsed again with deionized water. Decolorizer, ethanol, was applied and then the slide was left to sit for 15-30 seconds. After another rinse, the slides were flooded with counterstain, safranin, and left to sit for 30 seconds. Following this final stain, the slides were rinsed again then blotted. Once the slides dried, the staining pattern of the specific species, the general cell morphology, and organization were visualized using a light microscope.

Isolating Lactobacillus

Four female microbiology students inoculated selective media with their respective vaginal swabs. The medias used were blood agar and Man-Rogosa-Sharpe (MRS); both have been reported to be selective for *Lactobacillus* (Pendharkar et al. 2013). After 48 hours, any microbes that needed to be isolated were restreaked. Those that did not require isolation were analyzed using the same procedures as those listed above. Once the colonies that were re-streaked had spent 48 hours in the incubator, the analysis procedure were repeated on the resulting isolates.

Next, the isolates were prepared for 16s ribosomal DNA PCR. First, each colony was suspended in 20 microliters of sterile, deionized water. Then, the remaining PCR components were added to the mixture. These components include 10 microliters of GoTrg buffer, 1 microliter of dNTP's, 0.5 microliters of GF primer, 0.5 microliters of 149z R primer, 0.2 microliters of GoTag, and 17 more microliters of deionized water. Once this mix was prepared, the 16s ribosomal DNA components were amplified via PCR. Following this preparation, these genetic fragments were run through a gel. Due to ineffective primers, the gel did not produce accurate results, so this process was repeated. With confirmation that the PCR procedure was successful, the isolates' genetic information was be sent to a lab for DNA sequencing. The results are summarized in table 8.

Fusobacterium

F. Nucleatum ATCC 25586 and *F. Necrophorum* ATCC 255386 were streaked for isolation using a disposable sterile loop on Anaerobe Systems[2] Brucella Blood Agar (BRU) and Fusobacterium Selective Agar (FSA). These plates were incubated at 37°Celsius under anaerobic

[2] Media produced by Anaerobe Systems is manufactured and packaged in a deoxygenated environment.

conditions for 48 hours. Anaerobic conditions were met using an anaerobic chamber, with an internal environment comprised of 90% N2, 5% CO2, and 5% H2. BRU media was chosen as a positive control, supporting the growth of *Fusobacterium spp*. (Brucella Blood Agar- BRU, 2018). FSA is an enriched selective medium for the isolation and presumptive isolation of *Fusobacterium* species, chosen to inform the formulation of CERVIS media discussed in Chapter 5 (Fusobacterium Selective Agar- FSA, 2018).

3.3 Results

Lactobacillus

Culturing Vaginal Samples

Growth was achieved on the TSA plates for 15/16 samples. The observed size and shape of the colonies varied between the three respiratory conditions. Colonies grown under anaerobic conditions were the smallest and most abundant making them difficult to differentiate, while colonies on aerobic plates were dominated by large, circular colonies that we suspected were yeast given the aerobic growth conditions. After Gram staining the colonies from the TSA plates, it was found that almost every bacteria from these colonies was Gram positive. Additionally, all of the cultivated bacteria had a relatively similar morphology: cocci in either chains or clusters with positive catalase tests.

Isolating Lactobacillus

Limited microbial growth was attained on the MRS plates, and the analysis of colonies from these plates was inconsistent, so the data from these plates was discarded. Additionally, many of the colonies that grew on the aerobic plates were identified as yeast and our target bacteria *Lactobacillus* is an aerotolerant anaerobe. As a result, the plates grown in the aerobic condition were also discarded to help increase selectivity for *Lactobacillus.* Thus, only the data obtained from the analysis of the microbes on the anaerobic blood agar plates was enlisted to determine the species each group member cultured.

All members of the group achieved growth on blood agar media under both aerobic and anaerobic conditions. A majority of plates in the anaerobic condition appeared to have a film of condensed, small colonies while the colonies on the aerobic plates were larger, and more

defined. Though there were differences between vaginal samples, in general there was more limited growth on MRS plates under the varying oxygen levels. Although growth was evident in both aerobic and anaerobic conditions, we believe that many of the large colonies on our aerobic plates, for MRS and blood agar, were yeast. Consequently, the aerobic plates were discarded from the data set and not used for subsequent testing and isolation. The remaining anaerobic MRS plates were also discarded due to a lack of consistent growth.

PCR was run for seven of the isolated colonies. None of the sequenced colonies derived from subject's vaginal samples proved to be in the genus *lactobacillus.* The results are summarized in table 8. All identified species are commonly found in the vaginal microbiota, which as you know is fairly variable between individuals. The Streptococcus isolates are the closest relatives of *Lactobacillus*.

Table 3: Summary of Findings from Attempt to Isolate Lactobacillus

Sample	Primer	Edited Sequence Length (bp)	Primary Species ID (BLAST)	Primary Species ID (SeqMatch)	Notes
A	13;8F.ab1	X	X	X	13;8Fab.1: MIXED
	13;1492R	744	Corynebacterium aurimucosum	Corynebacterium aurimucosum	
B	14;8F.ab1	708	Streptococcus anginosus	Streptococcus anginosus	
	14;1492R	668	Streptococcus anginosus	Streptococcus anginosus	
C	15;8F.ab1	679	Actinomyces neuii	Actinomyces neuii	
	15;1492	721	Actinomyces neuii	Actinomyces neuii	

	R				
D	16;8F.ab1	623	Enterococcus faecalis	Enterococcus faecalis	
	16;1492R	670	Enterococcus faecalis	Enterococcus faecalis	
E	17;8F.ab1	729	Staphylococcus aureus	Staphylococcus aureus	
	17 ;1492R	707	Staphylococcus aureus	Staphylococcus aureus	
F	18;8F.ab1	690	Staphylococcus aureus	Staphylococcus aureus	
	18;1492R	767	Staphylococcus aureus	Staphylococcus aureus	
G	19;8F.ab1	695	Streptococcus oralis, pneumoniae	Streptococcus sanguinis	
	19;1492R	725	Streptococcus oralis, sanguinis, mitis, pneumoniae	Streptococcus mitis, oralis	
H	20;8F.ab1	X	X	X	10;8F.ab1: POOR
	20;1492R	716	Actinomyces urogenitalis	Actinomyces urogenitalis, naeslundii, howellii, oris	

Fusobacterium

Fusobacterium growth was observed on BRU and FSA. Small, white isolated colonies were observed after 72 hours. Larger colonies of the same morphology were observed on the BRU plate than the FSA plate.

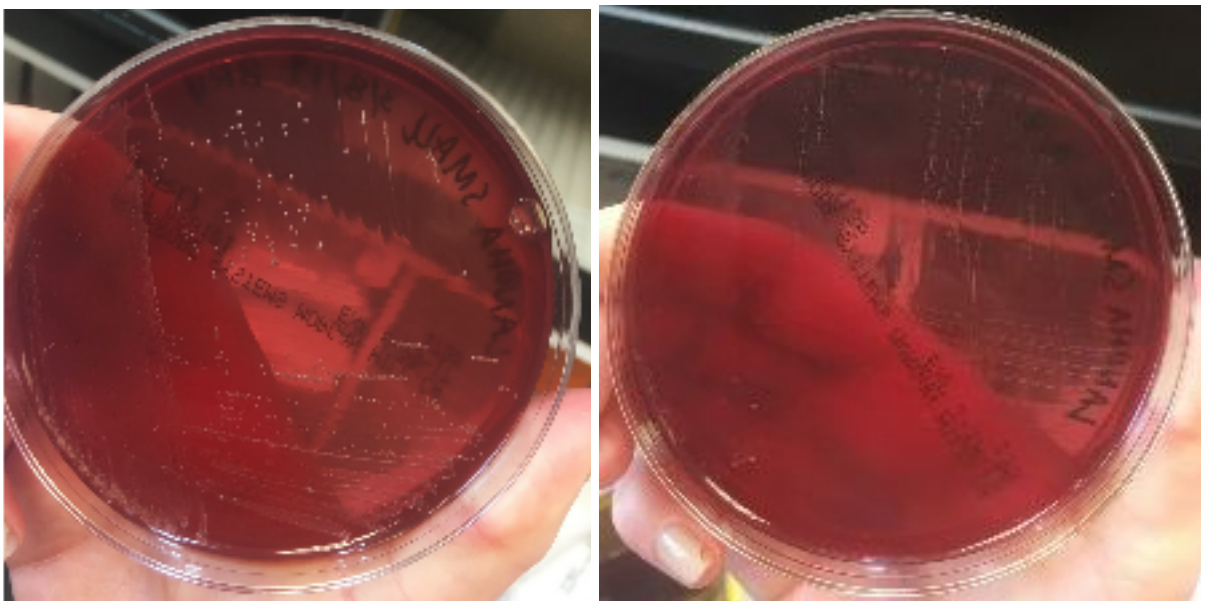


Figure 4: *F. nucleatum* Growth on BRU (left) and FSA (right)

3.4 Next Steps: potential pitfalls and future directions

The high prevalence of *lactobacillus* in the vaginal microbiome eliminated it as a potential biomarker for the final device. *Lactobacillus* was not isolated from any of the tested subject's vaginal samples, which may suggest a need for improving use of anaerobic culturing to be more likely to obtain *lactobacilli*.

F. Nucleatum and *F. Necrophorum* were determined strong microbial biomarker candidates based on their association with cervical cancer and our ability to cultivate these species in the lab. The other identified subsystems use *Fusobacterium* spp. as the bacterial biomarker of interest.

Microbial biomarkers for high risk HPV warrant investigation. This would allow the device to not only have the capabilities of detecting early stage cervical cancer, but also the ability to pick up on high risk HPV infections that are strongly linked to the development of cervical cancer

(Shannon et al, 2017). Studies show the potential of using other species found in the vaginal microbiota as an indicator of HPV infection. *Sneathia* spp. has been described as a possible microbiological marker of high risk HPV strains associated with the development of cervical cancer and warrants consideration as a secondary biomarker to increase test specificity in future device iterations (Lee et al., 2013).

CHAPTER 4: Assay Platform

4.1 Introduction

In developing the Assay platform, or self-contained design which would house the selective growth media described in Chapter 5, two common microbiology techniques were considered: the plate and the tube. In developing this self-contained design, the anaerobic growth requirement of *Fusobacterium* was the most important condition to be met in order to achieve bacterial growth. Therefore, the two designs were evaluated based on their ability to maintain anaerobic conditions.

4.2 Methods

Plate vs. Tube

The anaerobic environment created using jars described in Section 3.3 was not effective at culturing lactobacillus and would not be portable. Therefore, other options were explored to culture fusobacterium. The attempted anaerobic environment was created using Ziploc bags, a gas pack, and anaerobic indicators. The gas pack would effectively remove oxygen from the environment once the bag was opened or if oxygen leaked into the system. The anaerobic indicators were used to determine if anaerobic conditions were maintained. Plates were streaked with inoculated fusobacterium cultures and immediately placed into the anaerobic environment, keeping air exposure to a minimum.

Swab Selection

To determine the swab type that resulted in the least oxygen exposure a series of five different swabs without bacterial samples were tested in parallel experiments. In order to conduct the experiment, anaerobic transport media tubes were used, which include a chemical resazurin, which reacts with oxygen creating a color change. Each swab was submerged into the media tube and dipped then removed from the media tube. The swabs tested included: the Cervex brush, the cervical spatula, the cotton swab, the polyester swab, and the bristle brush. During testing each tube was exposed to oxygen for no more than 15 seconds, the test was performed at room temperature, and the tubes were checked after 24 and 48 hours.

Swab Mechanism

After the swab was selected the same test was repeated with this swab to further verify which swabbing mechanism would result in the least amount of oxygen entering the system. The swab was first submerged into the anaerobic transport media tube containing resazurin. In parallel, the same swab was dipped and then removed from the media tube. The results of both trials were compared.

4.3 Results

Plate vs. Tube

After attempting to create an anaerobic environment with Ziploc bags, the enclosure remained anaerobic for the first 24 hours, but no bacterial growth was observed. Furthermore, by 72 hours the anaerobic indicators changed color from pink to purple showing that the environment within the Ziploc bags was no longer anaerobic, as shown in Figure 5. No growth was observed. It was concluded that by the 72 hour mark the gas packs were no longer actively removing oxygen and that anaerobic conditions were no longer maintained. Overall, anaerobic conditions necessary to grow fusobacterium were not achieved.

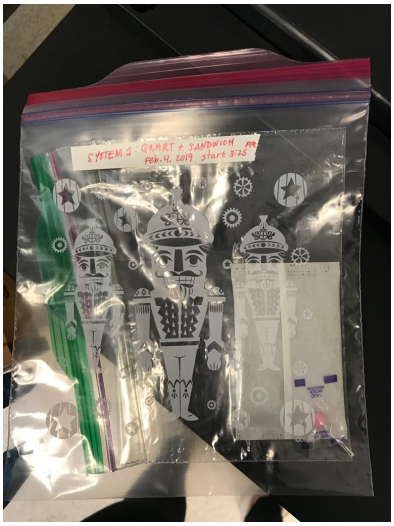
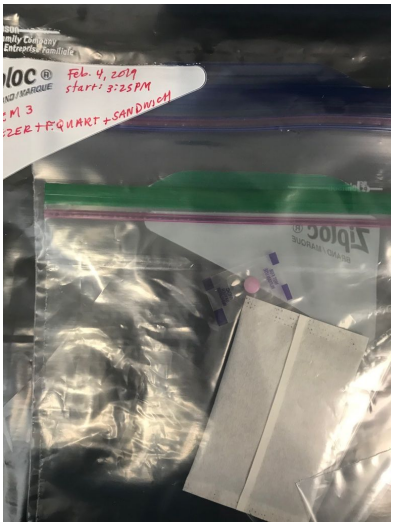
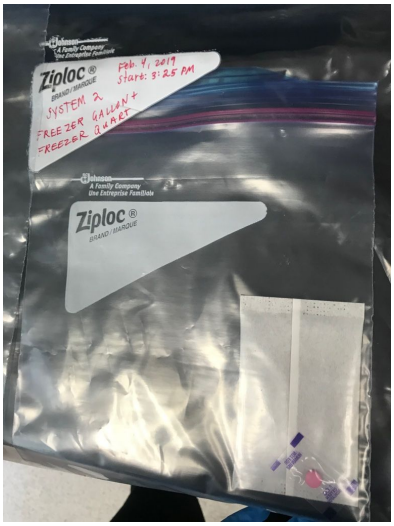
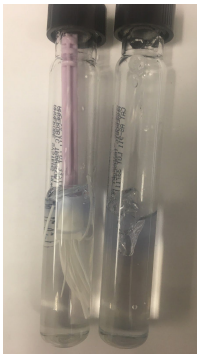
Sandwich Bag + Quart Bag	Freezer Bag + Sandwich Bag	Double Freezer Bag
		

Figure 5: Anaerobic Systems Experiment after 72 hours

Following the unsuccessful results creating an anaerobic environment to culture *Fusobacterium*, attempts to culture the bacteria in the anaerobic hood began. Successful growth on plates was then achieved as shown in Figure 3. However, it was concluded that it would be difficult to create an entirely anaerobic environment using the plates, as oxygen exposure occurred across the entire surface area of the plate, penetrating into the media. Therefore the tube design was pursued per results which are discussed below.

Swabbing Mechanism

Table 4: Results of Swab Submersion Experiments

	t=0	48 hours	72 hours
Cervex Brush			
Cotton Swab			

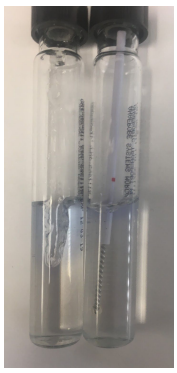
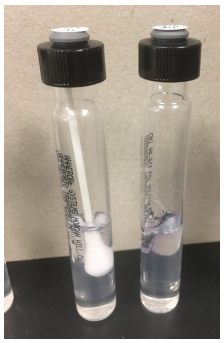
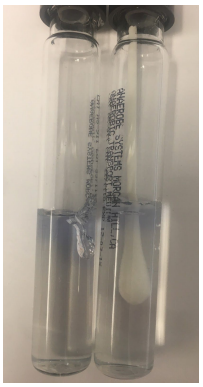
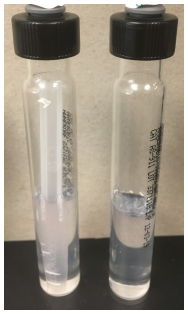
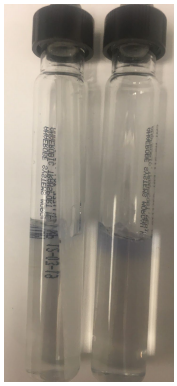
Bristle Brush			
Polyester Swab			
Cervical Spatula			

Table 4 above summarizes the results of the swab selection experiment. As indicated by the color change, submerging the swab in the tube was the best method to maintain anaerobic conditions. In conducting this experiment, the sizes of the cervex brush and cervical spatula were too big to be inserted into the tube, and needed to be cut to fit, making their size an issue. The cotton swab was also eliminated due to the toxic nature of the wooden handle which could prevent bacterial growth. Between the polyester swab and bristle brush, research supported that the bristle brush

collects a better sample than the polyester swab, so the bristle brush was selected. Table 12 below summarizes this selection.

Table 5: Final Swab Selection

	Size	Toxicity	Collection
Cervex Brush		✓	✓
Cervical Spatula		✓	✓
Cotton Swab	✓		✓
Polyester Swab	✓	✓	
Bristle Brush	✓	✓	✓

Table 13 shows the results of verifying the submersion swabbing mechanism as the best method to maintain anaerobic conditions. As shown by the images below, leaving the bristle brush submerged in the media resulted in the least oxygen exposure, indicated by the pink color change isolated to the top of the tube.

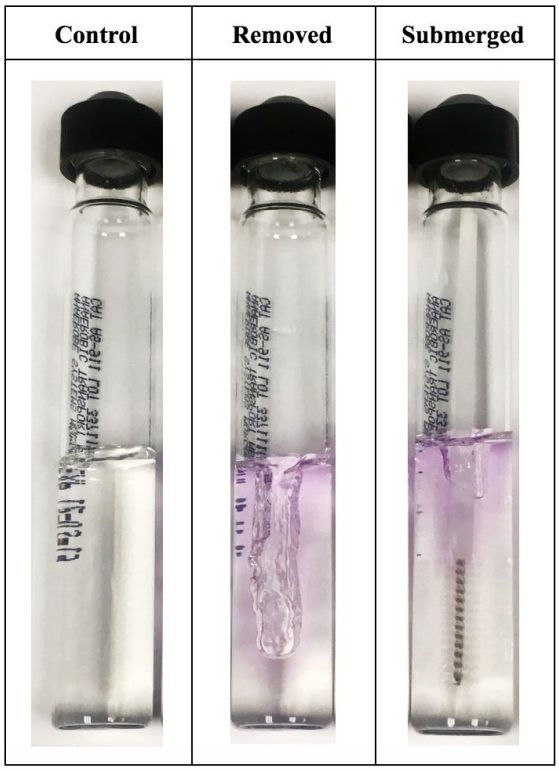


Figure 6: Sampling Mechanism Oxygen Exposure

These experiments informed the final design prototype. The design was decided to be a tube with semi-solid media to maintain anaerobic conditions and keep the swab submerged to create a self-contained test.

4.4 Next Steps: Potential Pitfalls and Future Directions

After deciding to move forward with the tube design, bacterial growth had yet to be observed. Given the results from the anaerobic transport media, it was reasonably concluded that conditions necessary for growth could be achieved as the bottom 70% of the tube remained anaerobic.

CHAPTER 5: Growth Media

5.1 Introduction

As noted in the introduction, the test for *fusobacterium* growth requires a selective media and an anaerobic environment. The media developed fulfills these criteria through the methods listed below.

5.2 Methods

Lab procedures focused on growing *fusobacterium* on both selective and non-selective solid media plates incubated at 37°C. Both BRU and FSA contain blood and therefore appear dark red. While this coloring actually increases the ease of growth observation on solid media plates, it makes growth observation very difficult in media tubes. Therefore, a novel media, CERVIS 1.0 was developed to include the selectivity of FSA without incorporating blood. FSA contains three antibiotics that select for fusobacterium: vancomycin, neomycin, and josamycin (Morgenstein, Citron, & Finegold, 1981). Thus, CERVIS 1.0 contained all three of these antibiotics to maintain the selectivity. To provide a nutrient rich media base, porcine Brain Heart Infusion (BHI) broth was also added to the media. Porcine was selected over bovine or other options for ease of future use. Some countries have laws against the transport of bovine material across borders for disease prevention and protection, so porcine material is a safer choice. BHI was selected primarily because it is transparent and prior research suggests that fusobacterium can grow optimally on this media (Han, 2006). The complete list of ingredients in the media is listed in Appendix Figure A.

In order to test the CERVIS 1.0, pure *fusobacteria* cultures of F. *nucleatum* and F. *necrophorum* were plated and incubated at 37°C until colony formation was observed. Approximately two colonies of each strain were taken from the plated cultures using a bristle brush, which was then used to inoculate the media. The inoculated sample tubes were then incubated at 37°C outside of the anaerobic chamber and growth was monitored over a period of 72 hours.

5.3 Results

The non-selective media, Blood Brucella Agar (BRU) successfully grew *Fusobacterium* cultures in the anaerobic chamber. The selective media, Fusobacterium Selective Agar (FSA), developed by Anaerobe Systems was also successful in growing *Fusobacterium* colonies, but the observed colonies were much smaller.

The first iteration of the CERVIS media, CERVIS 1.0, was a major milestone for the project's success. The media supported the growth of pure *fusobacteria* cultures of F. *nucleatum* and F. *necrophorum* in tubes incubated at 37°C both inside and outside the anaerobic hood. This proved that an obligate anaerobe could be grown in CERVIS media outside of a contained anaerobic chamber and showed that the media successfully maintained anaerobic conditions. Additionally, this proved that the media contained enough nutrients to support growth. However, bacterial growth was difficult to distinguish from other artifacts or air bubbles within the media, as shown in Figure 7.

Figure 7: Fusobacteria Growth in CERVIS 1.0 Media

The results of the tests using CERVIS 1.0 were difficult to interpret. Additionally, there was no confirmation that the organisms growing in the tube really were *Fusobacterium* spp. In order to

make a more accurate test where growth was easy to visualize, a secondary differential test for the presence of fusobacteria was deemed necessary.

CERVIS 2.0 was developed to address the limitations of CERVIS 1.0. To identify growth more easily and with better accuracy, components of a secondary test were implemented in the media. The secondary test, modeled after Triple Sugar Iron (TSI) tubes, confirms that any growth seen is actually fusobacterium. If enough cysteine is present in the media, fusobacterium can produce dihydrogen sulfide (H_2S) (Basic et al., 2015). This byproduct can then react with ammonium iron (iii) sulfate hexahydrate, which acts as an H_2S indicator and turns black when H_2S is present. Thus, the presence of fusobacterium, the media will turn a dark black color, in Figure 8 below, that is significantly different from the control golden color of the media in the absence of fusobacterium. L-cysteine also acts as an oxygen reducing agent, eliminating the need for DTT in CERVIS 2.0. resazurin was removed because the anaerobic conditions of the media were already tested and proved and adding this color change was thought to interfere with the color change from the secondary test. Additionally, magnesium sulfate heptahydrate was added to help solidify the Gelrite to maintain the semisolid consistency (Basic et al., 2017).

The same experiments outlined in the CERVIS 1.0 media above were performed with CERVIS 2.0 media to measure and compare its functionality. The results shown in Figure 8 display the growth of F. *nucleatum* (middle) and F. *necrophorum* (right) in CERVIS 2.0 media compared against the control, with no bacterial sample introduced, seen on the left.

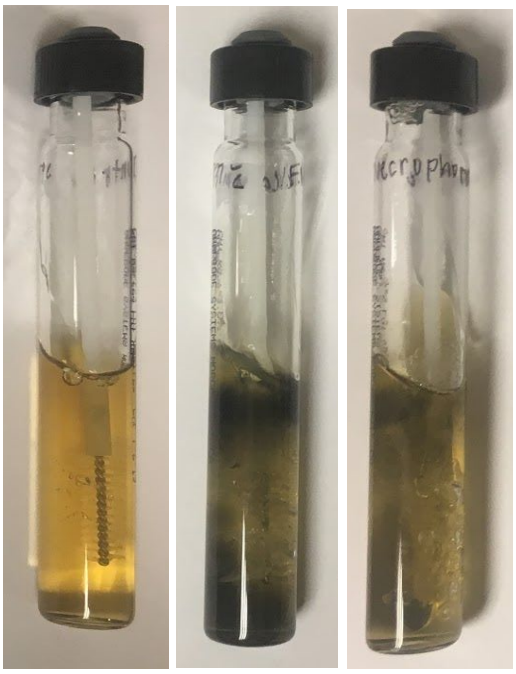


Figure 8: *Fusobacterium* Growth in CERVIS 2.0 Media

As depicted, the media changed from a transparent, golden color, seen in the control sample, to a murky black in both strains. This color change shows the product of the secondary test, which confirms the presence of fusobacterium growth in the tube. Again, this test was performed outside of the anaerobic chamber, confirming that the media maintained anaerobic conditions sufficient for fusobacterium growth. To confirm that no other bacteria from a vaginal swab would cause this color change, five members of the team performed vaginal swabs and inserted them into CERVIS 2.0 media, following the procedures outlined in Section 3.2. The results of this experiment are shown in Figure 9.

Figure 9: Vaginal Swabs in CERVIS 2.0 Media

While each tube with a vaginal swab looks slightly cloudier than the control tube, there is no color change, proving that no other presumably relevant bacteria would cause the color change indicating the results of the test.

5.4 Next Steps: Potential Pitfalls and Future Directions

CERVIS 2.0 was successful in growing fusobacterium selectively, but the sensitivity of the media is not yet known. Experimental plans to begin estimating the sensitivity of the media are outlined in Section 6.2.1. Additionally, the color change in the presence of fusobacteria was only observed at 37°C. At room temperature, some growth was observed but the media remained a golden color. This indicated the need for some form of incubation, which must be considered for the final device design and implementation.

It is not yet understood what other microbial species in the vaginal microbiome produce hydrogen sulfate as a byproduct and will in turn, invoke the reaction in the presence of L-cysteine to blacken the CERVIS media in the vial. Further testing is necessary to better

understand the validity of CERVIS as a viable screening method for early stage cervical cancer and what the risk of false positives is due to other bacteria that can grow in CERVIS media and invoke the reaction. To test this, samples from human cervical cancer patients must be collected and tested.

CHAPTER 6: Conclusion & Future Directions

6.1 Prototype

Over the course of this project, a biomarker that has the potential to indicate the presence of cervical cancer has been identified using emerging research on the vaginal microbiome, and a novel screening test capable of selectively detecting for this species of bacteria from a patient sample was designed. The scope of the project focused on the development of this prototype through the creation of a selective media that incorporated the antibiotics from FSA media and maintained anaerobic conditions through the use of a semi-solid media with oxygen scavengers. The physical form of the assay incorporated a patient sample through the use of a vaginal swab taken with a bristle brush. This sample applicator was selected from a group of five applicators tested on the criteria of size, toxicity, and sample collection. The screening method is minimally invasive as it only requires a single self-administered vaginal swab. The test is self contained as the swab is inserted directly into the media and sealed with a cap. These characteristics of the screening method help to minimize the need for clinically trained personnel to be present to conduct the test or analyze the result; hopefully, alleviating the need for women to seek out traditional clinical settings in order to gain access to preventative care. The final iteration of the proposed design of the screening test currently costs less than $5 to manufacture, with the hungate tube and cap being the most costly items. Reference Figure A in the Appendix for a complete cost analysis.

Figure 10: CERVIS Prototype

6.2 Sensitivity

To better understand the correlation of the bacterial samples from our initial tests compared to the vaginal microbiome of a diseased patient, calculated estimations were made to investigate the sensitivity of the CERVIS 2.0 prototype. It is known that 17% of the vaginal microbiome is composed of fusobacterium in the case of Cervical Cancer (Audirac-Chalifour et al., 2016). Bartlett et al., reports an average of 10^9 anaerobic bacteria in the average vaginal microbiome (Bartlett, 1977).

Quantification of Fusobacterium in the Vaginal Microbiome of a Cervical Cancer Patient:

$$17\% \; of \; 10^9 \; = \; 170,000,000$$

Mitra et al., notes the average bacterial load using a cytology (bristle) brush is 6.41. This value is reported on a log base 10 scale. Therefore, assuming that the bacteria taken up by one sample using the CERVIS 2.0 sampling device, 2570395 bacteria are collected by one swab.

Quantification of Fusobacterium in one swab from a Cervical Cancer Patient:

$$17\% \text{ of } 2{,}570{,}395 = 436{,}967$$

It is noted that these calculations are rough estimates, as the vaginal microbiome is highly variable between individuals due to factors including sexual activity, menarche, menses, age, pregnancy, infection, and birth control (Huang et al., 2014).

Next, the estimated number of fusobacterium in the diseased vaginal microbiome was compared to the colonies taken up by the swab during experimentation. It is estimated that one colony contains an average of 10^7 bacteria. For each plate of fusobacterium (*F. necrophorum and F. nucleatum*) 2 colonies were collected using the cytology (bristle) brush. Under this assumption 20,000,000 bacteria of pure *fusobacterium* spp. respectively were delivered into each tube.

In conclusion, 436,967 bacteria (estimated patient sample) < 20,000,000 bacteria (estimated experimental sample). Thus, these calculations are insufficient in correlating the vaginal microbiome to colonies on a plate. Further sensitivity testing is required.

6.2.2 Preliminary Sensitivity Experiments

To further estimate the sensitivity of the device, an experiment was designed under aerobic conditions to estimate the threshold of bacteria needed to cause the color change in CERVIS 2.0 media. The experiment is outlined as follows:

Using BHI media, create a suspension of cells from a colony of *F. necrophorum* and *F. nucleatum.* BHI broth from Anaerobe Systems is maintained anaerobically, which is why it was chosen as the solvent over saline typical of a dilution series. These cultures were used to roughly estimate cell density.

Serial dilutions were prepared using the cultures. To prepare the first dilution, add 1 mL of culture to 99 mL BHI (10^{-2} dilution). Next, 1 mL of the 10^{-2} dilution should be added to another 99 mL BHI to generate a 10^{-4} dilution. At this point, three further dilutions should be prepared (10^{-5} to 10^{-7}) adding new samples of *f. Nucleatum and f. necrophorum* to 4.5 ml BHI.

After completing the dilution series, a bristle brush should be used to take a sample from each dilution. The brushes should be inserted directly into the CERVIS 2.0 media and the tubes immediately resealed to maintain anaerobicity.

This experiment was not conducted due to unforeseen laboratory challenges. At the time of scheduled experimentation, both control strains of *fusobacterium* previously plated on FSA were dried, and well-defined colonies were not collectable by bristle brush. Thus, the starter culture could not be made. Moving forward, sensitivity testing is necessary to validate the integrity of the CERVIS 2.0 device.

6.3 Device Implementation

Human Subjects Research

There currently lacks adequate data to confirm that the bacteria introduced by a colony from a pure culture represents the vaginal microbiome of a disease state. In order to clinically scale up the device, a partnership with a research hospital is required to confirm device sensitivity and specificity. Following the required protocols for human subjects testing, CERVIS 2.0 will be

tested with patient samples from representative populations of the following categories: HPV negative, HPV positive, intraepithelial lesions, and cervical cancer.

Clinical Trial in the United States

The results from the human subjects research testing will inform a clinical trial conducted within the United States. To properly conduct a clinical trial, the IRB protocol and FDA 510-k filing will be completed. The prototype will be classified as an in vitro diagnostic Medical Device for self-testing, because it is intended by the manufacturer for use by lay persons and its noninvasive nature. Additionally, since other medically established means of diagnosis of cervical cancer exist, we will not need to file for an Investigational Device Exemption (IDE).

Sustainable Partnership with Aga Khan University

The clinical trial will be repeated in Kenya to guarantee the results are replicable within the target population. Variations in the microbiome are dependent partially on the individual's environment, and this trial will ensure that the microbiome is consistent enough that fusobacterium is an appropriate biomarker in this context.

Pilot Test within Target Community

Upon test validation, the device will be pilot tested in a community near Nairobi, Kenya in a manner that is culturally appropriate. This may be completed by using the device initially in a community setting, screening a number of women at a location they regularly frequent, similar to a blood drive. Results from this test will inform how the device is deployed on a larger scale.

6.3.1 Supplemental to Current Screening Tests

Per current research, the *Fusobacterium* strains of interest are only present in the vaginal microbiome of individuals with precancer and cancer. Although our device will never replace the current diagnostic standard, our device is a less-invasive and lower-costing alternative that is indicative of serious disease. For this reason, it is necessary to have a standard for referring women who test positive to seek further screening and subsequent treatment. The methods for further screening are outlined in Section 1.2, and the educational component included in the CERVIS implementation strategy will contain relevant information about these methods and available treatment options. Additionally, the targeted areas for pilot testing must have the means

to access such subsequent methods. As device deployment is scaled up, methods of transporting women from rural areas to more developed areas with a greater capacity for medical care must be explored.

CHAPTER 7: Engineering Standards

7.1 Manufacturability

Anaerobe Systems is the producer of the world's only true pre-reduced anaerobically sterilized (PRAS) culture media poured and packaged under anaerobic conditions (Anaerobic Chambers & Culture Media Manufacturer, 2018). This company is currently the independent manufacturer of CERVIS media. According to Anaerobe Systems product quality and development manager, it takes between 4 – 6 different people to produce one liter of media, all with varying hourly pay rates. The manufacturing procedure is outlined in the following paragraphs.

The Anaerobe Systems production manager first schedules the manufacturing and determines who will make the media. He/She also orders all necessary components and ensures the correct equipment is being used. Next, the mixing technician makes the media and starts the sterilization process. Once the media is sterilized, another technician adds any heat labile components like the antibiotics. Only a few people at Anaerobe Systems are authorized to do this since it takes a skilled person to perform this procedure correctly without contaminating or harming the media.

Next, a different production technician hand pours the media in pre-sterilized tubes. They need to be pre-sterilized beforehand since heat-labile components are added after the primary media is sterilized. They cannot be sterilized in the tubes since that will destroy the antibiotics. This increases the cost since a person needs to hand pour the media instead of just placing it on the automatic tube pouring machine. That machine can only be used for media that can be sterilized in the tube. The machine also pours tubes faster than a technician can. All the media is made within larger anaerobic chambers in production, which have their own electrical expenses and nitrogen gas consumption.

Once the tubes are poured, they need to be labeled and packaged in foil filled with nitrogen gas and sealed. Some cost considerations include electricity, including the AC unit to maintain the appropriate room conditions and the lights so employees can see. Additionally, autoclaves and a boiler are used to make the steam for sterilization.

Once the media goes to quality control for approval, a quality control technician conducts sterility and performance tests on the media. This requires specialized skills and equipment including an anaerobic chamber, incubators, and other general laboratory equipment like loops and saline blanks. All necessary organisms are preemptively cultured on a general media like Brucella blood agar (BRU). This machinery requires electricity to operate and the anaerobic chamber requires expensive tri-mix gas. Once all the testing is completed, the quality control manager approves and passes all media.

7.2 Ethics

The primary ethics question that arose from this project was: *Is it ethical for a team from the developed world with little first-hand knowledge of the cultural practices in the target location to develop an alternative screening method and, if so, on what basis?*

Developing and implementing a low-cost, minimally-invasive screening method that is culturally relevant will benefit the common good and improve social justice because it will limit the the need for more invasive procedures, increase the chances for successful treatment, and will reduce incidence and mortality rates for women living in low- and middle-income countries.

There are a variety of stakeholders that warrant consideration when addressing this question. The most important are the women living in Kenya who because of CERVIS will have a lesser chance at contracting late stage cervical cancer compared to women in other similar sub-Saharan countries. Additionally, community health workers, doctors, and volunteers involved in the care, delivery, and education concerning our device have a stake in our project. Our team from Santa Clara University, including students from the departments of bioengineering and public health science, and our advisors from biology, bioengineering, and public health science all have a stake because the researchers care about designing the device to work as intended and cause the most benefit and least amount of harm to our target community.

On a greater scale, the members of the cervical cancer research community including researchers and doctors from around the world have a stake because our device has the potential to reduce the burden of disease significantly, especially for individuals in previously unreachable contexts. Since the bacteria our device detects is indicative of other disease states in other microbiomes,

this device has the potential to screen for other diseases beyond cervical cancer. This extends the stakeholders beyond the cervical cancer community to dentists and other oncologists and researchers. The employees at Anaerobe Systems, a corporate partner helping manufacture CERVIS, hold a stake in terms of the viability of the product because they are interested in expanding their market (primarily microbiology researchers working with anaerobic bacteria) to helping people in the developing world. Society is always a stakeholder.

7.3 Other Emerging Work

Intellectual Ventures

A research team led by investigators from the National Institutes of Health (NIH) and Global Good has developed a computer algorithm that can analyze digital images of a woman's cervix and accurately identify precancerous changes that require medical attention. This artificial intelligence (AI) approach, called automated visual evaluation, has the potential to revolutionize cervical cancer screening, particularly in low-resource settings. This new technology could dramatically improve the screening capabilities of clinics in low-resource settings, as it requires minimal training, which makes it ideal for contexts with limited resources. However, many cultural barriers exist that prevent women from seeking out preventative care, and many women only go to the clinic if they are experiencing symptoms or are pregnant. Therefore, one limitation of this innovation is that it requires a clinical setting (AI approach outperformed human experts in identifying cervical precancer, 2019).

Unitaid Projects

From May 2018 to September 2018, Unitaid collected submissions for projects seeking to help eliminate the incidence of cervical cancer in low and middle income countries. Unitaid is aware of the need for expanded access to screening in order to reduce the incidents of cervical cancer deaths in these settings. Currently, Unitaid is developing two grants related to cervical cancer screening and treatment, and at this time both have not been published. The projects are moving into the final development stages and further information should be available in coming weeks (Preventing Deaths from Cervical Cancer, 2018).

7.4 Engineering Standards & Realistic Constraints

Economics, manufacturability, cultural impact, and health and safety are the engineering standards affecting device design.

Economics

Pilot testing is intended to take place in Nairobi, Kenya and for this reason, the economic landscape of Kenya must be evaluated. The low cost of CERVIS is aimed to address this challenge, enabling women of all socioeconomic backgrounds the ability to be screened for cervical cancer (See Appendix).

Manufacturability

The device is currently manufactured by Anaerobe Systems in Morgan Hill, CA. For long term scale-up and sustainability, manufacturing options in Kenya warrant consideration.

Cultural Impact

Religion plays an important role in the life of most Kenyans, and the concept of virginity is highly valued by both Christians and Muslims, the two most predominant faiths practiced in Kenya (Spronk, 2005). In earlier iterations of our device, team CERVIS explored tampons and menstrual cups as a means to collect blood samples from women that could be used to screen for cervical cancer. However, after talking to several nurses working in sub-Saharan Africa, it became clear that these items are not typically used due to a stigma that a "broken" hymen equates to a loss in virginity. Despite having no identifiable role in the woman's body, the hymen retains significant religious and moral value in Kenyan society (Odongo, 2015). The uptake of earlier detection methods was limited by the Kenyan belief that hymenal integrity signifies virginity, and women would not use earlier collection methods because of the fear of tearing the hymen.

Our current prototype requires a woman to self-swab her vagina using a small, soft bristle brush. Although this is less-invasive than a tampon, a woman using our device may have similar fears about virginity after inserting the swab. To address these fears, an educational component designed by nurses and educators working in the field to help women understand their anatomy, the benefits of screening, and how to use our device effectively would be necessary. A hymen

can be “broken” through sex, by using a tampon or a menstrual cup, doing sports, or visiting a gynecologist, and misunderstanding this information poses challenges in implementing cervical cancer screening interventions (Sayer, 2018).

Health and Safety

Health and safety is the most important factor under consideration. To ensure this goal is maintained and monitored, an educational program should be co-developed by educators, volunteers, and healthcare workers on the ground that helps to explain a woman’s anatomy and the importance of screening for cervical cancer in addition to creating an alternative low-cost screening method and deploying it in Kenya. A plan to increase access to subsequent screening and treatment options for women who test positive after using CERVIS must also be put into place.

Additionally, once a vaginal swab is administered and introduced to the media, CERVIS 2.0 becomes a biohazard that needs to be disposed of properly to maintain the health and safety of the community. Establishing clear, well-defined policies to ensure proper disposal must be put in place prior to device implementation.

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APPENDICES

Table A: Summary of Goals, Objectives, and Expected Results

Goals	Objectives	Expected Results
Create an alternative screening method for cervical cancer	Develop a working prototype	A final sketch of a proposed device
Device must not require a clinical setting	Develop a test that is easy to use without laboratory equipment or medical expertise	The final device can be self-administered as an at-home test
Device must be self-contained	Develop a handheld device that does not contain any loose components	Device will resemble a pregnancy test
Device must be low-cost	Create a device for less than existing methods	Cost will depend on final biomarker selection
Device must produce a binary output	Create an output that is easily interpreted in the field (+/-)	Device produces a binary output when introduced to a biomarker
Device must be deployable in low- and middle-income countries	Create sustainable partnerships with healthcare workers in the field	Partnerships formed throughout the year will be professional and provide information through interviews to inform the cultural relevance of the project

Table B: Funding Received

Funding Received	**School of Engineering**	**$2000**
	Xilinx Grant	**$500**
	Total Received	**$2500**

Table C: CERVIS Expenditures

Product Description	**Company/ Provider**	**Product Number**	**Unit Cost**	**Total Cost**	**Date Ordered**
FSA media	Anaerobic Systems	353136	9.18	45.9	1/11/19
Anaerobic pouches	Biomerieux	46968	73.93	128.01	1/17/19
Anaerobic Indicator	Biomerieux	46971	1.28	42.73	1/21/19
Fusobacterium nucleatum subsp.	ATCC	25586	133.26	133.26	1/29/19
Anaerobic Transport Medium	Anaerobic Systems	3381118	20.42	20.42	1/31/19
FSA media	Anaerobic Systems	353136	9.18	45.9	2/4/19

Fusobacterium nucleatum subsp.	ATCC	25586	133.26	133.26	2/12/19
Puritan Cytology Bristle Brushes	Marketplace Direct	252199	0.59	58.91	2/12/19
Anaerobic Chamber Gas	Praxair	88404127	640.24	640.24	4/8/19
			Total Spent	**1,230.27**	

Table D: 2018-2020 Academic Timeline for CERVIS

	Fall Quarter	**Winter Quarter**	**Spring Quarter**
Week 1	First meeting after summer recess with Primary Advisor Dr. Prashanth Asuri Partnership formed between BIOE students and EWH students	Working in Dr. Stephens microbiology lab is confirmed *Fusobacterium* is selected as the bacterial biomarker of interest EWH & BIOE students draft preliminary experiments for winter quarter lab work	CERVIS1.0 results collected and analyzed Second collaborative effort with Anaerobe Systems to design CERVIS2.0
Week 2	First meeting with all team members-	Accepted to present at	CERVIS 2.0Media Tested with Pure Strains

	brainstorming session on prospective analytes and biomarkers	Unite for Sight Conference Environmental Health and Safety form approved for Stephens lab Initial materials order placed Registration confirmed for Senior Design Conference presentation	of *Fusobacteria* spp. and samples from female group members Short presentation delivered to advisory board members (Bioinnovation & Design)
Week 3		Second Frugal Innovation workshop with Gregory Theyel	Final Frugal Innovation workshop with Gregory Theyel Unite for Sight Global Health & Innovation Conference
Week 4	Crashing brainstorming session- bacteria biomarker idea comes to fruition	Anaerobicity experiments run in lab; attempt to grow *fusobacterium* in a micro-aerobic environment	GHTC Paper submitted for review
Week 5	Dr. Craig Stephens joins the team- preliminary budget/list of material submitted to School of Engineering	CERVIS team runs the Kaiser Permanente San Francisco half marathon Colonies grown under different aerobic conditions analyzed using 16s	Sensitivity Testing

		ribosomal RNA sequencing Meeting with Dr. Ruscetti alters project trajectory- refocused on device design	
Week 6	First Frugal Innovation workshop with Gregory Theyel	Conversation with Lata Mukundan from CCSA for a discussion on the therapeutic potential of the human microbiome Dr. Ruscetti guided a training session to work in the anaerobic hood Control culture of *F. nucleatum* was plated and streaked for isolation on FSA under anaerobic conditions Meeting with Mike Cox and Hiram Lonzo at Anaerobe Systems inspires device prototype	Senior Design Conference
Week 7	Preliminary experiments run in attempt to cultivate lactobacillus	IEEE Conference Presentation at San Jose State University	Finalize Thesis Rough Draft
Week 8			Coordinate Next Steps

			for Project Continuation
Week 9	EWH students begin working on abstract for GHTC		Submit Thesis to Bioengineering Adviser for Revisions
Week 10	Group meeting with all advisors, EWH, and BIOE students; recess until post winter break	Experimental lab work in the anaerobic hood using CERVIS 1.0 Media	Final Thesis Submission

Table E: Information about Vaginal Microbiota

Species	Gram (-/+)	Fermentation products	Morphology	O2 tolerance	Other important characteristics	Source:
L. crispatus	+	H2O2 (can be used as antimicrobial agent) Lactic acid (lower environmental pH- another antimicrobial attack)	Rods	Obligate anaerobe-aerotolerant	-Approx 60% healthy vaginal biome -produce antibiotic products that target E.coli and Candida	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4985617/
G. vaginalis	gram variable	*G. vaginalis* ferments a wide variety	Coccobacilli (Short rods) Nonmotile	Facultative anaerobes	-Is the main cause of Bacterial	https://www.ncbi.nlm.nih.gov/

		of carbohydrates, producing acetic acid as an end product. *G. vaginalis* does not reduce nitrate or hydrolyze urea. Negative catalase test			Vaginosis -it has been found in 15% to 69% of women without signs or symptoms of vaginal infection -*G. vaginalis* can be sexually transmitted -Its role in pathogenesis may be through altering the microbial environment due to RBC lysis releasing iron metabolites or through altering the host response	pubmed/6389437 https://www.sciencedirect.com/topics/medicine-and-dentistry/gardnerella-vaginalis
L. Iners	+ Variable	Catalase negative lactic acid bacteria group (i.e. they convert	Rods	Facultative anaerobe		https://aem.asm.org/content/aem/early/2016/09/26/

		sugars to lactic acid).				AEM.02385-16.full.pdf https://www.cell.com/trends/microbiology/pdf/S0966-842X(16)30181-0.pdf
L. Gasseri	+	Catalase negative	Rods	anaerobic	More common in the colon- not super worried about this one	https://microbewiki.kenyon.edu/index.php/Lactobacillus_gasseri
L. Jensenii	+	Catalase negative	Rods	anaerobic	It is one of the predominant species (along with *Lactobacillus crispatus*) found in the female lower genital tract	https://microbewiki.kenyon.edu/index.php/Lactobacillus_jensenii

S. Agalactiae	+	Catalase negative Lactic acid	Spherical	Microaerophilic	- Causes meningitis in neonates and elderly - Occasionally colonizes female reproductive tract - Increase risk for premature rupture of membranes and transmission of infection to child	https://www.sciencedirect.com/sdfe/pdf/download/eid/3-s2.0-B978143772702900 1215/first-page-pdf https://www.sigmaaldrich.com/technical-documents/articles/analytix/streptococci-overview.html
L. Acidophilus		undergoes fermentation only		Anaerobic		https://jcm.asm.org/content/45/9/3145
F. Necrophorum	-	H2S	Rods	Anaerobic		

F. Nucleatum	-	H2S	Rods	Anaerobic		

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Anaerobe Systems
Bill of Materials Report
As of Apr 8, 2019

Filter Criteria includes: 1) Item ID's from WP-MED-723 to WP-MED-723. Report order is by Item ID. Report is printed in Detail format with with shortened descriptions.

Item ID	Item Description	Qty Needed	Qty on Hand	Est Cost
WP-MED-723	CERVIS MEDIUM			
. RM-0525	BRAIN HEART INFUSION-PORCINE	37.00	873.00	12.95
. RM-3480	YEAST EXTRACT TECH GRADE	5.00	1791.76	0.70
. RM-0930	CYSTEINE-SIGMA	1.00	2327.77	0.60
. RM-1580	JOSAMYCIN	0.00	0.21	3.99
. RM-3350	VANCOMYCIN	0.01	31.55	0.44
. RM-2020	NEOMYCIN TRISULFATE	0.16	43.23	0.31
. RM-1320	GELLAN GUM	4.00	4248.00	1.44
. RM-1720	MAGNESIUM SULFATE HEPTAHYD	0.10	1465.45	0.01
. RM-0152	AMMONIUM IRON II SULFATE HEXA	0.20	2.00	
WP-MED-723 Total				**20.44**

4/8/19 at 09:46:29.18 Page: 1

Anaerobe Systems
Bill of Materials Report
As of Apr 8, 2019

Filter Criteria includes: 1) Item ID's from WP-PACK-723 to WP-PACK-723. Report order is by Item ID. Report is printed in Detail format with with shortened descriptions.

Item ID	Item Description	Qty Needed	Qty on Hand	Est Cost
WP-PACK-723	CERVIS MEDIUM			
. RM-2415	POUCHES ANAEROBIC 6 x 7.25	1.00	34664.00	0.21
. WP-EA-723	CERVIS MEDIUM	10.00		
. . WP-MED-723	CERVIS MEDIUM	0.07		
. . . RM-0525	BRAIN HEART INFUSION-PORCINE	2.59	873.00	0.91
. . . RM-3480	YEAST EXTRACT TECH GRADE	0.35	1791.76	0.05
. . . RM-0930	CYSTEINE-SIGMA	0.07	2327.77	0.04
. . . RM-1580	JOSAMYCIN	0.00	0.21	0.28
. . . RM-3350	VANCOMYCIN	0.00	31.55	0.03
. . . RM-2020	NEOMYCIN TRISULFATE	0.01	43.23	0.02
. . . RM-1320	GELLAN GUM	0.28	4248.00	0.10
. . . RM-1720	MAGNESIUM SULFATE HEPTAHYD	0.01	1465.45	0.00
. . . RM-0152	AMMONIUM IRON II SULFATE HEXA	0.01	2.00	
. . RM-3079	TEST TUBES 16x100	10.00	42062.00	1.70
. . LABOR	Anaerobe Lab Labor Rate	10.00		
. . OVERHEAD	FACTORY OVERHEAD	10.00		
. . WP-1470	HUNGATE ASSEMBLED	10.00	54758.00	
. . . RM-1470	HUNGATE CAPS- BLACK	10.00	18400.00	0.50
. . . RM-1480	HUNGATE STOPPERS	10.00		1.40
. . . LBR-0020	CONTRACT CAP/STOPPER ASSEM	10.00		
. RM-1598	LABELS- AS THERMAL	1.00	86667.00	0.06
WP-PACK-723 Total				**5.30**

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Anaerobe Systems
Bill of Materials Report
As of Apr 8, 2019

Filter Criteria includes: 1) Item ID's from WP-EA-723 to WP-EA-723. Report order is by Item ID. Report is printed in Detail format with with shortened descriptions.

Item ID	Item Description	Qty Needed	Qty on Hand	Est Cost
WP-EA-723	CERVIS MEDIUM			
. WP-MED-723	CERVIS MEDIUM	0.01		
. . RM-0525	BRAIN HEART INFUSION-PORCINE	0.26	873.00	0.09
. . RM-3480	YEAST EXTRACT TECH GRADE	0.04	1791.76	0.00
. . RM-0930	CYSTEINE-SIGMA	0.01	2327.77	0.00
. . RM-1580	JOSAMYCIN	0.00	0.21	0.03
. . RM-3350	VANCOMYCIN	0.00	31.55	0.00
. . RM-2020	NEOMYCIN TRISULFATE	0.00	43.23	0.00
. . RM-1320	GELLAN GUM	0.03	4248.00	0.01
. . RM-1720	MAGNESIUM SULFATE HEPTAHYD	0.00	1465.45	0.00
. . RM-0152	AMMONIUM IRON II SULFATE HEXA	0.00	2.00	
. RM-3079	TEST TUBES 16x100	1.00	42062.00	0.17
. LABOR	Anaerobe Lab Labor Rate	1.00		
. OVERHEAD	FACTORY OVERHEAD	1.00		
. WP-1470	HUNGATE ASSEMBLED	1.00	54758.00	
. . RM-1470	HUNGATE CAPS- BLACK	1.00	18400.00	0.05
. . RM-1480	HUNGATE STOPPERS	1.00		0.14
. . LBR-0020	CONTRACT CAP/STOPPER ASSEM	1.00		
WP-EA-723 Total				**0.50**

Figure A: Anaerobe Systems Bill of Materials