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Testing the Inhibition of Uropathogenic Escherichia coli by Urinary Lactobacilli

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Testing the Inhibition of Uropathogenic *Escherichia coli* by Urinary Lactobacilli

by

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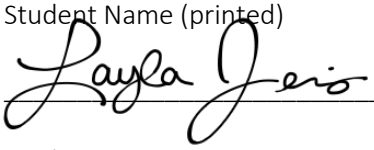

Student Signature

04/28/2023

Date

Dedication

This capstone is dedicated to my loving parents, my sister, and my brothers. Thank you for providing me with the home and endless love that support me to this day. I also dedicate this to my lab partner, Rayan Haque, who tirelessly worked with me this past year through all the ups and downs of these assays. And of course, I dedicate this work to the entirety of the Sysoeva Lab: you immediately welcomed me with open arms, guidance, and mentorship. I look forward to all the discoveries we shall make together in these next few years.

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Abstract

Urinary tract infections (UTIs) are some of the most common bacterial infections worldwide, with over 404.6 million infections occurring annually. The urinary tract hosts a urinary microbiome, or urobiome, that has been observed to play a role in both urinary health and disease, including UTIs. Lactobacilli have been identified as abundant commensals in the female urobiome, and their abundance and identity have been shown to correlate with a predisposition to recurrent UTIs. A previous project was successful in isolating several commensal *Lactobacillus* strains from human catheterized urine samples and demonstrated that most of them inhibit growth of the uropathogenic strains, such as the dominant uropathogenic *Escherichia coli* (UPEC). The goal of this project was to further test and understand how lactobacilli inhibit growth of UPEC. We hypothesized that altered acid resistance of test strains would affect their susceptibility to the inhibition by lactobacilli and their secretions. We tested for this inhibition in well-diffusion inhibition assays. Our results showed greater inhibition by full-cell cultures of lactobacilli compared to cell-free filtrates. Future work will continue to examine how lactobacilli inhibit UPEC, and how lactobacilli might be used as probiotic and prophylactic treatment.

Introduction

Urinary tract infections (UTIs) are one of the most common bacterial infections worldwide, with an estimated 404.6 million infections occurring annually (Zeng et al. 2022). UTIs are defined as an infection occurring at any point in the urinary tract that consists of the urethra, bladder, ureters, and kidneys. UTIs are caused by a variety of Gram-negative and Gram-positive bacteria, as well as some fungi (Flores-Mireles et al. 2015). The dominant infectious agent of UTIs is uropathogenic *Escherichia coli* (UPEC), accounting for over 80% of infections that are contracted outside a healthcare facility (Mobley, Donnenberg, and Hagan 2009). UPEC and other bacterial agents typically infect via an ascending route, starting at the urethra and traveling up to the bladder. In some cases, bacteria can continue to move up the ureter and into the kidneys, leading to the onset of more severe infections like pyelonephritis that can cause renal scarring or urosepsis (Bacheller and Bernstein 1997). Throughout their lifetime, males will have a 12% chance of developing a UTI and females will have a 40% chance (Kaur and Kaur 2020).

Presently, symptomatic UTIs are treated with antibiotics, but this presents several issues. Antibiotic use leads to the development of resistance in uropathogens. Commensal species can also be altered and permit new infectious agents to occupy emptied or weakened niches (Flores-Mireles et al. 2015). Moreover, during infection, UPEC can colonize host epithelial cells of the urinary tract, allowing it to evade antibiotic treatment and immune defenses (Kostakioti, Hultgren, and Hadjifrangiskou 2012). In children, serious, long-term effects of complicated UTIs include hypertension and progression to end-stage renal disease (Olson et al. 2017). These morbidities can manifest in all ages and sexes of individuals who suffer from UTIs. Concerns have warranted research into more sustainable remedies and prophylaxis of UTIs, including the study of a urinary microbiome, or urobiome.

Microbiomes found throughout the body are known to play a role in human health and disease (Pflughoeft and Versalovic 2012). Various studies of the urobiome have suggested a link between the urobiome and urinary health (Fouts et al. 2012; Nelson et al. 2010; Pearce et al. 2014). It was long assumed that urine was sterile due to traditional urine culture techniques (Zorc, Kiddoo, and Shaw 2005). However, the development of expanded quantitative urine culture (EQUC) procedures in the past decade have allowed for the detection and characterization of primarily bacteria that make up the urobiome (Hilt et al. 2013). In adult females, the urobiome has been repeatedly observed to be dominated by genera *Gardnerella, Corynebacterium, Streptococcus, Staphylococcus,* and, notably, *Lactobacillus* (Wojciuk et al. 2019). It should be noted that a recent study reclassified *Lactobacillus* into 23 novel genera, and the strains we test here remain in the *Lactobacillus* genus (Zheng et al. 2020; Qiao et al. 2022).

The *Lactobacillus* genus is made up of non-spore-forming, Gram-positive, non-motile, catalase-negative bacteria. In addition to the female urobiome, they are commonly found in the microbial communities of the mouth, vagina, and gastrointestinal (GI) tract. Most species are facultative anaerobes; as their name suggests, lactic acid is the dominant metabolic end-product due to their fermentation of glucose (Slover and Danziger 2008). They also produce acetate, ethanol, CO_2, formate, and succinate, though in lower concentrations than that of produced lactic acid. Fermentation products present in high concentrations around the cell are known to inhibit bacterial growth (Salvetti, Torriani, and Felis 2012). Lactobacilli are also known to produce bacteriocins, which are ribosomally synthesized peptides (<70 amino acids) that exhibit antimicrobial action (Zheng et al. 2014; da Silva Sabo et al. 2014). This action has been observed *in vivo* by bacteriocins produced by *Lactobacillus salivarius* (Corr et al. 2007). Studies have also

examined the use of lactobacilli as probiotics for gastrointestinal and genitourinary health (Reid and Burton 2002).

Among others, a recent study by Vaughn and collaborators (2021) was successful in isolating several species of *Lactobacillus* from catheterized urine specimens of adult, postmenopausal females, according to EQUC procedures. The obtained isolates included *Lactobacillus gasseri* and *Lactobacillus delbrueckii* strains. A previous project of this laboratory tested these isolates for their ability to inhibit various uropathogens *in vitro,* including UTI model strains and clinical isolates. Both *L. gasseri* 5006-2 S1 and *L. delbrueckii* 5010-2 S9 inhibited these species. The authors concluded that inhibition was due to a combination of factors derived from cells and low pH (Johnson et al. 2022).

On its way to urinary tract, UPEC passes through the vaginal opening, which is a well-described, high-acidity environment (Brumbaugh and Mobley 2012). Surviving the fluctuating pH of various environments to maintain infection led us to address how UPEC responds to acid stress. In *E. coli* there are several characterized pathways to resist acid stress including *btsT*, *ypdAB*, *phoPQ, evgAS,* the *gad* and *cad* systems, and recently described *sdaAB* pathway. *btsT* regulates the uptake of pyruvate as a carbon source via a pyruvate/H^+ symporter (Kristoficova et al. 2018). *ypdB* is a member of the *ypdAB* two-component system that promotes *btsT* expression (Behr, Fried, and Jung 2014; Fried, Behr, and Jung 2012). *phoPQ* is a two-component system that regulates genes such as those associated with acid-stress responses (Miyashiro and Goulian 2007; Zwir et al. 2005). *evgAS* is a two-component system that has also been observed to activate genes related to acid-stress responses such as *gadA*, *gadB,* and *gadC* (Masuda and Church 2003). *cadA* is a member of the *cadAB* operon, which converts lysine to cadaverine by using H^+ when conditions

are acidic (Fritz et al. 2009). *sdaA* and *sdaB* have recently been observed to be a method of acid tolerance by L-serine deamination (Wiebe et al. 2022).

The primary aim of this project was to further characterize and understand how lactobacilli inhibit the growth of UPEC. We tested *E. coli* mutants that were previously shown to have elevated or decreased acid resistance. We hypothesized that altered acid resistance of test strains would affect their susceptibility to the inhibition by lactobacilli and their secretions.

Materials and Methods

MRS Broth and Plate Preparation

Preliminary work suggested that *E. coli* was sensitive to the preparation of Man Rogosa and Sharpe (MRS) media. Specifically, it was observed that the amount of time the medium was kept at 121°C for sterilization affected the density of lawn growth on MRS agar. Hazy, reduced lawn growth of *E. coli* resulted in hazier, ill-defined zones of inhibition that could not be as accurately quantified. When comparing autoclave settings of 20- and 35-minute sterilization times, qualitative and quantitative observations suggested that 20 minutes promoted richer *E. coli* lawn growth and more clearly defined zones of inhibition in well-diffusion inhibition assays. Subsequent preparation of all MRS media was performed according to this standard.

To prepare MRS broth, 25 g of MRS broth powder were combined with every 1 L of deionized (DI) water. The broth was transferred to glass bottles and appropriately autoclaved. Bottles of MRS broth were stored at ambient temperature until use.

To prepare plates, 25 g of MRS broth powder and 15 g of agar were combined with every 1 L of DI water. The agar mixture was appropriately autoclaved. To minimize variation in diffusion rates due to well height (agar thickness), plates were pipette-poured with 25 mL of agar to achieve a thickness of approximately 5 mm; this was verified across all plates.

Preliminary observations suggested that if agar plates were not adequately dried prior to running the assay, absorption of *E. coli* liquid cultures was noticeably reduced. Therefore, if plates were to be used the next day, plates were allowed to sit agar-side-down with lids removed in the biosafety cabinet for approximately 30 minutes to dry after the agar had set; plates were then stacked (sets of ~15) agar-side-down and kept at ambient temperature until use. If plates were to

be used after 2 – 3 days, this drying process was skipped, and plates were immediately stacked agar-side-down (sets of ~15) after the agar had set and were kept at ambient temperature until use.

Strains Used and Experimental Groups

Lactobacillus Isolates. This project utilizes the same *Lactobacillus* isolates used in the previous project: *L. gasseri* 5006-2 S1 and *L. delbrueckii* 5010-2 S9. Both strains were isolated from healthy, post-menopausal women; *L. delbrueckii* 5010-2 S9 came from one with a history of rUTI. Both strains possess several bacteriocin genes (Johnson et al. 2022).

Background, CFT073, and SVS144 Group. The previous project tested the two *Lactobacillus* strains for the inhibition of various UTI models and of one nonpathogenic background strain MG1655 of *E. coli* (Johnson et al. 2022); the background strain and one UPEC strain (CFT073) were retested in this project **(Table 1)**.

A recent study by the Cruz-Vera group (Worthan et al. 2022) identified a ribosomal mutant, uL22 (K90D), of *E. coli* strain SVS1144. Overall, the mutant strain demonstrated reduced efficiency in the translation of genes involved in acid-stress response systems. This seemingly corresponded with an increase in the expression of acid resistance genes, including those in the glutamate-dependent acid-stress response pathway (*gadA, gadB*) (Worthan et al. 2022). This project retested the background, CTF073, and wild type- and mutant-SVS1144 strains alongside each other for inhibition by both *Lactobacillus* strains. Cultures and filtrates of each *Lactobacillus* strain were tested separately.

UTI89 Group. Similar tests were repeated with UTI model, UTI89 (Hultgren et al. 1986; Beebout et al. 2022), and three other strains derived from UTI89 **(Table 1)**. Each possessed different knockouts of the previously mentioned genes related to acid-stress response. These

strains were simultaneously tested against *L. gasseri* cultures and filtrates. They were then tested against a citrate buffer.

Strain	Genotype/Description	Source
Background, CFT073, and SVS1144 Group		
MG1655	Background	Lab stock
CFT073	UTI model (pyelonephritis isolate)	(Mobley et al. 1990); Mehreen Arshad, Northwestern University
SVS1144-WT	W3110 λ tnaP tnaC-UGA tnaA'-'lacZ	(Worthan et al. 2022; Stewart and Yanofsky 1985); Luis Cruz-Vera, University of Alabama in Huntsville
SVS1144-K90D	Ribosomal uL22 (K90D) mutant	(Worthan et al. 2022); Luis Cruz-Vera, University of Alabama in Huntsville
UTI89 Group		
UTI89	UTI model (cystitis isolate)	(Hultgren et al. 1986; Beebout et al. 2022); Maria Hadjifrangiskou, Vanderbilt University
UTI89-a	Δ*btsT* Δ*ypdB* Δ*phoPQ* Δ*evgAS*	
UTI89-b	Δ*gadA* Δ*gadB* Δ*cadA*	
UTI89-c	Δ*sdaA* Δ*sdaB*	

Table 1 | Escherichia coli *strains used, sorted by experimental group. Knockout UTI89 strains are designated in this work as "-a", "-b", and "-c".*

Bacterial Isolation and Liquid Culture Preparation

All strains were obtained from glycerol stocks kept at -80°C. *E. coli* strains were streaked out on LB agar plates and then incubated for 16-18 hours at 37°C. Lactobacilli were streaked out on sheep-blood agar plates and then incubated for 48 hours at 37°C within resealable plastic pouches to create a closed environment. When not in use, *E. coli* plates were sealed with Parafilm and stored at 4°C for up to 4 weeks; *Lactobacillus* plates were kept within resealable plastic

pouches and stored at ambient temperature for up to 2-3 days. Strains were regularly re-streaked to maintain this schedule and were only re-streaked from -80°C stocks if circumstances prohibited this.

A single colony from each strain's isolation was used to prepare its respective liquid culture. *E. coli* liquid cultures were prepared in individual culture tubes containing 8 mL of LB broth. *Lactobacillus* liquid cultures were prepared in individual culture tubes containing 8 mL of MRS broth. *E. coli* cultures were incubated for 24 hours at 37°C with agitation in a shaker at 250 rpm. Culture tube caps were closed loosely. *Lactobacillus* cultures were incubated for 48 hours at 37°C without agitation. Culture tube caps were closed and secured completely to promote anaerobic conditions.

Lactobacilli Spent Media Preparation

Following 48-hour incubation, *Lactobacillus* cultures were observed to have formed substantial cell "pellets" at the bottom of culture tubes. These were resuspended via vortexing for 5-10 seconds at low speed just until the culture achieved homogeneity. An aliquot of culture was taken and set aside for use in the assay before reading the remaining culture's optical density (absorbance at 600 nm (OD_{600})).

Cultures were then individually transferred to centrifuge tubes and centrifuged for 10 minutes at 4°C and 4000 rpm. The resulting supernatant of each culture was decanted into a sterile 5-cc syringe and the cell pellets were discarded. The supernatant was syringe-filtered through a 0.22 µm PES sterile filter. An aliquot of this filtrate was taken and set aside for use in the assay before measuring the remaining supernatant's pH with an electrode.

Buffer Preparation

A citrate-phosphate buffer was prepared to serve as a control for *Lactobacillus* filtrates, which were typically measured to be around a pH of 4. 1.1850 g of citric acid and 1.0927 g of disodium hydrogen phosphate were diluted to 100 mL with DI water. Measured pH was 3.92.

A citrate buffer was prepared to test for inhibition of *E. coli* by a more-acidic environment. 4.65 mL 0.1 M citric acid and 0.350 mL 0.1 M sodium citrate were mixed and diluted to 10 mL with DI water. Measured pH was 2.66.

Buffers were syringe-filtered through a 0.22 µm PES sterile filter before use in the assay.

Well-Diffusion Inhibition Assay

This protocol was replicated as it was performed in the previous work (Johnson et al. 2022). To produce a lawn of growth, 100 µL of liquid *E. coli* culture were pipetted onto an MRS agar plate and spread evenly across the surface by shaking sterile glass beads until culture had been distributed and absorbed **(Figure 1.1)**; glass beads were then tipped out of the plate and discarded. Sterile Pasteur pipettes were used to punch out individual wells with a diameter of approximately 6 mm **(Figure 1.2)**. These wells extended from the surface of the agar to the bottom of the plate. Well punch-outs were removed using ethanol-sterilized tweezers **(Figure 1.3)**. The resulting well was filled with 50 µL of the appropriate liquid: *Lactobacillus* culture, *Lactobacillus* filtrate, buffer, or MRS broth **(Figure 1.4)**. This volume was enough to fill the well almost completely, save for a small amount of headspace from the top (estimated <0.5 mm).

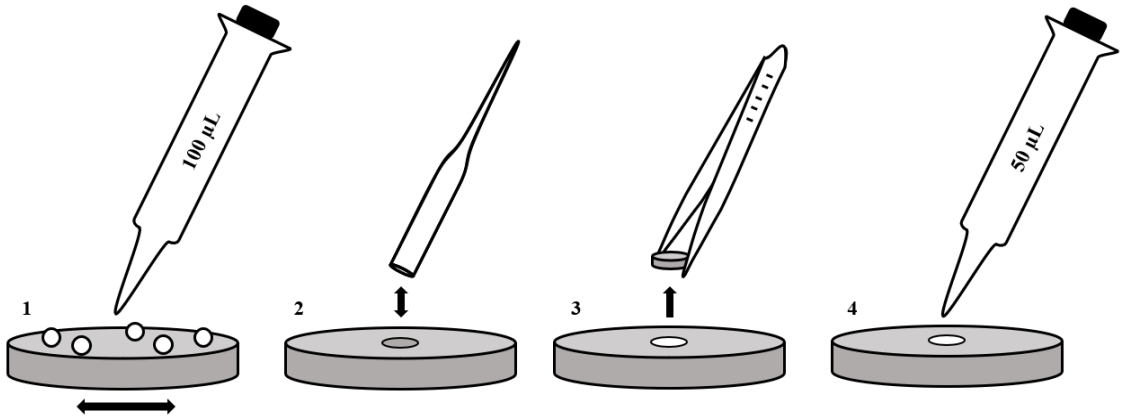


Figure 1 | Summary of steps for well-diffusion inhibition assays. Author illustrated.

Completed plates were allowed to sit agar-side-down with lids removed in the biosafety cabinet for approximately 30 minutes to allow liquid in the wells to dry. Lids were placed back onto the plates before inverting and then incubating for 16-18 hours at 37°C.

Plate Imaging and Zone Quantification

Following incubation, plates were imaged using the camera on a cell phone and then processed in GNU Image Manipulation Program (GIMP) (https://www.gimp.org). Pixel diameters of the plate (P_P), wells (W_P), and zones of inhibition (Z_p) were measured with the Ellipse Select tool set to a fixed 1:1 ratio. These diameters were compiled in a Microsoft Excel sheet for further analysis.

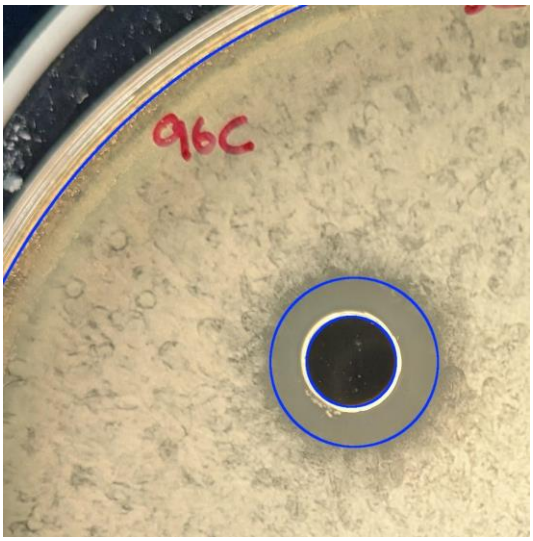
Millimeter diameters of the wells (W_{mm}) and zones of inhibition (Z_{mm}) were obtained using cells programmed with a conversion formula previously defined for use in similar assays:

$$W_{mm}\ or\ Z_{mm} = (W_P\ or\ Z_P) * \frac{85}{P_P}$$

Diameters of plates only encompassed the surface of the agar. Diameters of wells encompassed the inner wall, as close-up imaging occasionally revealed minor inconsistencies in outer well shape. This may have been a result of slight variances in the angle at which the well was punched out, or due to normal expansion/shrinkage of the agar during incubation.

While our standardized MRS media preparation increased their clarity, zones still consistently exhibited noticeably "frayed" and "hazy" edges that affected how they were quantified. Therefore, when measuring in GIMP, images of plates were zoomed in to view the zone more closely. This allowed for a more objective discernment as to where the zone of inhibition "ended," which was defined as the point at which no cell density could be visualized. It should be noted that this resulted in the exclusion of a supposed "partial inhibition" in which the perimeter of this definite zone was surrounded by a less-definite, "weaker" growth **(Figure 2).** Zones were quantified as areas instead of diameters because some results had "shifted" **(Background, CFT073, and SVS144 Strains by *Lactobacillus* Cultures).** Some zones did not surround the entire well and resembled a crescent shape.

Figure 2 | Example of ring measurements performed in GIMP. Measurements of the plate (partially shown; top left), definite zone of inhibition (larger circle; partially shown), and well (smaller blue circle) are shown. Pixel diameters were not influenced by the width of the drawn ring (5 px line width).



Results

Inhibition of Background, CFT073, and SVS144 Strains by *Lactobacillus* Cultures

Three biological replicates were done, each consisting of three technical replicates for each *E. coli* strain. Only one technical replicate of each *Lactobacillus* culture was prepared.

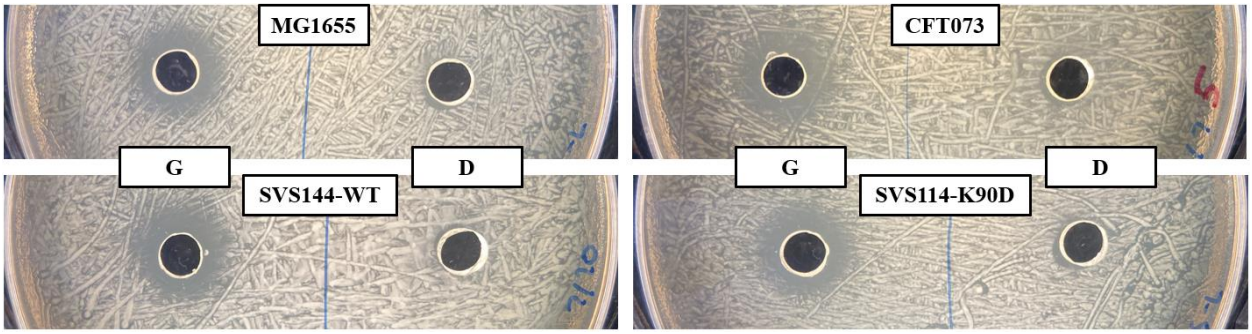
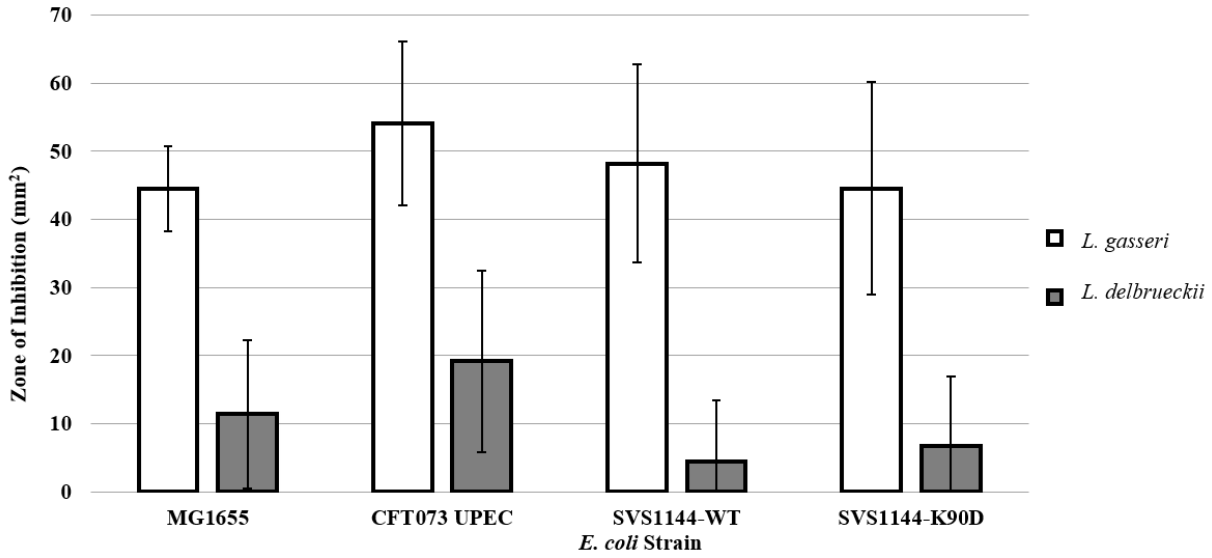


Figure 3 | Inhibition of background, CFT073, and SVS114 strains by *Lactobacillus* cultures. *L. gasseri* **(G)** and *L. delbrueckii* **(D)** both inhibited all *E. coli* strains; *L. gasseri* inhibited *E. coli* the most.

Qualitatively, *L. gasseri* inhibited all strains significantly more than *L. delbrueckii*. *L. delbrueckii* exhibited sharply-defined, crescent-shaped zones surrounded by weakened growth **(Figure 3)**. Quantitative measurements were able to be performed for this set of replicates **(Figure 4)**. Initially, these measurements suggested that CFT073 was the most inhibited, and that the SVS1144-wild-type strain had demonstrated slightly more inhibition than the mutant strain, as hypothesized. Paired, two-tailed *t*-tests were performed with this data, and these observations were not computed to be statistically significant at an alpha level < 0.05.

(a)



(b)

Strain	*L. gasseri*	*L. delbrueckii*
MG1655	44.5 ± 6.3	11.4 ± 10.9
CFT073	54.1 ± 12.0	19.2 ± 13.3
SVS1144-WT	48.2 ± 14.5	4.5 ± 8.9
SVS1144-K90D	44.6 ± 15.7	6.7 ± 10.2

Figure 4 | Measurements of inhibition of background, CFT073, and SVS114 strains by *Lactobacillus* cultures. **(a)** Overall summary of average areas (mm^2) of calculated zones of inhibition; includes all biological and technical replicates. **(b)** Table showing the corresponding average areas and their standard deviations.

Inhibition of Background, CFT073, and SVS144 Strains by *Lactobacillus* Filtrates

Five biological replicates were done, each consisting of three technical replicates for each *E. coli* strain. Only one technical replicate of each *Lactobacillus* culture was prepared and used to prepare filtrates. The average pH ± standard deviation of filtrates across all biological replicates was 4.06 ± 0.083 for *L. gasseri* and 4.29 ± 0.034 for *L. delbrueckii*.

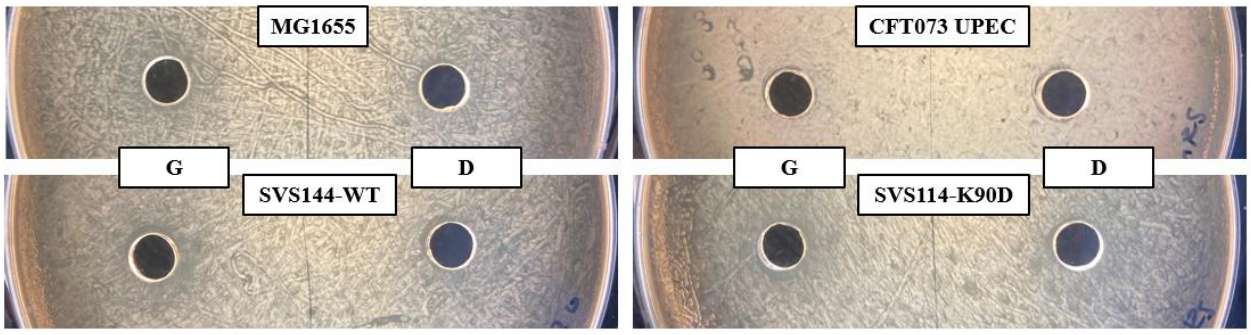


Figure 5 | Inhibition of replicate strains and SVS114 strains by *Lactobacillus* filtrates. *L. gasseri* **(G)** and *L. delbrueckii* **(D)** both inhibited E. coli, but not strongly.

Compared to initial culture experiments, both lactobacilli inhibited all strains significantly less. Qualitative observations could not determine if *L. gasseri* had continued to outperform *L. delbrueckii. L. delbrueckii* again exhibited some crescent-shaped zones surrounded by weakened growth, though these were not as definitive. Compared to initial culture experiments, CFT073 exhibited significantly limited inhibition by both lactobacilli. Quantitative measurements could not be performed according to our established method of quantification.

The effect of incubation time on zone characterization had come into question following initial results. One of these biological replicates was imaged at 16-, 18-, and 24-hour incubation and showed no significant qualitative differences. Quantitative measurements could not be performed according to our established method of quantification.

Inhibition of UTI89 Strains by *L. gasseri* Culture and Filtrate

Only *L. gasseri* was selected for testing because *L. delbrueckii* exhibited varied liquid culture success across biological replicates. Four biological replicates were done, each consisting of three technical replicates for each *E. coli* strain. Only one technical replicate of *L. gasseri* culture was prepared and used to prepare filtrates. The average pH ± standard deviation of filtrates across

all biological replicates was 4.01 ± 0.135. MRS served as a negative control, and the citrate-phosphate buffer of pH = 3.92 served as a positive control.

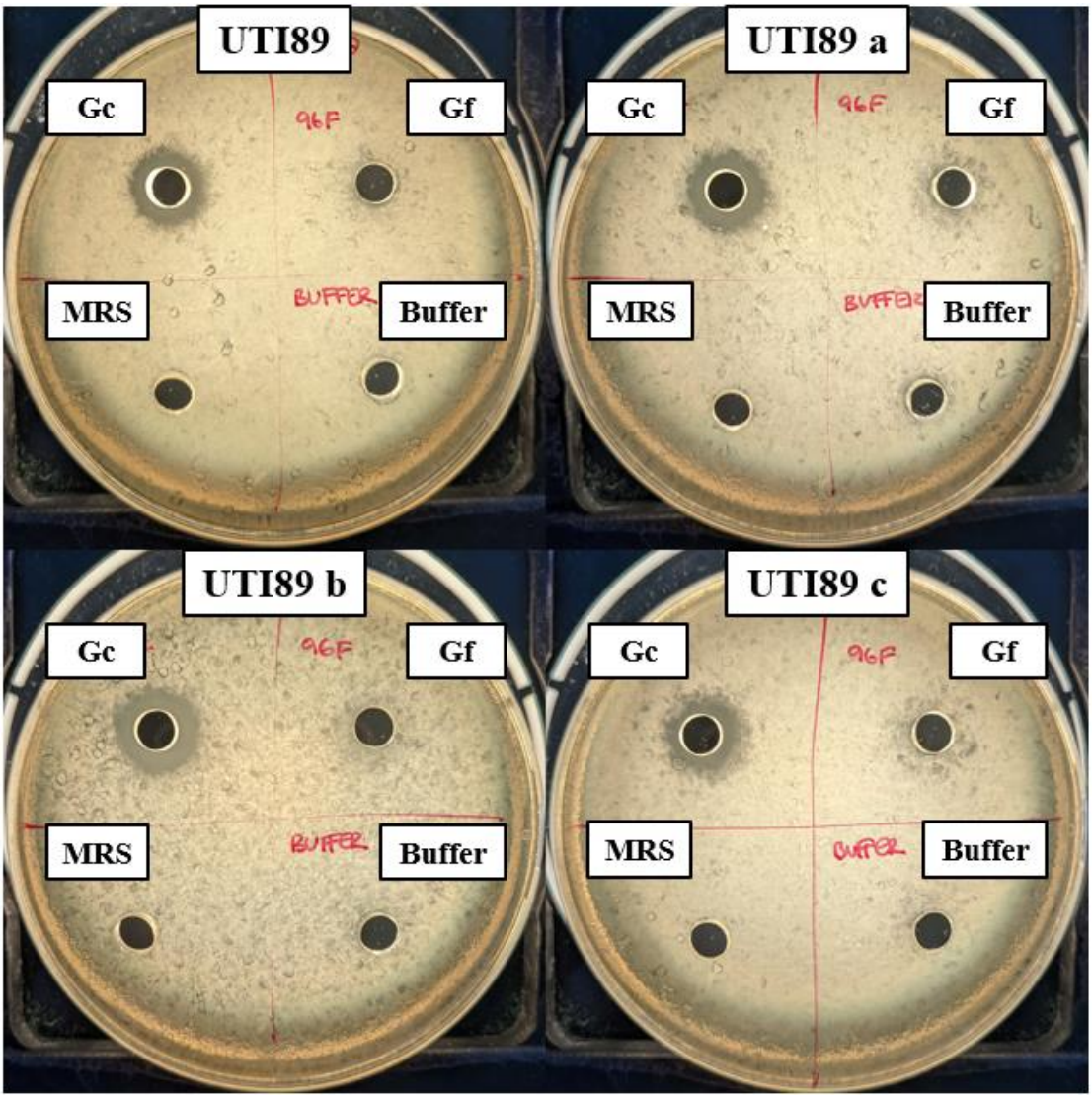
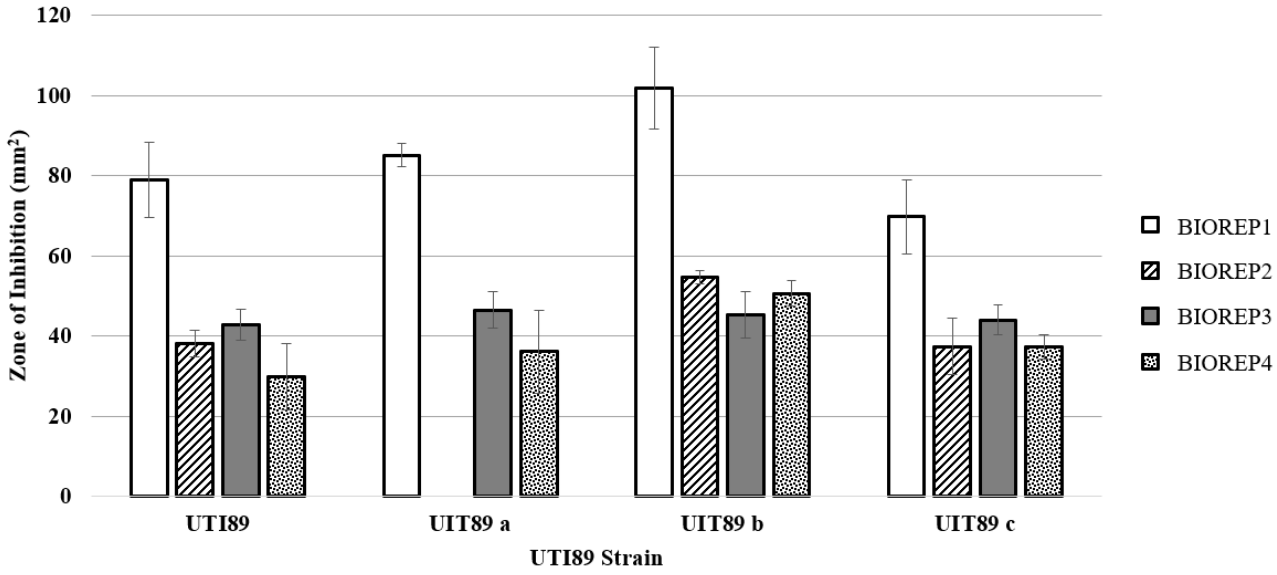


Figure 6 | Inhibition of UTI89 strains by *L. gasseri* cultures **(Gc)** and filtrate **(Gf)**.

L. gasseri inhibited all strains. Both filtrate and buffer inhibited all strains very weakly. Qualitatively, observations suggested that filtrate inhibited slightly better than buffer. **(Figure 6)**. Quantitative measurements were able to be performed for almost all plates **(Figure 7)**. For Biological Replicate 2 (BIOREP2), UTI89-a produced zones that were too hazy to be measured

with our established method of quantification. Initially, these measurements suggested that UTI89-b (Δ*gadA,* Δ*gadB,* Δ*cadA*) demonstrated the most inhibition, and that UTI89-c (Δ*sdaA,* Δ*sdaB)* demonstrated the least. Paired, two-tailed *t*-tests were performed with this data (excluding UTI89-a due to sample size discrepancy), but they were not computed to be statistically significant at an alpha level < 0.05.

(a)



(b)

Strain	BIOREP1	BIOREP2	BIOREP3	BIOREP4
UTI89	70.4 ± 8.43	34.0 ± 2.99	38.3 ± 3.47	26.7 ± 7.36
UTI89-a	75.9 ± 2.55	-	41.5 ± 4.07	32.2 ± 9.14
UTI89-b	90.8 ± 9.02	48.8 ± 1.48	40.4 ± 5.20	45.2 ± 2.80
UTI89-c	62.2 ± 8.28	33.3 ± 6.22	39.2 ± 3.28	33.2 ± 2.70

Figure 7 | Inhibition of UTI89 strains by *L. gasseri* culture and filtrate. **(a)** Overall summary of average areas (mm^2) of calculated zones of inhibition; includes all three technical replicates for each biological replicate. **(b)** Table showing the corresponding average areas and standard deviations.

Inhibition of UTI89 Strains by Citrate Buffer

Following these results, UTI89 strains were tested for inhibition by a low-pH environment simulated by the citrate buffer in the wells. One biological replicate was done, consisting of three technical replicates of each UTI89 strain. MRS broth served as a negative control. As observed with the citrate-phosphate buffer, results showed a similar pattern of weakened *E. coli* growth surrounding the citrate buffer wells, though not a complete zone of inhibition **(Figure 8)**. Quantitative measurements could not be performed according to our established method of quantification.

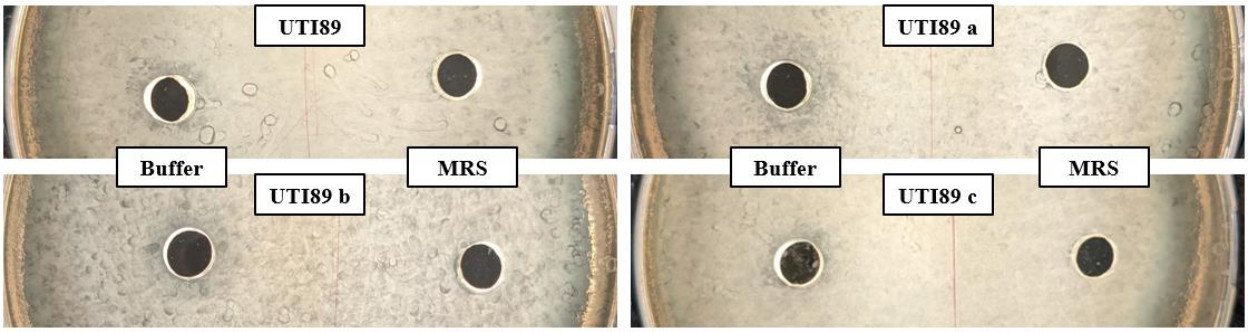


Figure 8 | Inhibition of UTI89 strains by citrate buffer (pH 2.66).

Discussion

In this project, our assays provide phenotypic characterization of UPEC inhibition by *L. gasseri* and *L. delbrueckii* isolates, both by cultures and filtrates. Our results continue to suggest that UPEC inhibition occurs by a combination of pH- and cell-dependent factors. They do not support our hypothesis that these acid-stress response pathways we tested are significantly involved in increasing or decreasing the fitness of UPEC in acidic environments.

For the background and CFT073 strains, our results contradict what was observed in the previous project. Specifically, our filtrate did not inhibit CFT073 to the same degree, and the resulting zones were not as clearly defined. In the previous work, filtrates had reached below pH of 4.00, while our filtrates did not. Notably, CFT073 also exhibited significantly denser and more robust growth in our filtrate-only set of assays, which may have also contributed to these results. All strains had been obtained again from freezer stocks prior to these experiments; it is suspected that a mutant of CFT073 had been isolated in the initial culture experiments. We had expected the SVS1144 mutant (increased *gadA* and *gadB* expression) to exhibit significantly reduced inhibition, although these results demonstrated only minimally reduced inhibition. The previous study had performed acid challenges on this strain via methods not entirely analogous to our methods. In their methods, 4-hour subcultures of SVS114 strains were added to liquid LB media at acidic (pH 2.6) or neutral pH and then incubated for one more hour. Final cultures were diluted and grown on neutral LB agar overnight (Worthan et al. 2022). It is possible that these differences contributed to these results, although it may be a more likely cause of inconsistencies in lawn growth that increased quantitative noise.

UTI89 experiments were more successful in quantification efforts, and variations in average measurements for each biological replicate were reduced. However, there was an extreme

variation in the magnitude inhibition of the first biological replicate compared to subsequent replicates. No observations made during completion of the assay were observed to have likely contributed to this, and it is unusual that only this first replicate had such high inhibition. Interestingly, UTI89-b (Δ*gadA*Δ*gadB*Δ*cadA*) qualitatively demonstrated greater inhibition compared to the other knockout strains, which would support our previous hypothesis regarding the SVS1144 mutant. These results suggest that the *gad* or *cad* systems might be contributing to UPEC's resistance to lactobacilli inhibition.

Despite our efforts to standardize our methods, our results were subject to high magnitudes of variance and inconsistencies in day-to-day replicates. While we intend to continue evaluating the efficacy of these well-inhibition assays, future work will focus on determining metabolites that are involved in the observed inhibition. Heated-filtrate experiments from the previous project will be performed again to determine if heat-sensitive components, such as peptides, play a significant role in the inhibition process. It is of interest to run inhibition assays in liquid media and to compare those results with inhibition on solid media. Efforts to design minimal media for these assays that allow for follow-up metabolic studies are underway.

In conclusion, this project confirms that urinary lactobacilli inhibit the growth of UPEC. This inhibition is strongest when examining the effects of whole-cell *Lactobacillus* cultures, and inhibition is weakest by filtrates. Therefore, we conclude that inhibition is not restricted to pH-dependent factors, but that it is a combination of cell-dependent factors as well. We observed *L. gasseri* to have produced significantly higher levels of inhibition compared to *L. delbrueckii*. These results reiterate the importance of studying urobiome composition and interactions. Our work contributes to the understanding of urobiome ecology and to developing targeted probiotics and prophylaxis for UTIs.

Reference List

Bacheller, Catherine D., and Jack M. Bernstein. 1997. “Urinary Tract Infections.” *Medical Clinics of North America* 81 (3): 719–30. https://doi.org/10.1016/s0025-7125(05)70542-3.

Beebout, Connor J., Gabriella L. Robertson, Bradley I. Reinfeld, Alexandra M. Blee, Grace H. Morales, John R. Brannon, Walter J. Chazin, et al. 2022. “Uropathogenic *Escherichia coli* Subverts Mitochondrial Metabolism to Enable Intracellular Bacterial Pathogenesis in Urinary Tract Infection.” *Nature Microbiology* 7 (9): 1348–60. https://doi.org/10.1038/s41564-022-01205-w.

Behr, S., L. Fried, and K. Jung. 2014. “Identification of a Novel Nutrient-Sensing Histidine Kinase/Response Regulator Network in *Escherichia coli*.” *Journal of Bacteriology* 196 (11): 2023–29. https://doi.org/10.1128/jb.01554-14.

Brumbaugh, Ariel R, and Harry LT Mobley. 2012. “Preventing Urinary Tract Infection: Progress toward an Effective *Escherichia coli* Vaccine.” *Expert Review of Vaccines* 11 (6): 663–76. https://doi.org/10.1586/erv.12.36.

Corr, S. C., Y. Li, C. U. Riedel, P. W. O’Toole, C. Hill, and C. G. M. Gahan. 2007. “Bacteriocin Production as a Mechanism for the Antiinfective Activity of *Lactobacillus salivarius* UCC118.” *Proceedings of the National Academy of Sciences* 104 (18): 7617–21. https://doi.org/10.1073/pnas.0700440104.

Flores-Mireles, Ana L., Jennifer N. Walker, Michael Caparon, and Scott J. Hultgren. 2015. “Urinary Tract Infections: Epidemiology, Mechanisms of Infection and Treatment Options.” *Nature Reviews Microbiology* 13 (5): 269–84. https://doi.org/10.1038/nrmicro3432.

Fouts, Derrick E, Rembert Pieper, Sebastian Szpakowski, Hans Pohl, Susan Knoblach, Moo-Jin Suh, Shih-Ting Huang, et al. 2012. “Integrated Next-Generation Sequencing of 16S RDNA and Metaproteomics Differentiate the Healthy Urine Microbiome from Asymptomatic Bacteriuria in Neuropathic Bladder Associated with Spinal Cord Injury.” *Journal of Translational Medicine* 10 (1). https://doi.org/10.1186/1479-5876-10-174.

Fried, L., S. Behr, and K. Jung. 2012. “Identification of a Target Gene and Activating Stimulus for the YpdA/YpdB Histidine Kinase/Response Regulator System in *Escherichia coli*.” *Journal of Bacteriology* 195 (4): 807–15. https://doi.org/10.1128/jb.02051-12.

Fritz, Georg, Christiane Koller, Korinna Burdack, Larissa Tetsch, Ina Haneburger, Kirsten Jung, and Ulrich Gerland. 2009. “Induction Kinetics of a Conditional PH Stress Response System in *Escherichia coli*.” *Journal of Molecular Biology* 393 (2): 272–86. https://doi.org/10.1016/j.jmb.2009.08.037.

Hilt, E. E., K. McKinley, M. M. Pearce, A. B. Rosenfeld, M. J. Zilliox, E. R. Mueller, L. Brubaker, X. Gai, A. J. Wolfe, and P. C. Schreckenberger. 2013. “Urine Is Not Sterile: Use of Enhanced Urine Culture Techniques to Detect Resident Bacterial Flora in the Adult Female Bladder.” *Journal of Clinical Microbiology* 52 (3): 871–76. https://doi.org/10.1128/jcm.02876-13.

Hultgren, S J, W R Schwan, A J Schaeffer, and J L Duncan. 1986. “Regulation of Production of Type 1 Pili among Urinary Tract Isolates of *Escherichia coli*.” *Infection and Immunity* 54 (3): 613–20. https://doi.org/10.1128/iai.54.3.613-620.1986.

Johnson, James A., Lydia F. Delaney, Vaishali Ojha, Medha Rudraraju, Kaylie R. Hintze, Nazema Y. Siddiqui, and Tatyana A. Sysoeva. 2022. “Commensal Urinary Lactobacilli Inhibit Major Uropathogens in Vitro with Heterogeneity at Species and Strain Level.” *Frontiers in Cellular and Infection Microbiology* 12 (June). https://doi.org/10.3389/fcimb.2022.870603.

Kaur, Rajanbir, and Rajinder Kaur. 2020. “Symptoms, Risk Factors, Diagnosis and Treatment of Urinary Tract Infections.” *Postgraduate Medical Journal* 97 (1154): postgradmedj-2020-139090. https://doi.org/10.1136/postgradmedj-2020-139090.

Kostakioti, Maria, Scott J. Hultgren, and Maria Hadjifrangiskou. 2012. “Molecular Blueprint of Uropathogenic *Escherichia coli* Virulence Provides Clues toward the Development of Anti-Virulence Therapeutics.” *Virulence* 3 (7): 592–93. https://doi.org/10.4161/viru.22364.

Kristoficova, Ivica, Cláudia Vilhena, Stefan Behr, and Kirsten Jung. 2018. “BtsT, a Novel and Specific Pyruvate/H^+ Symporter in *Escherichia coli*.” Edited by Ann M. Stock. *Journal of Bacteriology* 200 (2). https://doi.org/10.1128/jb.00599-17.

Masuda, Nobuhisa, and George M. Church. 2003. “Regulatory Network of Acid Resistance Genes in *Escherichia coli.*” *Molecular Microbiology* 48 (3): 699–712. https://doi.org/10.1046/j.1365-2958.2003.03477.x.

Miyashiro, T., and M. Goulian. 2007. “Stimulus-Dependent Differential Regulation in the *Escherichia coli* PhoQ/PhoP System.” *Proceedings of the National Academy of Sciences* 104 (41): 16305–10. https://doi.org/10.1073/pnas.0700025104.

Mobley, H L, D M Green, A L Trifillis, D E Johnson, G R Chippendale, C V Lockatell, B D Jones, and J W Warren. 1990. “Pyelonephritogenic *Escherichia coli* and Killing of Cultured Human Renal Proximal Tubular Epithelial Cells: Role of Hemolysin in Some Strains.” *Infection and Immunity* 58 (5): 1281–89. https://doi.org/10.1128/iai.58.5.1281-1289.1990.

Mobley, Harry L. T., Michael S. Donnenberg, and Erin C. Hagan. 2009. “Uropathogenic *Escherichia coli*.” *EcoSal Plus* 3 (2). https://doi.org/10.1128/ecosalplus.8.6.1.3.

Nelson, David E., Barbara Van Der Pol, Qunfeng Dong, Kashi V. Revanna, Baochang Fan, Shraddha Easwaran, Erica Sodergren, George M. Weinstock, Lixia Diao, and J. Dennis Fortenberry. 2010. “Characteristic Male Urine Microbiomes Associate with Asymptomatic Sexually Transmitted Infection.” *PloS One* 5 (11): e14116. https://doi.org/10.1371/journal.pone.0014116.

Olson, Patrick D., Lisa K. McLellan, Alice Liu, Kelleigh L. Briden, Kristin M. Tiemann, Allyssa L. Daugherty, Keith A. Hruska, and David A. Hunstad. 2017. “Renal Scar Formation and Kidney Function Following Antibiotic-Treated Murine Pyelonephritis.” *Disease Models & Mechanisms* 10 (11): 1371–79. https://doi.org/10.1242/dmm.030130.

Pearce, Meghan M., Evann E. Hilt, Amy B. Rosenfeld, Michael J. Zilliox, Krystal Thomas-White, Cynthia Fok, Stephanie Kliethermes, et al. 2014. “The Female Urinary Microbiome: A Comparison of Women with and without Urgency Urinary Incontinence.” Edited by Martin J. Blaser. *MBio* 5 (4). https://doi.org/10.1128/mbio.01283-14.

Pflughoeft, Kathryn J., and James Versalovic. 2012. “Human Microbiome in Health and Disease.” *Annual Review of Pathology: Mechanisms of Disease* 7 (1): 99–122. https://doi.org/10.1146/annurev-pathol-011811-132421.

Qiao, Nanzhen, Stijn Wittouck, Paola Mattarelli, Jinshui Zheng, Sarah Lebeer, Giovanna E. Felis, and Michael G. Gänzle. 2022. “After the Storm—Perspectives on the Taxonomy of Lactobacillaceae.” *JDS Communications* 3 (3): 222–27. https://doi.org/10.3168/jdsc.2021-0183.

Reid, Gregor, and Jeremy Burton. 2002. “Use of *Lactobacillus* to Prevent Infection by Pathogenic Bacteria.” *Microbes and Infection* 4 (3): 319–24. https://doi.org/10.1016/s1286-4579(02)01544-7.

Salvetti, Elisa, Sandra Torriani, and Giovanna E. Felis. 2012. “The Genus *Lactobacillus*: A Taxonomic Update.” *Probiotics and Antimicrobial Proteins* 4 (4): 217–26. https://doi.org/10.1007/s12602-012-9117-8.

Silva Sabo, Sabrina da, Michele Vitolo, José Manuel Domínguez González, and Ricardo Pinheiro de Souza Oliveira. 2014. “Overview of *Lactobacillus plantarum* as a Promising Bacteriocin Producer among Lactic Acid Bacteria.” *Food Research International* 64 (October): 527–36. https://doi.org/10.1016/j.foodres.2014.07.041.

Slover, Christine M., and Larry Danziger. 2008. “*Lactobacillus*: A Review.” *Clinical Microbiology Newsletter* 30 (4): 23–27. https://doi.org/10.1016/j.clinmicnews.2008.01.006.

Stewart, V., and C Yanofsky. 1985. “Evidence for Transcription Antitermination Control of Tryptophanase Operon Expression in *Escherichia coli* K-12.” *Journal of Bacteriology* 164 (2): 731–40. https://doi.org/10.1128/jb.164.2.731-740.1985.

Vaughan, Monique H., Jialiang Mao, Lisa A. Karstens, Li Ma, Cindy L. Amundsen, Kenneth E. Schmader, and Nazema Y. Siddiqui. 2021. “The Urinary Microbiome in Postmenopausal Women with Recurrent Urinary Tract Infections.” *Journal of Urology*, June. https://doi.org/10.1097/ju.0000000000001940.

Wiebe, Michelle A., John R. Brannon, Bradley D. Steiner, Adebisi Bamidele, Alexandra C. Schrimpe-Rutledge, Simona G. Codreanu, Stacy D. Sherrod, John A. McLean, and Maria Hadjifrangiskou. 2022. “Serine Deamination Is a New Acid Tolerance Mechanism Observed in Uropathogenic *Escherichia coli*.” Edited by Tracy Lyn Raivio and Scott J. Hultgren. *MBio* 13 (6). https://doi.org/10.1128/mbio.02963-22.

Wojciuk, Bartosz, Agata Salabura, Bartłomiej Grygorcewicz, Karolina Kędzierska, Kazimierz Ciechanowski, and Barbara Dołęgowska. 2019. “Urobiome: In Sickness and in Health.” *Microorganisms* 7 (11): 548. https://doi.org/10.3390/microorganisms7110548.

Worthan, Sarah B., Elizabeth A. Franklin, Chi Pham, Mee-Ngan F. Yap, and Luis R. Cruz-Vera. 2022. “The Identity of the Constriction Region of the Ribosomal Exit Tunnel Is Important to Maintain Gene Expression in *Escherichia coli*.” Edited by Amanda G. Oglesby. *Microbiology Spectrum* 10 (2). https://doi.org/10.1128/spectrum.02261-21.

Zeng, Zhilin, Juan Zhan, Kaimin Zhang, Huilong Chen, and Sheng Cheng. 2022. “Global, Regional, and National Burden of Urinary Tract Infections from 1990 to 2019: An Analysis of the Global Burden of Disease Study 2019.” *World Journal of Urology* 40 (January): 755–63. https://doi.org/10.1007/s00345-021-03913-0.

Zheng, Jinshui, Michael G. Gänzle, Xiaoxi B. Lin, Lifang Ruan, and Ming Sun. 2014. "Diversity and Dynamics of Bacteriocins from Human Microbiome." *Environmental Microbiology* 17 (6): 2133–43. https://doi.org/10.1111/1462-2920.12662.

Zheng, Jinshui, Stijn Wittouck, Elisa Salvetti, Charles M.A.P. Franz, Hugh M.B. Harris, Paola Mattarelli, Paul W. O'Toole, et al. 2020. "A Taxonomic Note on the Genus *Lactobacillus:* Description of 23 Novel Genera, Emended Description of the Genus *Lactobacillus* Beijerinck 1901, and Union of *Lactobacillaceae* and *Leuconostocaceae*." *International Journal of Systematic and Evolutionary Microbiology* 70 (4): 2782–2858. https://doi.org/10.1099/ijsem.0.004107.

Zorc, J. J., D. A. Kiddoo, and K. N. Shaw. 2005. "Diagnosis and Management of Pediatric Urinary Tract Infections." *Clinical Microbiology Reviews* 18 (2): 417–22. https://doi.org/10.1128/cmr.18.2.417-422.2005.

Zwir, I., D. Shin, A. Kato, K. Nishino, T. Latifi, F. Solomon, J. M. Hare, H. Huang, and E. A. Groisman. 2005. "Dissecting the PhoP Regulatory Network of *Escherichia coli* and *Salmonella enterica.*" *Proceedings of the National Academy of Sciences* 102 (8): 2862–67. https://doi.org/10.1073/pnas.0408238102.