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Interbacterial Competition Amongst Lactobacilli Isolated from Human Urinary Tract

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Interbacterial Competition Amongst Lactobacilli Isolated from Human Urinary Tract

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Abstract

The rise in antibiotic resistant uropathogens has led to an increase of interest in studying the human urinary microbiome to find alternative treatments of urinary tract infections (UTI) that do not rely on traditional antibiotic applications. Multiple studies suggest that a prominent lactic acid producing probiotic bacterium *Lacticaseibacillus rhamnosus*, that is also sometimes found in the urinary tract, can inhibit the growth of uropathogens and potentially prevent UTI. However, it is not currently known whether *L. rhamnosus* can inhibit other species of lactobacilli. In order to see the potential impact of introducing *L. rhamnosus* to the urinary bladder, the competitive relationships between urinary lactobacilli species must be examined. Here we tested competitive relationships amongst urinary lactobacilli using well-inhibition assays. Using this technique, 4 isolates of *L. rhamnosus* were tested for their ability to inhibit isolates of 4 other species of urinary lactobacilli: *Lactobacillus gasseri, Lactobacillus delbrueckii, Lactobacillus animalis,* and *Lactobacillus johnsonii.* Both, *L. rhamnosus* cells and spent medium filtrate of this bacterium, were tested to determine whether observed inhibition was cell-dependent. After testing, it was determined that all patient-derived strains of *L. rhamnosus* were capable of significantly inhibiting *L. delbrueckii* but showed low or no inhibition against the other three species. These results on *L. rhamnosus* competing with some urinary lactobacilli, combined with our previous observations that urinary *L. rhamnosus* strains are amongst the strongest inhibitors of uropathogens, suggest that *L. rhamnosus* might be a great candidate to explore in search of urinary probiotic.

Introduction

Urinary tract infections (UTI) are one of the most common bacterial infections accounting for 150 million cases per year across the globe and 6 billion dollars in direct healthcare expenditures[1]. Pathogens including *Escherichia coli, Enterococcus faecalis*, *Klebsiella pneumoniae,* and *Staphylococcus epidermidis* are frequent causes of UTI infections[2]. Due to the rising of antibiotic resistant pathogens, investigations into new treatment and preventative innovations have increased regarding the treatment of UTI infections in order to prevent UTI complications such as sepsis or renal failure and death[2-4]. One of these innovations is an attempt the use of probiotics to treat and prevent UTI infections. Probiotics are any safe and useful living microorganism that have positive effects on health and physiology[5]. Some well known of these probiotic bacteria are lactobacilli that are found naturally in the normal flora of the mouth, intestine, bladder, and female genital tract[6]. Studies have found that *Lactobacilli* have several anti-infective properties: the ability to adhere to urinary tract surfaces and inhibit the adhesion of urinary pathogens; the ability to deplete nutrients otherwise used by the pathogens; the production of inhibiting chemicals such as bacteriocins, organic acids, and hydrogen peroxide; and the ability to modulate the host immune response and microenvironment[3,7]. These properties explain how Lactobacillus species are able to outcompete uropathogens and, therefore, possibly become a preventative treatment of UTI infection.

A recent study that examined the urinary microbiome of pre-menopausal women suggested a correlation between increased prevalence of Lactobacillus species and a reduced risk of developing a UTI[3,5]. Several Lactobacillus species have been shown to inhibit uropathogens *in vitro* including *E. coli*, *K. pneumoniae*, and *E. faecalis*.[8] The strength of inhibition of uropathogens varies between *Lactobacillaceae* with species such as *Lacticaseibacillus rhamnosus* showed great inhibition and species such as *Lactobacillus jensenii* showed no visible signs of inhibition[8]. Many other species show intermediate inhibition levels including *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus delbrueckii*, *Lactobacillus johnsonii*, and *Lactobacillus animalis* strains[8].

In order to use *Lactobacillaceae* in a clinical setting, there have been multiple studies done that examine which *Lactobacillus* strains are the best candidates for clinical treatment[3, 9]. *L. rhamnosus* has been shown as a great candidate of probiotic treatment due to its affinity for adhesion to the urinary bladder wall and good growth characteristics[3]. Strains *L. rhamnosus* GG and *L. rhamnosus* GR-1 are two strains that are being frequently studied to determine their effectiveness for prophylaxis measure for UTI infection[5,7]. For instance, a study by Akgul and Karakan concluded that *L. rhamnosus* GR-1 and *L. fermentum* supplements have been shown to increase the resistance to urinary tract infections[7].

However, it is not known how introduction of *L. rhamnosus* into the urinary tract may affect urinary microbiome how this species and interact with other lactobacilli species present. These interactions are important for determining the ability of *L. rhamnosus* to inhabit and dominate the urinary microbiome. In this project, several lactobacilli species were isolated from adult female patients including *L. gasseri*, *L. delbrueckii*, *L. animalis*, *L. johnsonii* were tested against four strains (SL101, SL102, SL103, and SL105) of *Lacticaseibacillus rhamnosus* to assess their competitive and inhibition abilities. All these lactobacilli strains were isolated from postmenopausal females, This isolation and identification of these bacterial strains were conducted by Vaughan et al. using enhanced quantification urine culture (EQUC) detecting the microorganisms to isolate the urinary bacteria found in catheterized urine specimens were streak isolated and identified by Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF) and 16SA rRNA gene sequencing.

Materials and Methods

Bacterial Growth Lawn Experiments

In order to measure the zones of inhibition in the well-inhibition assays, a visible evenly spread lawn of bacteria must be attained. Initial experiments demonstrated that growing lactobacilli isolates on De Man, Rogoa, and Sharpe (MRS) plates required approximately 200 microliters of lactobacillus culture to be incubated in anaerobic conditions. Anaerobic conditions were obtained by storing the plates in a container containing an anaerobic pouch system with a colored indicator.

Bacterial Growth and Preservation

Vaughan and coworkers used published EQUC procedures to isolate urinary bacteria from catheterized urine specimens and the isolates were typed by MALDI-TOF[10-11]. **Table 1** details each strain and its respective Isolate ID. The Lactobacillus strains were cultured on Sheep Blood Agar plates for 48 hours in a 37 ºC incubator on MRS agar under anaerobic conditions. Each Lactobacillus strain then was inoculated in 8 mL of MRS liquid broth for 24 hours in a 37 ºC incubator at anaerobic atmospheric conditions and all tubes were stationary.

L. rhamnosus Cell Filtrate Production

In order to isolate the cellular secretions from the *L. rhamnosus* cells, tubes of MRS containing the different *L. rhamnosus* strains were centrifuged at 3,230 rcf for 10 min. The supernatant was then sterile filtered using a sterile syringe and a 0.22 µl PES filter into a new sterile tube.

Well-Inhibition Assays

Bacterial inhibition was tested using well-inhibition ssays performed on solid MRS agar plates. Using the overnight broths, the surface of the MRS agar plates were inoculated with 200 µL of either *L. delbrueckii*, *L. gasseri*, *L. johnsonii*, or *L. animalis*. The bacterial broth was uniformly spread over the agar plate to create a bacterial lawn using rolling glass beads. Wells were punched in the surface (6 mm) and created plugs were removed with sterile tweezers. Then, 50 µL of the *L. rhamnosus* culture or *L. rhamnosus* bacterial culture filtrate were added to the wells. The bacterial strain IDs and names are listed in **Table 1**. MRS and 0.5 mM HCL were used as controls for this experiment. The plates were sealed in an anaerobic environment at 37 ºC. After 24 hours of incubation, all plates were first imaged to quantify the zones of inhibition. Then, the areas of the wells and the zones of inhibition were measured by the graphical software GIMP (www.gimp.org) and finally the area of the wells (6 mm diameter) were subtracted from the area of the zones of inhibition.

Lactobacillus DNA Isolation

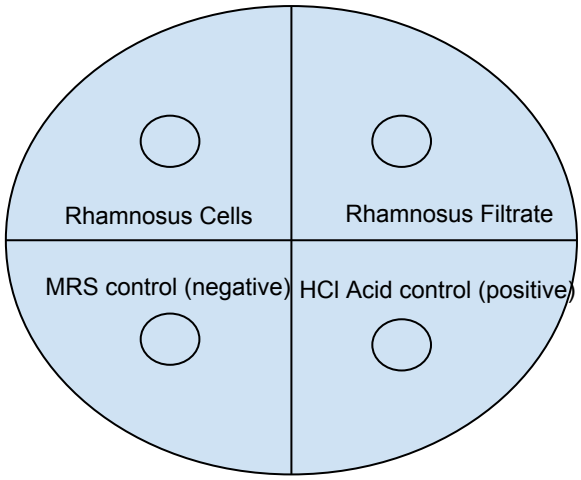
Genomic sequencing for the urinary lactobacilli needs to be conducted to start identifying genetic determinants of interbacterial competition. In order to perform this sequencing, cell pellets were created by culturing each lactobacillus species on sheep blood agar. Then, one bacterial colony of each strain was inoculated in a separate tube of 8 mL of MRS broth for 24 hours. After this incubation time, these tubes were centrifuged at 3,230 rcf for 10 minutes in order to collect the cells. The total DNA from these cell pellets was extracted using the Nanobind CBB kit using the provided specific gram-positive bacteria procedure. Two extractions were performed. The first extraction the DNA was resuspended in 120 microliters of EB elution buffer and for the second extraction the amount of EB buffer was reduced to 75 microliters in order to obtain more

concentrated DNA. The amount of DNA extracted was quantified using the Nanodrop UV-Vis Spectrophotometer.

Table 1: The stain species and their identification numbers used in this experiment using Vaughan et al.'s method of isolation and classification.

Samples Lab ID	Sanger 16s rRNA gene	Isolate ID/Other names
SL96	*Lactobacillus gasseri*	(S1) 5006-2
SL97	*Lactobacillus delbrueckii*	(S9) 5010-2
SL124	*Lactobaccillius animalis*	5008-1
SL114	*Lactobacillus johnsonii*	5037-1
SL101	*Lacticaseibacillus rhamnosus*	5011-2
SL102	*Lacticaseibacillus rhamnosus*	5011-3
SL103	*Lacticaseibacillus rhamnosus*	5024-3
SL105	*Lacticaseibacillus rhamnosus*	5038-2

A.



B.

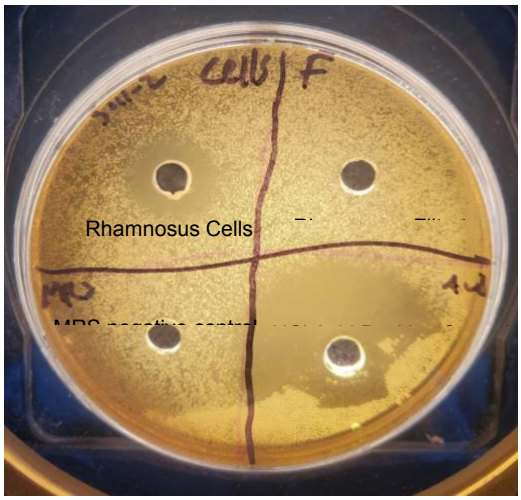


Figure 1. A. The Plate experimental design with a designated well for the *L rhamnosus* cells, *L. rhamnosus* Filtrate, Negative Control, and Positive Control. **B.** An example result plate with an *L. delbrueckii* Lawn.

Results

Well-inhibition Assays

The results of the area measurements of the bacterial cell well-inhibition assay can be seen in **Table 2** and **Figure 2**. Using the area readings of the four competing strains, it was observed that *L. delbrueckii* was significantly inhibited by *L. rhamnosus*. This inhibition was universal across all four strains of *L. rhamnosus:* SL101, SL102, SL103, and SL105. The rest of tested bacteria: *L. gasseri*, *L. johnsonii*, and *L. animalis* showed minimal to slight inhibition. However, the low bacterial concentration of the plate assays of *L. johnsonii* bacterial lawns made measuring the zone of inhibition difficult.

Table 2: Inhibition zone average for each combination of *L. rhamnosus* cells with different Lactobacillus strains.

Strain ID	*L. delbreuckii*	*L. gasseri*	*L. johnsonii*	*L. animalis*
SL101 cells	7.181 ± 2.142	0.869 ± 0.967	2.062 ± 1.123	2.427 ± 0.321
SL102 cells	7.39 ± 3.333	1.188 ± 0.386	2.234 ± 0.728	2.622 ± 0.629
SL103 cells	6.566 ± 1.651	0.737 ± 0.319	2.161 ± 1.257	2.291 ± 0.478
SL105 cells	6.854 ± 3.291	0.698 ± 0.570	1.885 ± 1.048	2.291 ± 0.478

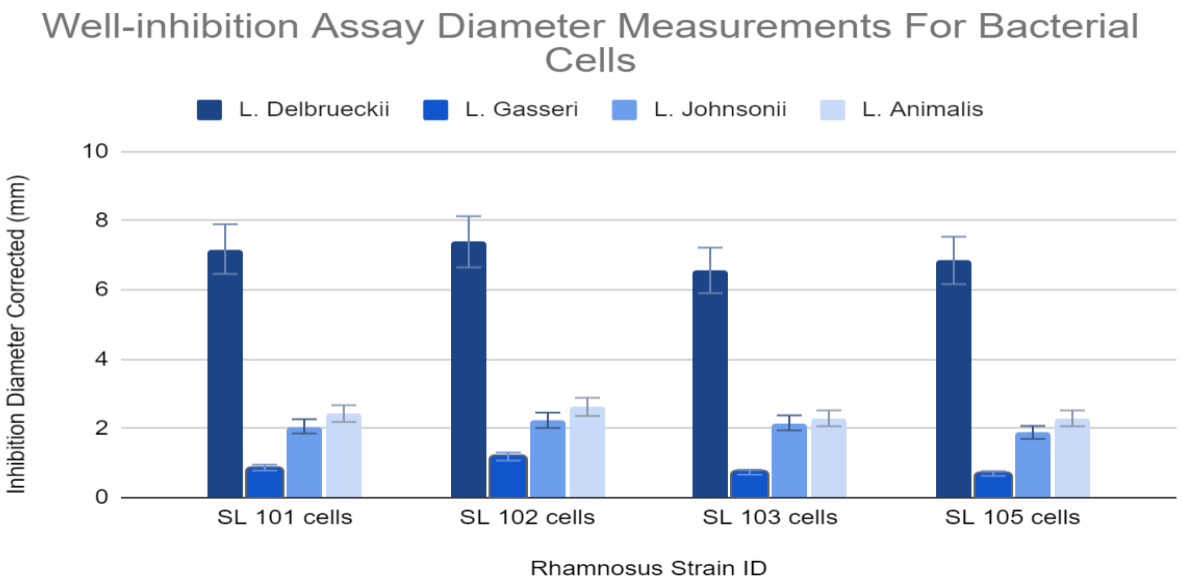


Figure 2: The diameter measurements for the different strains of *L. rhamnosus* cells growth inhibition of the lactobacillus species.

After the cell filtrate was extracted from cells using centrifugation and sterile filtration, they were placed in an adjacent well in the assay and were subjected to the same conditions as the well containing lactobacillus cells. The results of the zone inhibition of the cellular filtrate can be seen in **Table 3** and **Figure 3**. The cellular filtrate wells showed minimal to no apparent inhibition of the lawn bacteria. Additionally, there seemed to be little difference between the *L. rhamnosus* strains filtrate.

Table 3: Inhibition zone average for each combination of *L. rhamnosus* filtrate with different Lactobacillus strains.

Strain ID	*L. delbrueckii*	*L. gasseri*	*L. johnsonii*	*L. animalis*
SL101 filtrate	0.834 ± 0.422	0.646 ± 0.400	0.945 ± 0.147	0.378 ± 0.279
SL102 filtrate	1.091 ± 0.566	0.691 ± 0.141	1.115 ± 0.345	0.844 ± 0.379
SL103 filtrate	1.351 ± 0.704	0.447 ± 0.196	0.949 ± 0.214	0.537 ± 0.250
SL105 filtrate	0.738 ± 0.663	0.524 ± 0.327	0.734 ± 0.213	0.524 ± 0.118

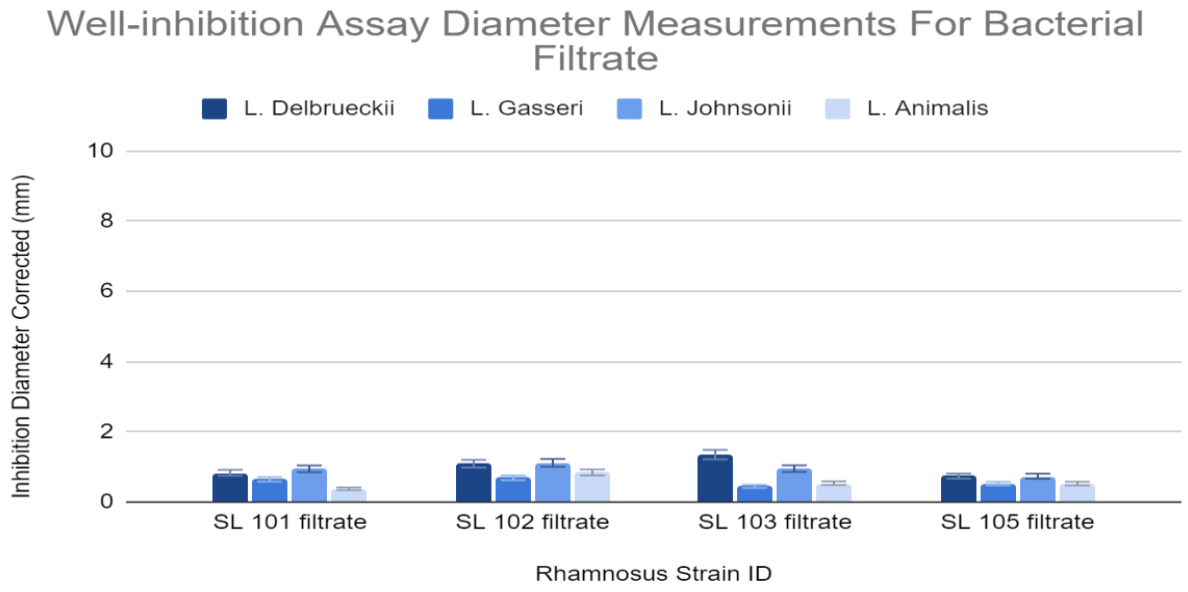


Figure 3: The diameter measurements for the different strains of *L. rhamnosus* cell filtrate growth inhibition of the lactobacillus species.

DNA Isolation from Lactobacilli for Whole genome sequencing

The DNA of each species of Lactobacillus was stored using an emulsion buffer and stored at -18 ºC freezer. The results for the amount collected of each species are shown in **Table 4**. The amount in order to sequence successfully is 2 micrograms in 15 microliters. These concentrations are too low to successfully sequence and future extractions will be performed to extract more DNA.

Table 4: the amount of DNA extracted from various Lactobacillus cells in (ng/µl) to be used in DNA sequencing.

Species Name	Concentration Extracted: First Extraction in 120 microliters EB buffer (ng/µl)	Concentration Extracted: Second Extraction in 75 microliters of EB buffer (ng/µl)	Total Amount Extracted (ng)
L. gasseri (SL96)	2 ng/microliter	5.4 ng/microliter	645 ng
L. delbrueckii (SL97)	4.9 ng/microliter	7.6 ng/microliter	1,158 ng
L. johnsonii (SL114)	2.7 ng/microliter	0 ng/microliter	324 ng
L. animalis (SL124)	46.7 ng/microliter	24.9 ng/microliter	7,471 ng
L. rhamnosus (SL101)	8.8 ng/microliter	36.2 ng/microliter	3,771 ng
L. rhamnosus (SL102)	11.4 ng/microliter	42.4 ng/microliter	4,548 ng
L. rhamnosus (SL103)	10 ng/microliter	0 ng/microliter	1,200 ng
L. rhamnosus (SL105)	12 ng/microliter	7.0 ng/microliter	1,965 ng

Discussion

Several studies have examined the inhibition of pathogenic bacteria by Lactobacillus bacteria; however, it is important to examine the interaction between probiotic candidates and commensal species. Introducing *L. rhamnosus* as a probiotic prevention of UTI will alter the urinary microbiome and perhaps make *L. rhamnosus* one of the primary species present in the urinary tract. The results indicate that strains of *L. rhamnosus* bacteria can inhibit the growth of one of the species of Lactobacillus tested. This competitive relationship is in the inhibition of *L. delbrueckii* which has shown significant inhibition zones around the *L. rhamnousus* bacteria wells. Additionally, this pattern of inhibition was universal across all strains of *L. rhamnosus* that were tested. This inhibition behavior appears to be a cell-dependent mechanism due to minimal to no inhibition being present surrounding the cell-free filtrate wells. It is not known why *L. delbrueckii* is extremely susceptible to *L. rhamnosus* inhibition. Despite all the strains of *L. rhamnosus* had consistent inhibition of *L. delbrueckii*, strain SL103 showed reduced effectiveness of inhibition compared to the other strains. This could be due to a genetic mutation that causes reduced effectiveness in inhibition. The DNA isolation shows that lactobacilli DNA can be isolated using the Nanobind CBB kit however despite the kit working for all gram positive bacteria it is not designed for the extraction of DNA from lactobacillus and could be a factor in the low DNA yields obtained. A method of making the existing DNA more concentrated by reducing the amount of EB buffer might be used in order to get the required DNA concentration for following sequencing.

Future Research

Using the DNA that was already extracted, future research will examine these bacterial species genetically to determine genetic differences between them as well as strains of *L. rhamnosus* used in this experiment. This genetic comparative analysis will also help to determine which mechanism causes this competitive inhibition. Additionally, a full genetic comparison will determine which genetic differences results in reduced ability of SL103 strain to inhibit *L. delbrueckii.* Because the results of this experiment have determined that the secreted enzymes and molecules are not responsible for the inhibition with other lactobacillus species this leaves cell-dependent inhibitors such as greater adherence ability, faster growth rate and consuming of resources, or another cell-activated mechanism. Determining the prominent mechanism by which *L. rhamnosus* inhibits lactobacillus can also give an indication of what additional factors inhibit uropathogens and what genetic factors cause this superior inhibition.

Future research should examine the introduction of *L. rhamnosus* in the human urinary tract and whether the concentration of *L. rhamnosus* increases over time. If *L. rhamnosus* concentration increases after introduction to the urinary microbiome or if it requires replenishment will also determine its effectiveness as a treatment alternative to UTI infections. However, this research must be conducted using environmental factors similar to that of the *in vivo* urinary tract in order to give an identical environment for the lactobacilli to grow and inhibit uropathogens. However, this will require a study using animal models or clinical studies with human patients and cannot be replicated *in vitro*.

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