Evaluating Lactobacillus Acidophilus as a Model Organism for Co-Culture Cancer Studies

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ABSTRACT

The causality dilemma between dysbiosis and cancer has given rise to numerous studies both exploring the mechanisms behind cancer progression and the associative shifts in the microbiota upon carcinogenesis. Aside from the hallmark study of Dr. Barry Marshall in establishing the true causal relationship between *Helicobacter pylori* and gastric adenocarcinoma, studies have only been successful in adding associative links of carcinogenesis mediated by bacteria to the literature. The current field is limited in its ability to establish causative relationships, and further work is needed to construct a reference community whose physiological responses reflect global community responses. In this thesis, the organism *Lactobacillus acidophilus* was selected as a pilot strain for the development of a novel framework to establish the fitness and physiological changes that occur when bacteria engage the human epithelial environment. The pilot strain was revived from the American Type Culture Collection (ATCC), verified through 16S rRNA Sanger sequencing, and grown in its conventional culture medium and human tissue culture medium to establish baseline growth rates and gauge its physiological responses to an *in vitro* tumor microenvironment. A set of standard conditions was proposed for growth under human tissue culture conditions. Finally, a metabolic study and spot plate assay were performed to elucidate the anabolic deficits and viability of this strain in human tissue culture medium, respectively. This research was performed to better understand the environmental and metabolic requirements for this pilot strain to inhabit the human epithelial environment and to establish a workflow that will set the foundation for an appropriate clinical study to demonstrate the causative relationship between dysbiosis and carcinogenesis.
DEDICATION

To Baba and Mama, whose sacrifice led to opportunity  
To Tota, my paragon of success and humility  
To Gido Samuel and Gido Aziz, leaders in their fields and families  
To my friends who provided encouragement every step of the way  
And to Pupsy and Mish-Mish, for always knowing what to say

“So do not fear, for I am with you; do not be dismayed, for I am your God. I will strengthen you and help you; I will uphold you with my righteous right hand.” Isaiah 41:10
ACKNOWLEDGMENTS

I would like to express my gratitude to those who made the completion of this thesis possible. My greatest thanks to Dr. Moore for always guiding me to the next logical step, for your patience when things didn’t go as planned, and for always helping me see the big picture of my work. To Dr. Andl, for your enthusiasm at every sign of progress and for introducing me to a field of research I’ve come to love. To Dr. Yooseph, for helping me overcome the many obstacles encountered early on in this research with validating our pilot strain identity. I would also like to thank the lab members of the Moore lab for taking the time out of their busy schedules to teach me, for encouraging me when my experiments failed, and for creating such a supportive work environment. Thank you to the members of the Andl lab for being such delightful collaborators, for their eagerness in providing me with the resources and supplies needed to complete this research, and for trusting me not to contaminate their human tissue cultures. To all, I am so thankful for your support.
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INTRODUCTION

The relationship between dysbiosis and carcinogenesis is still unclear, and there exists a causality dilemma of whether dysbiosis is a cause or result of cancer progression. Ecosystems such as the human microbiome are so complex that there are no ways to analyze the metagenomic or metabolic characteristics of individual strains found to inhabit the host microbiota, making it difficult to attribute true causal relationships between bacteria and cancer.

Demonstrated in 1984, there exists only one true established case of carcinogenesis mediated by bacteria.\(^1\) As the first two postulates had been established by the literature, in an attempt to fulfill Koch’s remaining postulates for pyloric *Campylobacter* (later recognized as *Helicobacter pylori*), Dr. Barry Marshall drank a cultured broth of *H. pylori* to establish a causative relationship between bacteria and gastritis, thought to lead to chronic infection, peptic ulceration, and gastric adenocarcinoma. An endoscopy performed on the tenth day after infection revealed histological evidence of gastritis and significant colonization by the bacterium, fulfilling Koch’s third postulate that “the inoculation of these germs, taken from the original animal, should produce the same disease in a susceptible animal.” Koch’s requirements for the final postulate, that “the germs should be found in the diseased areas so produced in the animal,” were met after subsequent Gram-stained specimens of the organism were identified as *H. pylori*.\(^1\) This groundbreaking study set aside the speculation of the existence of oncogenic bacteria. However, as research clearly cannot continue in this direction and experiments of this nature cannot be repeated, the current path of studies on bacterial carcinogenesis is limited in scope.

In our research, the speculative links between bacteria and cancers of the head and neck, particularly head-and-neck squamous cell carcinoma (HNSCC), have yet to be confirmed.

\(^1\)
HNSCC is the seventh most common form of cancer and comprises over 5% of all cancer cases worldwide. Both smoking and alcohol consumption are the greatest risk factors, and over 75% of all cases of HNSCC can be attributed to a combination of these elements. Viral factors, such as infection with carcinogenic strains of human papillomavirus (HPV), are an additional confirmed risk factor. Yet despite the range of well-established risk factors, HNSCC and related cancers have been seen to occur in those without a history of tobacco or alcohol use, and HPV is seen in only a moderate proportion of cases. The host microbiome has been implicated in the initiation and progression of HNSCC, and several mechanisms proposed to explain the carcinogenesis mediated by dysbiosis are rapidly gaining interest. Moreover, several studies noting associative changes in the microbiome have well-documented these shifts; however, the field is limited in establishing the causative relationship as was done with *H. pylori*. In an ideal situation, there exists a subset of variable, characterized microbes that can be used to gauge the physiological responses of a reference community. Work needs to be completed to highlight hallmark strains and construct a reference community whose physiological responses to stressor conditions will be telling of global community responses, which will set up the framework for a proper clinical study. The underlying premise is that microbes play a role, no matter how small, in carcinogenesis – the precedent being that humans are not sterile organisms. There are several studies that have characterized the local microbiota in regions of interest, but again the data is missing on direct causative relationships.

The research completed was performed in a unique setting – a bacterial physiology lab able to analyze bacterial responses located near our collaborator, a head-and-neck cancer lab positioned to emulate the human epithelial environment and analyze organotypic tissue.
responses. Together, collaboration between the two labs allowed us to characterize the growth behavior and physiological responses of individual strains thought to be hallmark strains of a reference community that is telling of the global host response. For this thesis, ATCC (American Type Culture Collection) 4796 Lactobacillus acidophilus was selected as a pilot strain as it is readily cultivable, found in normal and diseased tissue, and its complete genome is annotated.\textsuperscript{7,8} The specific strain was selected from a larger list of commensal and pathogenic bacterial strains evaluated in proposed models for HNSCC in a grant from our cancer collaborator lab. A novel framework was developed to establish the fitness and physiological changes that occur when bacteria engage the human epithelial environment. The specific aims of this research were as follows: 1) to establish baseline growth behavior of L. acidophilus in its conventional culture medium, and 2) to characterize the physiological responses of L. acidophilus to exposure to a human tissue culture medium. The completion of this thesis set the stage for characterizing the transcriptional and adaptive responses of this bacterium to the human epithelial environment.
MATERIALS AND METHODS

Pilot Strain Revival and Verification

The materials for strain revival and propagation, including deMan, Rogosa, and Sharpe (MRS) agar plates and broth, were prepared per ATCC recommendations (refer to Appendix B: MRS Composition and Preparation). The strain was retrieved from the Andl lab in freeze-dried double-vial preparation and was revived according to ATCC protocol. The freeze-dried pellet was rehydrated in 5 mL of MRS broth and streaked on two MRS agar plates; after 48 hours, isolated colonies were observed and inoculated in 1 mL of MRS broth. A series of several overnight (O/N) cultures was carried out and a final inoculation was used to prepare a 20% glycerol stock, which was placed in a -80°C freezer for joint lab use. A sub-stock was created from the previous freezer stock to create a dedicated freezer stock for the Moore lab.

To verify the pilot strain, 16S amplicon analysis was utilized. DNA was extracted using bead ablation followed by silica binding from an isolated colony derived from the Moore lab freezer stock and stored at -20°C (refer to Appendix C: Reagents for DNA Extraction). The isolated DNA was used in a PCR reaction using the “path_tail” set of primers for taxonomy (refer to Appendix D: Primers for Taxonomy). A portion of the PCR reaction was run on an agarose gel to roughly verify the length of the PCR reaction (~400-500 base pairs), and the remaining PCR product was stored at 4°C. Afterward, the PCR product was cleaned to remove primers, enzyme, and excess reagent using a silica column (refer to Appendix E: Reagents for PCR Cleanup). An Applied Industrial Microbiology (AIM) sequencing primer, which anneals to the 5’ portion of the Illumina tail and the sequencing read through to the other end, was then used for Sanger sequencing of the V3-V4 region (Figure 1). The cleaned PCR samples were
prepared and sent for Sanger sequencing. The results were received in .seq and .ab1 format, and the Sanger sequence was trimmed of bad base calls and primer sequences.

**Growth Study – MRS**

Preliminary growth studies were performed to determine both baseline growth rates and optimal growth conditions for the pilot strain. Additionally, the growth of *L. acidophilus* in its native medium, MRS broth, was analyzed under two different environmental conditions and three different culture volumes. The environmental conditions were reproduced using two different 96-well plate lids: low-evaporation and high-evaporation. This was done to vary the levels of humidity and gas exchange; the low-evaporation lids allow for, as the name suggests, lower rates of evaporation of the well volume, reducing the amount of gas exchange in the system. Furthermore, the culture volumes used – 50, 100, and 150 μL – allowed us to establish volume dependence on these cultures; different volumes simulate changes in the ratio of exposed aerobic and anaerobic regions, representing different rates of gas exchange. To analyze growth, turbidity measured at fixed intervals was used to estimate the biomass of each independent well. The turbidity of bacterial cultures linearly correlates with biomass – this has been observed in both bacterial and yeast cultures. In addition, bacterial density expresses the doubling time of a bacterial culture – or the division rate, if the assumption that all the cells are of constant average size is made. The outer edge wells of the plate were filled with water and the adjacent row was left empty to act as a thermal barrier for the culture wells. An O/N stationary culture was diluted 1/100 in fresh MRS media, vortexed, and quickly spun down. The three different culture volumes were pipetted into each well first, followed by the blank wells. The three replicate samples, with their respective blank cultures, were grown for 24 hours in a continuously shaking
plate reader at 37°C, and turbidity at a wavelength of 600 nm (OD600) was read every five minutes.

**Growth Study – CCM**

After baseline growth rates were established, as well as optimal culture conditions in a 96-well plate, growth of *L. acidophilus* in cell culture media was characterized to determine the microbe’s response to human cell conditions. The cell culture media (CCM) utilized, keratinocyte serum-free media (KSFM), was retrieved from the Andl lab and prepared with no antibiotics and supplied with human recombinant Epidermal Growth Factor 1-53 (EGF 1-53) and Bovine Pituitary Extract (BPE). The CCM was refrigerated at 4°C when not in use and allowed to reach room temperature before inoculation. Three replicate 100 μL samples of CCM – as established by the previous growth study – were inoculated with an O/N stationary culture, along with their respective blanks, and grown for 24 hours in a low-evaporation lid 96-well plate in a continuously shaking plate reader at 37°C, and turbidity at a wavelength of 600 nm was read every five minutes.

As discussed in greater detail below, no noticeable growth was observed in any of the CCM samples; therefore, a subsequent growth study was carried out to determine if CCM acts as an inhibitor or spectator of microbial growth. An O/N stationary culture in MRS was divided into two 500 μL aliquots. One aliquot was spun down, the supernatant was aspirated and discarded, and the pellet was resuspended in 500 μL of CCM and placed on ice to create a 1X CCM stock culture. A 1/100 dilution with this stock culture in fresh CCM was performed to create a 100% CCM inoculation. A 1/100 dilution with the stock culture was also inoculated in 490 μL of CCM and 500 μL of dH2O to create a 1:1 ratio of CCM and water. The second 500 μL aliquot was
used to create a 1/100 dilution in 490 μL of fresh MRS and 500 μL of CCM to create a 1:1 ratio of MRS and CCM. The second aliquot was also used to create a 1/100 dilution in 490 μL of fresh MRS and 500 μL of dH₂O to create a 1:1 ratio of MRS and dH₂O. Three replicate samples of 100 μL of each mixture, along with their respective blanks, were grown in a low-evaporation lid 96-well plate for 24 hours in a continuously shaking plate reader at 37°C, and turbidity at a wavelength of 600 nm was read every five minutes.

Additionally, a short meta-analysis was conducted to compare the major metabolic components in MRS and CCM to highlight any nutritional deficiencies in the media. The product information sheets for the proprietary KSFM compositions were collected, and literature for their original compositions was referenced.

**EZ Metabolic Study**

To further elucidate the metabolic requirements of *L. acidophilus* after a meta-analysis of media substrates, a synthetic, rich defined medium (RDM) kit was used to control and exclude major metabolite groups. A series of RDM compositions, along with negative controls, were created from the kit using pre-prepared aliquots of each major group: MOPS modified rich buffer, potassium phosphate dibasic solution, 20% glucose, ACGU solution, and supplement EZ mixture of amino acids and cofactors (refer to Appendix F: MOPS EZ Rich Defined Medium Kit Components). An O/N stationary culture was prepared from a freezer stock and inoculated into three experimental cultures: full RDM, full RDM minus ACGU, and full RDM minus supplement. Three negative cultures of MRS, CCM, and the full RDM, as well as previous experimental trials from the growth study to confirm the lack of growth, were also prepared. The cultures were placed in a 37°C shaking incubator and monitored for growth over 72 hours.
Spot Plate Assay

Upon completion of the growth studies of the pilot strain in human cell culture media, after which it was concluded that the media could not support growth, a spot plate assay (SPA) was performed to assess whether CCM is innocuous or harmful to *L. acidophilus*. For this portion of the experiment, an overnight stationary culture of *L. acidophilus* was serially diluted in both MRS and CCM to a countable concentration, plated on MRS agar, and the number of colonies produced, in terms of colony-forming units per milliliter (CFU/mL), were compared to assess their viability. The viability of these cultures in their respective media was analyzed in the incubation environment of the human cell – the Andl lab human cell incubator (37°C, 5% CO₂, no shaking). An isolated colony from an MRS agar plate was used to create an O/N stationary culture (~10¹⁰/mL), which was then diluted by a factor of 100 in two fresh aliquots of MRS and CCM – these “stock” cultures were incubated in the Andl lab and serially diluted for the assay. Dilutions based on observable turbidity were made each day to create new serial dilution stock cultures; the original dilution window was set at 10⁻², 10⁻⁴, 10⁻⁵, and 10⁻⁶ for both cultures. The remaining stock cultures were placed in the Andl lab human cell incubator. Four replicate samples of four appropriate serial dilutions were dispensed in 5 μL droplets in the following manner: a pseudo-drop was formed on the pipette tip and gently placed on the agar plate; the pipette tip was then inserted into the drop, and the micropipette was pushed till the second stop. The spots were left to dry on the lab bench for 30 minutes and later placed agar-side down in the Moore lab 37°C incubator. Growth was monitored after 12 hours and the plates were subsequently flipped agar-side up to prevent condensation from disturbing the spots. After an additional 12 hours, the stock cultures were removed from the Andl lab and turbidity was
observed to determine the next set of dilution windows. After the first round, similar turbidity to
the stock culture was noted in the MRS culture, so the dilution window was moved farther down
\(10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}\); no noticeable turbidity was noted in the CCM culture, so the original
dilution window was kept for an additional 24 hours to allow for delayed growth. After the
second round, once again, similar turbidity to the stock culture was noted in the MRS culture, so
the dilution window was kept the same. However, no growth was noted in the CCM culture, so
the window was moved to a more concentrated window \(10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}\), in hopes of
capturing colony formation. For the third and fourth round of the assay, similar turbidity from
previous rounds was noted in both cultures, so the former dilution windows were kept the same.
The plates were wrapped in parafilm to prevent drying and were refrigerated for storage.
RESULTS

Pilot Strain Revival and Verification

Upon NCBI BLAST, no complete match to the trimmed Sanger sequence was found; however, near-perfect matches were observed with several closely related *L. acidophilus* strains, with single mismatch results that suggested close strain-specific changes. Upon consultation with Dr. Yooseph, it was revealed that the ATCC 4796 genome, currently sequenced as part of the Human Microbiome Project (HMP), is currently in a draft stage and part of an NCBI cohort of *L. acidophilus* strains; the representative annotated strain is *L. acidophilus* North Carolina Food Microbiology (NCFM) (scaffold sequences, gene calls, and assemblies can be found online). A .fasta file of the ATCC 4796 16S rRNA sequence was used as a template to align the Sanger sequence, resulting in a 100% match and complete verification of the strain.

The trimmed Sanger sequence was also BLASTed against both related *Lactobacillus* (taxid:1578) and *L. acidophilus* (taxid:1579) organisms to support our confidence in the identification of the pilot strain. After the sequences were aligned to the parameters, two organisms from each group with identities less than 100% (~99.77%) were selected and aligned to the trimmed Sanger sequence and compared with the experimental chromatogram (Figures 1 and 2). Among related species in the *Lactobacillus* genus, the organisms *Lactobacillus casei* and *Lactobacillus crispatus* had a 99.77% identity to the Sanger sequence; both varied in position 24 with cytosine instead of adenine. Among related *L. acidophilus* strains, the two strains PP12 and C11 also resulted in 99.77% identity to the Sanger sequence. The PP12 strain varied in position 191 with cytosine instead of guanine, and the C11 strain varied in position 289 with guanine instead of thymine. These variable regions were compared to the Sanger chromatogram; in all
cases, the chromatogram peaks in the positions of interest were strong, distinct, and no other significant fluorescent signals were noted in the annotated template read.

Figure 1: Sanger sequence and chromatogram aligned to closely-related species
Growth Study – MRS

The data was imported, and the log2 transform and derivative values of each blank-corrected well were analyzed to determine optimal time windows for each environmental condition (Figures 7-11). The use of log base 2 – as opposed to log base 10 – allows for convenient representation of growth curves where one ordinate value represents a single division when log base 2 of the bacterial density is plotted against time. Time windows when the derivative remained at a maximum constant value, and where the log2 transform values versus time gave a linear regression line, represented the doubling time of each culture condition – these were used as a reference to compare the different volumes and environmental conditions. To find optimal windows, the graphed curve of the blank-corrected derivative values was smoothed in GraphPad Prism to the following parameters: 25 neighbors on each side, 2nd order of smoothing.
polynomial. This was done to achieve a rough estimate of the curve peak. Values in the time window from 755-790 minutes in the low-evaporation environment remained constant, whereas values in the time window from 940-980 minutes in the high-evaporation environment remained constant, represented by the peaks in Figure 8 and 9, respectively. The doubling time of each culture condition, represented by the reciprocal of the slope of the linear regression line of each culture condition during the selected time windows, was used as a reference to compare the different volumes and environmental conditions (Figure 12). Independent t-tests (p < 0.05) were conducted between each sample, revealing no significant difference in the doubling times between low-evaporation and high-evaporation replicate samples for 50 μL culture volumes, with a p-value of 0.1682. Additionally, there was no significant difference in the doubling times between the 50 μL and 100 μL culture volumes under low-evaporation lids. However, there was a significant difference in the doubling times between the two 100 μL cultures under both environmental conditions, with a p-value of 0.0005.
Figure 3: Turbidity of culture volumes of *L. acidophilus* under low/high-evaporation lids

Figure 4: Derivative values of *L. acidophilus* under low-evaporation lid
Figure 5: Derivative values of *L. acidophilus* under high-evaporation lid

Figure 6: Log2 transform values of *L. acidophilus* under low-evaporation lid
Figure 7: Log2 transform values of *L. acidophilus* under high-evaporation lid

Figure 8: Doubling times of culture volumes of *L. acidophilus*
**Growth Study – CCM**

As noted above, no significant growth was detected in the 100% CCM cultures; therefore, culture ratios of CCM and MRS, as well as MRS and H$_2$O, were inoculated and the log2 transform and derivative values of each blank-corrected well were analyzed (Figures 13-16). The GraphPad Prism smoothing operation was performed once more to give time windows where the derivative remained at a constant value. The derivative remained constant at the time window from 920-960 minutes for the 1:1 CCM-MRS cultures and from 1105-1145 minutes for the 1:1 MRS-H$_2$O cultures. Linear regression lines were generated for each culture condition and the reciprocal slope values were calculated; the doubling times between the two culture ratios were significantly different (p < 0.01) (Figure 17). Furthermore, literature was gathered on the metabolic substrates of each media for comparison.

![Figure 9: Turbidity readings of L. acidophilus cultures in ratios of MRS, CCM, and H$_2$O](image)

*Figure 9: Turbidity readings of L. acidophilus cultures in ratios of MRS, CCM, and H$_2$O*
Figure 10: Derivative values of *L. acidophilus* in 1:1 ratio of CCM-MRS culture

Figure 11: Derivative values of *L. acidophilus* in 1:1 ratio of MRS-H₂O culture
Figure 12: Log2 transform values of *L. acidophilus* in 1:1 culture ratios

Figure 13: Doubling times of *L. acidophilus* in 1:1 ratios of CCM-MRS and MRS-H₂O
EZ Metabolic Study

The growth of each culture after incubation was noted in Table 1, denoted by plus/minus signs to indicate levels of visible turbidity. No visible turbidity was noted in the three negative inoculations, MRS, CCM, and Full RDM, as well as in the O/N inoculations in both CCM and RDM w/o ACGU component. In both the 1:1 ratio of MRS and Full RDM and RDM w/o Supplement EZ, similar heavy turbidity was recorded; yet in the O/N inoculations in MRS and Full RDM, only moderate turbidity was observed.

Table 1: EZ Metabolic Study Observable Turbidity

<table>
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<tr>
<th>Tube Number</th>
<th>Culture Composition</th>
<th>Turbidity</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>MRS</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>CCM</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Full RDM</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>MRS + Stationary</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>CCM + Stationary</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Full RDM + Stationary</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Full RDM + MRS + Stationary</td>
<td>++</td>
</tr>
<tr>
<td>8</td>
<td>Full RDM w/o ACGU + Stationary</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Full RDM w/o Supplement EZ + Stationary</td>
<td>++</td>
</tr>
</tbody>
</table>

“-” no observable turbidity  
“+” mild turbidity  
“++” significant turbidity

Spot Plate Assay

After two consecutive 24-hour incubations and immediate refrigerator storage, the number of colonies from each spot were counted and recorded in Table 2. The colony counts were averaged and multiplied by their respective dilution factor as well as by the spot correction,
the percentage of one mL (1000 uL / 5 μL spots = 200), to calculate the number of colony-forming units per mL (CFU/mL). Spots with solid lawns were designated as too numerous to count (TNTC); standard deviation values were also calculated using the same mathematical correction. For plates with two replicates, the value with the smallest error was prioritized and used in the final graphical replication, with the exception of the 24-hour CCM culture (Figure 18). Upon analysis of the previous dilution, $10^{-2}$, the colony count average was 28.75; this suggests a severe undercount of the colonies upon a 100-fold dilution that yielded an average of 1.25 colonies. Additionally, in order to adjust the variable spread, the third colony count was omitted. The changes in viability, represented by CFU/mL in log base 10, as a function of incubation time in each respective media, were recorded graphically below. In the MRS culture, a significant bloom within the first 24 hours of inoculation was noted, followed by two consecutive decreases in viability. A similar trend was noted in the CCM inoculation where a significant decrease of viability was observed albeit not as severe as the MRS culture.
Table 2: SPA Colony Count (CFU/mL)

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>Dilution Factor</th>
<th>Spot Counts</th>
<th>Spot Correction</th>
<th>Average CFU/mL ± SD</th>
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<tbody>
<tr>
<td><strong>MRS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.00E+02</td>
<td>19, 14, 29, 12</td>
<td>200</td>
<td>3.25E+05 ± 7.72E+04</td>
</tr>
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*aOmitted in average CFU/mL (discussed below)*

Figure 14: Viability of *L. acidophilus* under *in vitro* human tissue culture conditions
DISCUSSION

Analysis of Results

The 16S small ribosomal subunit (16S rRNA) gene is composed of nine hypervariable regions termed V1-V9 with constant regions flanking either side; used for taxonomic research, this gene has been used to characterize the composition of bacterial communities in the human microbiome as well as soil and oceanic samples. Historically, sequencing the entire 16S rRNA gene was limited by the length and depth of sequencing, so many protocols now focus on sequencing shorter regions of the gene. In particular, V3 analysis was found to distinguish bacteria to the genus level, and primers flanking the V3-V4 segments provide an optimal target for sequencing and taxonomic classification. Moreover, analyses on the use of short sequencing reads in the V1-V9 region found that paired-end sequencing of either the V3 or V4 region provided the most taxonomic resolution. The set of path_tail primers used are modified Illumina sequencing primers for the amplification of the V3 and V4 regions of the 16S rRNA gene and have widely different primer melting temperatures (Tm). Originally, there was an extra cytosine at the 3’ end of the reverse primer; however, the Moore lab found that this led to greater underrepresentation of the reverse read, so it was removed. The forward primer was initially degenerate, containing a Q in the annealing portion of the forward primer; it was later removed to allow for greater specificity when originally used for identifying pathogenic bacterial strains.

After comparing the sequence differences between related *Lactobacillus* and *L. acidophilus* organisms on NCBI BLAST to the fluorescent base-pair calls on the chromatogram, we are confident in our ability to distinguish our pilot strain from closely related organisms. This verification process was integral in confirming that our studies utilized the same strain found in
normal and diseased tissue. A limitation of this method is that *L. acidophilus* is reported to have four ribosomal RNA loci mainly assembled around 260 kb, from which it follows that the V3 and V4 regions of these operons may vary in sequence.\(^8\) The reference genome lacks these annotations and we were unable to locate each individual read. However, the V3 and V4 regions were BLASTed against *L. acidophilus* NCFM, resulting in four matches – three 100% reads and one read with a single mismatch, confirming the number of ribosomal RNA loci in *L. acidophilus*.

The turbidity of *L. acidophilus* at OD600 was monitored over 24 hours in its established culture medium to determine both baseline growth rates and optimal growth conditions. The different plate lids and culture volumes allowed for the control of the levels of humidity and gas exchange, and doubling rates were calculated by taking the reciprocal of the slope of the linear regression line of log2 values where the derivative remained at a constant, maximum value. In Figure 3, we observe classic bacterial growth curves among the samples with distinct lag, exponential, and stationary phases. This behavior provides validation that the pilot strain behaves similarly to other microbes despite the range of potential environmental stressors. The final turbidity readings are greater in the larger culture volumes as well as the low-evaporation lids of each group of cultures, which suggests that *L. acidophilus* prefers low gas-exchange levels with the atmosphere and a primarily anaerobic environment. Moreover, the pilot strain reaches its maximum growth rate notably earlier under the low-evaporation lid, further supporting this claim. *L. acidophilus* is reportedly the least oxygen-tolerant species in the *Lactobacillus* genus, utilizing a strictly homofermentative pathway of metabolism to produce lactic acid.\(^{15}\) The *Lactobacillus* genus has been previously classified as an oxygen-tolerant group of organisms and
several studies have found that certain species grown in aerobic conditions may have greater acquired resilience to increased temperature and oxidative stress.\textsuperscript{16} For example, among the genus, \textit{L. casei}, \textit{L. plantarum}, and \textit{L. sakei} are among the most well-equipped organisms to survive in aerobic conditions, possessing increased gene density coding for enzymes involved in pyruvate metabolism, oxidative stress tolerance pathways, and components of the respiratory chain.\textsuperscript{16} However, \textit{L. acidophilus} is reported to undergo cell death when exposed to aerobic environments, often presented during the homogenization and agitation steps of probiotic yogurt production.\textsuperscript{17} While oxidative studies report that \textit{L. acidophilus} has a limited capability of scavenging reactive oxygen species, it contains low levels of NADH oxidase and NADH peroxidase, enzymes critical in oxygen elimination from the cell; experimentally, this has led to results demonstrating that increasing levels of oxygen lead to lower levels of lactic acid produced.\textsuperscript{17} A wide range of doubling times was observed, with the low-evaporation 100 μL culture averaging around 107 minutes; for reference, the standard \textit{Escherichia coli} MG1655 K-12 research strain has a doubling time of \~30 minutes at 37°C. Visualized in Figure 8, as there was a significant difference in the doubling times between the 100 μL cultures, this volume was considered as a baseline for future growth studies. As no significant difference was observed between the 50 μL and 100 μL cultures under low-evaporation lids, the larger volume will provide for less noise and is optimal for the analysis of subtle growth differences. Additionally, the low-evaporation lids provide for a more anaerobic environment catered to the strain’s metabolic and biochemical properties. Therefore, we propose that a 100 μL culture volume under low-evaporation lids be the standard condition for the pilot strain. With that said, the observation that \textit{L. acidophilus} grows best under anaerobic conditions and with limited gas-exchange may
prove helpful for future *in vitro* studies as the head-and-neck microenvironments are relatively hypoxic.\(^1^8\)

No significant growth was detected in cultures solely containing CCM, so cultures containing mixtures of CCM and MRS were inoculated and graphical representations of turbidity versus time were generated. In the 1:1 CCM-MRS cultures, the turbidity of each replicate well climbed to a maximum value; however, we also note the early death phase of these cultures – a phase not observed in the previous inoculations. This suggests that MRS may not be able to sustain growth for long periods of time, an idea further discussed in the spot plate assay. More importantly, based on both the turbidity readings and doubling times, the culture containing CCM does not appear to hinder growth as compared to the 1:1 MRS-H\(_2\)O replicates, which suggests CCM does not act as an inhibitor. In the 1:1 MRS-H\(_2\)O replicates, final doubling times were observed to be about two-thirds of the 1:1 CCM-MRS cultures. In the span of 24 hours, these replicates appear to take a longer time to reach exponential phase; this may be due to the lack of nutrients and an overall lower ratio of substrate to total culture volume. By the time the 1:1 MRS-H\(_2\)O replicates reach stationary phase, the 1:1 CCM-MRS culture is undergoing death phase. Not shown are the turbidity readings of *L. acidophilus* inoculated in pure CCM, as no recordable turbidity was observed – further confirmed by the EZ metabolic study. As the MRS media used in this research was undefined, containing several crude components such as beef extract, yeast extract, and proteose peptone, it was difficult to compare its exact substrates to human tissue culture medium. Therefore, a defined medium from a study analyzing the growth and metabolic requirements of *L. acidophilus* R-26 was referenced and used for comparison. Additionally, a reference medium from which KSFM is derived from, Molecular, Cellular, and
Development Biology (MCDB) 153 was used for comparison. Among the major metabolic requirements, the amino acids, vitamins, trace elements, bulk organic ions, and other organic compounds were relatively similar. However, the components listed were found exclusively in the defined medium of *L. acidophilus*: guanine, adenylic acid (adenosine-5'-phosphate), cytidylic acid (cytidine,(2’,3’)-phosphate), uracil, spermidine phosphate, Tween 80 (polysorbate 80), sodium citrate, potassium hydroxide, sodium thioglycolate, thymine, and deoxyguanosine. According to the reference, Tween 80, a surfactant that increases the efficiency of nutrient absorption in *Lactobacilli*, was essential for the growth of *L. acidophilus*, and this metabolic requirement could not be replaced with oleic acid from which it is derived from. The auxotrophic nature of *L. acidophilus* for uracil could not be established, however, no stable growth was observed upon its absence. Aside from that, KOH was only required to neutralize the media components to pH 7.0. But more importantly, thymine was required in the absence of folic acid. Deoxyguanosine was required in higher concentrations if thymine was not present. Non-essential components for growth included adenine, cytidylic acid, thioglycolate, spermidine phosphate, and guanine; pyridoxine and citrate were also non-essential components but were extremely practical for lasting growth. Overall, a large emphasis is placed on the metabolic requirements of *L. acidophilus* with regards to nucleotides and nucleobase derivatives, as they prove essential or beneficial to growth – an idea further explored in the EZ metabolic study with regards to the biosynthetic capabilities of *L. acidophilus*.

In order to determine not only the metabolic requirements but to also highlight the considerable anabolic deficits of *L. acidophilus*, an EZ metabolic study was performed using an RDM kit to exclude certain metabolic groups and to analyze growth in lieu of an undefined
medium, such as MRS. No growth was noted in Tubes 1-3, serving as negative controls, which verified the lack of contamination of the reagents used. Mild turbidity was observed in tube 4, which acted as a positive control to demonstrate the viability of the pilot strain in its established culture medium; no turbidity was observed in tube 5, which further confirmed the lack of growth and potential disruption of viability in CCM. Mild turbidity was also observed in tube 6; acting as a control for the remainder of the experiment, the pilot strain was inoculated in full RDM to confirm that the mixture provided all necessary metabolic components to support growth – therefore, the absence of growth upon removal of certain metabolites could be attributed to the removal of those respective components. Similar to the growth study of *L. acidophilus* in equal proportions MRS and CCM, the pilot strain was also inoculated in equal proportions of the full RDM and MRS in tube 7 to rule out the former as an inhibitor; heavy turbidity was observed which suggests not only that both cultures mediums are able to support growth but also that either the full RDM or a combination of both mediums may be optimal choices in future studies. In tube 8, the pilot strain was inoculated in the full RDM minus the nucleotide component to test if the pilot strain was auxotrophic for either purines or pyrimidines. No observable turbidity was noted, and further genomic analysis of the representative NCFM strain revealed complete pathways for the *de novo* synthesis of purines yet only partial biosynthetic pathways for pyrimidine synthesis could be assembled. Consequently, it has been noted that many *Lactobacilli* are auxotrophic for either purines or pyrimidines as many strains lack the ability to reduce ribonucleotides into deoxyribonucleotides for DNA synthesis. Moreover, in a literature review of nucleotide metabolism in *Lactobacilli*, the genes encoding pyrimidine biosynthesis were repressed upon growth in rich medium, such as that of the full RDM mixture –
while no conclusive mechanism has been attributed to this phenomenon, it further stresses the interdependence between several catabolic pathways and nucleotide biosynthesis. Therefore, it has been postulated that *Lactobacilli* utilize several transporters to utilize exogenous sources of nucleobases. In a nutrient consumption pattern study of *L. acidophilus* strain KLDS 1.0738, uracil and thymine had the highest consumption rates, and guanine and uracil were among the most abundantly consumed nucleotides. However, decreased consumption of adenine nucleotides, as well as 2’-deoxyguanosine monohydrate, glucose, and other vitamins, was associated with increasing growth rates, highlighting the more effective use and metabolic efficiency of pathways upon increased biomass. In tube 9, the supplement EZ mix was omitted from the culture to investigate if *L. acidophilus* is auxotrophic for all amino acids; however, significant turbidity was noted in this case. Several *in silico* studies have revealed that *L. acidophilus* strain NCFM is only capable of the *de novo* synthesis of cysteine, serine, and aspartate; from these three amino acids, seven other amino acids can be synthesized (no conversion pathways could be found for the other ten amino acids). These studies are further supported by the high degree of complexity of amino acid/protein transporters, as well as peptidases and proteases, found in the organism. In total, nine types of ATP-binding cassette (ABC) transporters were found. Therefore, it is likely that *L. acidophilus* may utilize and rely on this complex system of transporters for the supply of amino acids. These studies highlight the pilot strain’s degree of auxotrophy, further complimenting its presence and adaptation to the nutrient-rich environment of the upper gastrointestinal tract.

The viability of *L. acidophilus* was represented by CFU/mL, calculated using the colony count averages and applying dilution factor and spot corrections. In MRS, an initial bloom to a
turbid $10^9$ CFU/mL of the culture is noted in the first 24 hours, which provides evidence that MRS supports the growth of the pilot strain and also that *L. acidophilus* is viable in human tissue *in vitro* conditions, mainly 5% CO$_2$ exposure and no shaking – representative of a more anaerobic microenvironment. However, a 10-fold decrease in viability and death of a significant portion of the culture is noted around 48 hours. There are several potential explanations for this, the most prominent being that *L. acidophilus* is a homofermenter of carbohydrates to lactic acid – metabolic studies have concluded that growth stops below a pH of 3.0, which can be attributed to the buildup of lethal amounts of lactic acid.\textsuperscript{15} This is further supported by probiotic viability studies that found *L. acidophilus* and *L. casei* to be among the most sensitive probiotic organisms at low pH levels, with *L. acidophilus* demonstrating a significant decrease in viability at pH 2.\textsuperscript{22} A similar trend was noted in the CCM culture, as we see a significant decrease in viability albeit not as extreme. No decrease in viability after 24 hours of incubation was observed, which confirmed that CCM is not an inhibitor, as supported by the previous growth study. Notably, a small increase in viability was observed; this may be attributed to the change in environmental conditions in the Andl lab incubator explained above – this may have led to decreased gas exchange rates, catering to a more anaerobic microenvironment. The observation that both culture mediums can only support growth for a short period of time may prove valuable for future *in vitro* studies where the overgrowth and over-colonization of *L. acidophilus* on epithelial surfaces is not a desired outcome. On the other hand, if more robust growth is desired, MRS broth is an additional tool to achieve a desired colony concentration.

A workflow was established to evaluate the pilot strain’s response to human tissue culture media and this research has positioned the lab to characterize the transcriptional and
adaptive responses of the pilot strain to the human epithelial environment. The pilot strain revived from the ATCC was validated by Sanger sequencing to be the strain implicated in head and neck cancers, and baseline growth rates in conventional medium and standard conditions were established for the organism – a 100 μL culture volume under low-evaporation lids. These growth studies suggest that *L. acidophilus* may grow best anaerobically, proving helpful in future studies analyzing the anaerobic tumor microenvironment. Moreover, we have illustrated that human tissue culture media supports little, if any, growth of the pilot strain but more importantly does not act as an inhibitor. This has led us to highlight the anabolic deficits of *L. acidophilus* and analyze the metabolic requirements that need to be fulfilled in future experiments. Furthermore, monitoring the viability of *L. acidophilus* in co-culture conditions in a human cell incubator has allowed us to set parameters for the colonization of this bacterium on epithelial surfaces, giving us more control of bacterium concentrations in future *in vitro* experiments.

In the future, we would like to identify the four ribosomal RNA loci and mark them in the annotated reference genome for comparison to our V3-V4 Sanger sequence to allow for further confirmation of the pilot strain. Moreover, to further explore the increase in viability of the *L. acidophilus* in CCM, we would like to incubate two replicate samples of each plate under aerobic and anaerobic conditions; comparison of the CFU/mL of each plate will help confirm whether the increase in viability is due to the change in environmental conditions or if CCM allows for moderate growth of the bacterium. The remainder of this thesis summarizes the use of microbes in cancer treatment, including their proclivity to inhabit tumors and the use of bacteria-derived anticancer agents, and explores both aspects of the causality dilemma surrounding dysbiosis and carcinogenesis.
**Microbial-Based Cancer Therapy**

*Tumor Visualization and Treatment*

The idea that microbes can reside within tumors and cancerous tissues has been recognized for the last several decades. One hallmark study observed certain strains of *E. coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Vibrio cholerae* entering and replicating within tumor in mice, visualized with luciferase-catalyzed luminescence and green fluorescent protein fluorescence. These microbes were later observed to replicate solely within these tumors despite having been well-distributed within the first few minutes after injection. One prominent theory is that these microbes actively sought out the immune-free microenvironment of cancerous tissue. And contrary to previous reports, these microbes did not require any genetic mutations to alter their viability within the tissue. Moreover, numerous studies have concluded that the anaerobicity of the tumor microenvironment is the most prominent factor in bacterial aggregation – initial access and subsequent survival and replication was found to be based on the degree of tumor vascularization as well as the degree of immune system presence. In addition to the tumor-specific replication and real-time visualization of cytosolic vaccinia virus, one possible application of this microbial behavior is their use as a molecular diagnostic for cancer – coined a “tumor-seeking” ability as bacteria have been observed to migrate past mucosal barriers to enter tumors. Also, these bacteria and viruses can be designed not only as therapeutic gene carriers for the treatment of cancer but also as site-specific detectors and visualizers of primary and metastasized tumors. This ability has shown success in detecting small nodules, such as 0.5 mm\(^3\) nodules on the lung surfaces of tumor-bearing mice.
Cancer therapy utilizing microbes was dismissed early on due to complications regarding the dose required for efficient use in balance with toxicity and other adverse side-effects. First described by William B. Coley in 1890, the microbial-based treatment of cancer was explored using sterile filtrates from *Clostridium histolyticum*.²⁴ Coley noticed tumor regression and attributed it to anti-cancer bacterial enzymes present in the filtrate. This was further explored when *C. histolyticum* spores were injected into transplanted mouse sarcomas, resulting in oncolysis; however, toxicity still proved to be a problem, which was later resolved with daily subcutaneous antitoxin injections.²⁵ The obstacle of toxicity was overcome in 1967 when researchers using *Clostridium oncolyticum* M55 in transplanted tumors in animal models proposed three universal conditions for the success of microbial-based cancer therapy. Success was based on 1) maintaining a threshold in tumor size (3 cm³ or 2 g of tumor weight), 2) a spore dosage range of 10⁶-⁹, and 3) injecting spores both intratumorally and intravenously.²⁴ These conditions, in addition to combining the strain spores with antineoplastic antimetabolites and other chemotherapy agents, raising the intratumoral temperature, or reducing oxygen levels within viable limits, resulted in increased tumor lysis.²⁴,²⁶ These studies built the foundation of more modern techniques of microbial-based cancer therapy; and now, attenuated and precisely engineered strains of *Clostridium, Bifidobacterium, Salmonella, Mycobacterium, Bacillus*, and *Listeria* are now the primary candidates for targeting cancer cells due to their proclivity to live in the hypoxic cores of solid tumors – areas that chemotherapy and radiation have difficulty targeting.²⁴ For the last two decades, engineered Clostridial strains have remained the focus of microbial-based cancer therapy, particularly due to their efficacy when combined with radiation – this is critical as radiation is especially dependent on oxygenation. It has been established that
hypoxic cells are several times more resistant to ionizing radiation, so highly anaerobic, solid
tumor centers are difficult to target. Consequently, a combination of radiation and microbes may
effectively target all areas of a solid tumor. A combinatorial approach using Clostridium novyi-
NT spores, a highly oxygen-sensitive strain with its α-toxin removed, and a mixture of a DNA-
damaging drug and anti-vascular agent, was tested in avascular mice tumors that resulted in
extensive tumor necrosis – a treatment strategy coined combination bacteriolytic therapy
(COBLANT). Another genus that has shown promising anticancer effects is Bifidobacterium, the
most abundant genus in the human colon. Selected based not only on its selective growth in
hypoxic tumors masses but also due to its ability to heighten host immune response,
Bifidobacterium has been shown to enhance the activity of NK cells and magnify the activity of
IL-2, INFγ, and INFα.

Additionally, several genes cloned into Bifidobacterium plasmids have been used in gene
therapy; the plasmid pBLES100 has been used to clone cytosine deaminase, allowing
Bifidobacterium longum to convert the prodrug 5-fluorocytosine (5-FC) into 5-fluorouracil (5-
FU), a medication used heavily in the treatment of head-and-neck cancers – this highlights this
genus’ efficacy as both a tumor-specific gene and prodrug delivery system. A hallmark strain
of microbial-based cancer therapy is Mycobacterium bovis Bacillus Calmette-Guérin (BCG), a
standard of care for the treatment of bladder cancer. BCG is inserted using a urethral catheter to
stimulate an increasingly large immune response upon the initial binding to fibronectin
attachment protein and subsequent internalization by urothelial and inflammatory cells; this leads
to a surge of cytokine and neutrophil concentration, dependent on a functional immune
system.
**Bacteria-Derived Anticancer Agents**

An additional facet of microbial-based cancer treatment focuses on the use of bacteria-derived products as anticancer agents. Certain strains are able to produce a multitude of enzymes, cytotoxic factors, antibiotics, and other secondary metabolites that can be used to target several aspects of tumor formation and spread. For example, several bacterially derived products are capable of interfering with metastasis, a highly specific process heavily governed through chemokines. By inhibiting the binding of chemokine and adhesion receptors, bacterial proteins can prevent the migration of malignant cells. This was observed in a class of bacterial proteins known as staphylococcal superantigens-like (SSL), produced by *Staphylococcus aureus*; these proteins were found to bind to the CXCR4 chemokine receptors in human T acute lymphoblastic leukemia, lymphoma, and cervical carcinoma cell lines, thereby inhibiting tumor cell migration.

Bacterial enzymes, even those as ubiquitous from parts of catabolic pathways, are strong contenders for anticancer agents. For example, arginine is an amino acid heavily implicated in tumor initiation, tumor-cell adhesion, and tumor-induced immunosuppression; produced by *Mycoplasma arginine* (Ma-ADI), the enzyme hydrolase arginine deiminase (ADI) breaks down L-arginine into L-citrulline and ammonia and has been used for the treatment of both *in vitro* and *in vivo* tumors in renal cell carcinoma and hepatocellular carcinoma. These types of cancers do not express their own biosynthetic enzymes for arginine synthesis, such as argininosuccinate synthetase, making them susceptible to ADI. Moreover, bacterial antibiotics are heavily implicated as anticancer agents through their modulation of the proto-oncogene Ras and the subsequent anchorage of Ras on the plasma membrane. For instance, Ras requires farnesylation...
and the antibiotic Manumycin A, produced by *Streptomyces parvulus*, acts as a farnesyltransferase inhibitor (FTI) and has shown success in reducing the number of pancreatic tumors in mice and enhancing apoptosis in myeloid leukemia cells.\textsuperscript{35,36} There are hundreds of metabolites of interest that have yet to be studied, but all share the same general mechanism of action: targeting a specific pathway within a tumor cell line. The workflow for this type of research is a blend of combinatorial chemistry and assays to target major mechanisms such as DNA replication, cell division, and apoptosis.\textsuperscript{24}

**Dysbiosis: Cause of Cancer Progression**

A darker side compared to microbes’ use in cancer therapy exists as dysbiosis can cause cancer progression. Changes in the human microbiome have been linked to several disease states, the premise being that an imbalance in the number of “protective” and “harmful” species is thought to be the underlying cause of chronic disease – an alternative model to Koch’s postulates.\textsuperscript{37} Those who maintain a normal flora during cancer progression, as seen in cases of oral and colorectal cancer, have a much better prognosis than those who do not; this has also been reported to be the case in inflammatory bowel disease, obesity, diabetes, atopic dermatitis, and psoriasis.\textsuperscript{4,38,39} Once commensal strains of microbes can also become pathogenic, yet many strains are opportunistic and retain their pathogenicity and look for openings in the host immune system; pathogenic strains have been reported to associate with commensal biofilms to avoid triggering an immune response.\textsuperscript{2,40,41}

It has also been postulated that dysbiosis not only leads to disease persistence but also to progression.\textsuperscript{39} Similar to the established relationship between *H. pylori* and gastric adenocarcinoma, it is though that *Campylobacter* species can lead to the progression of
esophageal adenocarcinoma (EAC). Clinical trials investigating the pathogenicity of *Campylobacter concisus* and *Campylobacter rectus* presented numerous isolated strains, separated based on adherent and invasive pathotypes, that are thought to be involved in EAC progression. Esophageal inflammation has been associated with changes in the microbiota, such as Barrett’s esophagus (BE), gastroesophageal reflux disease (GERD), and cancer. In studies examining the development of EAC, alterations in the microbiome in GERD – and subsequent chronic exposure to these conditions – were found to be carcinogenic. Similarly, over 21 distinct bacterial species were noted in one patient sample with BE. Microscopic examination of esophageal biopsies revealed Gram-negative cocci and coccobacilli in close association with the distal squamous epithelium of the esophagus and Gram-positive cocci concentrated in the intestinal-type gland lumen. Additionally, dysbiosis can lead to widespread systemic diseases. In a study on smoking and the oral microbiome, oral dysbiosis was not only identified to lead to inflammation states such as periodontitis, but it was also associated with diseases of the lungs, digestive tract, and cardiovascular system. In particular, the oral pathogen *Porphyromonas gingivalis*, detected using immunohistochemistry and the presence of lysine-specific gingipain, was located in 61% of cancerous tissues; it was also found to be associated with oral squamous cell carcinoma (OSCC) tumorigenesis and esophageal squamous cell cancer (ESCC), and was selected as a candidate for an ESCC biomarker. There are several mechanisms proposed to explain the carcinogenesis mediated by dysbiosis: production of carcinogenic and genotoxic compounds, activation and metabolism of pro-carcinogenic compounds, induction of chronic inflammation, and influence on eukaryotic cellular signaling.
Production of Carcinogenic and Genotoxic Compounds

Several microbes are capable of promoting a host inflammatory response through the production of carcinogenic compounds as well as inducing genetic damage through genotoxic compound production. Many species are able to catalyze nitrosation reactions, the production of N-nitroso carcinogenic compounds from precursor nitrites, amines, amides, and a variety of organic compounds. This activity was observed in strains isolated from the human trachea, urine, blood, and feces from microbes such as *E. coli* (18 out of 19 total experimental strains), *Pseudomonas aeruginosa*, and the genus *Proteus*. For these reactions, it was proposed that endogenous nitrosation was dependent on the pKa of the respective amine.

Activation and Metabolism of Pro-Carcinogenic Compounds

Additionally, carcinogenesis by dysbiosis may be explained not only by the production of carcinogenic agents but also the activation and metabolism of pro-carcinogenic compounds, a process mediated through xenobiotic metabolic enzymes produced by certain bacterial species. A hallmark example of this process is the metabolism of ethanol into acetaldehyde – ethanol itself is not a carcinogen but acetaldehyde has been shown to be carcinogenic in both animal models and *in vitro* studies. The reaction is catalyzed by alcohol dehydrogenase, naturally produced and found in human epithelial tissue, the liver, lower intestinal tract, and kidneys. Yet it has been reported that acetaldehyde can be produced by oral bacteria. In a study analyzing the levels of acetaldehyde in patient saliva after ingestion of ethanol to mimic social drinking, significantly higher amounts of acetaldehyde were found in those compared to controls; moreover, acetaldehyde production was markedly lower after patients underwent a three-day use of chlorhexidine, an antiseptic mouthwash.
study, *in vitro* trials revealed a linear relationship between acetaldehyde production and ethanol concentration. These results were further confirmed by a study exploring bacterial taxa associated with smoking; researchers found that smoking increased abundance levels of Gram-positive bacteria, which are more significant contributors of ethanol metabolism. Thus, smokers were found to have greater acetaldehyde production, with estimates as high as 50-60% compared to controls. *Streptococci*, Gram-positive aerobic bacteria, yeast, *Neisseria* species, and many other strains are also reported to have high levels of alcohol dehydrogenase, resulting in DNA damage and mutagenesis to secondary epithelial hyperproliferation. Even outside of the oral cavity, the highest metabolic activity of ethanol into acetaldehyde has been noted in the lower intestine by fecal bacteria. This overwhelming amount of acetaldehyde has been found to decrease the number of functional cells in colonic crypts, leading to compensatory hyperproliferation and proclivity towards colorectal cancer.

*Induction of Chronic Inflammation*

While acute inflammation plays a role in the innate immune system to defend the body against cancer, chronic inflammation has a promoting effect on cancer, allowing for the selection and subsequent expansion of tumors. Bacterial cells alone can induce inflammation by stimulating the immune system, promoting the mutagenic effects of cytokines. Several examples of site-specific inflammation caused by microbes have been noted in the literature. A bacterial infection by *Propionibacterium acnes* has been associated with prostatic cancer through the induction of inflammation. Histological examination of resected prostatic tissue samples from patients with prostate cancer revealed significantly higher levels of inflammation in those culture-positive for *P. acnes*. Upon further analyses, the isolates of *P. acnes* from patient
samples differed both phenotypically and genetically from cutaneous isolates, suggesting that a specific inflammatory subtype/strain might be a responsible agent.\textsuperscript{53} Similarly, *Chlamydia pneumoniae* and *Streptococcus bovis* play a role in the inflammatory response and carcinogenesis in the lungs and colon, respectively.\textsuperscript{4} *C. pneumoniae*, an intracellular human pathogen that is responsible for acute respiratory infections, has been found to induce gene expression of proinflammatory cytokines, such as IL-8, IFN-\(\gamma\), and TNF-\(\alpha\) in a respiratory carcinoma cell line; furthermore, the inactivated form of this microbe could still induce significant cytokine gene expression.\textsuperscript{54} *S. bovis*, historically a low-grade pathogen, entered the spotlight when the bacterium was found in 25-80\% of patients with colorectal tumors; upon proteomic analysis, twelve represented proteins were found to trigger the release of IL-8 and E2 prostaglandins in human epithelial colonic Caco-2 cells.\textsuperscript{55} Lastly, strains of *Streptococcus* with inflammatory properties in malignant tissue from the oral region were isolated.\textsuperscript{56} Strains of *S. anginosus* and *S. mitis* were preferentially seen to infect cancerous esophageal tissues, thought to then initiate inflammation through the stimulation of basal and suprabasal cells; these microbes were also found to promote inflammatory cytokines in esophageal cell lines.\textsuperscript{56} This association had led to the proposed theory that clearance of these tissues from microbes may decrease local and even regional recurrence of esophageal cancers.\textsuperscript{4,56}

*Influence on Eukaryotic Cellular Signaling*

The final major mechanism of carcinogenesis mediated by dysbiosis is the influence of microbes on eukaryotic cellular signaling, a critical process in tumorigenesis.\textsuperscript{2,4,5,18,46} These pathways have been observed to be influenced by several bacterial species through the activation of mitogen-activated kinase pathways that increase the levels of cellular transformation; these
same mitogenic compounds are produced by neoplastic cells that promote neoangiogenesis and lymphangiogenesis, processes heavily upregulated in tumorigenesis.\textsuperscript{2,57} \textit{P. gingivalis}, a Gram-negative anaerobe, contains highly specific proteins and lipopolysaccharides that stimulate human fibroblasts to proliferate in \textit{in vitro} studies.\textsuperscript{4,58} For example, the mediator protein fibroblast-activating factor (FAF), isolated from the outer membrane vesicles of this microbe, is thought to act as a competence factor and has been observed to increase thymidine uptake in human gingival fibroblasts and proliferation in human skin fibroblasts and umbilical vein endothelial cells.\textsuperscript{4,58} Additionally, \textit{P. gingivalis} is seen to accelerate tumorigenesis through the upregulation of cyclins and activation of cyclin-dependent kinases (CDKs).\textsuperscript{46,59} Acting through long fimbriae (FimA adhesins), \textit{P. gingivalis} increased levels of phosphoinositide 3-kinase (PI3Ks) and phosphoinositide-dependent protein-serine kinase 1 (PDK1), leading to rapid acceleration through the S-phase of the cell cycle.\textsuperscript{46,59} \textit{P. gingivalis} has also been seen to both increase the expression of and activate proMMP9, the inactive form of matrix metalloproteinase 9, using gingipain proteases. MMP9 is a collagenase that is thought to promote cellular invasion in OSCC by degrading the extracellular matrix and basement membrane – a process involved in the metastasis of tumor cells.\textsuperscript{46,60} \textit{P. gingivalis} induced proMMP9 production by activating the ERK1/2-Ets1, p38/HSP27, and proteinase-activated receptor 2 (PAR2)/NFκB cellular pathways.\textsuperscript{46,60} One of the largest influences on cellular signaling is a microbe’s ability to promote proliferation through the secretion of virulence factors or by impeding apoptosis.\textsuperscript{2,4} For example, the toxin cytotoxic necrotizing factor 1 (CNF1) secreted by \textit{E. coli}, activates the p21 Rho GTP-binding protein through the deamidation of glutamine; this activation promotes the assembly of actin and contractility of epithelial cells – but more importantly, CNF1 was found to modulate
the activity of the Bcl-2 family of antiapoptotic proteins.\textsuperscript{61} Similarly, \textit{Mycoplasma fermentans}, a wall-less microbe, was shown to reduce the activity of TNF-\(\alpha\)-induced apoptosis in a human myelomonocyte cell line, mediated through a significant reduction in mitochondrial inner transmembrane potential.\textsuperscript{62} Interestingly, these effects were observed when cells were infected with either live \textit{M. fermentans} or treated with sonicated, inactive \textit{M. fermentans}.\textsuperscript{62}

**Dysbiosis: Effect of Cancer Progression**

Despite the clear focus on the mechanisms that bacteria use to propagate cancer, there is no clear consensus on shifts in the diversity of the microbiome, particularly the oral and esophageal microbiome, in association with cancer. Equally important are the changes in the microbiome as a result of cancer progression; the following literature review will explore the other side of the causality dilemma in the relationship between carcinogenesis and dysbiosis.

**Oral Microbiota**

With respect to the oral region, a healthy microbiome is characterized by the phyla \textit{Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria}, and \textit{Fusobacteria}, assessed using culture-independent methods such as next-generation 16S rRNA sequencing.\textsuperscript{5} However, different culture methods, such as 16S sequencing, biopsies, swabs, and saliva samples, have presented contrasting results with respect to shifts in the microbiome upon carcinogenesis. For example, in a study utilizing 16S rDNA hypervariable region amplicons to monitor abundance levels in both cancerous and pre-cancerous lesions, lower levels of \textit{Firmicutes}, such as \textit{Streptococcus}, and \textit{Actinobacteria}, such as \textit{Rothia}, were observed in those with oral cancer.\textsuperscript{5} Significant decreases in these same groups were observed in oral pre-cancerous lesions but not when compared to contralateral sites from control groups.\textsuperscript{5} Yet in a study analyzing the
microbiome in OSCC where DNA obtained from both biopsies and deep-epithelium swabs was sequenced, the genus *Fusobacterium* and *P. aeruginosa* was abundant compared to normal samples.\(^6\) *F. nucleatum* was observed in higher levels on OSCC lesion surfaces – while this conclusion had been reported in previous studies, researchers were able to designate the subspecies *polymorphum* and *vincentii* to OSCC.\(^6\) Even more disparate results were obtained in a study utilizing unstimulated saliva samples from OSCC-free and OSCC-positive subjects, where oral cancer subjects had significantly elevated levels of *Capnocytophaga gingivalis*, *Prevotella melaninogenica*, and *S. mitis*, compared to controls.\(^6\) Additionally, these species were capable of predicting the presence of OSCC lesions with 80% diagnostic sensitivity and greater than 82% specificity.\(^6\) Combined with earlier studies with the same resounding conclusions, these authors postulated that oral cancers such as OSCC have a greater effect on the composition of the salivary microbiome than both smoking and periodontal infections.\(^6\) This discontinuous trend is further promoted by a study utilizing V4-V5 PCR amplicons to analyze stimulated saliva samples from OSCC patient that revealed a greater abundance of the following genera: *Streptococcus*, *Gemella*, *Rothia*, *Peptostreptococcus*, *Lactobacillus*, and *Porphyromonas*.\(^6\) In regard to specificity, species such as *Exiguobacterium oxidotolerans*, *Pseudomonas melaninogenica*, *Staphylococcus aureus*, and *Veillonella parvula* were found exclusively in oral cancer tumors.\(^6\)

*Esophageal Microbiome*

The literature is relatively more consistent with respect to the effects of cancer on the esophageal microbiome. As summarized above, several reviews have noted the presence of the genus *Streptococcus* as well as the phylum *Firmicutes* to inhabit the healthy esophageal
microbiome. Assessments of the major species in association with cancer and other esophageal disease states have revealed enrichments of entire taxa or the presence of unique species. The analysis of rat esophageal samples using PCR coupled to electrospray ionization mass spectrometry and further confirmation by fluorescence in situ hybridization revealed several major genera and species present — the most notable being the increased prevalence of *E. coli* in EAC. Additionally, DNA extractions from gastric corpus tissues analyzing microbiota patterns in early ESCC and esophageal squamous dysplasia (ESD) revealed increase abundances of the orders Clostridiales and Erysipelotrichales. Studies have gone further to note associations between the esophageal microbiota not only with disease but also with cancer predisposing states in the esophagus and stomach. For instance, two predisposing states focused on for ESCC were 1) serum pepsinogen I/pepsinogen II ratio (PGI/II), a predictor of gastric cancer where lower ratios are a marker for chronic atrophic gastritis, and 2) ESD, the precursor to ESCC. An overall lower level of microbial richness, defined as the number of bacteria genera per sample, was associated with lower PGI/II ratios and those with ESD. Moreover, decreased abundance levels of the genera *Lautropia*, *Bulleidia*, *Catonella*, and *Cardiobacterium*, and a relative decrease in overall microbial diversity, were found in ESCC fasting saliva samples compared to controls.

**Universal Changes**

Among the noted site-specific shifts in the microbiota thought to be caused by carcinogenesis, studies have also focused on universal changes in the abundance of bacteria in cancer. One prominent theory is that these shifts are associated with changes in bacterial receptors, such as specific cell membrane glycoconjugates. Therefore, when cells undergo
dysplasia, the subsequent changes in cell receptors will bind specific bacteria – thought to be in the same highly selective manner that enzyme and substrate interact. Consequently, changes in cell receptors give rise to shifts in the composition of the microbiome, marking their additional potential use as biomarkers for cancer. In studies examining universal shifts in the microbiota, an increase in the levels of *Fusobacterium* in all cancer patients was observed. However, an overall loss of richness and diversity were noted in the microbiota of those with HNSCC. In a study utilizing 16S rRNA sequencing from previously selected species from culture isolates, patients with EAC retained similar species to controls but experienced overall decreases in microbial diversity – with the exception of an increased abundance of *Lactobacillus fermentum*.67

However, with regards to biofilm formation, the literature notes a wholly opposite trend – an extensive increase in bacterial diversity. In OSCC tumors, biofilms contain a wider variety of bacteria present. Biofilms present in keratinizing squamous cell carcinoma lesions harbored increased numbers of both aerobes and anaerobes compared to healthy mucosal surfaces. Specifically, the aerobic genera *Haemophilus, Enterobacteriaceae*, and *Streptococcus*, and the anaerobic genera *Veillonella, Fusobacterium, Prevotella, Porphyromonas, Actinomyces*, and *Clostridium*, proliferated in biofilms. Additionally, *Candida albicans* was found to colonize biofilms in about a third of all tumor sites.

Several overarching similarities and trends in microbiota shifts associated with carcinogenesis are an important facet of this causality dilemma. Most isolate species found in the microbiota were saccharolytic and acid tolerant and seen to thrive in the acidic tumor microenvironment. These organisms, such as *Streptococcus*, are thought to contribute to the
acidic and hypoxic tumor microenvironment. Moreover, the environment is thought to lead to several bacterial groups, such as *Actinomyces* spp. to be outcompeted by other commensals who favor acidic, hypoxic conditions; this was supported by a study that found relatively decreased abundances of the genus *Actinomyces* and phylum Actinobacteria in HNSCC.

**Future Directions of the Field**

Each year, tens of millions of individuals are diagnosed with cancer; and with cancer representing about a fifth of all deaths in the industrialized world, a coordinated, interdisciplinary response is vital to halt its progression. Bacteria have demonstrated their potential as a new avenue for cancer treatment – personalized vessels that preferentially target tumors, encode cloned genes, and can deliver cytotoxic drugs, miRNA, and antibodies. Yet they also offer both a potential explanation for an attributable cause of cancer and a way to predict cancer progression. It is proposed that bacterial abundance levels can be used as diagnostic biomarkers where shifts in the microbiome upon early cancer progression will be telling of carcinogenesis.

The oral and esophageal microbiome are reportedly similar in composition, with the oral microbiome being the focus of biomarker studies for disease in gastric cancer, pancreatic cancer, and even colorectal cancer. Consequently, the oral microbiome has been selected as a candidate biomarker for cancers of both the head and neck. For example, *Fusobacterium nucleatum*, an abundant resident of the oral microbiome, was analyzed for its prognostic significance in esophageal cancer; in a study utilizing qPCR to quantify 325 resected esophageal cancer tissue samples, *F. nucleatum* was found to significantly associate with tumor stage progression but not with any cultural or social habits such as tobacco or alcohol use. A study utilizing fasting saliva samples to analyze high-risk areas for ESCC in China found a correlation between altered
salivary microbiota and esophageal cancer risk. Whether these biomarkers are telling of a potential timeframe to eliminate the spread of cancer or simply represent the remains of a difficult battle, as more studies accumulate on the associative links between dysbiosis and cancer, the current field has limited itself to solely establishing speculative links. The premise of this interdisciplinary approach is that humans are not sterile organisms, and the fact that we disproportionately know little of the complex community we are intimately connected to has limited our ability to fulfill the final half of Koch’s postulates. While we cannot repeat the hallmark experiments of Dr. Marshall, the construction of a reference community that will both provide evidence of causative links and set the foundation of a clinical study is a step in the right direction for resolving the causality dilemma.
APPENDIX A: ABBREVIATIONS
ADI: Arginine Deiminase 
AIM: Applied Industrial Microbiology 
ATCC: American Type Culture Collection 
BCG: Mycobacterium bovis Bacillus Calmette-Guérin 
BE: Barrett’s Esophagus 
BPE: Bovine Pituitary Extract 
CCM: Cell Culture Media 
CFU/mL: Colony-Forming Units per Milliliter 
CNF1: Cytotoxic Necrotizing Factor 1 
EAC: Esophageal Adenocarcinoma 
EGF 1-53: Epidermal Growth Factor 
ESCC: Esophageal Squamous Cell Carcinoma 
ESD: Esophageal Squamous Dysplasia 
GERD: Gastroesophageal Reflux Disease 
HMP: Human Microbiome Project 
HNSCC: Head-and-Neck Squamous Cell Carcinoma 
HPV: Human Papillomavirus 
KSFM: Keratinocyte Serum-Free Medium 
MCDB: Molecular, Cellular, and Development Biology Media 
MMP9: Matrix Metallopeptidase 9 
MRS: deMan, Rogosa, and Sharpe 
NCFM: North Carolina Food Microbiology 
OD: Optical Density 
O/N: Overnight 
OSCC: Oral Squamous Cell Carcinoma 
PGI/II: Pepsinogen I/Pepsinogen II Ratio 
RDM: Rich Defined Medium 
rRNA: Ribosomal Ribonucleic Acid 
SPA: Spot Plate Assay 
SSL: Superantigen-Like 
Tm: Melting Temperature 
TNTC: Too Numerous To Count
APPENDIX B: MRS MEDIA COMPOSITION AND PREPARATION
Lactobacilli MRS Agar (70 g Formulation Per Liter)
- 10.0 g Proteose Peptone No. 3
- 10.0 g Beef Extract
- 5.0 g Yeast Extract
- 20.0 g Dextrose
- 1.0 g Polysorbate 80
- 2.0 g Ammonium Citrate
- 5.0 g Sodium Acetate
- 0.1 g Magnesium Sulfate
- 0.05 g Manganese Sulfate
- 2.0 g Dipotassium Phosphate
- 15.0 g Agar
- Dissolve in deionized water, heat with frequent agitation, and autoclave at 121°C for 30 minutes
- Final pH 6.5 +/- 0.2

Lactobacilli MRS Broth (55 g Formulation Per Liter)
- Consists of the same ingredients without the agar
- Dissolve in deionized water, heat with frequent agitation, and autoclave at 121°C for 30 minutes
- Final pH 6.5 +/- 0.2
APPENDIX C: REAGENTS FOR DNA EXTRACTION
**Lysis Buffer (200 μL)**
- 10 mM Tris-Cl
- 5 mM EDTA
- 0.5 mM SDS
- 10 μL/mL RNase A

**Gel Melting Buffer (50 mL)**
- 5.5 M Gu-SCN
- 100 mM Acetic Acid
- Adjust pH to ~5.0 with KOH (forming potassium acetate, K-OAc)

**Isopropanol (200 μL)**
- 95-100% isopropanol

**5X Column Wash Buffer (50 mL)**
- 25 mM Tris-Cl
- 100 mM NaCl
- 0.5 mM EDTA
- Adjust pH to ~8.2 with NaOH
- Prior to use, dilute to 1X with ethanol (95-100% EtOH)

**4X DNA Elution Buffer**
- 10 mM Tris-Cl
- 0.4 mM EDTA
- Adjust pH to ~8.1 with diluted HCl
- Prior to use, dilute to 1X with dH₂O
APPENDIX D: PRIMERS FOR TAXONOMY
Path Tail Forward

5’ - TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACTCTACGGGAGGCAGCAG - 3’

Path Tail Reverse

5’ - GTCTCGTGGGCTGGAGATGTATTAAGAGACAGCGTGGAATCTACAGGGGTATCTAATC - 3’

*Bold: where the primer anneals to the genome
*Italicized: sequencing primer annealing portion
APPENDIX E: REAGENTS FOR PCR CLEANUP BY COLUMNS
Gel Melting Buffer (50 mL)
- 5.5 M Gu-SCN
- 100 mM Acetic Acid
- Adjust pH to ~5.0 with KOH (forming potassium acetate, K-OAc)

Isopropanol (200 μL)
- 95-100% isopropanol

5X Column Wash Buffer (50 mL)
- 25 mM Tris-Cl
- 100 mM NaCl
- 0.5 mM EDTA
- Adjust pH to ~8.2 with NaOH
- Prior to use, dilute to 1X with ethanol (95-100% EtOH)

4X DNA Elution Buffer
- 10 mM Tris-Cl
- 0.4 mM EDTA
- Adjust pH to ~8.1 with diluted HCl
- Prior to use, dilute to 1X with dH₂O
APPENDIX F: MOPS EZ RICH DEFINED MEDIUM KIT COMPONENTS
10X MOPS Buffer (100 mL)
- 40 mM Tricine
- 0.1 mM Iron Sulfate
- 95 mM Ammonium Chloride
- 2.76 mM Potassium Sulfate
- 5 μM Calcium Chloride
- 5.25 mM Magnesium Chloride
- 500 mM Sodium Chloride
- $2.92 \times 10^{-6}$ mM Ammonium Molybdate
- $4.00 \times 10^{-4}$ mM Boric Acid
- $3.02 \times 10^{-5}$ mM Cobalt Chloride
- $9.62 \times 10^{-6}$ mM Cupric Sulfate
- $8.08 \times 10^{-5}$ mM Manganese Chloride
- $9.74 \times 10^{-6}$ mM Zinc Sulfate

10X Potassium Phosphate Dibasic Solution (10 mL)
- 132 mM

10X 20% Glucose Solution (10 mL)
- 20.00%

10X ACGU Solution (100 mL)
- 15 mM Potassium Hydroxide
- 1.99 mM Adenine
- 1.99 mM Cytosine
- 1.99 mM Uracil
- 1.99 mM Guanine

5X Supplement EZ (200 mL)
- 4.0 mM L-Alanine
- 26 mM L-Arginine HCl
- 2.0 mM L-Asparagine
- 2.0 mM L-Aspartic Acid, Potassium Salt
- 3.0 mM L-Glutamic Acid, Potassium Salt
- 3.0 mM L-Glutamine
- 4.0 mM L-Glycine
- 1.0 mM L-Histidine HCl H₂O
- 2.0 mM L-Isoleucine
- 2.0 mM L-Proline
- 50 mM L-Serine
- 2.0 mM L-Threonine
- 0.5 mM L-Tryptophan
- 3.0 mM L-Valine
- 4.0 mM L-Leucine
- 2.0 mM L-Lysine HCl
- 1.0mM L-Methionine
- 2.0 mM L-Phenylalanine
- 0.5 mM L-Cysteine HCl
- 1.0 mM L-Tyrosine
- 0.05 mM Thiamine HCl
- 0.05 mM Calcium Pantothenate
- 0.05 mM para-Amino Benzoic Acid
- 0.05 mM para-Hydroxy Benzoic Acid
- 0.05 mM 2,3-diHydroxy Benzoic Acid

Sterile dH₂O
- Fill to 1 L with ~580 mL dH₂O
REFERENCES


