Characterization of Inhibitory Molecules Produced by Lactobacillus Crispatus

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CHARACTERIZATION OF INHIBITORY MOLECULES PRODUCED BY LACTOBACILLUS CRISPATUS

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
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BY
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For my family and friends.  
Your support has been invaluable along this journey.
“I like thinking big. I always have. To me it’s very simple: If you’re going to be thinking anyway, you might as well think big.”

“Treat the word impossible as nothing more than motivation.”

- President Donald J. Trump
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LIST OF ABBREVIATIONS

1-B 1-Butanol
A(q) Aqueous Phase
BAP Blood Agar Plates
BCA Bicinchoninic Acid Assay
Benz Benzene
CFS Cell Free Supernatant
CFU(s) Colony Forming Unit(s)
Chl Chloroform
CO₂ Carbon Dioxide
dH₂O deionized Water
DMEM Dulbecco’s Modified Eagle Medium
EA Ethyl Acetate
EQUC Enhanced Quantitative Urine Culture
FPLC Fast Protein Liquid Chromatography
H+ Hydrogen Ion
H₂O Water
H₂O₂ Hydrogen Peroxide
H₂SO₄ Sulfuric Acid
HCl Hydrochloric Acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>KMNO₄</td>
<td>Potassium Permanganate</td>
</tr>
<tr>
<td>Lc40</td>
<td><em>Lactobacillus crispatus</em> clinical isolate UMB0040</td>
</tr>
<tr>
<td>LUTS</td>
<td>Lower urinary tract symptoms</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistant</td>
</tr>
<tr>
<td>MRS</td>
<td>de Man, Rogosa, Sharpe</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>NDM</td>
<td>New Delhi metallo-beta-lactamase</td>
</tr>
<tr>
<td>O(r)</td>
<td>Organic Phase</td>
</tr>
<tr>
<td>O/N</td>
<td>Overnight</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PE</td>
<td>Petroleum Ether</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>TSA/B</td>
<td>Tryptic Soy Agar/Broth</td>
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<tr>
<td>UPEC</td>
<td>Uropathogenic <em>Escherichia coli</em></td>
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ABSTRACT

*Lactobacillus* species are widely accepted as beneficial bacteria of the human microbiota\(^1-8\). *Lactobacilli* spp. are well documented to inhibit pathogens by production and secretion of hydrogen peroxide (H\(_2\)O\(_2\)), organic acids, and/or proteinaceous bacteriocins into their environment. Most research attributes bactericidal activity of cell free supernatant (CFS) to H\(_2\)O\(_2\) and/or lactic acid. Here, I demonstrate that CFS from a clinical isolate of *Lactobacillus crispatus* contains a molecule(s) that inhibits uropathogenic *Escherichia coli* (UPEC) colony formation independently of H\(_2\)O\(_2\) and organic acids. Physiologic concentrations of H\(_2\)O\(_2\) and organic acids produced by *L. crispatus* do not inhibit UPEC colony formation. Incubation of UPEC with *L. crispatus* CFS has a bactericidal effect on UPEC that begins at about 4 hours and results in undetectable CFUs by 16 hours, apparently the result of severe plasmolysis. Bactericidal activity of this CFS is effective against some Gram-negative and Gram-positive species including multidrug resistant (MDR) *E. coli*. The broad-spectrum activity shows CFS may have applicability as future therapeutic for treatment of various bacterial infections including MDR infections.

The active molecule(s) present in CFS is stable over a wide temperature range and can be stored for at least 3 months while retaining full bactericidal activity. The active molecule(s) is sensitive to dilution and increased salt
concentrations. The active molecule(s) from CFS can be isolated via organic extraction. Based on the extraction profile, the molecule is predicted to be a dipolar organic molecule(s). The majority of proteins and tri-peptides are removed during organic extraction yet extracts retain full bactericidal activity. I conclude that *L. crispatus* produces a very stable potentially novel dipolar, organic molecule with broad-spectrum activity that is concentration-dependent and salt-sensitive, and which results in plasmolytic activity against its target. Isolation and identification of this molecule(s) could lead to application as a decontaminant or therapeutic.
CHAPTER I

INTRODUCTION

Previous Wolfe Laboratory Research

*Lactobacillus* species are widely accepted as beneficial bacteria present in the gastrointestinal or vaginal tracts\(^1\text{-}^4\), and recently within the lower urinary tract\(^5\text{-}^8\). Using a novel culture technique, the Enhanced Quantitative Urine Culture (EQUC), members of the Wolfe laboratory have cultured organisms from the bladders of women with and without urinary bladder symptoms\(^5\text{-}^8\). *Lactobacillus crispatus* was the most commonly cultured species from healthy controls without lower urinary tract symptoms\(^9\). Intriguingly, *Lactobacillus crispatus* and *Escherichia coli* rarely co-occurred\(^9\). This finding led Travis Price, a former MS student in the Wolfe laboratory, to investigate interactions between commonly cultured *Lactobacillus* species and *Escherichia coli*, which is implicated in numerous disorders of the urinary and reproductive tracts.

Travis Price and Katherine Diebel, another former MS student in the Wolfe laboratory, have documented inhibition of uropathogenic *Escherichia coli* (UPEC) by a clinical isolate of *Lactobacillus crispatus*, Lc40\(^9\text{-}^10\). By first analyzing EQUC co-culture frequencies, Price noticed a low co-culture rate between *L. crispatus* and *E. coli* from samples obtained from the urinary bladder. Thus, he tested possible interactions between these species by performing a Spotted Lawn
assay. Price reported that Lc40 cells, but not cell free supernatant (CFS), inhibited the growth of uropathogenic and clinically obtained *E. coli* strains on agar plates. No zone of inhibition developed around the Lc40 cell spots, suggesting that inhibition by *L. crispatus* is contact-dependent. However, Price also determined that Lc40 CFS could inhibit the growth of UPEC in liquid culture and had considerably more activity against UPEC than did other species of *Lactobacillus*. This finding would suggest *L. crispatus* has multiple mechanisms for the inhibition of other bacteria or the molecule(s) responsible for inhibition is not diffusible across/through agar plates. Because lactic acid and H$_2$O$_2$ are diffusible, this observation would suggest that these molecules are not responsible for Lc40’s ability to inhibit UPEC growth on agar plates. His results using *L. gasseri* differed from *L. crispatus*. *L. gasseri* cell spots on *E. coli* lawns did not show a zone of inhibition surrounding the spotted cells. Similar to *L. crispatus*, *L. gasseri* CFS was ineffective under plated conditions. In contrast to *L. crispatus*, *L. gasseri* CFS partially inhibited UPEC in liquid cultures, but its effect was substantially less complete.

Katherine Diebel repeated the work of previous researchers, by heating CFS or treating CFS with catalase. In contrast to the former reports, there was no increase of viable *E. coli* CFUs incubated in treated CFS when compared to untreated CFS, suggesting that treatment of CFS does not decrease its efficacy. Diebel also validated that the bactericidal activity of CFS was pH
dependent against UPEC\textsuperscript{12} by showing CFS buffered above pH 4.78 lost its inhibitory effect\textsuperscript{10}.

Together, the research of Price and Diebel has warranted further investigation into the inhibitory molecules of CFS as well as the mechanism of action against target cells. If a secreted molecule(s) is responsible for inhibition then it may have potential application as a future therapeutic.

**Common Lactobacilli Isolated from the Urinary Bladder**

The most commonly cultured *Lactobacillus* species from urinary bladder samples of women were *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii\textsuperscript{13}*. This finding is consistent with research studying the vaginal microbiome\textsuperscript{14}, where the four most commonly occurring Lactobacilli are the same as the four species of the urinary bladder. Three of these *Lactobacillus* spp. are associated with a healthy flora of the vaginal microbiota\textsuperscript{14-17}. Research on the vaginal microbiota shows that an absence or decrease in Lactobacilli is associated with various disease states\textsuperscript{14-17}. Research into the urinary microbiota is consistent with findings of the vaginal niche\textsuperscript{14,18}.

*L. crispatus* is commonly used as a probiotic and is widely accepted as a beneficial bacterium\textsuperscript{15-17,19-20}. *L. crispatus* has been shown to have antimicrobial activity against many pathogenic bacterial species, including *E. coli*, *Gardnerella vaginalis*, and *Chlamydia trachomatis*\textsuperscript{1,19,21}. It also may have the ability to regulate the vaginal microbiota by maintaining a *Lactobacillus* dominant flora rather than converting to a non-(*Lactobacillus* dominant flora\textsuperscript{22}. *L. crispatus*
maintains a *Lactobacillus* dominant microbiota better than *L. gasseri* or *L. iners* predominant women. Beyond the urogenital environment, *L. crispatus* is often used in the production and processing of dairy and fermented food products. Use in the food industry is due to its anti-spoilage and anti-pathogenic properties. There have not been any documented detrimental aspects of *L. crispatus* colonization in various anatomical niches including both the urogenital and gastrointestinal tracts.

*L. gasseri* is another commonly used organism in probiotics. When used as a probiotic, it is speculated to assist in weight reduction in individuals who are overweight or obese. *L. gasseri* has also been proposed to prevent periodontal disease and assist in immune system regulation when present in the gastrointestinal tract. It has shown to be effective against pathogens such as *E. faecalis, H. pylori, and L. monocytogenes*. It does not appear to have any negative effects on human health.

*L. jensenii* has been genetically modified to express human cell surface proteins (e.g. CD4) or to secrete antiviral drugs (e.g. cyanovirin-N). Both altered isolates were shown to reduce HIV infectivity. *L. jensenii* also inhibits *Neisseria gonorrhoeae*. Similar to *L. crispatus*, *L. jensenii* is used during milk processing and fermentation processes due to antioxidant properties. In contrast to both *L. crispatus* and *L. gasseri*, *L. jensenii* has been reported to cause disseminated infective endocarditis. However, *L. jensenii*-induced endocarditis is rare.
Although it is one of the most commonly cultured Lactobacilli, *L. iners* has not been shown to have any inhibitory or protective potential\textsuperscript{16,22,32}. Women predominanted by *L. iners* are more susceptible to bacterial vaginosis than woman colonized by other *Lactobacillus* spp\textsuperscript{16}. Analysis of the *L. iners* proteome, may suggest that specific proteins produced are possibly associated with vaginal dybiosis\textsuperscript{32}.

In general, the female urogenital tract is predominantly colonized by only one species of *Lactobacillus*. Both *L. crispatus* and *L. gasseri* have the most documented benefits to human health without documentation of detriment. Based on research performed by Travis Price, clinical isolates of *L. crispatus* have greater inhibitory activity against pathogens than *L. gasseri*. *L. crispatus* is also the most widely recognized beneficial *Lactobacillus* species among published research.

**Hydrogen Peroxide and Organic Acids**

*Lactobacillus* spp. are well documented to produce hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and organic acids. Some species are also recognized to produce antimicrobial bacteriocins. H\textsubscript{2}O\textsubscript{2} acts by inducing oxidative stress on target cells leading to the damage of cellular components including proteins, lipids, and nucleic acids\textsuperscript{33}. Oxidative damage to nucleic acids results in irreversible alterations to the bacterial genome that ultimately results in death. Organic acids can act by permanently damaging target cell membranes or by permeabilizing the membrane, allowing antimicrobial molecules to enter target cells\textsuperscript{34}.
Bacteriocins are sorted into multiple groups, which act against their target using different mechanisms.

Both H\textsubscript{2}O\textsubscript{2} and organic acids are secreted by Lactobacilli into their surrounding environment. Studies focusing on products secreted by Lactobacilli have used CFS derived from spent culture media to evaluate the antimicrobial effects of these secreted molecules. While many studies state that some Lactobacilli produce bacteriocins\textsuperscript{35-40}, most of those studies attribute the bactericidal effects to lactic acid and/or H\textsubscript{2}O\textsubscript{2}\textsuperscript{2,11,41-42}. However, treatment of CFS with catalase or high temperatures to degrade H\textsubscript{2}O\textsubscript{2} results in only moderate decreases in CFS efficacy against uropathogenic \textit{E. coli} (UPEC), and not complete inhibition\textsuperscript{11,41-42}. Additionally, it has been demonstrated that de Man, Rogosa, and Sharpe (MRS) broth, the standard culture medium for Lactobacilli, facilitates the rapid decomposition of H\textsubscript{2}O\textsubscript{2}\textsuperscript{43}. Rodrigeuz \textit{et al.} supplemented MRS broth with 1-100mM H\textsubscript{2}O\textsubscript{2} and measured OD\textsubscript{405} over time. H\textsubscript{2}O\textsubscript{2} concentrations were determined to decrease over time by 90\% of the originally supplemented concentration within a 24-hour period. In contrast, distilled water or phosphate-buffered saline supplemented H\textsubscript{2}O\textsubscript{2} retained initially added H\textsubscript{2}O\textsubscript{2} concentrations when measured 24 hours after addition of H\textsubscript{2}O\textsubscript{2}. Based on these findings, it would seem unlikely that H\textsubscript{2}O\textsubscript{2} is responsible for the bactericidal effects observed in untreated CFS.

The previously cited studies\textsuperscript{2,11} did not determine the physiologic levels of lactic acid or H\textsubscript{2}O\textsubscript{2} produced by the \textit{Lactobacillus} species. These studies also did
not test physiologic concentrations of H$_2$O$_2$ or lactic acid, either individually or combined, against pathogens of interest. Instead, they tested arbitrary ranges of concentrations for each molecule to determine the minimum inhibitory concentration. The concentration of lactic acid produced by *Lactobacillus* spp. has been measured as ~62mM$^{11}$. This concentration has been shown to only minimally inhibit pathogen colony formation$^{11}$. Acetic acid concentrations have been measured in liquid cultures of *L. sanfrancisco* CB1 CFS as 13.8mM$^{44}$, but the concentration produced by other *Lactobacillus* spp. has not been cited. H$_2$O$_2$ production by various *Lactobacilli* has been measured with values ranging from 0.03 to 2.6mM$^{45}$. Inhibitory concentrations of H$_2$O$_2$ necessary to achieve inhibition required 2.5mM or greater, depending on the target pathogen$^{11}$. Additionally, the physiologically produced concentrations of H$_2$O$_2$ would degrade in MRS broth, according to findings of Rodriguez et al.$^{43}$.

When utilizing Dulbecco’s Modified Eagle Medium (DMEM) to neutralize the effects of lactic acid, CFS bactericidal activity was decreased, but still maintained some activity against some pathogens$^{2,11}$. Unfortunately, the pH of the media supplemented with DMEM was not documented. Antimicrobial activity of some Lactobacilli CFS has been shown to be pH dependent and therefore pH cannot be negated as a factor in pathogen viability$^{10,12}$. These investigators attributed inhibition solely to lactic acid and/or H$_2$O$_2$ and not the presence of other organic acids that may have been produced by Lactobacilli species. Research has shown that lactic acid and H$_2$O$_2$ can inhibit UPEC$^{11,41-42}$, but, to achieve
these results, greater concentrations than are physiologically relevant must be used. This suggests that a molecule(s) other than lactic acid or H₂O₂ may be responsible for the remainder of UPEC inhibition.

**Antimicrobial Peptides and Bacteriocins**

Many pathogens have developed antibiotic resistance with associated increases in morbidity and mortality⁴⁶. The increased rate of antibiotic resistance exceeds the development and identification of new antibiotic that may be effective against resistant strains. Consequently, only a limited cache of antibiotics exists to combat bacterial infections. Various bacterial species have been identified to produce molecules with bactericidal activity that may have application as new antibiotic treatments. For instance, recently researchers have identified lugdunin, a bacteriocidal molecule produced by *Staphylococcus lugdunensis* with efficacy against the colonization of methicillin-resistant *Staphylococcus aureus*, Glycopeptide intermediate-resistant *S. aureus*, vancomycin-resistant *Enterococcus*, and other infectious bacteria⁴⁷. *L. crispatus* CFS also may contain many excreted, secreted or sloughed factors, which would include possible antimicrobial peptides and/or bacteriocins. If *Lactobacillus crispatus* CFS contains a molecule that is effective at inhibiting a broad spectrum of pathogens, then the molecule may be effective against antibiotic resistant strains with downstream application as an antimicrobial agent.

Antimicrobial peptides (AMPs) are small molecular weight peptides with broad spectrum activity⁴⁸-⁵¹. Various AMPs have been identified and determined
to be produced by most organisms ranging from bacteria to mammals. AMPs tend to be amphipathic molecules and this characteristic allows for selective targeting of lipopolysaccharide membranes resulting in pore formation and loss of cellular membrane integrity. Researchers have also found correlations between AMPs and the mammalian immune response.²⁰-²¹

Four major classes of AMPs have been identified. AMPs are classified based on their target organism. The bactericidal activity of all AMPs involves the compromised integrity of cellular envelopes or membranes. Antiviral peptides act against viruses by integrating into the viral envelope.²²-²³ Antibacterial peptides target bacterial membranes and act by compromising the bacterial lipid bilayer membrane.²⁴-²⁵ Antifungal peptides can act by targeting either fungal membranes or internal cellular components.²⁶-²⁷ Antiparasitic peptides form pores in the membranes of parasites.²⁸-²⁹

Some bacterial species produce bacteriocins, which are proteinaceous molecules that have antimicrobial activity against closely related species.³⁵-³⁶,³⁹ Many bacteriocins have also been determined to be effectively bactericidal in nano- and picomolar concentrations. Beyond antimicrobial activity, some bacteriocins have been evaluated for their potential application as therapeutics including anticancer agents.⁶⁰ Bacteriocins are synthesized by ribosomes and are often encoded by plasmids, instead of the core genome of the producing bacterium.³⁸ Bacteriocins are classified based on numerous criteria, including but not limited to: method of production, mechanism of action, molecular weight, and
chemical properties\textsuperscript{37}. Classification of bacteriocins differs between Gram-negative and Gram-positive bacteria. Bacteriocins produced by Gram-negative species are generally classified based solely on size. Bacteriocins produced by Gram-positive species are classified based on the previously stated criteria and are grouped into Classes I-IV with some classes further separated into subclasses\textsuperscript{35,37-38}. As \textit{L. crispatus} is a Gram-positive bacterium, I will focus solely on Gram-positive bacteriocin classifications.

Class I bacteriocins are comprised of the lantibiotics that include the amino acid lanthionine and its derivatives. They are small (<5 KDa) in size and insensitive to protease degradation\textsuperscript{40}. They are synthesized by Gram-positive bacteria and post-translationally modified to form intramolecular ring structures\textsuperscript{40}. Intramolecular rings are formed through thioether bonds of internal cystine residues of lanthionine amino acids\textsuperscript{40}. Two types of lantibiotics exist\textsuperscript{40}. Type A lantibiotics are elongated, flexible, amphipathic peptides that act by forming pores in the cytoplasmic membrane of Gram-positive bacteria. By contrast, Type B lantibiotics have a rigid, globular form and inhibit peptidoglycan synthesis of Gram-positive bacteria.

The Class II bacteriocins are heat-stable, allowing them to retain structure and function at elevated temperatures ranging from 100°C-121°C\textsuperscript{37-39}. They do not contain lanthionine amino acids or its derivatives, have molecular weights <10 KDa, and tend to be amphipathic molecules with net positives charges. Class II bacteriocins are sub-categorized into subclasses based on genetic
consensus sequences. Class IIa are considered to be listeria-active bacteriocins but have overall broad range activity. However, the broad range of activity is not well defined. If this subclass acts on organisms that are phylogenetically distant from the producing organism than these molecules do not fit the definition of a bacteriocin. These act against their target by permeabilizing cellular membranes. Class IIb bacteriocins require two peptides for activity and compromise bacterial membranes by pore formation. Subclass IIc bacteriocins are cyclic, thiol activated peptides that require cysteine residues for activity. Two additional, but minor, subclasses are subclass IIId and IIe.

Class III bacteriocins are larger in size (>30 KDa) than Class I or II. This class contains proteins with enzymatic function. Subclass IIIa degrade cellular membranes leading to cell lysis. Subclass IIIb act by altering cell membrane potential leading to ATP efflux from cells, and ultimately cell death. Class III bacteriocins are heat-labile and denature with increased temperature.

Class IV bacteriocins do not have definitive classification criteria. Molecules placed into Class IV do not meet the criteria of the previously mentioned classes. These are considered complex bacteriocins and are generally conjugated with carbohydrates or lipids. Other characteristics of molecules in this class vary and there is not a currently identified consensus sequence allowing for grouping into Class IV.

*Lactobacillus* CFS has been tested on a wide range of bacteria with varying phylogenetic relatedness. However, *Escherichia* and
Salmonella, both phylogenetically unrelated to Lactobacillus, are susceptible to Lactobacillus CFS\textsuperscript{11-12,67}. Because bacteriocins are defined as only being bactericidally active against closely related organisms, the activity of Lactobacillus CFS does not fit into the classical definition of a bacteriocin. This suggests that the active molecule against these genera is not likely to be a bacteriocin.

Proteins and polysaccharides tend to be very immunogenic and therefore one would expect increased cytokine production if the inhibitory molecule were one of these two compounds. Nucleic acids and lipids tend to not induce an immune response. Researchers have incubated vaginal epithelial cells with CFS from L. crispatus reference strain CTV05 and documented that they do not exhibit an immune response, as measured by pro-inflammatory cytokine levels\textsuperscript{1}. Therefore, based on previous observations, it would be suggested that the inhibitory molecule present in Lc40 CFS is unlikely to be a protein or polysaccharide.

Lc40 CFS activity has been shown to be insensitive to heat and has broad spectrum activity against phylogenetically unrelated genera. Due to these results it would seem unlikely that the bactericidally active molecule(s) in Lc40 CFS is a bacteriocin, with the exception that it may be a Class IIa bacteriocin. I hypothesize that the bactericidal molecule(s) of Lc40 CFS is likely an antibacterial AMP.
Inhibition and Mechanism of Action

A mechanism of action for the inhibition of bacteria by *Lactobacillus* CFS has not been proposed. Spotted lawn assays were performed using Lc40 cells or filter sterilized CFS. Under plated conditions Lc40 cells, but not CFS, had an inhibitory effect against UPEC. Yet, liquid cultures containing CFS do have inhibitory effects. This may suggest Lc40 has more than one mechanism of inhibition or the molecule produced is either hydrophobic or has a low solubility.

Based upon data presented by others it seems unlikely bacterial inhibition by *L. crispatus* is due to classically defined bacteriocins, lactic acid, or H$_2$O$_2$. The necessity to use greater than physiologically produced concentrations of lactic acid and H$_2$O$_2$ to achieve inhibitory effects shows that inhibition cannot be attributed to either of these molecules$^{1-3,11,44-45,68,70}$. Due to the heat stability of CFS and phylogenetic distance between *L. crispatus* and *E. coli*, the inhibitory effect is also not likely to be due to a bacteriocin, peptide, or protein. If the active molecule(s) is a bacteriocin then it is either a Class II bacteriocin or a previously unidentified class. It is being suggested that *L. crispatus* produces a potentially novel molecule, or class of bacteriocin not previously identified, which I intend to identify.

If *L. crispatus* produces a novel molecule that could be used as a decontaminant or therapeutic, then we must understand the mechanism of action. Knowledge of the mechanism would help evaluate the spectrum of activity against pathogenic and non-pathogenic bacterial strains. If this molecule acts
against pathogens with little or no damage to commensal bacteria of the host, then this could be a good therapeutic. However, similar to many antibiotic treatments, if this molecule eliminates commensal/symbiotic bacteria of the host, then it may have negative downstream effects on host health. As a mechanism of action for the inhibition of pathogens has not been investigated, the morphology and integrity of target cells has not been characterized. This should be a first step in order to understand the mechanism by which inhibition of colony formation occurs.

Through my research, I intend to identify the bactericidal molecule(s) present in CFS and determine a mechanism of action.
CHAPTER II
MATERIALS & METHODS

Bacterial Strains and Culture Conditions

Culture Conditions

*Escherichia coli*  
*E. coli* (CFT073, NU14, or UMB901) was plated on Blood Agar plates (BAP) from a frozen stock. Plates were then incubated at 37°C aerobically for 24 hours. After 24 hours, a single colony was obtained from BAP and used to inoculate 6 mL of Tryptic Soy Broth (TSB). The culture was then incubated at 37°C aerobically on a shaker for 24 hours.

*Enterococcus faecalis*  
*E. faecalis* clinical isolate UMB891 was plated on BAP from a frozen stock. Plates were incubated in 5% CO\textsubscript{2} at 37°C for 48 hours. After 48 hours, multiple colonies were transferred using a sterile cotton swab from BAP into 6 mL TSB supplemented with 5% FetalPlex™ animal serum complex (Gemini Bio-Products). The inoculum was incubated at 37°C in 5% CO\textsubscript{2} for 48 hours.

*Lactobacillus crispatus*  
Clinical isolate Lc40 of *L. crispatus* was plated onto BAP from a frozen stock and incubated in 5% CO\textsubscript{2} at 37°C for 48 hours. After 48 hours, multiple colonies were transferred from BAP into 6 mL de Man, Rogosa, and Sharpe (MRS) Broth\textsuperscript{71}. The culture was then incubated statically in 5% CO\textsubscript{2} at 37°C for 48 hours.
**Staphylococcus aureus** S. aureus clinical isolate UMB910 was plated in BAP from a frozen stock and incubated aerobically at 37°C for 24 hours. After 24 hours, a single colony was obtained to inoculate 6mL TSB. The inoculum was then incubated aerobically at 37°C statically.

**Sample Preparation**

*E. coli, E. faecalis, and S. aureus* 100 µL of overnight cultures (O/N) were added to 900 µL of TSB. The optical density (OD600) of these cultures was then obtained using the Eppendorf BioPhotometer® D30 with TSB as a blank. All samples were then standardized to a final an OD600 of 1.0.

*L. crispatus* 48-hour liquid cultures were centrifuged at 3500 rpm for 10 minutes to pellet bacterial cells. The supernatant liquid was then filter sterilized using a 0.22µm filter to remove bacterial cells and obtain a cell free supernatant (CFS). To determine CFS activity over Lc40 growth OD600 was measured every six hours. 48-hour liquid cultures were standardized to an OD600 of 0.05 in 6 mL of MRS. Measurements were obtained using MRS medium as a blank. Samples were measured by adding 100 µL of culture to 900 µL of MRS to measure a 1:10 OD600.

**Inhibitory Molecule Concentrations**

**Hydrogen Peroxide Concentration**

Hydrogen peroxide concentrations were determined by titration\textsuperscript{72}. CFS was prepared as indicated in Methods Sample Preparation. Hydrogen Peroxide (H$_2$O$_2$) concentration in CFS was determined by titration of potassium
permanganate (KMNO₄) into CFS diluted in water and sulfuric acid (H₂SO₄). KMNO₄ is a purple solution, whereas the CFS solution is clear. As KMNO₄ is titrated into the CFS solution, it will gradually turn from a clear to pink solution, but will revert rapidly back to clear. Once an equivalence point is reached, the solution will remain pink. The volume of KMNO₄ needed to reach equivalence is then used to determine the concentration of H₂O₂ in CFS. Sterile water and a commercial 3% H₂O₂ solution were used as negative and positive controls, respectively.

**Organic Acid Concentration**

Acetic and lactic acid concentrations in CFS were determined using commercially available kits from Boehringer Mannheim.

**Protein Quantification**

The bicinchoninic acid assay (BCA) was followed per supplier instructions to quantify protein concentrations in MRS and CFS. The samples were read at 562 nm using Molecular Devices SpectraMAX 250 plate reader and SOFTmax Pro software. Bovine Serum Albumin was used as the standard.

**Quantification of Conditioned Medium Effect against E. coli**

**UPEC Incubation with CFS, Conditioned-Medium, and MRS**

The volume of overnight UPEC cultures grown in TSB needed for an OD₆₀₀ of 1.0 in a final volume of 200 µL was determined. The determined volume was added to 100 µL CFS, conditioned medium, or MRS. Cultures were then brought to a final volume of 200 µL by addition of TSB. The mixed samples
result in a final UPEC OD600 of 1.0 (~1.0x10^8 cells). Samples were prepared in 96 well plates. Wells were covered with Parafilm™ to prevent evaporation of samples. Plates were then incubated at 37°C aerobically on a shaker overnight.

**Serial Dilutions**

Serial dilutions were performed in 96 well plates with each well containing 180 µL Phosphate Buffered Saline (PBS) or dH₂O. 20 µL of sample were added to the first row using a multi-channel pipette. Samples were then mixed by pipetting samples ~30 times before transferring 20 µL to the next row. Pipette tips were changed between sample transfers. This row was mixed using the above method and 20 µL transferred to the next well. 1:10 serial dilutions were carried out by repeating this process through the 8th row of the 96 well plate (Figure 1).

![Figure 1. Serial dilutions performed in a 96-well plate](image)

**Cell Quantification**

Samples from each well were plated onto Tryptic Soy Agar (TSA) plates. Plates were divided into 4 equal sections, one for each dilution factor of a sample
(Figure 2). 10 µL of each sample dilution were spotted onto the TSA plates in the section respective of their dilution. Triplicates of samples were spotted in the same quadrant. The spots were allowed to dry before plates were inverted and incubated at 37°C aerobically for 24 hours. After 24 hours colonies were counted and used to quantify average cells/mL by the following formula:

\[
\frac{\text{Cells}}{\text{mL}} = \text{cell count} \times (10^{\text{dilution factor}+2})
\]

Additional dilution factors are added because 10 µL of a 1:10 dilution is plated resulting in additional dilution factors. The mean and standard deviation of triplicates were then calculated.

**Figure 2. Plating of serial dilution spots from a 96-well plate.**

**Survival Graphs**

Graphs were constructed using the mean of cell counts under each condition at the 24-hour time point. Red dotted lines on each graph represent the detection limit of colony-forming units following serial dilution. The first dilution
plated is a 1:10 dilution and 10µL of each dilution are plated. Therefore, the limit of detection was calculated as 10^3 cells.

**CFS Concentration and Dilution**

**CFS Concentration**

CFS concentration was performed using the Savant Integrated SpeedVac System ISS110. 1 mL aliquots of CFS were placed into the SpeedVac, which uses heat and centrifugation to concentrate all non-liquid components within the CFS. Once the liquid was evaporated, then the components were re-suspended in 50 µL (20X) or 100 µL (10X) of H₂O at pH 3.7 or 7.0.

**CFS Dilution**

CFS was diluted by adding either H₂O pH 3.7 or PBS pH 3.7 to non-concentrated CFS to obtain specific dilution factors, e.g. 90% CFS in a final volume of 500 µL = 450 µL CFS and 50 µL H₂O/PBS.

**CFS Fractionation**

**Fast Protein Liquid Chromatography**

An Amersham Biosciences FPLC was used to perform CFS fractionation by size. 200 µL of CFS was injected into and run through a Superose 6 10/300 GL column in a Tris buffer pH 8.0 at 0.6 mL/min and collected in 1 mL fractions. Additional runs were performed using PBS pH 3.7 rather than Tris. The first run was performed at an elution rate of 0.6 mL/min collected in 1 mL fractions. The second was run at rate of 0.06 mL/min and collected in 300 uL and/or 1 mL fractions.
Liquid-Liquid Organic Extraction

Liquid-liquid organic extraction was performed following a modified Method\textsuperscript{76}. Solvents were added starting at a 1:1 ratio to 10X concentrated CFS samples or MRS. Samples were mixed by vortexing and then centrifuged to separate organic and aqueous layers. The two layers were then separated by pipet into sterile Eppendorf tubes. Sample liquids were then evaporated overnight by SpeedVac and re-suspended as previously mentioned.

Immunofluorescent Stains

Live/Dead Staining

Cells incubated for 24 hours with Lc40 CFS were stained using the Invitrogen LIVE/DEAD BacLight Bacterial Viability Kit\textsuperscript{77}. This kit uses SYTO-9 and propidium iodide nucleic acid fluorophores. SYTO-9 readily enters cells with and without damaged cell membranes. Propidium iodide is a larger molecule and only enters cells with compromised membranes. Fluorophore properties are listed in TABLE 1.

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Color</th>
<th>Molecular Weight</th>
<th>Excitation</th>
<th>Emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syto9\textsuperscript{78}</td>
<td>Green</td>
<td>400</td>
<td>480nm</td>
<td>500nm</td>
</tr>
<tr>
<td>Propidium Iodide\textsuperscript{79}</td>
<td>Red</td>
<td>668</td>
<td>490nm</td>
<td>635nm</td>
</tr>
<tr>
<td>FM 4-64\textsuperscript{80}</td>
<td>Red</td>
<td>608</td>
<td>515nm</td>
<td>640nm</td>
</tr>
<tr>
<td>Hoescht 33342\textsuperscript{81}</td>
<td>Blue</td>
<td>616</td>
<td>361nm</td>
<td>497nm</td>
</tr>
</tbody>
</table>

TABLE 1. Fluorophore Properties.
Membrane and Nucleic Acid Stains

Sample Preparation UPEC cultures were standardized to an OD600 of 1.0 prior to being incubated in CFS or MRS. 200 µL of sample were obtained hourly and centrifuged at 13500 rpm for 1.5 minutes. Supernatants were removed by pipette and samples were washed with 100 µL dH₂O. Samples were centrifuged again at 13500 rpm for 1.5 minutes. Supernatants were removed by pipette and samples were re-suspended in 100 µL H₂O.

Slide Preparation and Sample Staining 10 µL poly-L-Lysine was spotted on microscope slides and allowed to sit for 1 minute. Liquid was then suctioned off by vacuum pipette. Spots were washed 2x with H₂O and dried. 10 µL of sample was applied to Lysine spotted regions and allowed to sit for 10 minutes. Excess sample was then suctioned off and the plates were dried. 0.05 µg Hoescht nucleic acid dye was added to samples in a dark room and incubated for 10 minutes. Excess dye was suctioned off and samples were washed once with H₂O. 0.01 µg FM 4-64 membrane specific fluorophore was then added to samples, incubated for 30 seconds then excess dye removed and samples were washed once. Coverslips were added to samples.

Imaging

Samples were imaged using the Leica DMIRB microscope at 1000X and images were captured using MagnaFire 2.1C software. Images were obtained in the red, blue, and bright fields. Images were merged using ImageJ software to obtain final composite images.
Statistics

One-way ANOVA and/or Mann-Whitney U tests were performed on raw data to determine statistical significance between variables. Statistical significance will be denoted as follows: * = p<0.05, ** = p<0.01.
CHAPTER III

DETERMINATION OF KNOWN OR PROSPECTIVE BACTERICIDAL
MOLECULES PRESENT IN LACTOBACILLI CELL FREE SUPERNATANT

Introduction & Rationale

Using a novel culture technique, the Enhanced Quantitative Urine Culture (EQUC), researchers have cultured organisms from the bladders of women with and without urinary bladder symptoms\textsuperscript{5-8}. Based on the organisms cultured from these specimens, the co-culture frequency of various organisms was determined\textsuperscript{9}. The most commonly cultured \textit{Lactobacillus} species from these samples were \textit{L. crispatus}, \textit{L. gasseri}, \textit{L. iners}, and \textit{L. jensenii}. Intriguingly, of these species, \textit{L. crispatus} had a significantly lower co-culture frequency with \textit{Escherichia coli} and spotted lawn assays showed inhibition of uropathogenic \textit{E. coli} (UPEC) by \textit{L. crispatus} cells, but not by \textit{L. gasseri}\textsuperscript{9}. The inhibition of UPEC by \textit{L. crispatus}, however, did not result in a zone of inhibition surrounding the \textit{L. crispatus} cell spot and \textit{L. crispatus} cell free supernatant (CFS) did not show inhibitory activity when spotted onto a UPEC lawn. In UPEC liquid cultures, however, \textit{L. crispatus} CFS inhibited growth and colony formation when plated\textsuperscript{9}. The ability of CFS to inhibit UPEC in liquid culture but not on agar plates suggests the production of a secreted molecule that is concentration dependent and/or not diffusible.
Lactobacilli are a Gram-positive genus commonly cultured from the gastrointestinal and urogenital tracts of women. Lactobacilli are widely recognized as beneficial by providing an additional barrier beyond the host defenses that inhibit pathogen colonization\(^1-^4\). Most previous research attributes bactericidal activity of Lactobacilli to the production of hydrogen peroxide (H\(_2\)O\(_2\)) and organic acids, predominantly lactic acid\(^1-^3,^11,^44,^46,^68,^70\). Certain *Lactobacillus* *spp.* also have been recognized to produce bacteriocins responsible for pathogen inhibition\(^2,^4,^35,^82\).

The concentration of lactic acid produced by *Lactobacillus* *spp.* has been measured as ~62mM\(^11\). This concentration has been shown to only minimally inhibit pathogen colony formation\(^11\). Acetic acid concentrations have been measured in liquid cultures of *L. sanfrancisco* CB1 CFS as 13.8mM\(^44\), but the concentration produced by other *Lactobacillus* *spp.* has not been cited. H\(_2\)O\(_2\) production by various Lactobacilli has been measured with values ranging from 0.03 to 2.6mM\(^45\). Inhibitory concentrations of H\(_2\)O\(_2\) necessary to achieve inhibition required 2.5mM or greater, depending on the target pathogen\(^11\).

**Hydrogen Peroxide Concentration in CFS**

If H\(_2\)O\(_2\) were responsible for the bactericidal activity of CFS, then we would expect concentrations greater than the concentrations shown to inhibit pathogens\(^11\). Measurement of H\(_2\)O\(_2\) in CFS had a mean value of 11.51 +/- 0.000006 µM (TABLE 2), lower than concentrations measured\(^45\) by previous researchers, and at least 10-fold lower than concentrations documented to result
in pathogen inhibition\textsuperscript{11}. This result would suggest $\text{H}_2\text{O}_2$ is unlikely to contribute to the bactericidal activity of CFS.

**Organic Acid Concentration in CFS**

CFS was prepared as indicated in Methods Sample Preparation. Organic acid concentrations were determined using commercially available kits from Boehringer Mannheim\textsuperscript{73-74}. In short, each kit utilizes enzymes that convert acetate or lactate to molecules that can be measured by UV-Vis Spectrometry. Optical density (OD\textsubscript{600}) measurements of converted molecules can then be used to determine concentrations of acetic and lactic acids present in CFS.

If acetic or lactic acids contribute to the bactericidal activity of CFS, as with $\text{H}_2\text{O}_2$, we would expect concentrations similar to or in excess of concentrations mentioned prior\textsuperscript{11}. Acetic and lactic acid concentrations averaged $7.26 \pm 0.059$ mM and $9.52 \pm 0.011$ mM, respectively (TABLE 2). The acetic acid concentration in CFS was considerably lower than the cited inhibitory concentrations\textsuperscript{44}. The lactic acid concentration present in CFS also was much lower than concentrations documented to be necessary for pathogen inhibition\textsuperscript{11}. As with $\text{H}_2\text{O}_2$, the concentration of organic acids produced by *L. crispatus* are unlikely to contribute to pathogen inhibition.
### Table 2. Concentrations of known inhibitory molecules in Lc40 CFS.

Hydrogen peroxide concentrations were determined by titration. Organic acid concentrations were determined by UV-Vis spectrophotometry. H$_2$O$_2$ and organic acid concentrations in CFS were lower than concentrations determined to exhibit inhibitory activity against pathogens.

<table>
<thead>
<tr>
<th>CFS Molecule</th>
<th>Inhibitory Concentration</th>
<th>Average Lactobacilli Concentration</th>
<th>Lc40 Produced Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen Peroxide</td>
<td>2.5 mM$^{11}$</td>
<td>0.03 – 2.6 mM$^{45}$</td>
<td>11.51 +/- 0.000006 µM</td>
</tr>
<tr>
<td>Lactic Acid</td>
<td>70 mM$^{11}$</td>
<td>62 mM$^{11}$</td>
<td>9.52 +/- 0.011 mM</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>8.33 mM$^{44}$</td>
<td>13.8 mM$^{44}$</td>
<td>7.26 +/- 0.059 mM</td>
</tr>
</tbody>
</table>

H$_2$O$_2$ and Organic Acid Conditioned-Medium against UPEC

To determine each molecule’s contribution to CFS bactericidal activity, 12 µM H$_2$O$_2$, 7.5 mM Acetic acid, and 8.5 mM Lactic acid were individually added to MRS medium (pH 3.7) and incubated with UPEC overnight. The molecules also were combined in MRS at the same final concentrations to determine their combined bactericidal activity. As a positive control, CFS was added to UPEC cultures as previously described and incubated overnight. CFUs were quantified at 0- and 24-hour time points to determine the killing activity of conditioned medium. MRS conditioned with H$_2$O$_2$ and/or organic acids at the measured concentrations did not inhibit colony formation by UPEC (Figure 3). Thus, we conclude that the bactericidal activity cannot be due to H$_2$O$_2$ and organic acids.
Figure 3. Contributions of physiologic concentrations of H$_2$O$_2$ and organic acids to bactericidal effects of L. crispatus CFS. 12µM H$_2$O$_2$, 8.5mM lactic acid, and 7.5mM acetic acid were added to MRS medium pH 3.7 at concentrations listed in Table 1. MRS supplemented with physiologically produced concentrations of H$_2$O$_2$ and organic acids did not result in bactericidal activity against UPEC.

**Temperature and Time Stability**

It has been shown here that H$_2$O$_2$ and organic acids are not responsible for bactericidal effects of CFS. To begin investigating the type of molecule responsible for bactericidal activity, as well as test molecule stability, CFS was subjected to different storage temperatures and times. Proteins tend to denature with extreme decreases or increases in temperature$^{69,83}$. Boiling or cooling CFS would, therefore, denature most proteins present within CFS and negate their bactericidal effect against UPEC. Finally, lactic acid and hydrogen peroxide degrade over time and at different temperatures. The half-life of lactic acid ranges from 8-21 days, while hydrogen peroxide spontaneously decomposes to O$_2$ and H$_2$O over time. The decomposition rate of H$_2$O$_2$ is increased with both light exposure and at higher temperatures$^{84}$. 
CFS was prepared as previously described. CFS samples were then subjected to 100°C, 25°C, or 4°C temperatures. 100°C samples were held at constant temperature for one hour then stored at 25°C. 4°C samples were stored at 4°C. Samples from each temperature condition were then stored for either one or fourteen days prior to UPEC incubation.

When UPEC was incubated with temperature- or time-conditioned CFS, there was no change in bactericidal activity when compared to untreated CFS (Figure 4). These results suggest typical proteins do not contribute to the bactericidal activity of CFS.

I have previously shown that bactericidal activity is preserved when CFS is stored at different temperatures for up to 2 weeks. I have not determined if CFS is stable when stored for longer than two weeks. Therefore, I stored CFS at room temperature (25°C) for three months and then tested its activity against UPEC. CFS retains full bactericidal activity against UPEC when stored at room temperature up to three months (Figure 5).
Figure 4. CFS held up to 2 weeks at different temperatures retains its bactericidal activity against UPEC. CFS samples were subjected to 100°C, 25°C, or 4°C temperatures and then stored for either one or fourteen days. 100°C samples were held at constant temperature for one hour before being stored at 25°C for the specified times. 4°C samples were stored at 4°C for the specified times. Conditioned CFS retained full bactericidal activity when compared with unconditioned CFS.

Figure 5. CFS Activity after storage at room temperature for three months. CFS retains full bactericidal activity against UPEC when stored up to three months at room temperature (25°C).
CHAPTER IV
IDENTIFICATION OF THE INHIBITORY MOLECULE(S) IN CFS CONTRIBUTING TO BACTERICIDAL ACTIVITY

Introduction & Rationale

To determine the type of molecule contributing to the bactericidal effects against UPEC, enzymatic treatment was considered. Most enzymes are only effective within narrow pH ranges and rarely under acidic conditions. The pH of CFS after filter sterilization is 3.7. At this pH, enzymatic treatment would be ineffective. Furthermore, CFS was previously determined to be ineffective at more neutral or basic pH. In one study, bactericidal activity of CFS was regained after re-acidification of CFS. Therefore, neutralization, enzymatic treatment, and re-acidification may be a viable approach to degrade specific classes of molecules and determine the class of the bactericidally active molecule(s) in CFS.

Organic extraction is a common technique used to fractionate and/or purify organic compounds. In liquid-liquid extraction, solvents are added to a liquid sample and mixed. After centrifugation, the mixture resolves into two separate phases, an aqueous phase and an organic phase. The aqueous phase contains remnants of the original sample. Molecules from the original sample sharing properties with the chosen solvent will migrate into the organic phase.
Solvents are chosen based on their miscibility in water and/or other properties, such as polarity. General properties of solvents include miscibility, organic versus inorganic, polar versus non-polar, and protic versus aprotic. Miscibility refers to the ability of a solvent to mix with water. Organic solvents contain carbon atoms whereas inorganic solvents do not. Polar solvents have a net charge. Dipolar solvents move electrons between atoms and therefore have a small moving charge throughout the molecule. Non-polar solvents have no net charge. Protic solvents have the capability to donate H+ ions, whereas aprotic solvents cannot.

Solvent properties are not mutually exclusive and some solvents have many of the above properties. Polar protic solvents have a polar group on one end (e.g. –OH) and a non-polar group on the other. Dipolar aprotic solvents have a slight moving charge and cannot donate H+ ions to sample molecules. Non-polar solvents tend to be hydrophobic and immiscible in water. Characteristics of select organic solvents are listed in Table 3.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>IUPAC Name</th>
<th>Solvent Type</th>
<th>Miscible with H₂O</th>
<th>Polar</th>
<th>Polarity Index</th>
<th>Protic or Aprotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Butanol</td>
<td>n-Butanol</td>
<td>Alcohol</td>
<td>Immiscible</td>
<td>Polar</td>
<td>0.586</td>
<td>Protic</td>
</tr>
<tr>
<td>Acetone</td>
<td>Acetone</td>
<td>Ketone</td>
<td>Miscible</td>
<td>Dipolar</td>
<td>0.355</td>
<td>Aprotic</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>Acetonitrile</td>
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<td>Miscible</td>
<td>Dipolar</td>
<td>0.460</td>
<td>Aprotic</td>
</tr>
<tr>
<td>Benzene</td>
<td>Benzene</td>
<td>Hydrocarbon</td>
<td>Immiscible</td>
<td>No</td>
<td>0.111</td>
<td>aprotic</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Chloroform</td>
<td>Chlorinated Solvent</td>
<td>Immiscible</td>
<td>Slightly</td>
<td>0.259</td>
<td>Aprotic</td>
</tr>
<tr>
<td>Ethyl Acetate</td>
<td>Ethyl Acetate</td>
<td>Ester</td>
<td>Immiscible</td>
<td>Dipolar</td>
<td>0.228</td>
<td>Aprotic</td>
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<tr>
<td>Petroleum Ether</td>
<td>n-Pentane</td>
<td>Hydrocarbon</td>
<td>Immiscible</td>
<td>No</td>
<td>0.009</td>
<td>Aprotic</td>
</tr>
</tbody>
</table>

Fast Protein Liquid Chromatography (FPLC)

I attempted FPLC to identify a fraction with bactericidal activity. This method would facilitate purification and size range determination of bactericidally active molecules. Three separate runs were performed with different eluents and elution rates. No active fractions were identified (Figures 7-9).

Size Exclusion Spin Columns

I used low-binding centrifuge spin columns in an attempt to fractionate samples by size exclusion and determine an approximate size range of the active bactericidal molecule(s). 3-100kD columns were used. Size fractionation of CFS resulted in fractions without bactericidal activity (data not shown). Size determination at this time has been unsuccessful.
Figure 7. FPLC of CFS Eluted in Tris pH 8.0. The sample was eluted at a rate of 0.6mL/min and collected in 1mL fractions. Fractions were incubated with UPEC for 24 hours. Fractions did not exhibit bactericidal activity.

Figure 8. FPLC of CFS Eluted in PBS pH 3.7. The sample was eluted at a rate of 0.6mL/min and collected in 1mL fractions. Fractions were incubated with UPEC for 24 hours. Fractions did not exhibit bactericidal activity.
The sample was eluted at a rate of 0.06mL/min and collected in 300µL fractions. Fractions were incubated with UPEC for 24 hours. Fractions did not exhibit bactericidal activity.

CFS pH Neutralization and Acidification

To treat CFS with enzymes targeting specific types of molecules, CFS would need to be neutralized and re-acidified. CFS was neutralized to pH 7.0 using 1N NaOH then acidified to pH 3.7 using 1N HCl. The volume of NaOH/HCl required resulted in less than a 10% dilution of CFS. However, neutralized and re-acidified CFS lost all activity against UPEC (data not shown).

CFS Dilution and Concentration

CFS Dilution

I suspected that FPLC was unsuccessful due to dilution of the active molecule(s) in CFS. To determine if CFS bactericidal activity was concentration-dependent, CFS was diluted in 10% increments in either dH₂O or phosphate-buffered saline (PBS) adjusted to pH 3.7. Each dilution was incubated with UPEC overnight and CFUs quantified at 0 and 24 hour time points.

When CFS was diluted by 75% (50% prior to adding UPEC culture), it lost all bactericidal activity against UPEC (Figure 10). FPLC would dilute CFS.
substantially more than 75% and therefore this loss of activity would explain why no active fractions could be identified from FPLC experiments. Bactericidal activity appears to be salt-sensitive. Dilutions performed in PBS exhibited greater activity against UPEC than dilutions performed in dH₂O (Figures 11 & 12). Together, these results show CFS bactericidal activity is salt-sensitive and concentration-dependent.

**Figure 10. CFS dilutions in H₂O pH 3.7.** CFS was diluted in 10% increments prior to being added to UPEC cultures. Dilution in H₂O shows a logarithmic decrease in bactericidal activity against UPEC and CFS loses all activity at a 50% concentration.
Figure 11. **CFS dilutions in PBS pH 3.7.** CFS was diluted in 10% increments prior to being added to UPEC cultures. CFS loses bactericidal activity when diluted 50%.
CFS Concentration

To determine if loss of activity due to CFS dilution could be controlled, CFS was concentrated. CFS was aliquoted into Eppendorf tubes in 1 mL volumes. Samples were then lyophilized by SpeedVac overnight. Samples were re-suspended in 50-100 µL H₂O pH 3.7 to obtain 10-20X concentrated CFS. Concentrated CFS was then incubated with UPEC (Figure 13). Concentrated CFS showed complete bactericidal activity against UPEC. Therefore,
experiments resulting in dilution could be used to determine characteristics and purify the bactericidal molecule(s) in CFS.

![Bar graph showing survival log CFU/mL for MRS, 1X CFS, and 20X CFS](image)

**Figure 13. Concentrated CFS activity against UPEC.** CFS was concentrated 20X by SpeedVac. Concentrated CFS retains full bactericidal activity against UPEC.

**CFS Activity on Agar Plates**

Price and Diebel previously determined that CFS only showed bactericidal activity in liquid culture but not on agar plates\(^9\)-\(^{10}\). CFS activity has also been determined to be pH-dependent with a loss of activity above pH 4.5 but in one study acidification of CFS restored activity. To determine if inactivity of CFS of plates was due to pH, I measured the pH. The pH of TSA plates is ~7.5 prior to addition of CFS while CFS pH is 3.7. After plating 100µL of CFS pH is measured as 3.7 but after CFS dries the plate pH increases to 7.0. pH was measured after the plate was incubated for 24 hours and pH remains at 7.0. These results may explain the lack of activity observed when CFS is spotted onto plates.
I attempted to regain CFS bactericidal activity on agar plates by performing lawn assays. UPEC overnight cultures were standardized to OD 1.0 and used to plate a lawn on TSA plates. Plates were allowed to dry. Sodium Citrate buffer was adjusted to pH 3.7, 4.7, 5.7, or 6.7. 6mm paper disks were saturated with 10µL sodium citrate or 10X CFS. Sodium citrate and CFS saturated disks were placed adjacent to one another on dried UPEC plates. CFS and sodium citrate-only disks were applied as controls. To determine whether inhibition occurred below disks, I removed the disks using sterilized tweezers. I then used a sterile loop to scrap the agar where the disk sat previously. The sample was then struck onto a secondary fresh TSA plate and incubated aerobically overnight. If inhibition occurred, I would expect to see either a zone of inhibition surrounding CFS disks or a clearing below the disk and a lack of growth on secondary plates.

Acidification by paper disks did not restore CFS activity on agar plates (Figure 14). Samples from each plate grew UPEC overnight, showing that inhibition did not occur beneath CFS- or sodium citrate-impregnated disks (data not shown). Lack of activity cannot be due to CFS concentration because I used 10X CFS. CFS activity has been detected in liquid culture, but not plated cultures. This may suggest the active molecule(s) may be sensitive to oxidation, as liquid cultures do not have the same oxygen exposure as plated cultures do.

I used an alternative approach to verify inactivity of CFS on agar plates. TSA was acidified using HCl to pH 3.6 prior to autoclaving and pouring plates.
TSA with 1.5% agar did not solidify under acidic conditions. Agar concentration was increased to 2.5% in an attempt to ensure solidification of TSA plates. TSA with 2.5% agar concentration did not solidify. Therefore, attempts to create acidic TSA plates were unsuccessful and at this time CFS activity has not been detected on plates.

Figure 14. CFS Activity on Agar Plates Acidified by Sodium Citrate Paper Disks. Localized acidification does not promote in bactericidal activity against UPEC on agar plates. Sodium citrate (NaC) impregnated disks (left) and CFS impregnated disks (right).
Solvent Extraction

Once concentrated CFS was determined to exhibit full bactericidal activity against UPEC, fractionation techniques resulting in CFS dilution could be utilized. Liquid-Liquid Solvent Extraction was used to fractionate CFS and MRS. In short, organic solvents have properties that differentiate them from water and allow molecules present in a sample to move from an aqueous phase (water) to an organic phase (solvent). In general, only molecules sharing properties with the solvent will migrate to the organic phase. This method allows for both fractionation and characterization of molecules that migrate to the organic phase.

20X concentrated CFS was used in all extractions. Solvent was added to CFS and the mixture was vortexed. The samples were then centrifuged at 3,500 rpm for 10 minutes to separate aqueous and organic layers. Layers were separated into Eppendorf tubes and placed in the SpeedVac overnight to evaporate water and solvent from samples. Samples were re-suspended in 1 mL H$_2$O pH 3.7 and incubated with UPEC.

The bactericidal molecule(s) of CFS were soluble in polar solvents, but not in non-polar solvents. When using 1 Solvent:1 sample ratios, incomplete extraction by several solvents was evident by bactericidal activity in both aqueous and organic fractions (Figure 15a). Moreover, acetone and acetonitrile did not form an organic phase when added to MRS. Increased solvent volume (5:1) resulted in more complete extraction of bactericidal molecules in the organic phase (Figure 15b).
To further purify the bactericidal molecule(s), sequential extraction was performed using 1-butanol followed by ethyl acetate. The solvents were chosen for the differences in their polarities and abilities to donate protons. Sequential extraction of CFS resulted in a 1-butanol organic ethyl acetate aqueous phase that lost activity. In contrast, the 1-butanol organic ethyl acetate organic phase retained full bactericidal activity against UPEC (Figure 16a).

Protein concentrations were measured in MRS, 1X 24-hour CFS, 1X 48-hour CFS, and 10X 48-hour CFS sequential extracts. The 1-butanol aqueous phases contained proteins from samples, while the 1-butanol organic and ethyl acetate extracts did not contain proteins (Figure 16b). The bactericidal molecule(s) of interest appears to be (di)polar, non-proteinaceous, and organic in nature.
Figure 15. Molecules contributing to CFS bactericidal activity are soluble in select organic solvents. Solvents were added to 20X CFS. Aqueous and organic layers were separated after extraction and solvents evaporated by SpeedVac. Fractions were then re-suspended in H₂O pH 3.7 and incubated with UPEC. Molecules with bactericidal activity were soluble in 1-butanol, ethyl acetate, acetone, and acetonitrile. Acetone and acetonitrile were miscible in water and did not form organic layers when added to MRS. a) 1 CFS:1 solvent incomplete extraction occurred. b) With increased solvent volume (1 CFS:5 solvent), more complete extracts were obtained, as evidenced by inactive aqueous phases.
Sequential Extraction of CFS in 1-Butanol and Ethyl Acetate removes proteins and tri-peptides from CFS. Sequential extraction of 20X CFS was performed using 1-butanol and ethyl acetate. 1-BA = 1-Butanol Aqueous phase, 1-BO = 1-Butanol Organic phase, EAA = Ethyl Acetate Aqueous phase, EAO = Ethyl Acetate Organic phase. a) Sequentially extracted fractions retain bactericidal activity against UPEC. b) Sequential extraction removes proteins and tri-peptides from MRS and CFS. Protein concentrations were determined by BCA colorimetric assay.
CHAPTER V
CHARACTERIZING AND DETERMINING CFS MECHANISM OF
BACTERICIDAL ACTIVITY AGAINST UPEC

Introduction & Rationale

CFS of Lactobacilli has been shown to have broad spectrum activity against a wide array of pathogens \(^{2-4,11-12,34,44-45,47,67-69}\). Due to this broad spectrum of activity, Lactobacilli may produce a molecule(s) with decontamination or therapeutic properties. If the molecule(s) has potential for use in ecological or medical fields, understanding the mechanism of action is necessary prior to application.

Many pathogens have developed antibiotic resistance with associated increases in morbidity and mortality \(^{46}\). The increased rate of antibiotic resistance exceeds the development and identification of new antibiotic that may be effective against resistant strains. Consequently, only a limited cache of antibiotics exist to combat bacterial infections. Various bacterial species have been identified to production molecules with bactericidal activity that may have application as new antibiotic treatments. For instance, recently researchers have identified lugdunin, a bacteriocidal molecule produced by Staphylococcus lugdunensis with efficacy against the colonization of methicillin-resistant Staphylococcus aureus, Glycopeptide intermediate-resistant S. aureus,
vancomycin-resistant *Enterococcus*, and other infectious bacteria. If *Lactobacillus crispatus* CFS contains a molecule that is effective at inhibiting a broad spectrum of pathogens, then the molecule may be effective against antibiotic resistant strains with downstream application as an antibiotic.

Various *Lactobacillus* spp. have been investigated for their bactericidal activity. Inhibitory activity has been attributed to H$_2$O$_2$, organic acids, or bacteriocins. As shown previously, these molecules do not or are unlikely to be responsible for the bactericidal activity of *L. crispatus*. The capability of Lactobacilli to produce multiple bacteriostatic or bactericidal molecules would suggest that the genus has multiple mechanisms of action against pathogens. One study has shown that when a spotted lawn assay is performed using *L. crispatus* cells on a UPEC lawn, UPEC does not grow within the circumference of the cell spot, but a zone of inhibition did not form around the cell spot. CFS spotted on the UPEC lawn did not inhibit UPEC growth. This would suggest either cell contact-dependent inhibition, production of a non-diffusible bactericidal molecule, or concentration-dependence of the bactericidal molecule. Here, we investigate the mechanism by which *L. crispatus* inhibits UPEC colony formation independent of cell contact.

CFS of *Lactobacillus* spp. has been documented to have inhibitory activity when Lactobacilli are incubated in MRS for only 24 hours. Inhibitory activity, however, was not completely bactericidal as evidenced by the presence of viable target pathogens. Other research has shown complete bactericidal effects of
CFS against target pathogens after incubating *Lactobacillus* in MRS for 48 hours\(^4,9\)\(^{-10},44,70\). This information suggests two possibilities: 1) bactericidal molecules are concentration-dependent, or 2) bactericidal molecules are only produced during late-exponential growth or stationary phase.

In previous studies, a mechanism of action has not been proposed or investigated. A first step to understand the mechanism of action would be to observe the morphology of CFS incubated cells. The morphology of cells can elucidate the type of damage occurring in cells and can assist in determining a mechanism of action.

**Kinetics and Susceptibility**

**Kinetics**

*Lactobacillus spp.* CFS has been shown to inhibit pathogens within 24 hours of incubation\(^2,4,9\)\(^{-11},35,44\)\(^{-45},67,82\). However, the rate of killing has not been determined. This knowledge is necessary if the active molecule is to be used in ecological or medical fields for dosing purposes. Thus, UPEC was incubated in CFS and colony-forming units were quantified over time to determine the kinetics of bactericidal activity. Incubation of UPEC with CFS had a bactericidal effect that began within 4 hours and resulted in undetectable CFUs by 16 hours. The bactericidal effect was exponential over time with an initial decrease of \(3.1 \times 10^6\) cells between 0- and 4-hour time points. Cell death was rapid; by 4 hours of incubation in CFS, 70.00% of UPEC cells had died and by 6 hours 99.99% of
cells were dead. Additionally, in all experiments performed, no UPEC CFUs have been observed when incubated in CFS (Figure 17).

![Figure 17. CFS kills UPEC logarithmically over time. Uropathogenic E. coli (UPEC) was incubated with either L. crispatus cell free supernatant (black) or MRS medium (grey). a) UPEC incubated in CFS decreased in viability logarithmically over time. Loss of viable UPEC cells began within 4 hours and were undetectable by 16 hours of incubation. The limit](image)

**CFS Activity based on L. crispatus Growth Phase and Incubation Time**

It remains unclear if the bactericidal molecule(s) is constitutively produced, produced only during a specific stage of growth, or when it reaches inhibitory concentrations. It is also unknown if incubating L. crispatus longer results in increased bactericidal activity. If the active molecule(s) is constitutively produced but is concentration-dependent, then I would expect to see a gradual increase in CFS activity with increased incubation time. If the active molecule(s) is not produced constitutively, then I would expect to see a sudden increase in bactericidal activity.
48-hour liquid cultures of *L. crispatus* were standardized to an OD600 of 0.05 in 6 mL of fresh MRS. OD was measured every 6 hours to obtain a growth curve. CFS was obtained from cultures at each time point. UPEC was incubated in CFS from each time point and quantified after 24 hours of incubation to assess bactericidal activity.

CFS did not have any bactericidal activity against UPEC until late exponential/early stationary phase (Figure 18). This rapid increase in bactericidal activity supports production of sufficient concentrations of bactericidal molecule(s) during late exponential growth of *L. crispatus*.

I suspected the partial bactericidal activity observed against pathogens by previous research was due to *Lactobacillus* incubation time in MRS. To determine if bactericidal activity of *Lactobacillus* was due to incubation time or was growth phase-dependent, CFS was obtained from *L. crispatus* cultures every 24 hours and incubated with UPEC.

To determine when CFS gains activity and during which growth phase *L. crispatus* either begins production of the active molecule(s) or when minimum inhibitory concentration is reached, a growth curve was performed and CFS obtained at each time point. UPEC was incubated with CFS obtained at each time point and CFUs quantified.

Because 48-hour CFS has complete bactericidal activity against UPEC, it was suspected that 24-hour CFS would show partial, but not complete activity as seen by previous researchers. Contrary to previous findings, 24-hour CFS did
not exhibit any bactericidal activity against UPEC. However, 48-, 72-, and 96-hour CFS showed complete bactericidal activity against UPEC (Figure 19). Longer incubation time did not exhibit an increased rate of bactericidal activity within 4 hours of incubation. I conclude that CFS only becomes bactericidally active when *L. crispatus* reaches early stationary phase and that longer *L. crispatus* incubation time does not result in increased activity against UPEC.

**Figure 18. Bactericidal activity of CFS against Lc40 growth.** Growth curve of *L. crispatus* clinical isolate Lc40. Bactericidal activity of Lc40 CFS obtained at each measured growth curve time point. Lc40 CFS has a rapid increase in bactericidal activity against UPEC between 36 and 42 hours suggesting bactericidal molecule production occurs in late exponential/early stationary phase.
Figure 19. Effect of *Lactobacillus* incubation time on bactericidal activity. *L. crispatus* was incubated in MRS up to 96 hours. CFS was obtained from the culture every 24 hours and incubated with UPEC. MRS and 24-hour CFS exhibited no bactericidal activity against UPEC. 48-, 72-, and 96-hour CFS exhibited similar killing activity to one another and resulted in full bactericidal activity against UPEC.

**UPEC Susceptibility**

In previous experiments, UPEC cultures had been standardized to an OD600 of 1.0, which represents stationary growth phase. CFS had not been tested against UPEC in exponential growth phase and therefore it remained unclear if UPEC only experienced susceptibility to CFS during stationary phase or if CFS was effective against UPEC independent of growth phase.

I inoculated TSB with an overnight UPEC (standardized to OD600 of 0.05) culture and grew the culture aerobically until it reached an OD600 of 0.4, which is representative of mid-exponential growth phase. UPEC and CFS were mixed in a 1:1 ratio as previously mentioned and CFUs were quantified at 0- and 24-hour
time points. UPEC was susceptible to the active molecule(s) in CFS regardless of growth phase (Figure 20).

**Figure 20. Susceptibility to CFS based on UPEC growth phase.** CFS completely inhibits UPEC independent of UPEC growth phase.

### Susceptibility of Select Gram-negative and Gram-positive Clinical Isolates

*Escherichia coli, Staphylococcus aureus, and Enterococcus faecalis* are known or suspected pathogens. *S. aureus* and *E. faecalis* are recognized for their resistance to antibiotic treatment. Research has shown that antibiotic-resistant strains of *S. aureus* are susceptible to CFS treatment. *E. faecalis* susceptibility has not been investigated.

Three *E. coli* strains were tested: CFT073 and NU14, both implicated in pyelonephritis, and a clinical isolate (Ec901). Clinical isolates of *S. aureus* (Sa910) and *E. faecalis* (Ef891) also were tested. The clinical isolates were obtained from urine samples obtained during previous Wolfe laboratory studies.
Cultures were standardized to an OD600 of 1.0. Samples were incubated in CFS as previously mentioned.

Bacteria incubated in MRS did not exhibit any change in CFU after 24 hours. In contrast, *E. coli* and *S. aureus* samples incubated in CFS showed complete loss of CFUs by 24 hours. *E. faecalis* appeared largely resistant to CFS with only a slight, but significant, loss of CFUs by 24 hours of incubation (Figure 21).

CFS susceptibility testing was further expanded in an attempt to differentiate between commensal and pathogenic bacteria including UPEC with plasmid mediated resistance to β-lactam antibiotics. Commensal bacteria tested were *L. gasseri*, *L. jensenii*, and *S. epidermidis*. Pathogens tested were *E. coli*, *K. pneumoniae*, *P. mirabilis*, *P. aeruginosa*, *B. cereus*, *E. faecalis*, and *S. aureus*. Two of the *E. coli* isolates tested were transfected with a plasmid containing bla$_{NDM-1}$ which encodes for the New Delhi metallo-beta-lactamase 1 (NDM-1).

NDM-1 is an enzyme that confers resistance to β-lactam antibiotics. B-lactam antibiotics include penicillins, penams, cephalosporins, monobactams, and carbapenams. This family of antibiotics has broad-spectrum activity against both Gram-negative and Gram-positive bacteria. B-lactams are generally used as an initial antibiotic to treat bacterial infections.

CFS had bactericidal activity against all tested species with the exception of *B. cereus* (TABLE 4). CFS only had partial bactericidal activity against *P. aeruginosa*, *E. faecalis*, and *S. epidermidis*. CFS had full bactericidal activity
against the remaining tested bacteria: *E. coli* (CFT073, NU14, and NDM+ clinical isolates Acl25 and F35231-1), *K. pneumoniae*, *P. mirabilis*, *L. gasseri*, *L. jensenii*, and *S. aureus*.

Therefore, CFS appears to inhibit certain isolates of both Gram-negative and Gram-positive bacteria. There was not a correlation between susceptibility to CFS and whether a bacterium is deemed commensal or pathogenic. Additionally, CFS had full bactericidal activity against multidrug-resistant bacteria (MDR). Due to this potential broad-spectrum activity, the active molecule(s) is not likely to be a bacteriocin, which tend to be active against only closely related organisms. Results instead suggest that the active molecule(s) in CFS is most likely to be an antibacterial AMP.
Figure 21. CFS activity against select Gram-negative and Gram-positive clinical isolates. Samples were incubated in CFS for 24 hours. *E. coli* and *S. aureus* (Sa910) were susceptible to CFS but *E. faecalis* (Ef891) showed substantial resistance.
Table 4. Susceptibility of select Gram-positive and Gram-negative organisms to CFS. Organisms were incubated in CFS or MRS for 24-48 hours depending on the isolate’s recommended incubation time. Most organisms were susceptible to the bactericidal activity of CFS with the exception of Br. cereus. ++ = complete bactericidal activity, + = partial bactericidal activity, - = no bactericidal activity.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Commensal or Pathogen</th>
<th>Gram-negative, Gram-positive</th>
<th>Susceptibility</th>
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<tr>
<td><em>Escherichia coli</em></td>
<td>Pathogen</td>
<td>Negative</td>
<td>++</td>
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<tr>
<td>Ac125</td>
<td>Pathogen – NDM+</td>
<td>Negative</td>
<td>++</td>
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<tr>
<td>F35231-1</td>
<td>Pathogen – NDM+</td>
<td>Negative</td>
<td>++</td>
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<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>Pathogen</td>
<td>Negative</td>
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<td><em>Proteus mirabilis</em></td>
<td>Pathogen</td>
<td>Negative</td>
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<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Pathogen</td>
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<tr>
<td><em>Bacillus cereus</em></td>
<td>Pathogen</td>
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<td><em>Enterococcus faecalis</em></td>
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<td>Commensal</td>
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<td><em>Lactobacillus jensenii</em></td>
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<td><em>Staphylococcus epidermidis</em></td>
<td>Commensal</td>
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CFS of Other Lactobacilli

A *L. crispatus* isolate with low bactericidal activity against UPEC had previously been identified\(^{10}\). However, I was unable to culture this isolate and replicate these results. If another isolate with low bactericidal activity could be identified, then its genome could be sequenced and compared to an isolate with high bactericidal activity. Variation in the genomes could identify genes of interest that may be responsible for production of the bactericidal molecule(s).

CFS was obtained for three *L. crispatus* and one *L. jensenii* clinical isolates. UPEC was incubated in CFS for 24 hours and CFUs quantified. CFS from all Lactobacilli showed complete bactericidal activity against UPEC (Figure 22).
CFS Activity of different \textit{Lactobacilli}. UPEC was incubated in CFS from \textit{L. crispatus} (Lc) or \textit{L. jensenii} (Lj) for 24 hours and CFUs quantified. CFS of each \textit{Lactobacillus} isolate tested showed complete inhibition of UPEC.

**Imaging**

As mentioned earlier, an initial step toward understanding the mechanism of inhibition against UPEC should be observation of cell morphology. CFS may cause damage to cells that is evident in bright field, immunofluorescent, and/or transmission electron microscopy. Each of these techniques can elucidate the type of damage occurring to cells that results in loss of viability within 24 hours.

Bright field microscopy will allow for basic analysis of cell morphology by observation of overall cell structure and cytoplasmic content. For example, damaged cells may show atypical morphology or blebbing. Immunofluorescent microscopy allows for targeted imaging of specific cellular components such as membranes and nucleic acids. Transmission electron microscopy (TEM) allows for detailed observation of cell membranes and organization of intracellular components.
**Phase Contrast Microscopy**

UPEC incubated in CFS or MRS for 24 hours were observed at 1000X under phase contrast microscopy. Treated cells appeared smaller and more round compared to controls incubated in MRS medium (Figure 23). Zooming in on images revealed decreased cytoplasmic density in CFS-incubated cells when compared to MRS-incubated cells.

![Image of phase contrast microscopy](image)

**Figure 23. Phase contrast microscopy of UPEC incubated in CFS or MRS (1000X).** UPEC incubated in CFS (right) or MRS (left) for 24 hours were observed at 1000X under phase contrast microscopy. CFS-incubated cells have atypical morphology and decreased intracellular density.

**Immunofluorescent Microscopy**

**Live/Dead Staining** UPEC cells incubated in CFS or MRS were stained using the BacLight Bacterial Viability Kit™. The kit uses two nucleic acid dyes, Syto9 and propidium iodide. Syto9 is a green fluorescent dye that readily enters all cells. Propidium iodide is a larger red dye that can only enter cells with a compromised membrane.
After 24 hours of incubation, cells were stained following the supplier’s protocol. MRS incubated cells appeared green, suggesting intact membranes (Figure 24). Approximately 70% of cells incubated in CFS stained red with propidium iodide, suggesting compromised membranes.

**Membrane and Nucleic Acid Staining** UPEC cells incubated in CFS or MRS were stained using FM 4-64 membrane and Hoescht 33342 nucleic acid fluorophores to observe changes in morphology and membrane permeability over time. FM 4-64 (red) stains plasma membranes and the fluorophore is internalized rapidly over time\(^\text{80}\). Hoescht 33342 (blue) binds the minor groove of DNA preferentially at A-T rich double-stranded regions\(^\text{81}\). I experimentally determined that the Hoescht fluorophore only entered UPEC cells with compromised membranes. CFS- and MRS-incubated UPEC were stained at 0-, 6-, 12-, and 18-hours after incubation.

After 6 hours of incubation, CFS-incubated cells appeared smaller than MRS-incubated cells (Figure 25), consistent with phase contrast images. MRS-incubated cells localized FM 4-64 within the membrane, while CFS-incubated cells showed diffusion of FM 4-64 into the cytoplasm, suggesting increased rate of internalization potentially due to compromised membrane integrity. Decreased fluorescence intensity of CFS-incubated cell membranes, compared to MRS-incubated cells, supports a loss of membrane integrity. The Hoescht fluorophore was able to penetrate CFS-incubated UPEC membranes by 12 hours and was strongly fluorescent at 18 hours, suggesting increased DNA binding. Hoescht did
not enter MRS-incubated cells at any time point. As with Live/Dead staining, these results suggest CFS-incubated cells have compromised membranes. Hoescht fluorescence in CFS-incubated cells suggests the minor groove of DNA and A-T rich double-stranded regions maintain their structural integrity at 12 and 18 hours after incubation. Compromised membrane integrity at 12- and 18-hour time points correlates with loss of CFU in Figure 17.

I attempted to perform the previous procedure to compare UPEC against a Gram-positive bacterium. This could not be achieved, as the Hoescht fluorophore entered untreated Gram-positive cells (Figure 26). This suggests exclusion of Hoescht in UPEC cells is due to the presence of an outer membrane that is initially impenetrable to the Hoescht fluorophore. Because Hoescht enters untreated S. aureus cells, a Live/Dead Stain also would not be a viable option either as Hoescht and propidium iodide are similar in size and these cells would fluoresce red, suggesting they are dead when that would not be the case.
Figure 24. Live/Dead Stain of UPEC incubated in MRS or CFS. UPEC cells incubated in CFS or MRS were stained after 24 hours. CFS-incubated cells have compromised membranes as evidenced by entry of propidium iodide into cells (red).
Figure 25. Membrane and Nucleic Acid Staining of CFS- and MRS-incubated UPEC. UPEC was stained with FM 4-64 membrane fluorophore (red) and Hoescht 33342 nucleic acid fluorophore (blue). CFS-incubated cells showed altered morphology by 6 hours of incubation and compromised membranes by 12 hours of incubation.
Figure 26. Membrane and Nucleic Acid Staining of *E. coli* versus *S. aureus*. Hoescht fluorophores readily enter Gram-positive *S. aureus* but not Gram-negative *E. coli* prior to incubation in CFS.

**Transmission Electron Microscopy**

Transmission Electron Micrographs (TEM) were obtained of UPEC cells incubated with CFS or MRS. MRS-incubated cells appeared ellipsoid with diffuse cytoplasm (**Figure 27a**). CFS-incubated cells were smaller with irregular morphology compared to MRS-incubated cells. The cytoplasm of CFS cells appeared granular and less dense than MRS-incubated cells. Increased magnification showed that CFS-incubated cells have undergone plasmolysis with compromised cytoplasm and plasma membranes (**Figure 27b/c**).
Figure 27. Incubation of UPEC in CFS compromises the integrity of cell cytoplasm and membranes. TEM of MRS-incubated UPEC (left) versus CFS incubated UPEC (right) shows plasmolysis with compromised integrity of cytoplasm and periplasm in CFS-incubated cells. a) 3000X, b) 8000X, c) 13000X magnifications.
CHAPTER VI
DISCUSSION AND CONCLUSION

I propose that *Lactobacillus crispatus* produces a potentially novel dipolar, organic molecule with broad-spectrum activity that is concentration-dependent and salt-sensitive. This currently unidentified molecule(s) effectively and reproducibly inhibits colony formation of UPEC, apparently by a mechanism that causes plasmolysis. Identification of this molecule(s) is of high importance as it may contribute to human health either as a decontaminant or as a therapeutic in an era when antibiotic resistance is increasing.

I have shown that a urinary isolate of *L. crispatus* (Lc40) produces concentrations of H$_2$O$_2$ and organic acids significantly lower than those required to inhibit pathogen growth or survival (Table 2). MRS medium conditioned with physiologically produced concentrations of H$_2$O$_2$ and/or organic acids did not result in decreased UPEC viability when compared to unconditioned MRS medium (Figure 3). Surprisingly, previous researchers have not performed this simple experiment, i.e., to measure physiologically produced concentrations of H$_2$O$_2$ and organic acids and to determine their contributions to CFS bactericidal activity. Previous researchers have either measured concentrations of these molecules and not tested the physiologically produced concentrations or tested ranges of concentrations to determine at which concentration inhibition would
occur. Furthermore, the bactericidal activity of CFS was stable even upon boiling and over a range of storage times (Figures 4 & 5). Since H₂O₂ and organic acids degrade with heat and with time, these observations support my contention that H₂O₂ and organic acids cannot be responsible for the observed bactericidal activity against UPEC. In light of my results, it seems counterintuitive to attribute bactericidal activity of CFS to H₂O₂ or organic acids using the methods of previous researchers and therefore I suggest previous research be re-analyzed and re-interpreted.

Because the bactericidal activity of CFS was extremely stable, I also conclude that the bactericidal molecule(s) is not likely to be proteinaceous in nature. Proteins tend to denature with exposure to extreme temperatures and temperature-conditioned CFS stored up to two weeks did not exhibit a decrease in activity compared to unconditioned CFS (Figure 4). Moreover, 1-butanol organic extraction removed the majority of proteins and peptides from CFS, yet bactericidal activity was retained (Figure 16). I conclude that the active molecule(s) is likely not proteinaceous. Together, these results suggest the bactericidal activity of L. crispatus CFS is not due to H₂O₂, organic acids, proteins, peptides, or bacteriocins; which have been previously credited for pathogen inhibition.

Bacteriocins have many classes defined by various criteria. Based solely on the definition of a bacteriocin it appears unlikely that the active molecule(s) in CFS is a bacteriocin. The molecule(s) has broad-spectrum activity against
distantly related genera such as Escherichia, Klebsiella, Proteus, and Pseudomonas (Table 4). Based on this definition alone the only Classes of bacteriocin the active molecule(s) could fall into are Subclass IIa or Class IV. Subclass IIa is supported by the heat stability of the molecule(s) when full activity is retained after heating CFS for one hour at 100°C (Figure 4). However, as previously stated, the active molecule(s) acts against distantly related organisms and is unlikely to be a traditionally characterized bacteriocin. Classes I and III are composed of amphipathic molecules and should therefore be soluble in both polar and non-polar solvents. This is not the case. The active molecule(s) was only soluble is polar solvents suggesting the bactericidal molecule(s) is polar in nature (Figures 15 & 16). Additionally, Class III bacteriocins result in cell lysis.

CFS’ mechanism of action against UPEC is shown to result in cellular plasmolysis (Figure 27). Class IV bacteriocins have more variability in their characteristics and the molecule(s) may fit into this class. However, as stated the molecule(s) act against distantly related genera and is most likely not a bacteriocin. AMPs are more loosely defined and characterized based solely on target organism. Therefore, I would suggest that the bactericidal molecule(s) in CFS is an antibacterial AMP.

The bactericidal activity of CFS was sensitive to dilution and salt concentration (Figures 10-12). Samples diluted in PBS, which has increased salt concentration compared to H₂O, retained activity at lower CFS concentrations (Figure 12). Retention of activity in saline conditions is relevant to use of the
active molecule(s) as a therapeutic agent. The human body has greater salinity than H₂O, making this result physiologically relevant; however, actual salinity varies based on anatomical tissue type and location. Once the identity of the active molecule(s) is determined, then its concentration in 1X CFS should be measured. Based on dilution experiments, an approximate minimum inhibitory concentration could be approximated and tested.

The pH of urine from healthy individuals ranges from 5.0-7.0. At this pH, the bactericidal molecule(s) would be inactive. Lactobacillus spp. have been shown to reduce the pH of the medium they are grown in. I would speculate that in the bladder the genus would also reduce the pH of the environment. The volume and surface area of the bladder are significantly larger than that of a test tube. Therefore, I suggest that, rather than acidifying the entire bladder, L. crispatus reduces the pH of its microenvironment. The localized acidification of the microenvironment would allow for the bactericidal activity of secreted antimicrobial molecule(s).

Incubating L. crispatus for 48 hours was required for maximum bactericidal activity, as bactericidal activity did not occur until 42 hours of L. crispatus growth; however, the effect was incomplete at this stage of growth (Figure 18). Yet, incubating L. crispatus longer than 48 hours did not alter the kinetics of bactericidal activity (Figure 19). This suggests the active molecule(s) is only produced or reaches effective concentrations during late exponential growth or in early stationary phase. Production or effective concentrations result
only after *L. crispatus* reaches late exponential growth. Holding *L. crispatus* in stationary phase beyond 48 hours did not result in increased bactericidal activity against UPEC, further supporting production or effective concentration during late exponential growth. To determine whether the molecule(s) is produced constitutively 24-hour CFS could be concentrated and tested against UPEC to evaluate bactericidal activity. If concentrated 24-hour CFS has bactericidal activity then it would be hypothesized that *L. crispatus* constitutively produces and secretes bactericidal molecule(s) rather than only producing the bactericidal molecule(s) at late exponential growth phase. An alternative is a possible saturation effect, where concentration beyond an undetermined threshold does not exhibit increased efficacy or production of a very stable molecule(s) only during late exponential growth and production does not continue through stationary phase. Another variable may be the pH of *L. crispatus* growth medium over incubation time. *L. crispatus* reduces the pH of MRS by 48 hours of incubation. To determine if lack of bactericidal activity prior to 42 hours of incubation is a pH effect, the pH of the growth medium would need to be measured over time and bactericidal activity of CFS determined at each time point. However, determination of when the cells produce the molecule(s) and concentrations produced over *L. crispatus* growth must wait until the molecule(s) is identified.

The active molecule(s) of CFS was soluble in polar/dipolar solvents, including 1-butanol, acetone, acetonitrile, and ethyl acetate (Figure 15). This
suggests that the active molecule(s) is polar in nature. 1-butanol, acetone, and ethyl acetate contain oxygen atoms that are not involved in linkage within their primary structure (Figure 6). Acetonitrile contains a terminal triple bonded nitrogen atom. Acetone, acetonitrile and ethyl acetate are unsaturated (i.e., having double or triple bonded atoms). These properties suggest the active molecule(s) in CFS may form hydrogen or polar covalent bonds with the electronegative atoms of solvents, facilitating migration into the organic phase. The molecule(s) was soluble in alcohol (1-butanol), ester (ethyl acetate), and ketone (acetone) solvents, but not in hydrocarbon or chlorinated solvents (Table 3). This diverse solubility does not assist in classification of the molecule(s). However, based on the solubility profile of the bactericidal CFS molecule(s), I conclude the molecule(s) is a (di)polar organic molecule.

Attempts to fractionate CFS by size were unsuccessful (Figures 7-9). FPLC failure was likely the result of high dilution factors that occur during the liquid chromatography process. Size exclusion spin columns did not result in dilution of CFS, but still resulted in fractions without bactericidal activity. It is possible the active molecule(s) may be bound to spin column membranes, even though low-binding membranes were used. These results may also suggest CFS bactericidal activity requires more than one molecule. During size exclusion, molecules may be separated and unable to form an active complex. Furthermore, bactericidal activity may be due to a complex composed of molecules of different sizes. An alternative is that one molecule requires a
cofactor for activity. When CFS was diluted, the cofactor may not have been present in high enough concentrations for activity. Because the active molecule(s) can be extracted and retains full bactericidal activity, if a cofactor is required it must possess similar properties to that of the active molecule(s), but may also be of a different molecular weight.

Attempts to show CFS activity on agar plates were unsuccessful (Figure 14). Tryptic soy contains dibasic potassium phosphate buffer that maintains plate pH at approximately pH 7.3. pH measurements of TSA and CFS plated on TSA showed that acidic pH conditions are not retained after CFS dries on TSA. The increase in pH is most likely due to the buffer in TSA and explains why bactericidal activity has not been observed in plated conditions. Researchers have conducted experiments with TSA buffered between pH 3.9-8.0, showing TSA can be acidified. Plates acidified below 3.9 did not solidify even with increased agar concentration. Therefore, CFS activity on acidified plates could not be evaluated. Localized acidification by use of paper disks did not promote CFS activity. Dibasic potassium phosphate buffer present in TSA may neutralize the acidic paper disks and could explain the lack of CFS activity on agar plates. To further investigate bactericidal activity on agar plates, a different medium or modified TSA without dibasic potassium phosphate would need to be used. If a buffer were to be used in the medium then it would have to be a buffer that is capable of maintaining medium pH at 3.7. If activity is still not observed in plated conditions then the active molecule(s) in CFS may be sensitive to oxidation. CFS
is exposed to increased oxygen concentrations in plated conditions compared to liquid cultures, but further experiments must be performed to evaluate sensitivity to oxidation.

*L. crispatus* CFS showed efficacy against some species implicated in various diseases and recognized to exhibit troubling rates of antibiotic resistance ([Figure 20, Table 4](#)). CFS had full bactericidal activity against NDM+ UPEC with resistance to β-lactam antibiotics, which include carbapenems, cephalosporins, and penicillin derivatives ([Table 4](#)). Not only does this support broad-spectrum activity but also shows the potential application as a treatment for MDR bacterial infections. Carbapenems, cephalosporins, and penicillins are traditionally used as a first line therapeutic for bacterial infections. With increased resistance, a decrease in the rate of new antibiotic development, and a limited cache of antibiotics it is critical to identify and develop new therapeutics against the increasing prevalence of MDR bacterial infections. In a limited study, a low efficacy isolate of *L. crispatus* was not identified ([Figure 22](#)). The CFS of the *Lactobacillus* isolates tested all showed complete inhibition of UPEC. However, in this preliminary screen, H$_2$O$_2$, organic acids, proteins, and bacteriocins could not be excluded as contributing factors to the observed inhibition, as tests were not performed to evaluate the concentration of each molecule in their CFS. These data suggest the active molecule(s) produced by *L. crispatus* has broad-spectrum activity and may be ubiquitous among other *L. crispatus* clinical isolates tested.
Incubation of UPEC with CFS had a bactericidal effect that was exponential over time beginning within 4 hours and resulted in undetectable CFUs by 16 hours (Figure 17). Cell death was rapid; by 4 hours of incubation in CFS, 70% of UPEC cells had died and by 6 hours 99.99% of cells were dead. Additionally, in all experiments performed, no UPEC colony-forming units have been observed when incubated in CFS. This result suggests cells cannot develop resistance to the active molecule(s) in CFS under conditions used. It is possible that cells are exposed to extremely lethal concentrations of the active molecule(s) and are unable to develop resistance due to rapid cell death. Experiments would need to be conducted using sub-lethal concentrations, such as 60% CFS, and cells passaged through multiple generations to determine if resistance can be developed by UPEC. The bactericidal molecule(s) present in CFS has also been shown to be extremely stable retaining full bactericidal activity when stored for up to three months at room temperature (Figure 5). Stability is of critical importance when considering use of this molecule(s) as a therapeutic. That the CFS can be lyophilized or stored as a liquid up to three months and still retain full activity could lead to its application as a therapeutic in underdeveloped countries where the storage of a liquid or refrigerated therapeutic may not be feasible.

Observation of CFS-treated UPEC by phase-contrast microscopy showed smaller cells with a rounded morphology compared to UPEC incubated in MRS (Figure 23). Cells also appeared to have decreased cytoplasmic density,
suggesting intracellular components may be degraded or leaking from cells. Live/Dead staining showed a majority of CFS-incubated cells had compromised membranes, allowing propidium iodide (PI) to enter cells and stain nucleic acids (Figure 24). This also showed that nucleic acids are retained within CFS-treated cells after a 24-hour incubation period. Immunofluorescent staining with FM 4-64 membrane and Hoescht 33342 nucleic acid fluorophores showed an increased number of cells with FM 4-64 internalization at 6 hours in CFS-incubated cells, suggesting membranes are compromised by this time. Whether membrane damage is the cause of death or a secondary effect to cell death has not been determined at this time. Hoescht was seen to enter CFS-incubated cells by 12 hours (Figure 25). CFS-incubated cells had increased fluorescence at 18 hours compared to MRS-incubated cells, which do not internalize Hoescht at any time point. I could not determine if Gram-positive species show the same permeability when incubated with CFS as Gram-negative E. coli, as S. aureus readily internalized both Hoescht and FM 4-64 prior to incubation with CFS (Figure 26). This result is consistent with results of Gram-positive bacterium research, where Hoescht is used in experiments as a nucleic acid fluorophore for live cells. To determine if Gram-positive species show similar damage to E. coli after incubation in CFS, we would need to obtain TEMs to observe detailed cytoplasmic and morphological differences compared to MRS-incubated cells. Alternatively, we could use a different fluorophore, one that permeates Gram-negative and Gram-positive cells in a similar manner. PI intercalates between
intact double-stranded DNA. Hoescht binds the minor groove of DNA at A-T rich regions. The ability of PI and Hoescht to bind the DNA of CFS-incubated cells suggests DNA architecture was not affected by the active molecule(s) in CFS since DNA remained double-stranded and A-T rich minor groove regions were apparently intact. Closer analysis by TEM of UPEC incubated in CFS revealed atypical morphology compared to MRS-incubated cells (Figure 27). Cells showed signs of plasmolysis with compromised plasma membranes and granulated cytoplasmic contents. Granular cytoplasmic contents could explain the appearance of less dense cytoplasm observed by phase contrast imaging (Figure 23).

Much still needs to be learned about the bactericidal activity of *L. crispatus* CFS. Many characteristics of the bactericidal molecule(s) in CFS have been determined; however, the identity of the molecule(s) has still not been elucidated. Identification of the molecule(s) will assist in further determining characteristics of the molecule(s), potential *L. crispatus* genes responsible for production of the molecule(s), a mechanism of action, minimum inhibitory concentration, and potential applications.

Characterization could include the classification and structure of the molecule(s). Determination of class and structure will provide information about how the molecule(s) acts against its target and in which anatomical or ecological niches, if any, it would be active. If the molecule(s) is only active in specific anatomical or ecological niches then modification of its structure could result in a
molecule(s) with more diverse activity in various niches. As the active molecule(s) is pH-sensitive, its structure could be modified to enable activity in a more pH neutral environment.

Determination of the gene responsible for production of the molecule(s) is another important step toward use of the molecule(s) as a therapeutic. Once the gene is determined, it can then be introduced into a bacterium with a shorter generation time. Prior to introduction, however, the mechanism of action would need to be determined. Insertion of the gene into a susceptible bacterium would ultimately result in decreased production of the active molecule(s) compared to *L. crispatus*. By determining the mechanism of action we may be able to alter the genome of a bacterium deeming it resistant; and consequently, the ability to produce the active molecule(s) more rapidly than *L. crispatus*. If the molecule(s) can be used as a decontaminant or therapeutic then more rapid production (and potentially higher yield) could help to result in quicker distribution as well as minimize cost.

Based on the results presented here, I suggest that *L. crispatus* produces an extremely stable, potentially novel dipolar, organic molecule with broad-spectrum activity that is concentration-dependent, salt-sensitive and that results in plasmolytic action against its target. Identification of the molecule(s) could lead to its application as a decontaminant or therapeutic contributing to human health.


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Giuseppe became interested in research while considering applying to medical school. His thesis work is on the antimicrobial effects of molecules produced by *Lactobacillus crispatus* in the laboratory of Dr. Alan J. Wolfe.

After completion of his Master’s degree in Infectious Disease and Immunology at Loyola University Chicago he will begin a MBA and pursue employment with the Government.