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Lactobacillus crispatus Produces a Bacteridical Molecule That Kills Uropathogenic E. Coli

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LACTOBACILLUS CRISPATUS PRODUCES A BACTERICIDAL MOLECULE THAT KILLS UROPATHOGENIC *ESCHERICIA COLI*

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ABSTRACT

As many as 1 in 2 women will have at least one urinary tract infection (UTI) in their lifetime. UTIs can cause complications in pregnancy and decrease quality of life, and their treatment and prevention are expensive. Uropathogenic *E. coli* (UPEC) is the primary cause of UTI. The probiotic and bactericidal capacities of gut and vaginal *Lactobacillus* isolates have been studied, but the same attention has not been paid to urinary strains. These urinary isolates of *L. crispatus* appear to have a greater killing capacity against UPEC and this bactericidal activity does not depend on the cells themselves, consistent with the hypothesis that they secrete a molecule with anti-UPEC activity. In the future, this bacterium could be useful as a probiotic and molecules it produces could be used as antibacterial compounds.

The SCS of one urinary isolate of *L. crispatus* killed several logs of UPEC within 2 hours of exposure. This isolate creates a more acidic environment than isolates of other *Lactobacillus* species, but the killing of UPEC was not due to low pH alone, as buffered of the SCS delayed but did not eliminate the bactericidal effect. This effect became stronger after the SCS was left to sit for 24 hours. The molecule was not heat sensitive.

A urinary *L. crispatus* isolate produces a unique soluble molecule that can kill up to 9 logs of UPEC within 24 hours. The molecule may be an antimicrobial peptide or bacteriocin. Further experiments are required.

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CHAPTER ONE:

INTRODUCTION

As many as 1 in 2 women will have at least one urinary tract infection (UTI) in their lifetime. UTIs can cause complications in pregnancy and decrease quality of life, and their treatment and prevention are expensive (24). The ultimate goal of studying the bladder microbiota is to find ways to prevent growth of uropathogens that cause UTIs, to prevent dysbiosis, and to promote a balanced and healthy bladder microenvironment. Uropathogenic *E. coli* (UPEC) is the primary cause of UTI (25).

Lactobacilli are commonly found in the mouth, GI tract, and genital tract. Previous studies by the Wolfe lab have shown that *Lactobacillus* species, especially *L. crispatus, L. gasseri, L. jensenii,* and *L. iners*, are also found in urine obtained from the bladder (26, 27). In contrast to other *Lactobacillus* species, *L. crispatus* was found to be statistically associated with women with no lower urinary tract symptoms (26), and was only rarely isolated along with *E. coli*, much less often than the other *Lactobacillus* species (27). These results support the hypothesis that *L. crispatus* is part of a healthy bladder flora and/or that its presence can prevent blooms of uropathogenic *E. coli*. It has been shown that a vaginal suppository of *Lactobacillus crispatus* can protect against recurrent UTIs caused by UPEC (28), and vaginal *Lactobacillus* isolates have been shown to produce by-products that inhibit the growth of UPEC (3). The probiotic and

bactericidal capacities of gut and vaginal *Lactobacillus* isolates have been studied, but the same attention has not been paid to urinary strains.

Literature Review

The genus *Lactobacillus* consists of Gram-positive, microaerophilic, rod-shaped bacteria. They are associated with mucosal surfaces in animals, such as the gut and vaginal epithelia (1). Many studies have investigated the bactericidal and/or inhibitory activity of various strains of *Lactobacillus*. *Lactobacillus* species can inhibit the growth of other bacteria through competitive exclusion, interference with adhesion, and use of secreted factors such as organic acids, hydrogen peroxide, bacteriocins, and other antimicrobial molecules (16).

All *Lactobacillus* species produce lactic acid and many produce other organic acids as well. *In vitro* studies have shown that the bactericidal activity of some *Lactobacilli* depends on the pH of the cell culture. The anti-*E. coli* activity of vaginal fluid correlated with low pH and a high lactic acid content (2). In a study of spent culture supernatants of probiotic, urogenital strains *L. rhamnosus* GR-1 and *L. reuteri* RC-14, lactic acid and the culture supernatants downregulated promoter activity of genes that encode molecules critical for adherence to the urothelium by uropathogenic *E. coli* (UPEC) strain C1212 (3). It has been shown that some *Lactobacillus* species can prevent pathogens from adhering to host cells (4). The effect of *Lactobacilli* on Shiga toxin-producing *E. coli* depends on lactic acid production, resulting in a bacteriostatic phase at lower lactic acid concentrations and a bactericidal phase at higher concentrations (5).

Though many studies implicate lactic acid in the antagonistic activity of *Lactobacillus* species, it rarely works alone. Lactic acid permeabilizes the outer membrane of Gram-negative bacteria, making them vulnerable to the activity of other molecules (6). One study suggests a synergistic action of lactic acid and unidentified proteinaceous substances in the bactericidal activity of *L. acidophilus* HN027, *L. rhamnosus* DR20, and *Bifidobacterium lactis* DR10 against *E. coli* O157:H7 (7). The bacteriocin nisin has broad-spectrum activity against Gram-positive and Gram-negative bacteria specifically when the outer membrane of the Gram-negatives is compromised (8).

Hydrogen peroxide is another product of many *Lactobacilli*. *L. crispatus* and *L. jensenii* inhibit the growth of *N. gonorrhoeae* on plates through the production of H_2O_2 (9). Vaginal *E. coli* colonization is significantly more frequent in women who lack H_2O_2-positive *Lactobacilli* (10). A highly concentrated H_2O_2-producing *Lactobacilli* culture is toxic to *Gardnerella vaginalis* (11). A strong association exists between species associated with bacterial vaginosus and species inhibited by H_2O_2-producing *Lactobacilli* (12). Another group studied twenty-two vaginal *Lactobacillus* strains for production of lactic acid, H_2O_2, and bacteriocin. They found that 80% of these *Lactobacillus* strains produced bacteriocin that inhibited *G. vaginalis*, while 60% produced lactic acid, H_2O_2, and bacteriocin (13).

One study investigated the killing activities of the human intestinal strain *Lactobacillus johnsonii* NCC933 and the human vaginal strain *Lactobacillus gasseri*

KS120.1 against several pathogens, including the UPEC strain CFT073, and found that a co-operative effect of lactic acid and hydrogen peroxide kills UPEC (14).

Bacteriocins are protease-sensitive peptides and proteins produced by bacteria. Generally, bacteriocins exhibit antimicrobial activity against a narrow range of closely related bacteria, but some have a wider range against Gram-positives, and they are not frequently active against Gram-negatives (15). The bacteriocin family includes a wide variety of peptides and proteins, with various molecular weights and mechanisms of action (16). Three classes of bacteriocin exist. Class I bacteriocins, also known as lantibiotics because they contain the non-canonical amino acid lanthionine, are heat-stable peptides that weigh less than five kilodaltons. Nisin, mentioned above, is a lantibiotic. Class II bacteriocins are also heat-stable peptides weighing less than five kilodaltons, but they lack lanthionine. Some class II bacteriocins are formed by a complex of two distinct peptides. Class III bacteriocins are heat-sensitive and weigh greater than thirty kilodaltons.

Like nisin, many bacteriocins require the presence of an organic acid to be active against Gram-negative bacteria. However, this is not always the case. *L. acidophilus* produces a small bacteriocin that maintains activity across a very wide pH range against a narrow range of bacteria including *E. coli* (17).

L. salivarius M7 produces salivaricin B, a broad spectrum bacteriocin, and *L. acidophilus* M46 produces acidocin B, which has a narrow activity spectrum within the genus *Lactobacillus* (18). A bacteriocin may be responsible for the inhibitory effect *L. casei/rhamnosus* (the species could not be identified) and *L. acidophilus* exert on UPEC

strains, a heat-sensitive effect retained under pH buffer and with a molecular weight greater than 12kDa – 14kDa (19).

Lactobacillus species also produce antimicrobial molecules other than bacteriocins. One group described a microcin, a low molecular weight peptide, insensitive to proteases, whose production is mediated by plasmids (20). This molecule is produced by *Lactobacillus* species GG, is heat stable, and inhibits a wide range of species including *E. coli* in a pH range of 3 – 5. The authors speculate that the molecule might be a short chain fatty acid, rather than a peptide. Another group described another inhibitory molecule insensitive to protease treatment. It has a wide range of activity, is heat stable, and is produced by *L. casei* and *L. helveticus* (21). Another wide-range, protease-insensitive molecule is secreted by *L. acidophilus* LA1, independent of lactic acid production (22). Recently, a study of proteins active against *E. coli* found in cervicovaginal lavage found that four corresponded to *L. crispatus* or *L. jensenii* proteins. The group described these proteins as distinct from bacteriocins due to their resistance to proteinase K. They also found the spent culture supernatant from *L. jensenii* to be bactericidal against *E. coli*, even when buffered to pH 6.3 (23).

Our lab has demonstrated that the spent culture supernatant (SCS) of certain urinary isolates of *L. crispatus* can kill UPEC (27). These isolates appear to have a greater killing capacity against UPEC than urinary isolates of *L. jensenii* or *L. gasseri*. The killing is not dependent on contact between *L. crispatus* cells and UPEC, and UPEC does not need to be present to somehow induce *L. crispatus* to produce the active molecule, consistent with the hypothesis that they secrete a molecule with anti-UPEC

activity (27). The bactericidal molecule must be excreted, secreted or sloughed off the surface. The molecule could be unique and never studied before or alternatively, it could be a well-known product of lactic acid bacteria.

Whereas lactic acid is produced by *L. gasseri* and *L. jensenii*, which do not possess the bactericidal activity of *L. crispatus*, it is possible that some urinary isolates of *L. crispatus* produce more lactic acid. *Lactobacillus* species also secrete biosurfactants, bacteriocins, and anti-microbial peptides (16, 29). Urinary *L. crispatus* may kill *E. coli* using lactic acid, hydrogen peroxide, a bacteriocin, a molecule similar to one of the many previously described, or a unique molecule. In the future, this bacterium could be useful as a probiotic and molecules it produces could be used as antibacterial compound.

CHAPTER TWO:

MATERIALS AND METHODS

Culture Conditions

Lactobacillus isolates were grown on blood agar plates in anaerobic conditions at 37C for 48 hours. Colonies were then inoculated into de Man, Rogosa and Sharpe broth (MRS) and grown in CO_2 at 37°C for 48 hours. *E. coli* isolates were grown on tryptic soy agar (TSA) plates in ambient conditions at 37°C overnight. Colonies were inoculated into tryptic soy broth (TSB) and grown in ambient conditions at 37°C overnight. Other bacterial isolates from the urinary culture collection were grown on blood agar plates in anaerobic conditions at 37°C for 48 hours. Colonies were inoculated into TSB and grown in CO_2 at 37°C for 48 hours. The *Staphylococcus aureus* and *S. epidermidis* strains (generous gifts from Dr. Alonzo) were grown on TSA plates, then inoculated into TSB and grown in ambient conditions at 37°C overnight.

Bacterial Survival Assay

L. crispatus and NU14 were grown as described above. The *L. crispatus* was centrifuged for 2 minutes at 13000 rpm. The spent culture supernatant (SCS) was removed and filter-sterilized. Two mL each of NU14 and SCS were combined in a new

test tube, which was placed on a shaker at 37°C in ambient conditions. The negative control was two mL each of NU14 and MRS combined and maintained under the same conditions as the tubes containing SCS. Aliquots of 100 µl were taken at chosen time points. The samples were serially diluted and spread on TSA plates, which were placed at 37°C in ambient conditions for 24 hours. Colonies were counted and the CFU/mL of NU14 calculated.

To test the effect of heat on killing, the SCS was heated in epi tubes on a heating block for either ten minutes or 60 minutes at 100°C.

To test the effect of pH on killing, the SCS was separated into 3 mL aliquots. Equal parts KH_2PO_4 and K_2HPO_4 were combined to make a buffer. The aliquots were prepared as follows: aliquot 1, no buffer added, pH 3.7; aliquot 2, added 1mL buffer, pH 4.8; aliquot 3, added 2mL buffer, pH 5.74; aliquot 4, added 3mL buffer, pH 6.1. MRS was then added to each aliquot to bring the concentration of SCS to 50% in each, as follows: 1 mL aliquot 1 plus 1 mL MRS; aliquot 2 plus 2 mL MRS; aliquot 3 plus 1 mL MRS; aliquot 4, no MRS added. Two mL of each aliquot were added to 2mL NU14 for the experiment.

To test the effect of pH alone, I made an acidic solution of MRS and lactic acid. I used DL-lactic acid to make a lactic acid solution with the concentration of 65mM. This had a pH of 2.27. I added this solution to MRS until the mixture had a pH of 3.9. The final concentration of lactic acid in this mixture was 43.3mM.

To test the effect of catalase on SCS, I added catalase to LC040 SCS to a concentration of 2.5mg/ml, then let it sit at 37°C for three hours before use.

To test for a co-operative effect of lactic acid and hydrogen peroxide, I made the solution of MRS and lactic acid as described above, then added hydrogen peroxide until I had a final concentration of 10 mg/ml.

Lawn Competition Assay

LC040 and uropathogens were grown as described above. All uropathogen cultures were standardized to an O.D. of 1.000. 700µl of TSB were added to a TSA plate, 50µl of uropathogen were added to the TSB, the liquid was spread over the plate, and the plate was allowed to dry. I made a 10x concentration of the LC040 culture. Once the plate was completely dry, I added a 10µl spot of LC040. A 10µl spot of MRS broth was the negative control. Once the spots had completely dried, the plate was incubated at 37°C in CO_2 for 24 hours. The results were recorded and characterized.

Ethidium Bromide Assay

Uropathogens and LC040 were grown as previously described. LC040 SCS was separated via centrifugation and filter-sterilized. I mixed 450µl uropathogen and 450µl SCS in a microcentrifuge tube and let each duplicate mixture sit at the bench for 20 minutes, 1 hour, 2 hours, 4 hours, or 24 hours. This enabled me to determine which amount of time to use in future experiments with each uropathogen. The activity of SCS may be apparent within 20 minutes in some uropathogens, while for others the mixture should sit for up to 24 hours. I then diluted ethidium bromide 1:10 and added 100µl to the

tube, let the mixture sit for 5 minutes, performed a series of washes with PBS to remove the unbound ethidium bromide, and transferred the final mixture to a clean tube. I photographed the result under UV light. If DNA is exposed to the mixture, ethidium bromide will have bound and the liquid will glow under UV light. For a positive control, I heated the uropathogen at 100°C for 10 minutes and added TSB. For a negative control, I added MRS instead of SCS.

CHAPTER THREE:

RESULTS

Background

Summer intern Nikita Patel, under the supervision of Travis Price, spotted *L. crispatus* isolates onto lawns of *E. coli* strains (including several strains of UPEC) and observed no *E. coli* growth in the spotted zone. To characterize this behavior, Travis mixed strains of UPEC strain NU14 with filter-sterilized spent culture supernatants (SCS) of three different isolates each of three different *Lactobacillus* species (*L. crispatus, L. jensenii,* and *L. gasseri*) and of another commonly isolated urinary bacterium (*Gardnerella vaginalis)* (Figure 1). Two hours of incubation with *L. crispatus* SCS reduced UPEC's colony forming units (CFU) per milliliter (mL) by approximately 6 orders of magnitude. In contrast, it took 24 hours of incubation with SCS from the other two *Lactobacillus* species to reduce CFU/ml by two orders of magnitude. *G. vaginalis* SCS had no effect that does not depend on cell-cell contact. Thus, Travis determined that *L. crispatus* exhibits a species-specific bactericidal activity. At the time, we believed this activity was unlikely to result strictly from its production of lactic acid and hydrogen peroxide as the other *Lactobacillus* species also produce these two compounds. Furthermore, this bactericidal activity does not require cell-to-cell contact, as the SCS sufficed to kill UPEC. I tested the hypothesis that *L. crispatus* produces some secreted

bactericidal molecule and that the presence of another bacterium is unnecessary to stimulate its production.

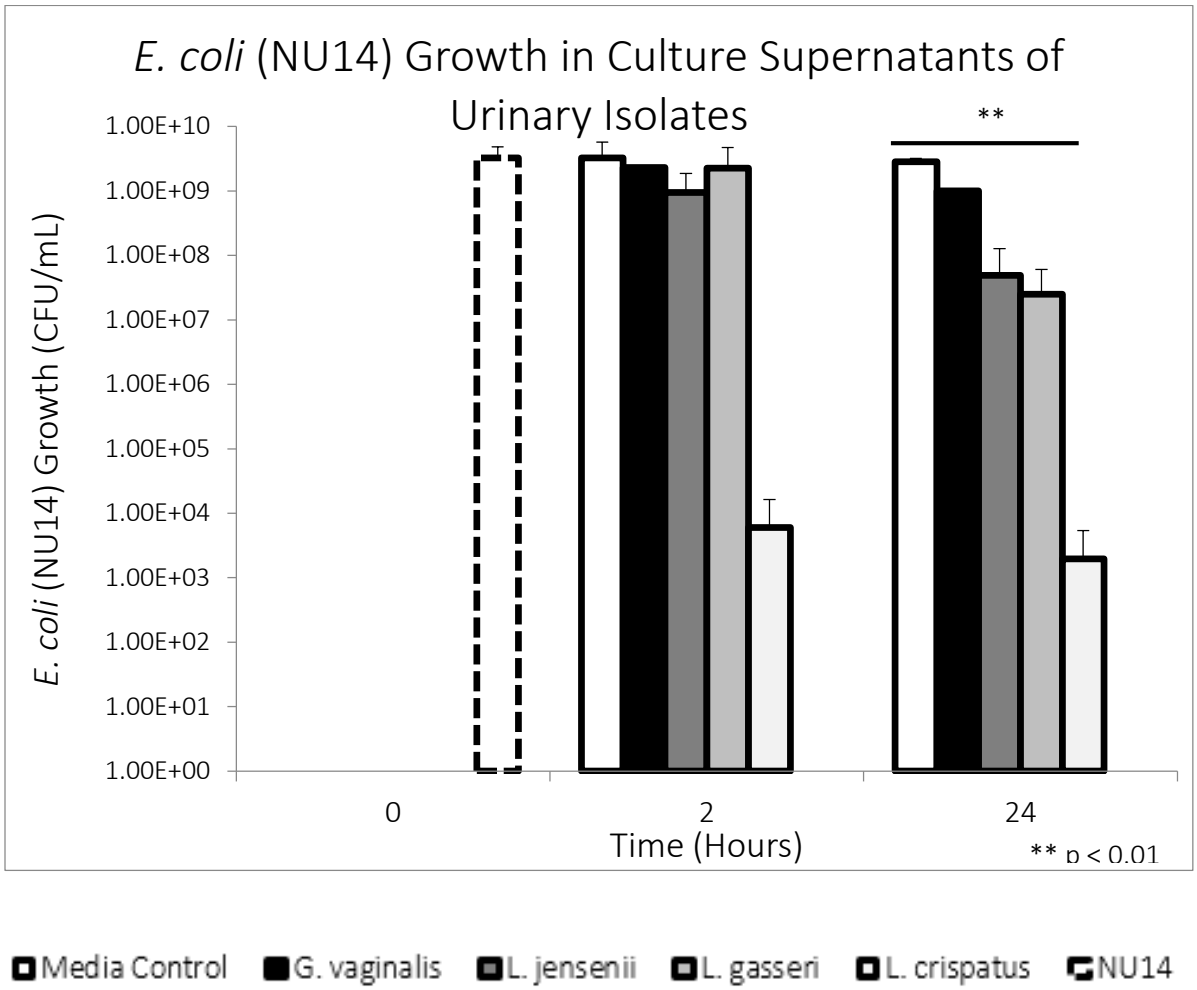

Figure 1: *E. coli* survival in culture supernatant of urinary isolates over time. Each bar shows the survival, in logs, of *E. coli* 2 or 24 hours after the addition of spent culture supernatant from one of the listed bacteria. Each bar is an average of several experiments using multiple isolates of the particular bacteria. The dotted bar represents the amount of *E .coli* living in the culture before the addition of supernatant.

Screening Urinary Isolates Against Uropathogens

Screening more urinary isolates of *L. crispatus* for their bactericidal activity would enable us to separate them into categories depending on whether they have high or

low activity. If necessary, the genomes of high and low activity isolates could be sequenced and those sequences could be compared for genetic differences that could account for their difference in activity.

I compared the bactericidal capacity of three different *L. crispatus* isolates (Figure 2). Of the three, LC040 had the greatest effect, reducing UPEC CFU/ml by about four logs after two hours incubation. By 24 hours, both LC040 and LC044 reduced CFU/ml below the level of detection. In contrast, LC020 had no significant activity against UPEC. I conclude that urinary isolates of *L. crispatus* can exhibit different levels of bactericidal activity. Because LC040 exhibited the strongest activity, I use it as my positive control. Because LC020 exhibited the least activity, I use it as a negative control or a comparison isolate where one is needed.

I found a urogenital *L. crispatus* strain ATCC-33197 in our lab's strain collection and compared its activity to that of LC040 (Figure 3). ATCC-33197 has bactericidal activity similar to that of LC040.

If LC040 has similar activity against other uropathogens, I would be able to determine whether clear differences exist between affected and unaffected groups of uropathogens. This is the first step in elucidating the type of activity that is involved. Some known bactericidal molecules target the cell wall or membranes. These tend to separate Gram-positive and Gram-negative bacteria broadly into susceptible or resistant groups.

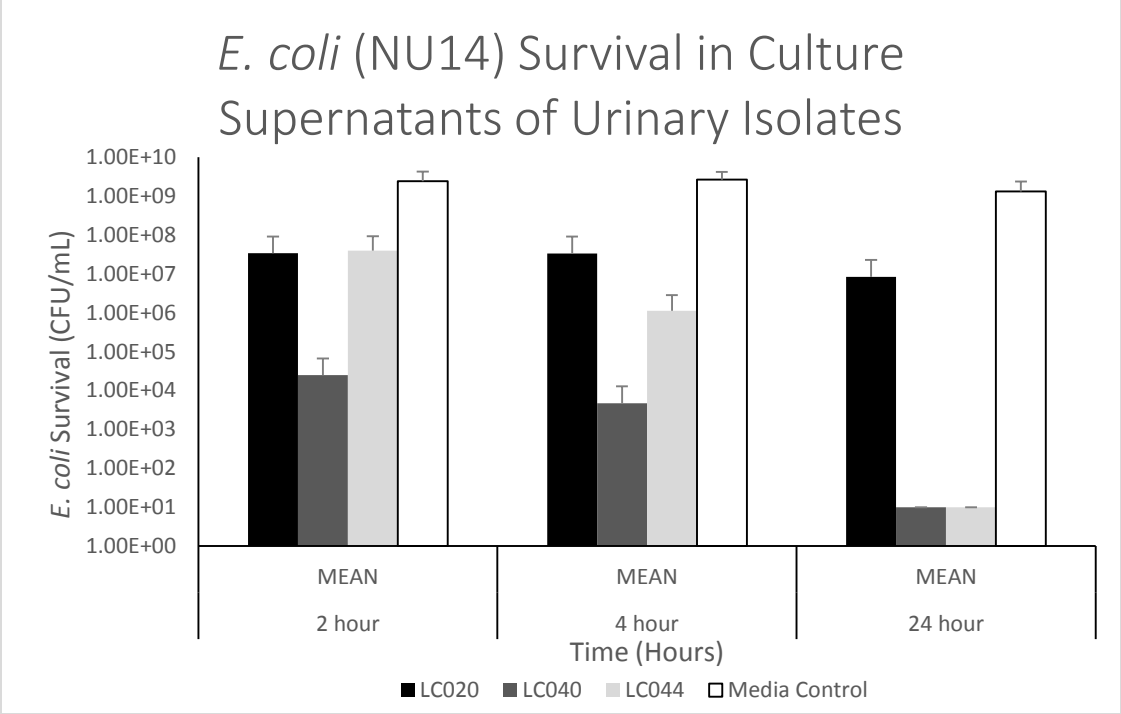

Figure 2: *E. coli* survival in culture supernatants of three *L. crispatus* urinary isolates over time. Each bar shows *E.* coli survival after the addition of spent culture supernatant. LC020, LC040, and LC044 are clinical isolates of *L. crispatus*. This graph shows averaged data from three experiments.

To screen a prioritized set of uropathogens and other urinary isolates for their sensitivity to LC040, I used the lawn competition assay used previously by Travis Price and Nikita Patel (Table 1). No clear pattern emerged between sensitive and resistant uropathogens. In addition to the Gram-negative *E. coli*, LC040 killed a number of Gram-positive Firmicutes, including multiple *Staphylococcus* species and *Streptococcus anginosus*. However, LC040 did not kill *Klebsiella pneumoniae*, a close relative of *E. coli*, nor did it kill *Enterococcus faecalis* or *Streptococcus agalactiae*, relatives of the sensitive Firmicutes.

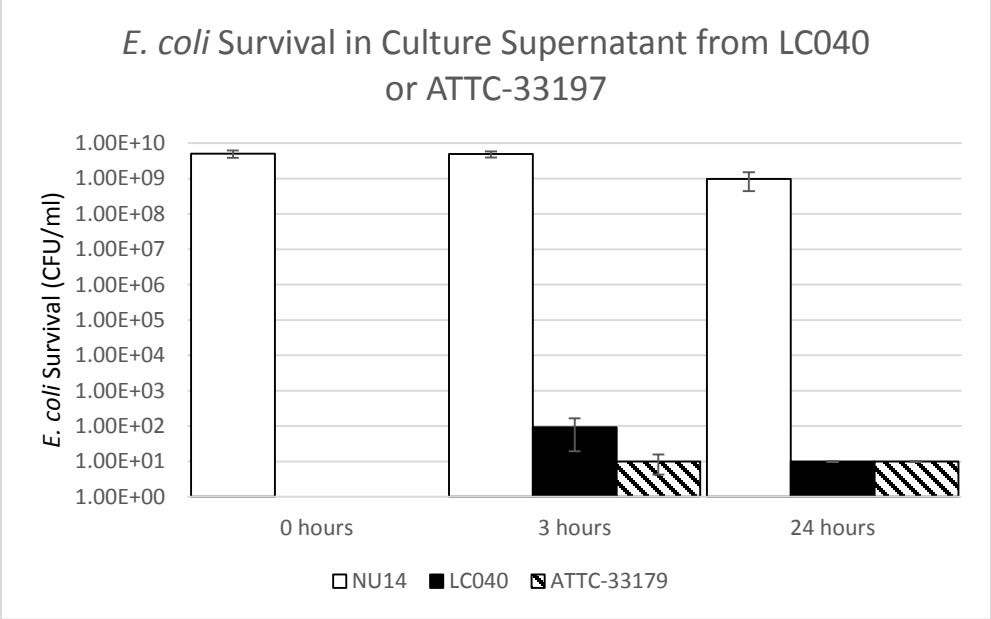

Figure 3: *E. coli* survival in supernatants from LC040 and ATCC-33197 over time. "NU14" is the control with media added.

	Killing on Plate	Number of Isolates Tested
E. coli	+	5
Proteus mirabilis	+	1
Klebsiella pneumoniae	-	3
Staphylococcus aureus	+	7
Staphylococcus haemolyticus	+	2
Streptococcus agalactiae	+ / -	2 / 2
Streptococcus anginosus	+	2
Enterococcus faecalis	+ / -	1 / 3
Candida albicans	+	1

Table 1: Results of lawn competition assay on various urinary isolates. All isolates listed were tested two or more times. Each assay tested activity of LC040.

A noticeable ring formed around the spot of LC040 on a lawn of *Staphylococcus aureus*, indicating a substance able to diffuse through the agar. I therefore performed the

bacterial survival assay with *S. aureus* (Figure 4). LC040 killed *S. aureus* in liquid culture, but at a much slower rate than it killed UPEC. Using the lawn assay, I tested a number of different *S. aureus* strains, including several strains of MRSA (Table 2). All MRSA strains tested were killed by LC040 on the plate.

MW2
MRSA 2395
MRSA 2404
Newman
LAC

Table 2: list of *S. aureus* strains borrowed from Dr. Alonzo.

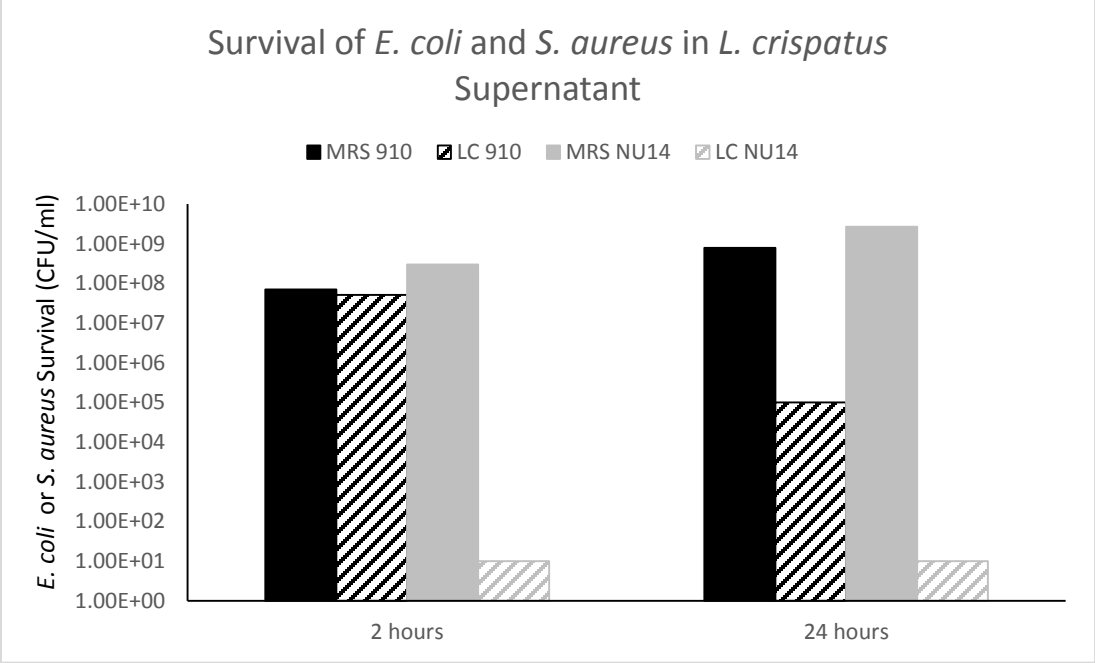

Figure 4: Comparison of survival of *E. coli* and *S. aureus* in *L. crispatus* culture supernatant over time. "LC 910" is the *S. aureus* with supernatant added, "LC NU14" is the *E. coli* with supernatant added, and "MRS 910" and "MRS NU14" are the controls with media alone added to each bacteria.

Because no clear sensitivity pattern has emerged and because the LC040-induced death of the Gram-negative bacterium *E. coli* did not resemble the LC040-induced death of the Gram-positive bacterium *S. aureus*, our current hypothesis is that LC040 possesses two distinct bactericidal mechanisms.

Traits of the Bactericidal Molecule

Because different *Lactobacillus* species and different *L. crispatus* isolates might produce differing amounts of lactic acid, I measured the pH of the SCSs of LC040 grown in MRS, LC040 grown in buffered MRS, LC020, two isolates of *L. gasseri,* and one isolate of *L. jensenii* (Table 3). The SCS of LC040 has a lower pH than that of LC020, which was in turn lower than that of the other *Lactobacillus* species isolates. However, the pH of LC040 grown in buffered media was comparable with that of LC020. A comparison of bacterial survival assay results using SCS from LC040 grown in buffered media and SCS from LC020 shows that by 24 hours both SCSs have killed the UPEC strain NU14 below the level of detection (Figure 5).

L. gasseri 59	4.2
L. gasseri 56	4.18
L. jensenii 847	5
LC040	3.72
LC040 in buffered MRS	4.3
LC020	4.1

Table 3: pH measurements for SCS of various bacterial cultures.

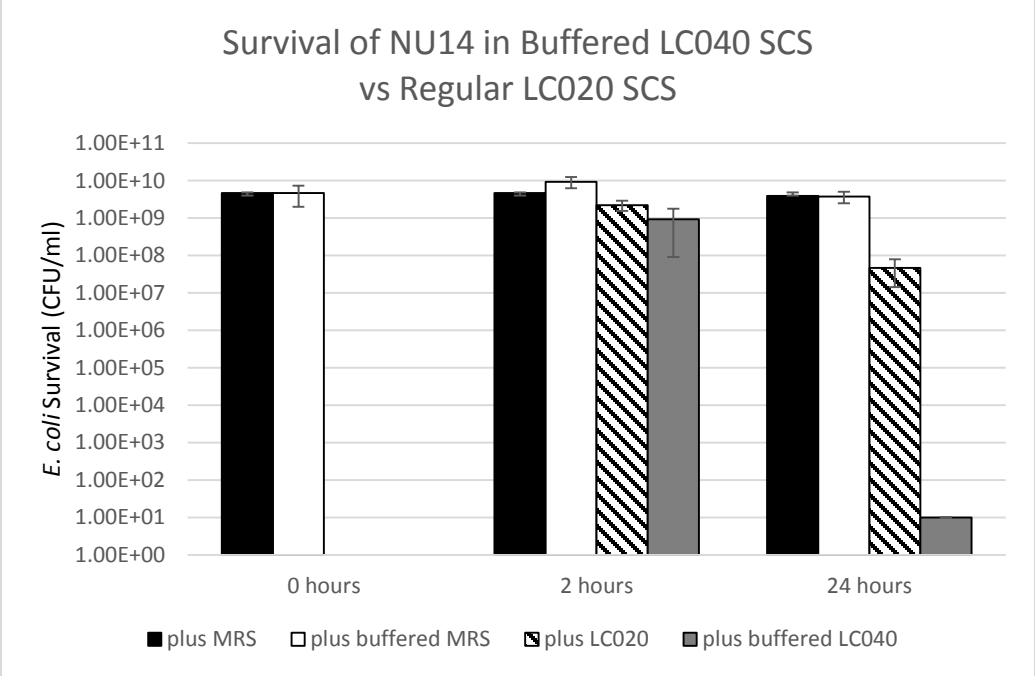

Figure 5: Comparison of *E. coli* survival in SCS from LC040 grown in buffered MRS and in SCS from LC020 grown in regular MRS. The buffer used was KH_2PO_4/K_2HPO_4. It was added while the media was made. LC040 grown in buffered MRS has a pH of 4.3, while LC020 grown in regular MRS has a pH of 4.1.

To determine whether the low pH of the SCS caused the death of NU14, I added different amounts of the buffer KH_2PO_4/K_2HPO_4 to aliquots of LC040 SCS to achieve several higher pHs and tested the survival of NU14 in these new solutions (Figure 6). Even at the lowest buffered pH, 4.78, the SCS lost its ability to kill *E. coli*. Combined with the data from Figure 5, I conclude that the bactericidal activity of LC040 SCS is pH-sensitive with a threshold between the pHs of 4.3 and 4.78. This could be because low pH is sufficient to kill NU14 or it could be that the putative bactericidal molecule is active below a certain pH.

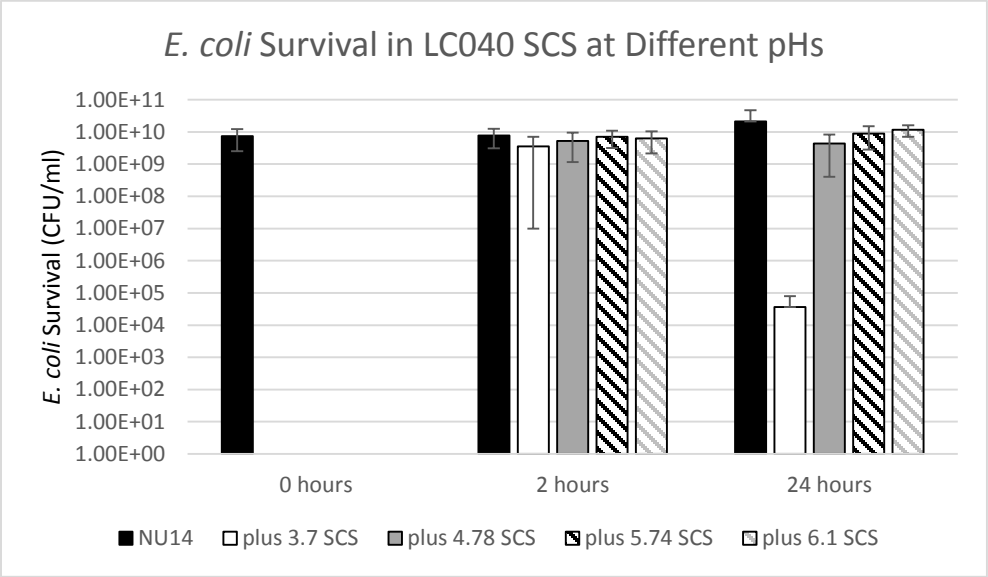

Figure 6: *E. coli* survival in buffered SCS from LC040. The buffer used is KH_2PO_4/K_2HPO_4. The untampered SCS had a pH of around 3.7. The buffered SCSs had pHs of 4.78, 5.74, and 6.1.

If pH alone is enough to kill NU14, a mixture of MRS and lactic acid should kill NU14. I tested this hypothesis using a mixture of MRS and 65mM DL-lactic acid, a concentration recommended by Atassi and Servin (14). This mixture had a pH of 3.9. I compared this mixture to LC040 SCS (Figure 7). By 24 hours, the lactic acid mixture had not killed NU14. Therefore, I conclude that NU14 is killed either by a co-operative effect of lactic acid and hydrogen peroxide or by a unique molecule.

To test whether hydrogen peroxide is required for the function of LC040 SCS, I added catalase to the SCS for a final concentration of 2.5 mg/ml and left the mixture for three hours, as in Kang et al., 2004 (30). The SCS with and without catalase killed NU14 equally well (Figure 8).

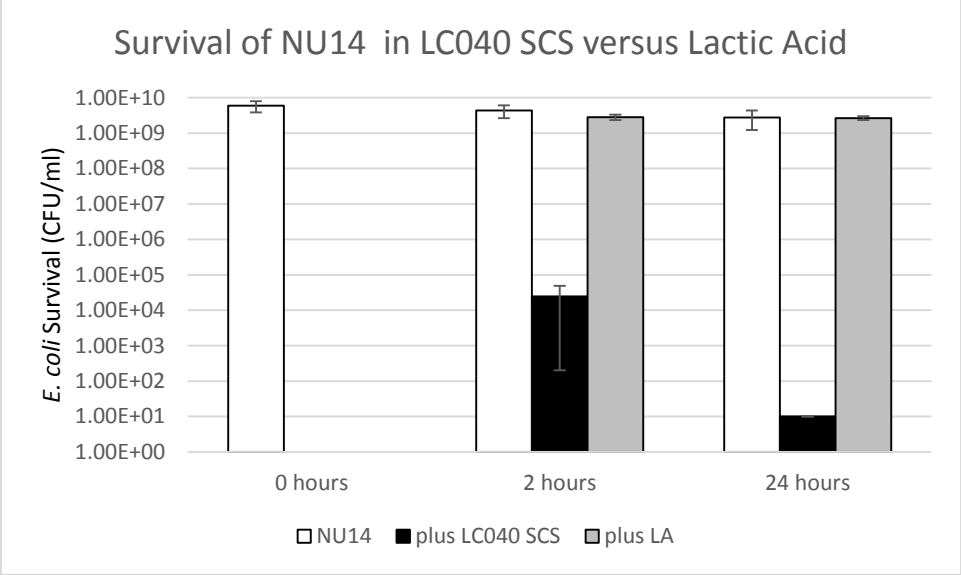

Figure 7: Survival of NU14 in LC040 SCS versus survival in a mixture of MRS and lactic acid. The pH of LC040 SCS was 3.7. The pH of the lactic acid mixture was 3.9.

To ensure that LC040 kills NU14 with a unique molecule/mechanism, I made a mixture of MRS, lactic acid, and hydrogen peroxide and tested its effect on NU14. This mixture contained a final concentration of 43.3 mM lactic acid as in an earlier experiment. I added hydrogen peroxide to a final concentration of 10 mg/ml. This concentration was one of several used in a similar experiment in Atassi and Servin (14). I compared the activity of this mixture to that of LC040 SCS (Figure 9). The combination of lactic acid and hydrogen peroxide was not sufficient to kill NU14. Therefore, I conclude that there must be one or more unique molecules in the supernatant that kills NU14.

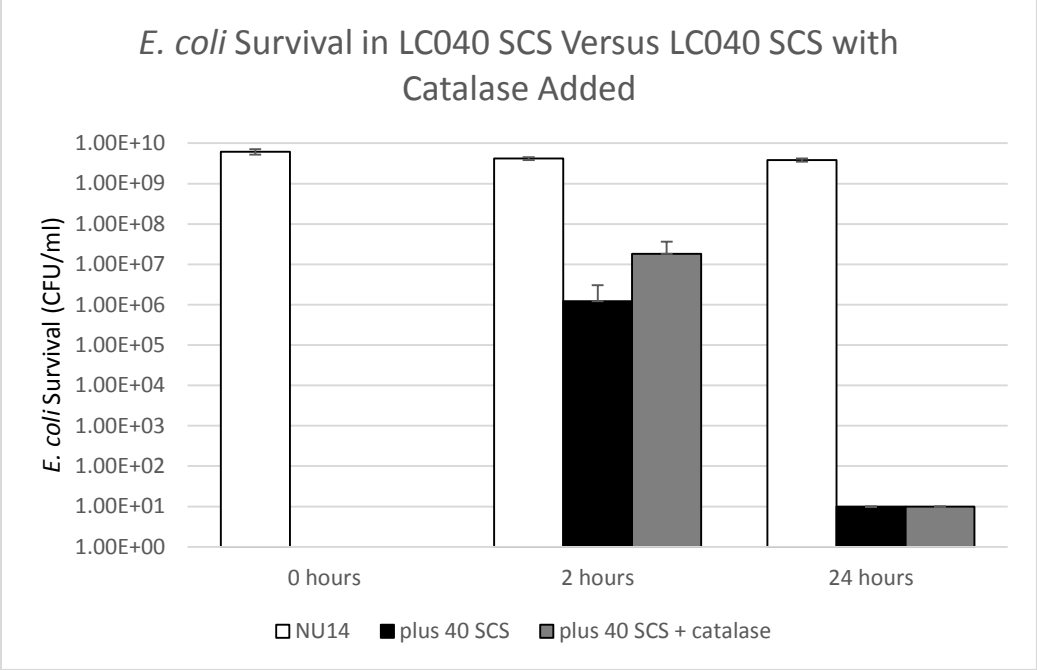

Figure 8: Survival of NU14 in LC040 SCS versus survival of NU14 in LC040 SCS with catalase added. Catalase was added to a final concentration of 2.5 mg/ml and the SCS was incubated at 37°C for three hours before being added to the NU14.

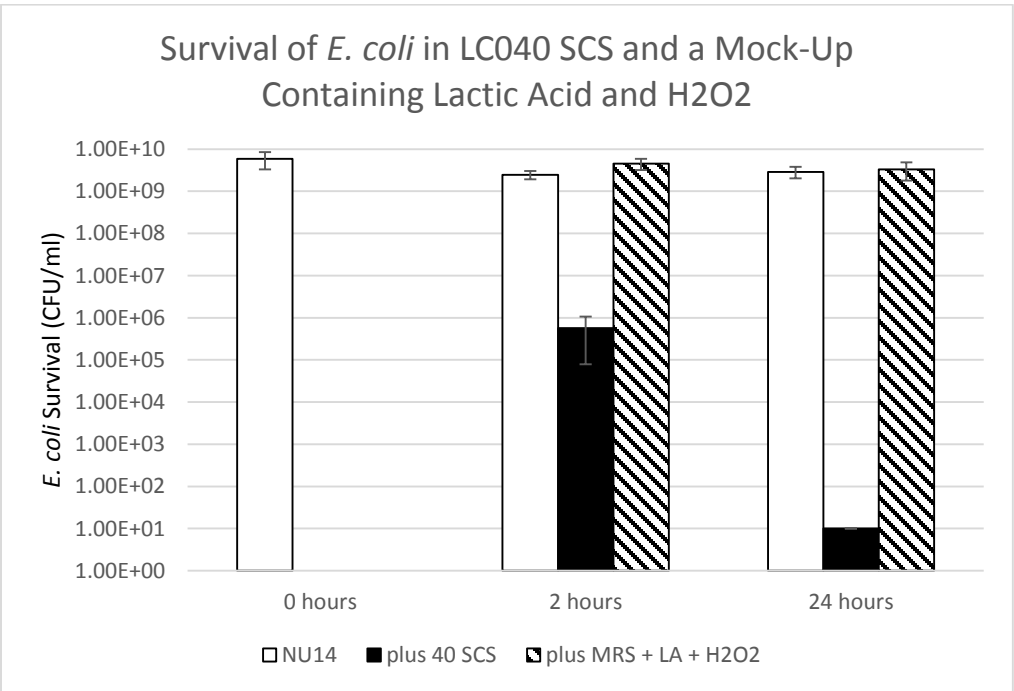

Figure 9: Survival of NU14 in LC040 SCS or in a combination of MRS, 43.3 mM lactic acid, and 10 mg/ml hydrogen peroxide.

Determining the stability of the active molecule will provide information concerning the nature of molecule. Bacteriocins and antimicrobial peptides are heat-stable (15). Some must be processed to become active (31). Although lactic acid and peroxide are heat stable, it is unlikely that they would become more active over time.

Heating supernatant at 100°C for 10 minutes should denature the majority of proteins. Therefore, I did another bacterial survival assay to determine whether the SCS was as functional after heating as before (Figure 10). I found that the heated and untreated SCS kill at comparatively the same rate. The difference in degree of killing at 3 hours was only present in one trial of the experiment, while the other two trials showed no difference. I also tested the activity of SCS after heating it for one hour at 100°C (Figure 11). I did not repeat this experiment. The single experiment showed that the SCS continues to function after this longer period of heat exposure. As heating did not destroy the activity, then the bactericidal molecule is unlikely to be a protein, unless it is a very stable one.

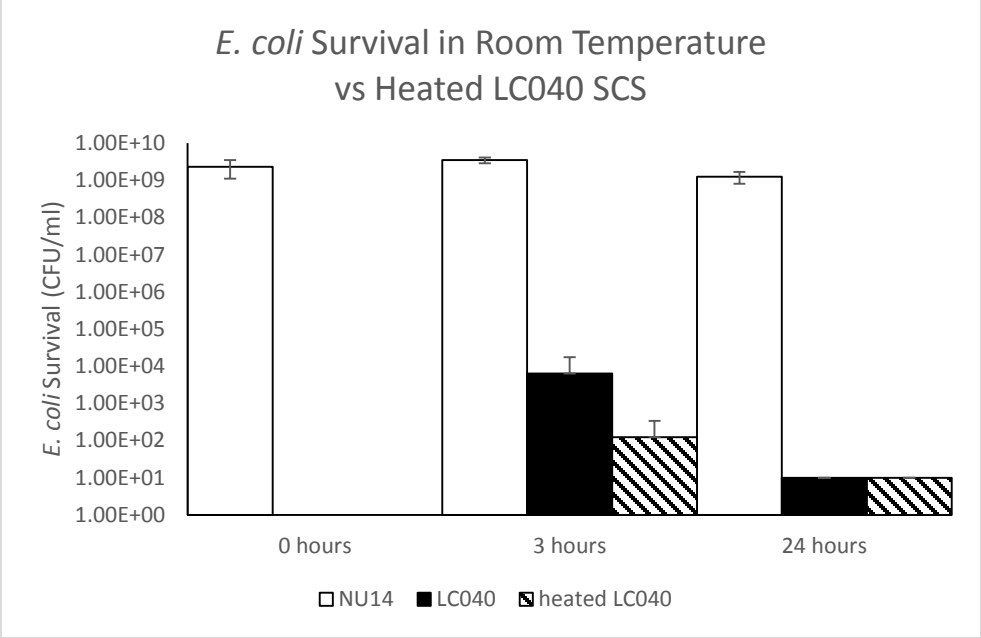

Figure 10: Comparison of *E. coli* survival in room temperature supernatant versus supernatant heated at 100°C for 10 minutes.

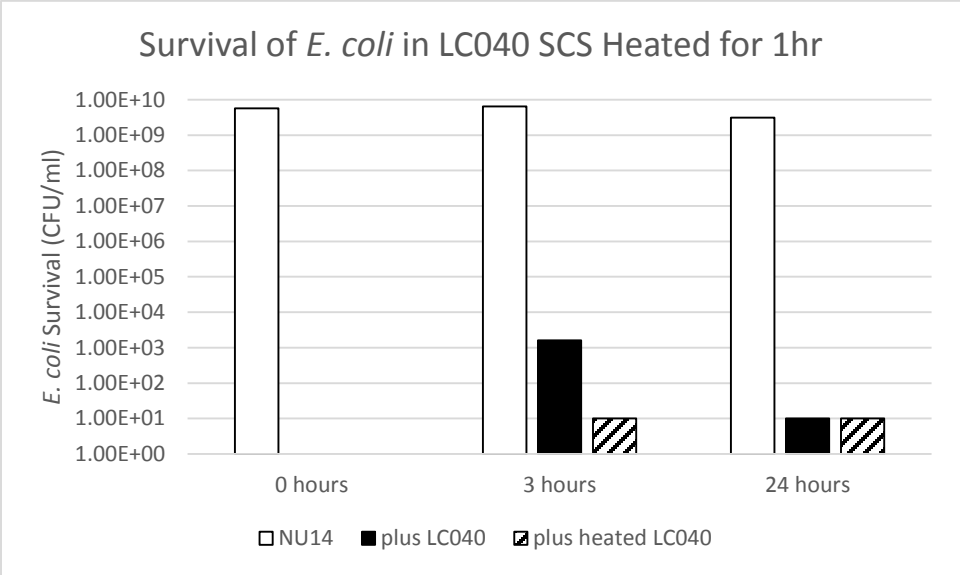

Figure 11: *E. coli* survival in LC040 SCS heated for 60 minutes at 100°C. Experiment performed only once.

Because some molecules lose their activity over time, I tested LC040 SCS and LC020 SCS after leaving them to sit for 24 hours. I found that SCS becomes more potent over time, regardless of whether it was stored at room temperature or 4°C (Figure 12). LC040 killed 9 logs of NU14 in 2 hours instead of killing 4 logs, and LC020 killed 9 logs in 24 hours instead of killing 2 logs. Because the *L. crispatus* cells had been filtered out and therefore cannot be making more of the molecule, the molecule itself must become more active. It may be activated by a cleavage event, either by another molecule or by the molecule itself. This is unlikely to be due to any change in pH over time (Table 3).

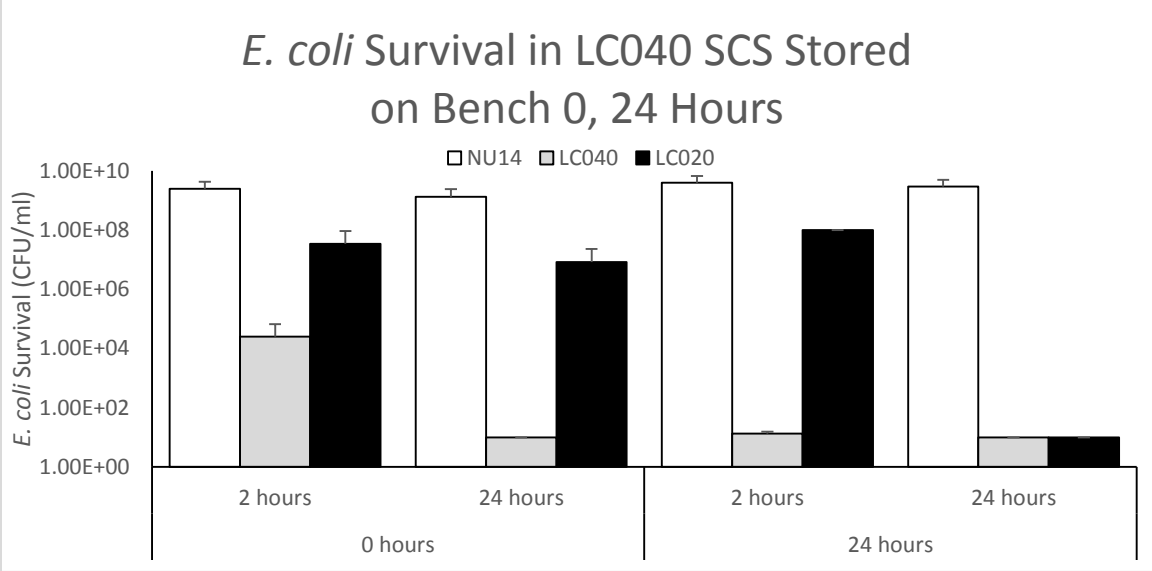

Figure 12: Comparison of *E. coli* survival in culture supernatant stored on the bench for 24 hours and culture supernatant used immediately after separation from *L. crispatus* cells. Data for *E. coli* survival in culture supernatant stored in the refrigerator for 24 hours closely resembles the bench data.

The Ethidium Bromide Assay

To test bactericidal activity against greater numbers of uropathogens, we needed a simpler, more rapid assay than the lawn competition assay. This assay also would be

useful to test bactericidal activity outside of this project, especially when determining the putative activity of other combinations of urinary isolates.

Travis established the ethidium bromide assay on the principle that since ethidium bromide binds to DNA, it can be used to detect the presence of free or accessible DNA in a mixture of cells, either because the cells have lysed or because their membranes have been compromised. Therefore, bound ethidium bromide indicates the presence of dead cells.

I modified the protocol to better remove the excess ethidium bromide through extra washes and swapping the mixture to a new tube before photographing. I then worked to determine how long a sample of NU14 needed to be exposed to LC040 SCS before enough DNA was present in the solution to be visibly bound by ethidium bromide (Figure 13). I did the same with *S. aureus* (Figure 14). However, when I measured the pH of the SCS, I realized that the acidity could kill enough bacteria to produce positive results in this assay. Originally I had planned to buffer the SCS to remove the effect of acidity and leave the effect of the molecule itself. However, I found that low pH was necessary to the function of the molecule, as described above (Figure 6), so I could not use the ethidium bromide assay to separate the activity of the acid from the molecule. This assay could be useful to screen for *L. crispatus* isolates that kill *E. coli* or to screen for killing interactions between other urinary isolates in our collection.

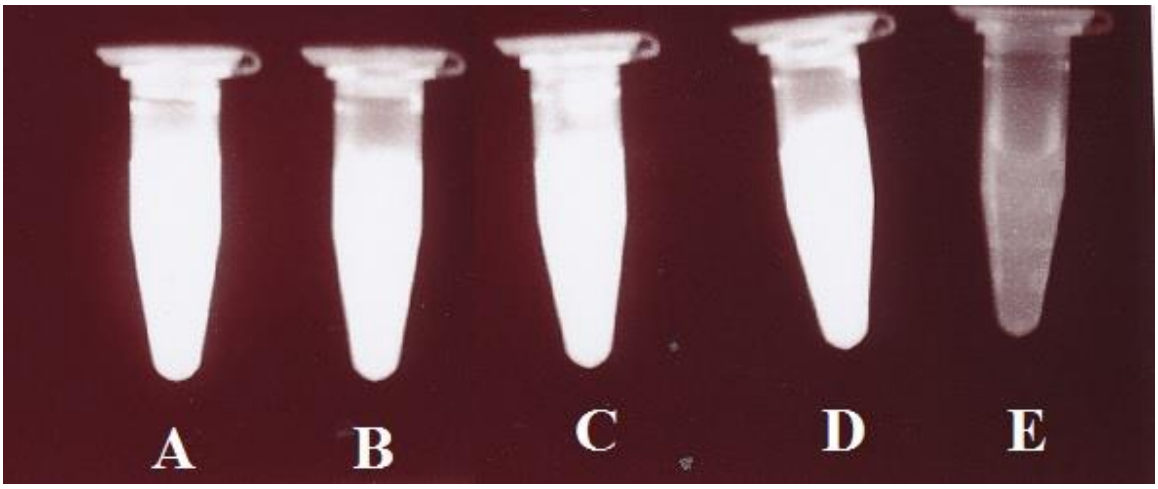

Figure 13: Ethidium bromide assay testing the effect of LC040 SCS on NU14. A) Positive control: NU14 heated at 100°C for 10 minutes; B-D) SCS added to NU14 for a series of time periods: B) 20 minutes, C) 1 hour, D) 2 hours; E) Negative control: MRS added to NU14.

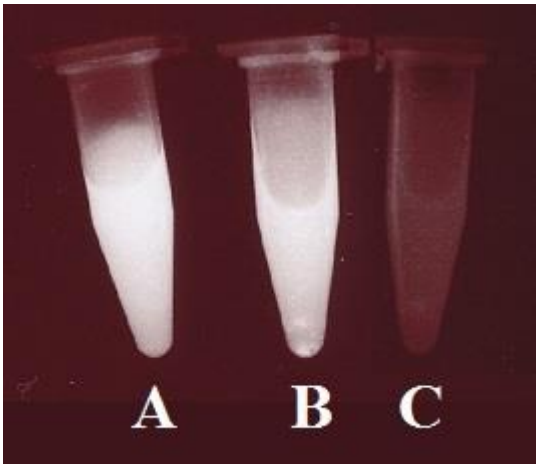

Figure 14: Ethidium bromide assay testing the effect of LC040 SCS on *S. aureus*. A) Positive control: *S. aureus* heated at 100°C for 10 minutes; B) SCS added to *S. aureus* for 24 hours; C) Negative control: MRS added to *S. aureus.*

CHAPTER FOUR:

DISCUSSION

Identity of the Bactericidal Molecule

Lactobacillus species kill or inhibit the growth of other bacteria through secretion of organic acids, hydrogen peroxide, bacteriocins, and other antimicrobial molecules (16). On the basis of the results of my research, I can eliminate some of the options and speculate on the identity of the molecule.

When I added buffer to the SCS of isolate LC040 to test its activity at various pHs, I found that activity was dependent on a low pH (Figure 6). LC040 SCS normally possesses at an average pH of 3.7. Grow of LC040 in buffered media resulted in an SCS with a pH of 4.3 and retention of its bactericidal activity (Figure 5). Somewhere between pH 4.3 and pH 4.8, the pH is too high and the activity is lost. Thus, the bactericidal activity of LC040 SCS requires a pH below 4.8. Giuseppe Pistone, a Master's student in our lab, will explore the range of pH between 4.3 and 4.8 to determine where the SCS loses its bactericidal activity.

Loss of activity above a certain pH could mean that low pH kills UPEC. However, a biologically relevant concentration of lactic acid, bringing the media to a pH of 3.9, was not sufficient to kill UPEC (Figure 7). Therefore, pH (caused primarily by excretion of lactic acid) must act together with some molecule or condition. Lactic acid

has been shown to be bactericidal in co-operation with hydrogen peroxide. Thus, I added catalase (which degrades hydrogen peroxide) to LC040 SCS, but it retained its bactericidal activity (Figure 8). A mixture of lactic acid and hydrogen peroxide added to media also did not kill UPEC (Figure 9). Therefore, hydrogen peroxide is not necessary for the bactericidal activity of LC040 SCS and a mixture of hydrogen peroxide and lactic acid is not sufficient.

Thus, the bactericidal activity of LC040 SCS is not due to lactic acid alone, nor is it due to hydrogen peroxide. Because the unknown molecule is heat-stable (Figure 10), it is very unlikely to be a protein, as boiling for 10 minutes would denature most proteins. Bacteriocins are heat-stable, so the bactericidal molecule could be a bacteriocin. Bacteriocins are susceptible to proteases, so the next step in identifying the molecule should be to add a protease to the SCS. Because of the low pH, pepsin would be the best choice of protease. Most other proteases work best at a more neutral pH. Giuseppe intends to perform this experiment shortly.

Many bacteriocins are only active against a narrow range of bacteria closely related to the bacterium that secreted the bacteriocin (15). In the case of bacteriocins from *Lactobacillus* species, this narrow range is therefore within the Gram-positive phlya. However, lactic acid can permeabilize the outer membrane of Gram-negative bacteria (6). There are examples of *Lactobacillus* products that only kill Gram-negative bacteria in a narrow pH range or in the presence of high concentrations of lactic acid or another permeabilizing agent (7, 8). I speculate that this weakening of the Gram-negative outer membrane may be the reason lactic acid is necessary for the bactericidal activity of

LC040 SCS. However, it is also possible that the bactericidal molecule is modified in some way in the presence of lactic acid or cannot fold correctly at a more basic pH. Given the results of this project so far, I cannot make a firm conclusion.

High Performance Liquid Chromatography (HPLC) should be used to determine the size of the active molecule by fractionating the SCS and testing each fraction for bactericidal activity. If the molecule is both susceptible to protease and has a low molecular weight (<5 kDa), it may be a bacteriocin. The classification of bactericidal molecules can be complicated. For example, most bacteriocins have a narrow range of bactericidal activity confined to bacteria closely related to the bacterium that produces the bacteriocin (15). However, some bacteriocins have a wider molecular weight range. Class I and class II bacteriocins weigh less than five kilodaltons, but class III bacteriocins weigh greater than thirty kilodaltons (15). Where size is concerned, there does not seem to be a strict definition of what can be classified as a bacteriocin.

Lactobacillus species also produce bactericidal or inhibitory molecules that can be generally classified as antimicrobial peptides (16). If the molecule produced by LC040 is not sensitive to protease, it may not be a bacteriocin but may instead be a unique molecule. Lack of sensitivity to protease may also indicate that the molecule is not a peptide at all. After separating the SCS into fractions via HPLC, the active fraction should be sent for mass spectrometry analysis.

Molecule that kills S. aureus

When *L. crispatus* was spotted onto a lawn of UPEC, the killing zone was restricted to the spot itself. When *L. crispatus* was tested on a lawn of *S. aureus*, the killing zone extended out from the spotted inoculum, indicating diffusion of the bactericidal molecule. In the bacterial survival assay, however, LC040 SCS alone killed *S. aureus* to a lesser degree than it killed UPEC (Figure 4). These two differences suggest the possibility that UPEC and *S. aureus* are killed by different molecules/mechanisms. This is supported by the knowledge that many bactericidal molecules produced by *Lactobacillus* species have a narrow range of activity (16).

To determine whether *S. aureus* and UPEC are killed by different molecules, I would subject the LC040 SCS to the same conditions as I did for anti-UPEC activity (for example, boiling the SCS for 10 minutes or buffering the SCS). If this bactericidal activity was not heat-stable and/or pH independent, it would indicate that *S. aureus* was killed by a different molecule. After the anti-UPEC bactericidal molecule is identified and isolated, it could be tested against *S. aureus*. The molecule could also be removed from the LC040 SCS and the remainder could be tested against *S. aureus*.

LC020 v LC040

LC020 SCS and LC040 SCS have different levels of anti-UPEC activity (Figure 2). This could be because (1) they produce different amounts of the bactericidal molecule, (2) they produce different amounts of acid (LC040 SCS has a lower pH), or (3)

they produce different bactericidal molecules altogether. The first and second options seem more likely to me than the third.

To distinguish these three possibilities, I would propose to first perform the same experiments to test heat-stability and pH-dependence of the LC020 SCS. Once the bactericidal molecule in the LC040 SCS is identified, it should be possible to determine whether it is present in the LC020 SCS.

Lack of Killing Pattern

LC040 kills both Gram-negatives and Gram-positives on the plate in lawn survival assays (Table 1). However, even within the limited number of bacteria I tested, there are some noticeable questions. For example, LC040 killed every strain of *E. coli* tested, including lab strains of UPEC (NU14 and CFT073), and several urinary isolates, but LC040 did not kill the closely related *Klebsiella pneumoniae*. Within the Gram-positives, I found that some isolates of certain species (namely *Streptococcus agalactiae* and *Enterococcus faecalis*) could be killed while other isolates of the same species could not. In the case of *E. faecalis*, one isolate was killed and three were not, suggesting to me that I may have read the plates wrong for that one isolate. It can be difficult to tell whether killing has occurred on a spot or whether the lawn was diluted in that spot or the lawn was simply uneven. These experiments must be repeated and extended, something Giuseppe intends to do.

Future Directions

As he takes on this project, Giuseppe has begun to repeat some of my experiments and do some of his own. He has repeated many of my experiments using the UPEC strain CFT073 instead of NU14, and has found that LC040 SCS also kills CFT073. He has found that the bactericidal molecule remains stable after two weeks of storage. Through experiments focusing on many more time points than I sampled, he has found that the amount of *E. coli* killed by around two hours is not consistent, but the amount killed by six hours is much more consistent. This could explain why some of my experiments show much less killing at two hours than others.

There are many questions left to answer regarding this bactericidal molecule. What is the nature of the molecule and what is its mechanism of killing? Does the molecule require a low pH because lactic acid weakens the outer membrane of *E. coli* or because the molecule can only fold correctly at a low pH? Is there a cut-off pH after which the environment is too basic for the molecule to function or is the transition more gradual? Does a single molecule kill both Gram-negatives and Gram-positives, or are there more than one? Why is LC040 SCS more potent than LC020 SCS? My lab mates, Travis Price and Krystal Thomas-White have struck up collaborations to sequence the genomes of many of our urinary isolates, including *L. crispatus.* These sequenced genomes should help guide our efforts to identify the bactericidal molecule(s).

In conclusion, I found that some urinary isolates of *L. crispatus* produce a heat-stable, pH-dependent, bacteridical molecule that kills uropathogenic *E. coli.* Other members of the lab will continue to study it. Hopefully, the compound could be used as

an antimicrobial therapy to fight urinary tract infections. Alternatively, the bacterium itself could one day be used as a probiotic to fight urinary tract infections.

BIBLIOGRAPHY

1. Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., et al. (2006). Comparative genomics of the lactic acid bacteria. *PNAS*, 103, 15611-15616.

2. Valore, E. V., Park, C. H., Igreti, S. L., and Ganz, T. (2002). Antimicrobial components of vaginal fluid. *AJOG*, 187, 561-568.

3. Cadieux, P. A., J. P. Burton, E. Devillard, and G. Reid. (2009). Lactobacillus by-products inhibit the growth and virulence of uropathogenic Escherichia Coli. *Journal of Physiology and Pharmacology*, 60, 13-18.

4. Reid, G., Cook, R. L., and Bruce, A. W. (1987). Examination of strains of lactobacilli for properties that may influence bacterial interference in the urinary tract. *Journal of Urology,* 138, 330-335.

5. Ogawa, M., Shimizu, K., Nomoto, K., Tanaka, R., Hamabata, T., Yamasaki, S., Takeda, T., and Takeda, Y. (2001). Inhibition of in vitro growth of Shiga toxin-producing Escherichia coli O157:H7 by probiotic Lactobacillus strains due to production of lactic acid. *Int J Food Microbiol*, 68(1-2), 135-140.

6. Alakomi, H. L., Skyttä, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., and Helander, I. M. (2000). Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. *Appl Environ Microbiol*, 66, 2001-2005.

7. Gopal, P. K., Prasad, J., Smart, J., Gill, H. S. (2001) *Int J Food Microbiol*, 67, 207-216.

8. Stevens, K. A., Sheldon, B. W., Klapes, N. A., and Klaenhammer, T. R. (1991). Nisin treatment for inactivation of Salmonella species and other gram-negative bacteria. *Applied and Environmental Microbiology*, 57, 3613-3651.

9. St Amant, D. C., Valentin-Bon, I. E., Jerse, A. E. (2002). Inhibition of Neisseria gonorrhoeae by Lactobacillus species that are commonly isolated from the female genital tract. *Infect Immun*, 70, 7169-7171.

10. Gupta, K., Stapleton, A. E., Hooton, T. M., Roberts, P. L., Fennell, C. L., and Stamm, W. E. (1998) Inverse association of H_2O_2-producing Lactobacilli and vaginal Escherichia coli colonization in women with recurrent urinary tract infections. *J Infect Dis*, 178, 446-450.

11. Klebanoff, S. J., Hilier, S. L., Eschenbach, D. A., and Waltersdorph, A. M. (1991). Control of the microbial flora of the vagina by H_2O_2-generating Lactobacilli *J Infect Dis*, 164, 94-100.

12. Hillier, S. L., Krohn, M, A., Rabe, L. K., Klebanoff, S. J., and Eschenbach, D. A. (1993). The normal vaginal flora, H_2O_2-producing Lactobacilli, and bacterial vaginosis in pregnant women. *Clin Infect Dis*, 16, S273-S281.

13. Aroutcheva, A., Gariti, D., Simon, M., Shott, S., Faro, J., Simoes, J.A., Gurguis, A., Faro, S. (2001). Defense factors of vaginal lactobacilli. *American Journal of Obstetrics and Gynecology*, 185: 375-379.

14. Atassi, F. and Servin, A. L. (2010). Individual and co-operative roles of lactic acid and hydrogen peroxide in the killing activity of enteric strain Lactobacillus johnsonii NCC933 and vaginal strain Lactobacillus gasseri KS120.1 against enteric, uropathogenic and vaginosis-associated pathogens. *FEMS Microbiol Letters*, 304, 29-38.

15. Parada, Jose Luis, Carolina Ricoy Caron, Adriane Bianchi P Medeiros, and Carlos Ricardo Soccol. (2007). Bacteriocins from Lactic Acid Bacteria: Purification, Properties and Use as Biopreservatives. *Brazilian Archives of Biology and Technology*, 50.3, 521-42.

16. Servin, A. L. (2004). Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. *FEMS Microbiology Reviews*, 28, 405-440.

17. Zamfir, M., Callewaert, R., Cornea, P. C., Savu, L., Vatafu, I., and De Vuyst, L. (1999). Purification and characterization of a bacteriocin produced by Lactobacillus acidophilus IBB 801. *J Appl Microbiol*, 87, 923-931.

18. Ten Brink, B., Minekus, M., van der Vossen, J. M., Leer, R. J., and Huis in't Veld, J. H. (1994). Antimicrobial activity of lactobacilli: preliminary characterization and optimization of production of acidocin B, a novel bacteriocin produced by Lactobacillus acidophilus M46. *J Appl Bacteriol*, 77, 140-148.

19. McGroarty, J. A. and Reid, G. (1988). Detection of a Lactobacillus substance that inhibits Escherichia coli. *Can J Microbiol*, 34, 974-978.

20. Silva, M., Jacobus, N. V., Deneke, C., and Gorbach, S. L. (1987). Antimicrobial substance from a human Lactobacillus strain. *Antimicrob Agents Chemother*, 31, 1231-1233.

21. Vescovo, M., Scolari, G. L., Caravaggi, L., and Bottazzi, V. (1993). Antimicrobial compounds from Lactobacillus casei and Lactobacillus helveticus. *New Microbiol*, 16, 171-175.

22. Bernet-Camard, M., Liévin, V., Brassart, D., Neeser, J., Servin, A. L., and Hudault, S. (1997). The human Lactobacillus acidophilus strain LA1 secretes a nonbacteriocin antibacterial substance(s) active in virto and in vivo. *Applied and Environmental Microbiology*. 63, 2747-2753.

23. Kalyoussef, S., Nieves, E., Dinerman, E., Carpenter, C., Viswanathan, S., et al. (2012) Lactobacillus proteins are associated with the bactericidal activity against E. coli of female genital tract secretions. *PLOS One*, 7.

24. NIDDK. "Urinary Tract Infection in Adults." National Institute of Diabetes and Digestive and Kidney Diseases, May 2012. Web.

25. Ronald, A. (2002). The etiology of urinary tract infection: traditional and emerging pathogens. *The American Journal of Medicine*, 113 (1A), 14S-19S.

26. Pearce, M., Hilt, E., Rosenfeld, A., Zilliox, M., Thomas-White, K., Fok, C., et al. (2014). The Female Urinary Microbiome: a Comparison of Women with and without Urgency Urinary Incontinence. *mBio*.

27. Price, Travis. *The Contribution of the Female Urinary Microbiota to Lower Urinary Tract Symptoms.* MS Thesis. Loyola University Chicago, 2015.

28. Herthelius, M., S. L. Gorbach, R. Moellby, C. E. Nord, L. Pettersson, and J. Winberg. (1989). Elimination of Vaginal Colonization with Escherichia Coli by Administration of Indigenous Flora. *Infection and Immunity*, 57.8, 2447-451.

29. Todar, Kenneth. (2008). "Lactic Acid Bacteria." *Online Textbook of Bacteriology.* Web.

30. Kang, D.-K., Oh, H. K., Ham, J.-S., Kim, J. G., Yoon, C. H., Ahn, Y. T., and Kim, H. U. (2004). Identification and characterization of hydrogen peroxide-generating Lactobacillus fermentum CS12-1. *Asian-Aust J Anim Sci*, 18, 90-95.

31. Izadpanah, Arash, and Richard L. Gallo. (2005). Antimicrobial Peptides. *Journal of the American Academy of Dermatology*, 52.3, 381-89.

32. Brogden, Kim A. (2009). Antimicrobial Peptides: Pore Formers or Metabolic Inhibitors of Bacteria? *Nature Reviews: Microbiology*, 3, 238-50.

33. Kwok, Louisa, Ann E. Stapleton, Walter E. Stamm, Sharon L. Hillier, Cheryl L. Wobbe, and Kalpana Guptas. (2006). Adherence of Lactobacillus crispatus to Vaginal Epithelial Cells from Women With or Without a History of Recurrent Urinary Tract Infection. *The Journal of Urology*, 176, 2050-054.

34. Ojala, T., Kankainen, M., Castro, J., Cerca, N., Edelman, S., Westerlund-Wikstrom, B., et al. (2014). Comparative genomics of Lactobacillus crispatus suggests novel mechanisms for the competitive exclusion of Gardnerella vaginalis. *BMC Genomics*, 15: 1070.

35. Whiteside, Samantha A., Hassan Razvi, Sumit Dave, Gregor Reid, and Jeremy P. Burton. (2015). The Microbiome of the Urinary Tract - a Role beyond Infection. *Nature Reviews: Urology*, 12, 81-90.

VITA

Katherine Diebel was born in Chicago, IL and raised in Western Springs, IL. She became interested in studying biology in middle school. She attended Luther College in Decorah, IA, graduating in 2012 with a Bachelor of Art in biology and mathematics. While at Luther, she interned for a month in a *C. elegans* lab at University of Iowa. That lab experience, along with her course work at Luther, sparked her interest in microbiology and her desire to work in a laboratory specifically related to microbiology.

Upon finishing her MS in microbiology and immunology, she plans on joining the workforce, preferably in a health care context.