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The Ability of *Lactobacillus* spp. to Limit Extracellular ATP Release by Urogenital Bacteria

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Microbiology and Immunology

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Abstract

Urgency urinary incontinence is a common symptom of overactive bladder (OAB) and urinary tract infection (UTI), which can be triggered by extracellular adenosine triphosphate (eATP) through purinergic signaling. Current treatments for OAB and UTI fail to consider the potential impact of eATP. It is hypothesized that certain commensal *Lactobacillus* spp. can modulate levels of eATP released by pathogenic bacteria. This project examined bacterial modulation of eATP during growth, the impact of prebiotics on lactobacilli growth, and studied its reduction in an *in vitro* bladder infection model. Release and uptake of ATP by a range of commensal and pathogenic bacteria was observed. *Lactobacillus crispatus* ATCC 33820 and *Lactobacillus gasseri* KC-1 able to reduce supplemented and *E. coli* IA2-derived ATP, was enhanced in culture with select prebiotics. This research builds a greater understanding of the handling of eATP by bacteria and suggests a prebiotic-probiotic application that could potentially improve bladder health.

Keywords

Urinary tract infection (UTI), *Lactobacillus crispatus* ATCC 33820, *Lactobacillus gasseri* KC-1, *Escherichia coli* IA2, urinary urgency incontinence (UUI), overactive bladder (OAB), microbiota, prebiotic, extracellular adenosine triphosphate (eATP), purinergic signalling.

Summary for Lay Audience

A urinary tract infection (UTI) can occur in any part of the urinary system (kidneys, ureter, bladder, or urethra). Not all UTIs have symptoms, but common ones are frequent urination, pain, or burning with urination. Uropathogenic *Escherichia coli* frequently cause this condition. Overactive bladder (OAB) is a collection of symptoms where one feels a frequent and urgent need to urinate regardless of bladder volume and consequently may experience urinary incontinence. Urgency urinary incontinence is the sensation to urinate regardless of bladder volume, a symptom of both conditions. It is now becoming apparent that UTI and OAB are somewhat associated with disruption of the normal commensal bacteria, the microbiota. Each individual has a diverse microbiota containing trillions of microbes that are compositionally different based on their location. For example, a healthy urogenital system (urinary tract and genital tract) has a microbiota dominated by *Lactobacillus* species.

Commensal and pathogenic bacteria release adenosine triphosphate (ATP) into the environment while they grow. All living things utilize ATP, which is a universal energy molecule and is used to power cellular functions. Studies have shown that it is an important signaling molecule for cell and host nervous systems. The release of ATP during growth allows a bacterium to communicate with the host, influence the environment, and be taken up later as an additional nutrient source. Several urinary-associated microbes and pathogenic *E. coli* can release relatively large amounts of ATP, which may trigger the host nervous system and potentially contribute to the feeling of urgency associated with UTIs and OAB. However, some commensal lactobacilli take up ATP during growth or produce substances that could reduce the adverse effects of ATP on the bladder. These lactobacilli can potentially be administered as probiotics (live microorganisms that, when administered in adequate amounts, confer a health benefit on the host) to reduce urinary ATP. Probiotics can be supported, or beneficial indigenous strains promoted, by prebiotics. Prebiotics are substrates selectively utilized by microorganisms conferring a health benefit. The use of lactobacilli and prebiotics to address abundant urinary ATP was a previously unexplored and promising method for urgency symptom management.

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The Burton and Reid labs are unique; they challenge students to take charge of their projects and drive their own research. Students are afforded the freedom to explore and develop as scientists and collaborate to broaden their ideas. I would like to thank the past and present members of the Burton and Reid labs. In particular, thank you to Dr. Ryan Chanyi for teaching me how to be a competent researcher. I will be forever grateful to Wongsakorn Kiattiburut for working with me on all ATP-related ideas, splitting late-night experiments, providing delicious food, and helping me with everything cell-related. Thank you to Scarlett Puebla-Barragán for all of the office chats, figure ideas, and support you have given me through my research and writing. Thank you to Shannon Seney for always having the bacteria we need and keeping the lab running. Thank you to Johnny Chmiel for constantly pushing me to think bigger and better with my experiments, all of the across-the-desk chats and the thousands of edits.

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List of Abbreviations

ACh	Acetylcholine
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BHYE	Brain heart infusion with yeast extract
Ca ²⁺	Calcium
CFU	Colony forming units
eATP	Extracellular ATP
FBS	Fetal bovine serum
FOS	Fructooligosaccharides
GOS	Galactooligosaccharides
HMO	Human milk oligosaccharides
LAB	Lactic acid bacteria
LB	Lysogeny broth
MRS	de Man, Rogosa and Sharpe
MUI	Mixed urinary incontinence
NO	Nitric oxide
OAB	Overactive bladder
PBS	Phosphate buffered saline
qPCR	Quantitative polymerase chain reaction

SUI	Stress urinary incontinence
TRP	Transient receptor potential
UI	Urinary incontinence
UPEC	Uropathogenic <i>Escherichia coli</i>
UTI	Urinary tract infection
UUI	Urgency urinary incontinence
VDMP	Vaginally defined media with peptone
TH	Todd-Hewitt
ZOI	Zone of inhibition

Chapter 1

1 General Introduction

1.1 Focus of this thesis

Overactive bladder (OAB) and urinary tract infection (UTI) are common conditions that affect millions globally and have similar symptom presentations^{1,2}. While these diseases are quite different, they may share a common link. Urgency urinary incontinence (UUI) is a symptom of both, where the patient feels an urgent need to urinate regardless of bladder fullness and is rarely associated with the presence of uropathogenic bacteria. Adenosine triphosphate (ATP), which is a known biomarker for UTIs, plays a significant role in bladder regulation^{3,4}. Current treatments for UUI and UTI fail to reduce extracellular ATP (eATP) impact on the host. Bladder-associated potentially pathogenic bacteria and the urothelium release ATP, which affects the uroepithelium and may be responsible for several bladder symptoms⁵. Even when an infection is not present, bacterial strains in the urinary microbiota can release ATP and potentially contribute to UUI⁵.

The release of ATP during the growth and establishment of infection by uropathogenic bacteria may act as a virulence factor, triggering Ca^{2+} influx and smooth muscle contraction through purinergic signaling^{5,6}. Purinergic signaling by ATP can regulate and control bladder contraction thus creating a favorable environment for the bacteria by disrupting host shedding and clearance of infection. Additionally, ATP release can potentially cause a reduction of antimicrobial and bacteriocin production by commensal organisms that inhibit *Escherichia coli* and reduce infectivity⁷⁻¹¹. In theory, decreased bacteriocin production could limit environmental competition, further enabling the establishment of infection.

Lactobacillus crispatus ATCC 33820 and *Lactobacillus gasseri* KC-1, when co-cultured with *E. coli* IA2, lower the level of eATP, suggesting that these strains can potentially consume ATP from culture⁵. Addressing the higher levels of eATP in the bladder environment with lactobacilli uptake could reduce UUI and UTI symptoms previously unaddressed.

The goal of this project was to build a greater understanding of the role that eATP plays in urogenital infections and explore the potential of a microbial therapeutic approach via a prebiotic-probiotic application to reduce the effects of that ATP on the host. This work builds

upon our previous studies⁵ and continues to focusing on *L. crispatus* ATCC 33820 and *L. gasseri* KC-1 to reduce the high levels of ATP released during uropathogenic bacterial growth. It is hypothesized that a greater understanding of this unique interaction could lead to better management of UUI and UTI related symptoms.

1.2 Microbial role in bladder health

1.2.1 Human microbiota

The human microbiota is the entire collection of microorganisms that have colonized the human body¹²; microbiome describes abiotic and biotic factors, including environmental factors and microorganism genomes¹³. The microbiota consists of commensal, opportunistic pathogens and pathogenic microorganisms, which vary based depending on the body site. The microbiota can influence susceptibility to infectious disease, and the host-microbiome interaction can impact infection outcomes¹². Commensal members of the microbiota aid in host resistance against infectious microorganisms through pathogen inhibition and exclusion^{14,15}. The microbiota plays a crucial role in host-infection interaction and therapeutic modulation of the microbiome with probiotics, and prebiotics can treat and prevent infectious diseases.

1.2.2 Bladder microbiota and its protective role

The bladder and urinary tract above the distal urethra in females were considered sterile until 2011 when a microbiota was characterized^{16,17} but this microbiota has not been considered when addressing urological conditions. Since then, studies have found that the urinary microbiota differs substantially between healthy and diseased individuals¹⁸. The urinary microbiome differs between sexes, with females having greater abundance of *Lactobacillus* and *Prevotella* and males having greater *Streptococcus* presence¹⁹. Studies of the bladder microbiota are limited due to the differences in the bladder and urethra microbiota. The standard sampling method of voided urine does not accurately capture the bladder microbiota, and the ideal method of catheterized urine is more invasive and less accessible^{19,20}.

Despite the anatomical isolation of the bladder and vagina, they share an interconnected urogenital microbiota, distinct from the gastrointestinal microbiota^{21,22}. The bladder and vagina have highly similar microbial compositions that include commensal and pathogenic microorganisms²². A healthy urogenital tract has a unique microbiota that is dominated by *Lactobacillus* spp.²³⁻²⁵. Lactobacilli dominance is essential to a healthy urogenital microbiota; the most common species are *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus iners*, and *Lactobacillus jensenii*^{23,24,26}. The urogenital microbiota composition (type and abundance) is impacted by age, hormones²⁷, environmental, and external factors (antibiotics, birth control, intercourse, etc.)²⁸. During sexually contracted infections, there is a depletion of *Lactobacillus*; specifically, *L. crispatus*²⁹. Interconnectivity of the bladder and vaginal microbiota could mean that similar *Lactobacillus* loss occurs during infection of the bladder, leading to an imbalance and increasing infection susceptibility. Decreased diversity of the microbiome and/or decrease of lactobacilli abundance in the urogenital tract results in a higher risk for urogenital infections¹⁸.

1.2.3 Probiotics

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host³⁰. Health Canada acknowledges several bacterial species as being associated with probiotic properties: *Bifidobacterium* (*adolescentis*, *animalis*, *bifidum*, *breve* and *longum*), *Lactobacillus* (*acidophilus*, *gasseri*, *johnsonii*, and *salivarius*) and *Lacticaseibacillus* (*casei*, *fermentum*, *paracasei*, *rhamnosus*, and *plantarum*)³¹. * All these species have been relatively well studied and are generally thought to confer health benefits³⁰. However, this does not account for strain differences within the same species, and strains should be designated when calling a product probiotic.

Probiotic strains can positively impact the host through enhancing the epithelial barrier, temporarily adhering to the intestinal mucosa, inhibiting pathogen adhesion, competitively excluding pathogens, modulating the host immune system and producing anti-microbial substances, amongst other potential mechanisms^{30,32,33}. However, probiotic mechanisms of

*The genus *Lactobacillus* was split into 25 genera including the embedded genus *Lactobacillus* in April 2020¹³⁷

action are generally assessed at the strain level, and while many share certain positive attributes, no single strain has every known probiotic feature.

Strains used as probiotics have been extensively characterized to produce an array of anti-microbial substances that contribute to their probiotic effect, including lactic acid, acetic acid, formic acid, phenyllactic acid, benzoic acid, other organic acids, short-chain fatty acids, hydrogen peroxide, carbon dioxide, acetaldehyde, diacetyl, acetoin, bacteriocins and bacteriocin-like inhibitory substances, and others³⁴. Bacteriocin production may be a critical beneficial trait in probiotics as these compounds allow competition in the diverse microbial community³⁵. Bacteriocins are typically anti-microbial peptides released by a broad range of bacteria during the stationary phase, inhibiting or killing similar or closely related bacteria. Thus, bacteriocin production by probiotic strains may function in several ways to aid in host immunity: facilitating a producer's introduction into an established niche, inhibiting invading competitive or pathogenic strains, or modulating the microbiota composition³⁵. In addition, anti-microbial molecules, both bacteriocins and others released from probiotic strains, are believed to directly inhibit a range of pathogenic bacteria and aid in the competitive exclusion of pathogens in the human microbiome³⁶.

1.2.4 Prebiotics

The idea of prebiotics originated from a study on carbohydrate consumption, which positively impacted lactobacilli in the microbiota³⁷; this study showed that diet could impact and modify the microbiota. Prebiotics were described in 1995 as "nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health"³⁸. The idea of supplementing diets to promote healthy microbiomes has since focused on substrates targeted to *Bifidobacterium* and *Lactobacillus* to build on the probiotic concept, though prebiotics may impact a much broader range of beneficial microorganisms³⁹. "Dietary prebiotics" are currently defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit". This effect can occur in the gut or elsewhere³⁹.

Prebiotics are mainly fructans (fructooligosaccharides (FOS) and inulin) and galactans (galactooligosaccharides or GOS), as they specifically promote *Bifidobacterium* and *Lactobacillus* but not pathogenic microorganisms⁴⁰. Recently human milk oligosaccharides (HMOs) have also been classified as prebiotics^{39,41,42}, and consumption leads to higher levels of Bifidobacteriaceae and Bacteroidaceae⁴³. Unlike most dietary fibres, which promote a broader range of microorganisms, prebiotics specifically enhance health-promoting microorganisms.

1.2.5 Probiotic and prebiotic roles in the female urogenital tract

Consumption of probiotics can impact and modify the vaginal and bladder microbiota to increase the level of commensal microorganisms⁴⁴, creating an unfavourable environment for pathogenic bacteria⁴⁵. Consumption of probiotics *Lactocaseibacillus rhamnosus* GR-1 and *Limosilactobacillus reureri* RC-14* can help shift an unhealthy vaginal microbiota, leading to significant increases in lactobacilli and reduction of pathogenic microorganisms while actively taking an oral probiotic⁴⁶. Prebiotics could be used to supplement probiotics, orally or topically, and stimulate health-promoting species already present in the urogenital tract. However, there is limited research on this combination in urogenital environments. Many probiotic products include species of lactic acid bacteria (LAB), *Bifidobacterium* and *Lactobacillus*. The probiotic activity of LAB in the vaginal environment is primarily due to lactic acid excretion, which reduces pH (≤ 4.5), making it an inhospitable environment for most pathogenic bacteria⁴⁷. The production of antibacterial molecules such as bacteriocins and hydrogen peroxide also makes them an ideal urogenital probiotic^{35,48}. Anti-microbial molecules, both bacteriocins and others released from many lactobacilli strains, can inhibit uropathogenic *E. coli* (UPEC) and reduce colonization of uroepithelial cells^{48,49}. Despite the strong evidence to suggest a probiotic lactobacilli treatment can effectively limit urogenital infections, there are very few targeted probiotic strains currently available and further clinical studies are needed⁵⁰.

* Both bacteria have been reclassified from *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reureri* RC-14¹³⁷.

1.3 Urogenital tract and diseases

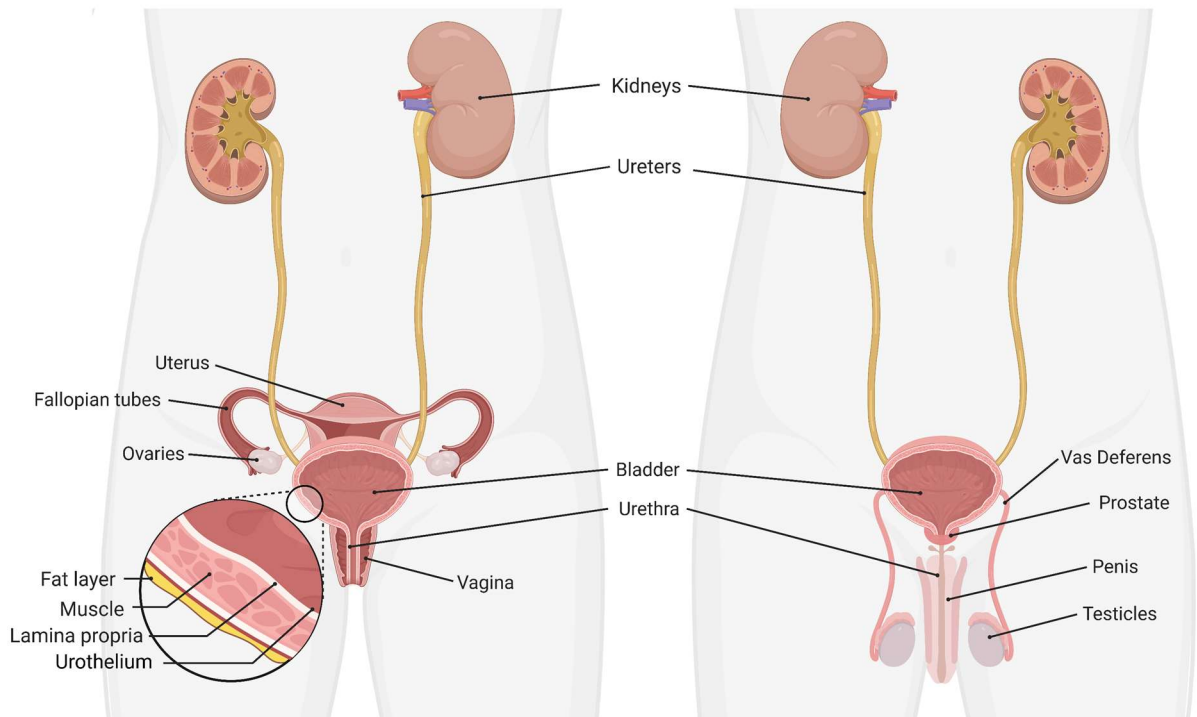
1.3.1 Urogenital tract

The urogenital tract includes all organs in the reproductive and urinary system (**Figure 1-1**). The normative female reproductive system includes the ovaries, fallopian tubes, uterus, and vagina; the normative male reproductive system includes the testicles, duct system, seminal vesicles, prostate gland and penis. The urinary system includes the kidneys, ureters, bladder, and urethra. Females and males share the same urinary system, however, individuals with shorter urethras (generally females) are at greater risk for bladder infections.⁵¹

1.3.2 Functional anatomy of the urinary system

The upper urinary tract consists of the kidneys and ureters, while the lower urinary tract contains the bladder and urethra¹⁸. Kidneys produce urine which travels through the ureters to the bladder; when the bladder is full, the smooth muscles in the wall tighten and expel urine through the urethra. Three main layers make up the bladder wall; urothelial, lamina propria, and muscularis propria (detrusor) (**Figure 1-1**). The urothelium, the interior lining, is a mucosal layer of transitional cells inside of the bladder. The lamina propria is a thin layer of connective tissue containing blood vessels, nerves and glands, surrounding the urothelium. Finally, the outer layer comprises three smooth muscle layers responsible for the contraction and relaxation of the bladder.

The interior layer of the bladder was initially thought of as just a barrier to separate the bacteria and interior bladder environment from the lower nerves and smooth muscle tissues. It is now understood that the uroepithelial (urothelium) layer acts as a mechanosensory conductor, transmitting physical and chemical signals from the bladder to the underlying layers⁵². Uroepithelial cells express numerous receptors/ion channels that release neurotransmitters into the lumen propria to excite the associated nerves and lower muscular tissues⁵³. Similarly, the urothelial cells can receive neurotransmitters from the nerves in the lumen propria. As the bladder fills, it distends, stretching the urothelium and inducing the release of neurotransmitters to regulate bladder contraction: ATP⁵², acetylcholine (ACh)⁵³ and nitric oxide (NO)⁵⁴. The response to physical and chemical stimuli and release of



signaling molecules by the urothelium demonstrates that it is vital in regulating nerve and bladder function and not simply a barrier as it was once thought⁵⁵.

Figure 1-1. Female and male urogenital tract. The reproductive tract and urinary tract together make up the urogenital tract. The urinary tract structures are the same regardless of sex, and the male urethra is typically longer. The reproductive system is closely located to the urinary tract, and the male urethra is part of both. The bladder wall constructing the outside is divided into three distinct layers. Created with BioRender.com

1.3.3 Bladder physiology

The bladder has two phases: urine storage and urine voiding. Bladder filling and urine storage occur at low pressure while the bladder is relaxed and internal and external sphincters are contracted. The sympathetic nervous system controls the storage phase; the norepinephrine-releasing hypogastric nerve triggers relaxation of the smooth muscles and engages the external sphincter⁵⁶. The internal sphincter is constantly engaged and requires internal pressure to release⁵⁶. As the bladder volume increases, the pressure triggers stretch receptors in the urothelium, leading to nerve signaling bladder fullness and the need for release^{52,56}. Disruption to the storage phase often results in lower urinary tract symptoms, including urgency, frequency, and urge incontinence⁵⁶. Urine voiding requires simultaneous contraction of the bladder and relaxation of the urethra and internal sphincter. When the bladder is full, the urothelium signals the parasympathetic nervous system through ACh, activating the parasympathetic motor neurons, which contract the smooth detrusor muscles, causing increased pressure which releases the internal sphincter. Simultaneously the somatic motor neurons inhibit the external sphincter allowing urine to pass through. ATP and other neurotransmitters released by the nervous system, urothelial cells, or bacteria within the bladder environment trigger smooth muscle contractions^{57,58}. Disruption of the voiding phase is associated with hesitancy, weak stream, feeling of incomplete bladder emptying and post-urination leakage⁵⁶. In a healthy bladder, these phases allow controlled storage and release of urine; however, physical and psychological disorders can disrupt signaling and lead to bladder dysfunction and disease⁵⁶.

1.3.4 Urinary tract diseases and disorders

Many conditions affect the bladder: bladder dysfunction, UTI, bladder stones, incontinence (urge, stress, functional, overflow, mixed), interstitial cystitis, cystitis, neurogenic bladder, OAB, cancer and asymptomatic bacteriuria. Many of these conditions present with similar symptoms such as nocturia, frequency, dysuria, urgency, incontinence, hesitancy, straining, weak stream, abdominal pain, intermittency, incomplete emptying and postmicturition dribbling. Over half of individuals presenting with lower urinary tract symptoms are misdiagnosed with UTI when they have OAB due to the high similarity of symptoms⁵⁹. Bladder problems can affect both sexes or be specific to females or males. Individuals with

uteruses can develop bladder problems due to shorter urethras, pregnancy and childbirth, pelvic floor weakening, trauma or injury, or menopause. Individuals with prostates face bladder problems associated with prostate health and disease. Both sexes experience an increased frequency of bladder diseases due to ageing, infection, blockages of the urinary tract, chronic coughing, constipation, and obesity². Nervous system injury or disease, including Alzheimer's, stroke, Parkinson's, multiple sclerosis, spinal cord injury, diabetes and anxiety, can negatively impact and damage bladder nerves and muscles. With many urinary tract diseases and disorders, there is a shift in the urinary tract microbiome⁶⁰. The urinary microbiome changes observed with infection or insult could be addressed with probiotics, prebiotics and dietary intervention to help revert the microbiome to a healthy state.

1.3.4.1 Overactive bladder

OAB is a complex of symptoms, UUI, frequency, and nocturia, characterized by unstable bladder contractions. OAB is a disruption of the storage phase due to the involuntary contraction of the smooth muscles of the bladder. The predominant symptom of OAB is UUI, the sensation to urinate regardless of bladder fullness¹. This condition is frequently undertreated and underreported¹. Globally, OAB affects 1.5-36.4% of populations, 10% in Canada⁶¹, with prevalence depending on age and sex⁶². The economic burden of OAB in America in 2020 was \$82.6 billion and is estimated to increase as the population ages⁵⁷. Individuals suffering from OAB often have decreased quality of life, lower work productivity, and higher anxiety and depression⁶³.

There are several treatment options for OAB, including fluid management, behavioural modification, bladder training, pelvic floor exercises, drug therapy, neuromodulation and surgery; however, treatments are often unsatisfactory⁶⁴. The goal behind treatment is to improve the quality of life for the patient as there is no cure, only symptom reduction. Behavioural therapy is the most common course of treatment followed by pharmacological administration; mainly antimuscarinics⁶⁵ which limit ACh binding to muscarinic receptors⁵¹. Unfortunately, there is no truly effective drug treatment; those available have very low efficacy, and the side effects generally deter long-term use⁶⁴.

Despite active research and drug development, there has been limited improvement in treatment. There is no set definition of 'failure' or 'success' in a patient suffering from OAB, as how the treatment impacts the patient's quality of life varies greatly and cannot be standardized. Responses to treatments are also widely impacted by comorbidities, drug metabolism, concurrent therapies and more. These factors are not always obvious and make it hard to determine if a treatment will be successful in an individual patient. A greater understanding of what causes the symptoms could result in new and more successful ways to address OAB.

1.3.4.2 Urinary tract infections

UTIs are among the most common infectious conditions globally, mainly impacting females and the elderly – UTI prevalence increases with age. There are approximately 150 million cases annually worldwide, with 50% of females and 5% of males contracting a UTI in their lifetime², and they cost over \$6 billion world wide⁶⁶. Most commonly, UTIs are caused by bacteria, predominantly UPEC strains^{67,68}. Risk factors for UTIs include sexual intercourse, previous UTIs, new/multiple sexual partners, urethral length, certain birth control types, and menopause^{69,70}. Many are caused by the ascension of pathogens from the rectum along the perineal skin and then through the vagina or directly up the urethra to the bladder. Symptomatic UTIs present with malodorous urine, abdominal pain, dysuria, frequency and urinary urgency⁷¹.

There are four different types of UTIs, dependent on infection location: urethritis (urethral infection), cystitis (lower urinary tract), urethritis (ureter infection due to blockage or infection migration), and pyelonephritis (an infection spread to the kidneys or from a urinary tract blockage resulting retrograde flow of infected urine). An infection involving the urethra and bladder is termed a lower UTI, while an infection of the ureters or kidney is an upper UTI. Most UTIs are uncomplicated lower tract infections, occurring in otherwise healthy, non-pregnant, pre-menopausal individuals with a normal functioning urinary tract. Complicated UTIs are associated with catheters, blockages, and functional abnormalities, resulting in increased colonization and decreased efficacy of therapy. Recurrent UTI occurs after a previously documented infection has been resolved, when an individual has two or more infections in 6 months or three or more infections in 12 months^{72,73}. Healthcare-

associated UTIs are the most common form of hospital-acquired infections⁷² and lead to more prolonged hospitalizations, increased morbidity and mortality⁶⁹.

A UTI is diagnosed with clinical presentation of symptoms and urine microbial evaluation. To identify the causative pathogen and suggest an ideal treatment both urinalysis and urine culture with sensitivity should be used; however, immune markers in urine alone are often used to identify infection in clinic⁷⁴. Treatment is determined based on infection and severity of symptoms, as well as any predisposing host factors. Uncomplicated and complicated UTIs are often treated with hydration and antibiotics, removal of any obstructions in the urinary tract, and catheter removal or replacement^{74,75}. Antibiotic treatment is often empirical before proper diagnosis had been acquired. The drug selection should depend on the infection location (urethra, bladder, ureter or kidney), which pathogen is believed to be responsible for the infection, and if there is any drug resistance⁷⁴⁻⁷⁶. The most commonly used antibiotics are trimethoprim/sulfamethoxazole, nitrofurantoin, fosfomycin, cephalexin and ceftriaxone. Frequently, fluoroquinolones are prescribed as a first choice instead of being reserved for more serious cases.

The majority of orally ingested antibiotics are highly concentrated in the urine, making them highly effective at clearing bacteria from the lower urinary tract. When kidney tissues are infected, antibiotics that can penetrate the tissue are needed⁷⁷. Though antibiotics effectively treat UTIs, they can disrupt the vaginal microbiome for up to 6 months resulting in a reduced *Lactobacillus* population and increased uropathogenic presents in the urethra⁷⁸. Individuals who experience recurrent UTIs often require suppressive antibiotic therapy, long-term prophylaxis or post-coital prophylaxis. Increased risk of side effects associated with long-term antibiotic use makes this a less ideal treatment⁷³. Intravaginal estrogen can be used in postmenopausal individuals to reduce recurrent UTI risk which is associated with thinning vaginal epithelium and a tendency for dysbiosis⁷³. The research on non-antibody prophylaxis options is limited; however, *Lactobacillus* probiotics^{79,80}, methenamine⁸¹, cranberry polyphenols⁸² and D-mannose⁸³⁻⁸⁵ are sometimes added to treatments and can be beneficial in the prevention of UTI.

1.3.5 Urgency urinary incontinence

Urinary incontinence (UI), defined as involuntary loss of urine⁸⁶, is a common symptom associated with bladder and pelvic floor dysfunction. There are three primary types of UI: stress UI (SUI), urgency UI (UUI) and mixed UI (both SUI and UUI); SUI is the involuntary loss of urine on effort or physical exertion or on sneezing or coughing (activity-related UI); UUI is the involuntary loss of urine associated with urgency⁸⁶; urgency is the complaint of a sudden compelling desire to pass urine which is challenging to control⁸⁷. UUI is potentially caused by physiological disruption of the detrusor muscle in the bladder wall and/or muscles in the pelvic floor, which leads to leaking and the urgency sensation⁸⁸. Abnormal neuromuscular signaling and/or function of the bladders storage and voiding phase also leads to UUI; this can be chronic and is poorly understood⁸⁸. This symptom can occur in males and females. In males, it is more frequently associated with prostatic enlargement or damage than muscle dysfunction as in females⁸⁸. The symptom is frequently defined as only present in a 'non-infected' bladder⁸⁷; however, UUI can result after or during a UTI⁶¹, meaning it can be seen in an infective environment^{16,17,89}. The specific global impact and economic burden of UUI is hard to ascertain due to the overlap of UI conditions and OAB patients; however, millions of individuals suffer the effects of UUI, and the economic burden is substantial⁶². As the current population ages, there is a projected increase in UUI prevalence and UUI associated comorbidities⁶².

Treatment of associated conditions often address UUI. If treatment does not impact the UUI symptom, it could be considered chronic and require further lifestyle changes or surgery⁹⁰. With chronic UUI, the goal tends to be symptom reduction rather than complete resolution; treatment to decrease symptoms is unique to the patient and their lifestyle⁸⁸. Similar to OAB, treatments target lifestyle changes, timed voiding, bladder training, weight loss, and fluid optimization⁹¹. There are pharmacological treatments for UUI, however, they fail or are unsatisfactory in approximately half of the patients⁶⁵. Lifestyle changes often give the best result when dealing the chronic UUI, as long as the patient is willing to work with their clinical team⁸⁸. Microbiota disruption is commonly found in individuals suffering from UUI, suggesting bacteria may play a significant role⁸⁹. Further research is needed to understand the

overlap and role that UUI plays in UTI and OAB and the role of microorganisms in UUI symptoms.

1.3.6 Establishment of UTI

Uncomplicated UTI is predominantly caused by *E. coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterococcus faecalis* and *Staphylococcus saprophyticus*⁹². As noted above, bacteria are introduced to the urinary tract from the rectum, peritoneum, sexual intercourse, or some forms of birth control (**Figure 1-2**). Adherence to host cells through flagella and pili appears important for the establishment of bacterial infection⁹³. Upon introduction to the urinary tract, bacteria migrate up the urethra towards the bladder. At the bladder, bacterial flagella, pili and adhesins promote colonization and bind directly to the bladder epithelium. During infection, UPEC strains can reside in fusiform vesicles within urothelial cells to avoid elimination with voiding and re-emerge during distention of the bladder⁵⁵. Host inflammatory responses to infection of the urinary tract include neutrophil infiltration to clear extracellular bacteria. However, bacteria can evade the immune response through biofilm formation, host cell invasion and morphological modification. Immune evasion allows further bacterial multiplication and infection progression. Bacteria produce toxins and proteases to damage host cells and release nutrients which promote bacterial survival⁹². Damaged bladder epithelial cells will shed off, exposing bacteria hiding in the wall and aiding in bacterial clearance during voiding. Cell shedding, in part, explains how some UTIs self-resolve without antibiotics. When the bladder distends with urine and during storage, remaining bacteria thrive in the nutrient-rich environment. From the bladder, bacteria can migrate up the ureters towards the kidneys⁹², where they can infiltrate and damage the renal epithelium and easily cross the tubular epithelial barrier and access the bloodstream, causing bacteremia.

In a complicated UTI, the introduction of bacteria and urethra migration are similar; however, the bladder, ureter or kidney must be compromised. Catheter placement and stones induce a host response, resulting in fibrinogen accumulation, ideal for fibrinogen-binding proteins expressed by pathogens⁹². When there is an obstruction, biofilms on medical devices or epithelia shield bacteria from neutrophils, antibiotics, and other stressors while promoting growth^{92,94}. Biofilm formation is common in uropathogenic bacteria, especially in complicated UTIs associated with catheters and other obstructions^{2,95}.

Several virulence factors contribute to UPEC strains effectively establishing a UTI. Expression of type 1 pili plays a role in binding to the epithelium with tip adhesin FimH, binding to mannose epitopes and paucimannosidic glycans conjugated to uroplakin Ia. Once bound, bacteria can invade cells⁹⁶, where they multiply to form intracellular bacterial communities, avoiding host immune responses (outside and inside the cell), and antibiotics⁹⁶. Upon bursting from the cell, the pathogens invade neighbouring cells and repeat the process. UPEC can also establish quiescent intracellular reservoirs, 4-10 non-replicating bacteria encased in F-actin, which can remain viable for months in lower transitional cells allowing reinfection^{92,96}. While in the bladder, UPEC releases molecules to promote its survival and control its environment. Toxin α -hemolysin lyses cells through pore formation, causing shedding and releasing host nutrients and iron; siderophores scavenge iron and allow uptake into the bacteria^{92,96}. Cytotoxic necrotizing factor 1 induces host cell anti-apoptotic and pro-survival pathways to prevent apoptosis and further bacteria colonization^{92,96}. Extracellular UPEC filamentous morphology can be modified, making the bacterium more resistant to neutrophil killing⁸⁶. In the kidneys, pyelonephritis-associated p-pili bind globoside-containing glycolipids on renal tissues, and the PapG adhesion tipping the pili modulates the host immune response to prevent opsonization and clearance. Cell adhesion, toxins, immune evasion, and intracellular colonization make UPEC strains ideal urinary pathogens.

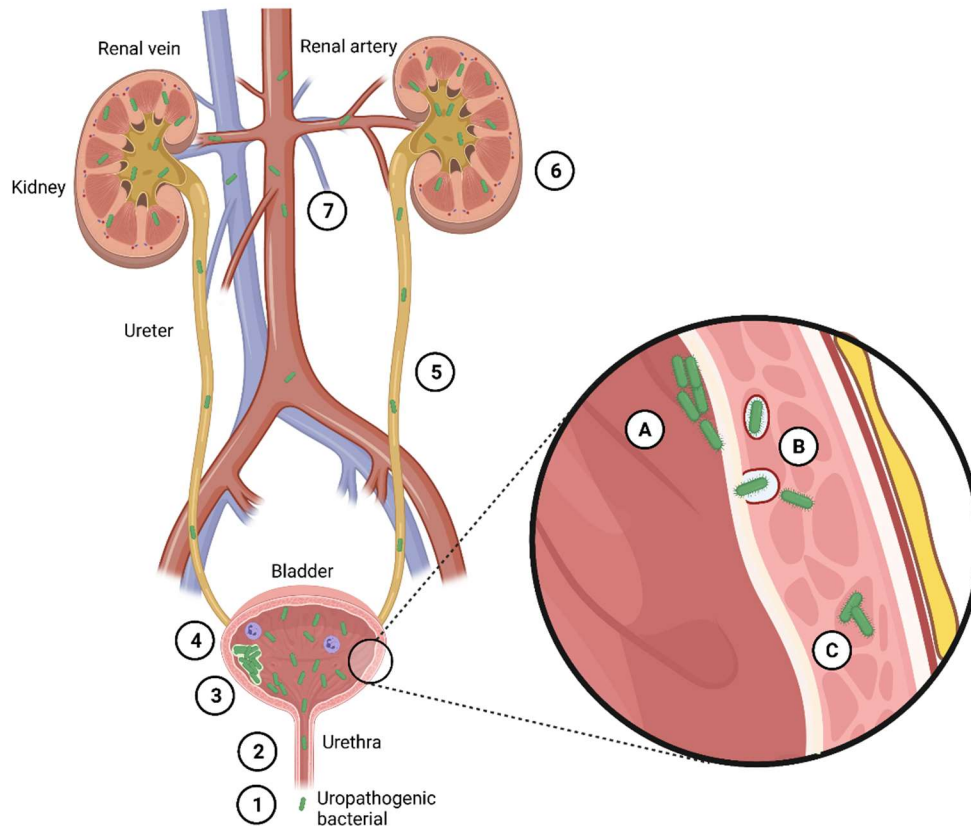


Figure 1-2. Establishment of urinary tract infection. Uncomplicated UTI initiated after contamination of the periurethral area (1), colonization of the urethra and migration to the bladder (2). Expression of pili and adhesins allows colonization of urothelium umbrella cells (A). Bacteria work to evade immune response through host cell invasion (A-C) and biofilm formation (3). Adherent bacteria invade cells and are expelled back into the lumen or escape from the vesicle into the cytoplasm (B). Bacteria can replicate in the cytoplasm, burst out of the cell, invade adjacent cells, or create quiescent intracellular reservoirs that may seed further UTIs (C). Host inflammatory responses, including neutrophil infiltration (4), work to clear extracellular bacteria. If bacteria persist, they can produce toxins to damage host cells releasing nutrients to promote survival and ascension to the kidneys (5). Kidney colonization and tissue damage initiated by bacteria (6), untreated this can progress further to bacteremia (7). Created with BioRender.com

1.3.6.1 Addressing bacterial infection

As previously described, antibiotics are most frequently used to treat UTI of which most are broad spectrum. There are other methods that have been devised to reduce recurrent UTIs. These include modulation of host immune responses, interfering with UPEC adhesion to urothelial cells, preventing and disrupting biofilms, and limiting invasion into host cells. Several vaccines under development target UPEC specifically, allowing a heightened adapted immune response against the O-antigens⁹⁷, siderophores⁹⁸, surface antigen YncE⁹⁹, and *E. coli* extract¹⁰⁰; though currently, none are available for patient use. Estrogen supplementation targets vaginal epithelial thinning and can lower recurrent UTI rates in postmenopausal women⁹⁶. Modification of the bladder pH with organic acids and vitamin C has a bactericidal effect further enhanced with methenamine which hydrolyzes formaldehyde and ammonia^{81,96}. Polymeric phenolic proanthocyanidins (isolated from cranberries) can reduce P-fimbrial adhesion *in vitro*, but so far, cranberry extract studies do not support consistent UTI reduction⁹⁶. Reducing adhesion by targeting and binding FimH with high levels of D-mannose in the urine prevents urothelial binding *in vitro*^{84,96}, but again strong evidence is lacking that this can be effective clinically. Restoration of the urinary microbiota though supporting lactobacilli could be beneficial²¹. Probiotic strains show promise in reducing pathogen loads, stimulating the immune system and modulating the environment with hydrogen peroxide, surfactants, bacteriocin-like molecules and anti-adhesion molecules^{23,36,79,97,101,102}. These treatments could potentially be used in conjunction with an antibiotic regimen to help treat and prevent UTIs.

1.3.7 Disrupted urogenital microbiomes and bladder disease

It is now appreciated that UUI and UTI are associated with imbalances in the urogenital microbiota^{89,103,104}. Current treatments were not designed to protect the beneficial microbes nor to appreciate significant differences between the microbiota of healthy individuals and those with urological disease^{89,103}.

Comparisons of the urinary microbiome of women with UUI to those without showed a significant difference in abundance and frequency of bacteria⁸⁹. In women with UUI, *Lactobacillus* abundance was lower, and *L. gasseri* was more common than *L. crispatus*⁸⁹.

Gardnerella is associated with a disrupted microbiota, commonly found in women with bacterial vaginosis and individuals with UUI, but not in healthy individuals⁸⁹. Uropathogenic species were also more frequently found in UUI patients⁸⁹. However, it is unknown if this shift in microbiota contributes directly to UUI or is a result of the condition. The urogenital microbiota is also altered in patients with UTI and recurrent UTIs^{59,79}. Not only do pathogens disrupt the microbiome, so too do antibiotic treatments resulting in pathogenic bacteria lingering for months⁷⁸. More research is needed to understand the differences between urinary microbiomes in healthy and infected individuals, which could lead to better therapeutic options.

1.3.8 Impact of eATP in the bladder

It has long been recognized that ATP is a universal intracellular energy source involved in cellular respiration, metabolism and cellular processes. Beyond its role as an energy storage molecule, eATP is a known purinergic neurotransmitter⁶ that can modulate host nervous systems, host immunity, and acute and chronic inflammation^{105,106}. Higher ATP levels are associated with several human bladder conditions, including UTI and OAB^{3,107,108}. For over 50 years UTI has been diagnosed by rapid test for ATP in urine³. The UUI associated with OAB could be triggered by high ATP concentrations stimulating the bladder epithelium at lower volumes¹. ATP plays a crucial role in the bladder's neural and motor function through purinergic signaling⁶. Bladder stretching stimulates urothelial-derived ATP release, and as the bladder fills, the ATP levels rise, with the highest concentrations observed in a full bladder¹⁰⁹. Uroepithelial and sub-urothelial cells purinergic (P2) receptors, ionotropic P2X and metabotropic P2Y receptors are responsible for urothelial ATP release in response to the increased levels of eATP¹¹⁰⁻¹¹³. In a healthy bladder, eATP binds P2X3 receptors to alert the bladder of fullness. In a patient with UUI, ATP levels in a reduced volume bladder are significantly higher and could trigger the urgency sensation¹⁰⁹. Higher ATP concentrations in disordered bladders can impact bladder function, increasing urination frequency, inducing pain and full bladder sensations with lower volumes^{4,114}. Blocking or knocking out the P2X receptor can reduce bladder symptoms caused by increased ATP levels^{114,115}. Despite this, current treatments do not use ATP reduction or P2X blocking to address bladder conditions.

1.3.9 The role of calcium in the bladder

Calcium (Ca^{2+}) ions play a regulatory role in the contraction of the smooth detrusor muscle in the bladder wall¹¹⁶. In the urothelium, transient receptor potential (TVR) channels (TRPV1 specifically) and purinergic receptors control intracellular Ca^{2+} concentrations and are sensitive to neurotransmitters such as ATP¹¹⁷. Neurotransmitter release and cellular processes can be regulated through Ca^{2+} influx and blocking ion channels inhibits neurotransmitter release. When the action potential is reached at a nerve terminal, voltage-dependent Ca^{2+} channels open, and the greater extracellular concentrations cause Ca^{2+} to enter the terminal. Following Ca^{2+} influx, there is a rapid release of neurotransmitters into the surrounding area. Purinergic receptors are triggered to release ATP by increased eATP levels, and with greater ATP there is an associated increase in intracellular Ca^{2+} leading to cellular depolarization (an excitatory phase)¹¹⁸. As bladder Ca^{2+} increases, sub-urethral parasympathetic postganglionic nerve terminals release neurotransmitters ACh and ATP, exciting cholinergic muscarinic receptors and triggering detrusor contraction and micturition¹¹⁸. Over activation of these pathways at lower urine volumes may be related to urgency conditions¹¹⁶. Potentially, Ca^{2+} channel antagonists, such as NO^{119} , or GABA^5 could be used to suppress and regulate contractions associated with increased urinary ATP.

1.3.10 Bacterial release of neurotransmitters to regulate their environment

The gut microbiota interacts with the host by releasing neuroactive molecules such as γ -aminobutyric acid, serotonin, histamine, dopamine and norepinephrine¹²⁰. These and other neurotransmitters can modify gut physiology and impact the nervous system and brain. Since the discovery of the urogenital microbiota, there has been minimal research on host-bacterial interactions; however, bacteria known to release neurotransmitters are found in the urogenital tract. The lower urinary tract connects to the pelvic parasympathetic nerves, lumbar sympathetic nerves and pudendal nerves¹¹⁷. Purinergic neurotransmission at these nerves can control smooth muscle contractions, and increased purinergic function can lead to unstable bladder conditions. Thus, neuroactive molecules produced by urinary microbes could similarly impact the bladder environment by interacting with the host nervous system.

Several studies have shown that bacteria release ATP into the extracellular environment during exponential and log phase growth^{121,122}. As noted above, bacteria can also reduce eATP levels during growth^{5,122}. During infection, the higher ATP levels in the urine can be attributed to bacteria, however there is also host release of ATP in response to bacterial ATP¹²³. As a crucial lower urinary tract neurotransmitter, bacterial ATP could control smooth muscle contraction similarly to host ATP. Uropathogenic *E. coli* IA2 and *Gardnerella vaginalis* 14018 can trigger Ca²⁺ influx and smooth muscle contractions of myofibroblasts through purinergic signaling via extracellular ATP release⁵. Compared to *E. coli* IA2, *G. vaginalis* 14018 releases significantly more ATP⁵ which could contribute to urgency in OAB patients who have an uninfected bladder. However, certain lactobacilli can reduce ATP levels in media supplemented with bacterially relevant concentrations. Additionally, *Lactobacillus* supernatant can reduce smooth muscle contractions triggered by uropathogenic bacterial supernatant⁵.

The release of extracellular ATP may be associated with bacterial cellular respiration. A study found that bacterial mutants without cytochrome *bd* oxidase release less ATP compared to wild-type¹²⁴. Increased ATP release is seen in *E. coli* which has up to six *bd*-type oxygen reductases, compared to lactobacilli which may have only one¹²⁴. The difference in oxygen reductases suggests that they could play a role in the level of ATP bacteria can release. Another study demonstrated that glucose is essential for ATP secretion¹²⁵. Taken together, these studies imply a potential causal link between cellular respiration, nutrient availability and ATP release/uptake.

1.3.11 The potential role of eATP released by bacteria during infection, and detrusor muscle control in the bladder

The role of ATP in bladder contraction and urgency symptoms has been well documented and understood. However, prior to work done in our lab, it has not been related to the urogenital microbiota. The association between bacteria releasing and uptaking ATP during growth, the known neuron interaction between microbiome and host, and the bacterial levels of ATP in urine suggest that bacteria could utilize ATP to modulate the bladder environment during infection. During UTI, uropathogenic bacteria could release neurotransmitters (ATP) to target the urothelium purinergic receptors. Bacterial ATP stimulates the release of host

ATP, increasing Ca^{2+} , creating an energy potential that releases ACh and ATP in sub-urothelial layers to target detrusor receptors and causes urgency and bladder contractions at low urine volume.

1.4 Potential therapeutic use of lactobacilli against eATP

1.4.1 Benefits of lactobacilli

Lactobacilli play a crucial role in maintaining a healthy urogenital tract. When disease occurs, there is generally a reduction or shift of the dominant *Lactobacillus* species; with this change, the host loses the commensal benefits previously provided. The ability of lactobacilli to protect the host is multi-factorial^{126–128}.

In addition to previously identified probiotic benefits, lactobacilli may play a role in reducing ATP from the environment. When *L. crispatus* ATCC 33820 was co-cultured with uropathogenic *E. coli* IA2, lower levels of eATP were found, suggesting consumption of eATP⁵. The high levels of ATP released by *E. coli* IA2 appear to cause smooth muscle contractions through purinergic signaling, and *L. crispatus* ATCC 33820 supernatant can decrease these contractions⁵. Furthermore, *Lactobacillus* has been shown to have a cytotoxic effect on *E. coli in vitro*, suggesting that it produces a bacteriocin-like molecule to target and inhibit or kill the pathogen^{7,8}.

1.4.2 Host introduction of lactobacilli and prebiotics

The ability to manipulate the urogenital microbiota and restore it to one associated with homeostasis is not a simple task. Introduction of a probiotic strain could be achieved via topical application (to the external distal urethra and vagina), oral consumption or a catheter^{46,129,130}; a non-invasive method is preferred. Lactobacilli strains for urogenital tract health should be selected based on their ability to survive and grow at this interface and in urine, plus properties that allow them to interfere with pathogenic bacteria¹³¹. Both *L. rhamnosus* GR-1 and *L. fermentum* RC-14* are good candidates^{46,130,132}, though others likely

* Reclassified to *Lactocaseibacillus rhamnosus* GR-1 and *Limosilactobacillus reureri* RC-14¹³⁷.

exist. The addition of an appropriate prebiotic, such as lactulose¹³³, may also promote recovery of the lactobacilli in the vagina and stimulate the probiotic strains^{46,84,134–136}.

1.5 Rational and hypothesis

In summary, ATP plays a role in both healthy and diseased bladder states. Uropathogenic and bacteria associated with urogenital disorders release ATP during growth. Consequently, more studies are required on regulation and reduction of ATP in the bladder to address symptoms and provide relief to UTI and UUI patients. The ability of lactobacilli to reduce ATP might provide a means to limit pathogenic impact on the host and urogenital microbiome. It is hypothesized that *Lactobacillus* spp. can modulate extracellular ATP levels released by bacteria in a urinary environment. It is hoped that this thesis will add to our understanding of the role that eATP plays in urogenital infections and provide a path towards novel approaches to treatment and disease management.

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Chapter 2

2 Bacterial release and consumption of ATP during growth

This chapter focuses on the adenosine triphosphate (ATP) levels released and taken up by pathogenic and commensal bacteria during growth. The goal was to understand which microorganisms can produce, sequester, or regulate extracellular ATP (eATP). This research also identifies the potential of eATP for uropathogenic swarming bacteria to utilise and follow as a chemoattractant.

A portion of the bacterial ATP screening work was completed by undergraduate student Jennifer Chen during her fourth-year thesis project. Undergraduate student Vibusan Shanthirasegaram completed the directional growth assays during his fourth-year project. Dr. Jeremy Burton supervised both, and their research and experiments were directed with my assistance.

2.1 Abstract

ATP is a crucial energy source that is utilized by all living organisms. It is an energy storage molecule that powers intracellular processes. Several bacteria have been shown to secrete ATP extracellularly, modulating the host environment as a neurotransmitter and potentially representing a virulence factor. In this study, ATP release during growth was assessed in 27 bacterial strains, including commensal, uropathogenic and other pathogenic bacteria. In addition, the swarming patterns of uropathogenic *Proteus mirabilis* were assessed in the presence of ATP and bacterial supernatants, to understand if bacterial eATP could impact another organism in the environment. The results showed that eATP concentrations were strain-specific and varied significantly within a species. Pathogenic organisms generally had greater levels of ATP release compared to commensals tested. The level of eATP increased and peaked during log-phase growth, then decreased in the stationary phase in *Escherichia coli* IA2 and *Lactobacillus crispatus* ATCC 33820. In addition, there was an observable directional growth change by *P. mirabilis* associated with ATP and bacterial supernatant. This study suggests that eATP release is specific for each bacterial strain and could supplement nutrients in the stationary phase. Greater concentrations of eATP in pathogens

support the idea that bacterial eATP can modulate the environment and represent a novel virulence factor.

2.2 Introduction

All living organisms require and utilize ATP as an energy source and storage molecule. The energy storage molecule is generated through cellular respiration and can be used to power cellular functions necessary for survival, growth and replication. It is not yet fully understood how bacteria release and take up ATP during growth¹⁻³. Generally, eATP concentrations peak around exponential phase growth then reduce in stationary phase¹⁻³. Both intracellular and eATP levels are well documented in *E. coli*, with significantly higher intracellular levels of 1-5 mM. The amount of intracellular ATP changes in response to environmental and physiological pressures on the bacterium⁴⁻⁸. Pathogenic as well as commensal species can release ATP, though higher levels are observed in pathogenic bacteria^{3,9,10}. The amount of ATP released may be linked to glucose availability and cellular respiration^{3,11}. Beyond the role played in bacterial survival and growth, eATP is interacting with the host environment.

Host or bacterial ATP can act as a neurotransmitter, triggering a range of signals in humans¹². In the gastrointestinal tract, bacterial eATP can modulate the environment by promoting bacterial-host cross-talk, controlling immune cell differentiation, and mediating inflammation^{9,13}. In the bladder, ATP concentrations rise as urine volume increases, which causes bladder distention and eventually triggers muscle contractions, bladder voiding and micturition¹⁴⁻¹⁶. In human urinary and gastrointestinal tracts, eATP signals through purinergic receptors P2, specifically P2X and P2Y¹⁷⁻²⁰; these responses can be reduced by blocking the P2X receptor in environments with higher eATP concentrations^{15,21}. These findings suggest that bacteria could use ATP to alter their environment during growth, acting either as a virulence factor or stimulating host-bacterial communication in commensals.

The mechanism of bacterial ATP secretion is unknown; however, several methods of secretion are observed in eukaryotic cells and other microorganisms. Eukaryotic cells release ATP through pore-forming channel-mediated release, active vesicle-mediated exocytosis and non-specific release²². Fungi such as *Candida albicans* secrete ATP via conductive channels and are histatin5 dependent, which leads to the death of neighbouring *C. albicans* cells²³.

Saccharomyces cerevisiae releases ATP via *MCD4P* glucose-dependent membrane protein through vesicular trafficking initiated by ATP uptake into the Golgi compartment²⁴. In *S. cerevisiae*, similar to *E. coli*, eATP concentrations are linked to glucose availability^{11,25}. Cytochrome *bd* oxidase is vital to ATP secretion in *Salmonella enterica*; as in *E. coli*, mutants lacking *bd* oxidase subunits had significantly reduced eATP levels compared to wildtype³. The cytochrome *bd* oxidase creates a proton motive force by pumping protons over the inner bacterial membrane, which catalyzes the final step in cellular respiration. The reduction in ATP release by *E. coli* with lower glucose availability and cytochrome *bd* oxidase mutants suggests that glycolysis and respiration are linked to higher ATP release than fermentation. Furthermore, this demonstrates that *E. coli* can build up ATP intracellularly and release it with functional respiration. Potentially, excess ATP can be released into the environment for storage until later, when intracellular levels are depleted.

None of the previously identified ATP release mechanisms have been observed in bacteria, and the necessary genomic pathways are mostly absent. However, there are several proposed mechanisms through which ATP could be released and taken up by bacteria: porins, mechanosensitive channels, outer membrane vesicles, efflux pumps/secretion systems or lytic mechanisms during growth²⁶. General porins in the outer bacterial membrane could facilitate eATP uptake passively or by a gradient into the cell periplasm where there is constant ATP, though none have been identified. Mechanosensitive channels secrete ATP and other small metabolites when bacteria are exposed to hypertonic osmotic shock^{27,28}; however, this has not been observed in unstressed bacterial growth. Finally, outer membrane vesicles play a known role in bacterial pathogenesis and contain ATP, which can be released extracellularly, though this is not fully understood²⁹.

The role that outer membrane vesicles play in eATP fluctuations is unclear. There is a steady amount of ATP in the periplasmic space which could be from production or uptake from the periplasm. An outer membrane vesicle could facilitate ATP transport from the periplasmic space to the extracellular environment^{26,29}. During cell division and cell lysis ATP could also leak out, leading to increased eATP concentrations post log-phase growth²⁶. There could also be unidentified efflux pumps or secretion systems in bacterial membranes specific to ATP²⁶.

In addition to releasing ATP, several bacteria can consume ATP from their environment. When media is supplemented with ATP, *E. coli* K-12, *S. enterica* SE2472, *L. crispatus* ATCC 33820, and *Lactobacillus gasseri* KC-1 can reduce or eliminate the supplemented concentration during growth^{3,10}. After presumed uptake, ATP can be found in the periplasm where it is hydrolyzed²⁹. Hydrolysis of ATP could feasibly occur on the outer membrane³, or there could be another unidentified mechanism of consumption. There is a steady ATP concentration in the periplasmic space, which is hydrolyzed in a linear eATP-dependent manner²⁹. Assuming ATP enters into the bacterial periplasm, there is no known mechanism by which it could be transported into the cytosol or transported out from the cytosol²⁶. Further research is needed to understand how bacteria reduce eATP concentrations and the cellular machinery used in this process.

The bacterial release of ATP may be a virulence factor that helps to modulate the environment during infection. Bacterial eATP impacts the host through immune cell modulation⁹, bladder contraction control¹⁰, induction of host cell ATP release^{10,29}, induction of pain^{15,19}, and triggering acute and chronic inflammation^{13,30}. Biofilm formation, a common uropathogenic virulence factor especially in complicated UTIs^{31,32}, is promoted by ATP³³⁻³⁵. Intracellular ATP is used as a nutrient source and a signaling molecule during biofilm formation^{1,34}. Similarly, increased eATP levels could be a nutrient source or bacterial signaling molecule. A common uropathogen, *P. mirabilis*, forms biofilms on catheters allowing the infection to progress with limited host interactions³⁶. Unique swarming motility of *P. mirabilis* could facilitate migration from the periurethral region up the catheter surface to the bladder³⁷. Higher levels of eATP released by bacteria or the bladder could aid in *P. mirabilis* infection establishment. Biofilm formation can be reduced *in vitro* by decreasing environmental ATP with the application of apyrase³⁸ (a calcium-activated enzyme that catalyzes the hydrolysis of ATP to yield AMP and inorganic phosphate). In the bladder, high levels of eATP can trigger Ca²⁺ influx³⁹; application of apyrase could potentially lower the eATP levels and their negative impact on the host.

Our lab has shown ATP enhances the growth of *E. coli* IA2 and *L. crispatus* ATCC 33820 in media, leading to a reduction in eATP¹⁰. This suggests that a commensal bacterium which consumes ATP could be introduced to the bladder, or a species that is already present could be promoted to reduce pathogenic eATP, thus contributing to a healthy urogenital tract. Host

purinergic receptor activation by bacterial eATP could play a vital role in virulence, and the increased eATP might act as a signaling molecule to other microorganisms or as a nutrient source.

The discovery of bacterial ATP secretion is relatively recent¹, and there has not been much research into a release mechanism or host interactions. However, there has been a recent increase in studies on bacterial eATP and its role in growth¹³. Increased eATP concentrations are associated with urinary tract infection (UTI) and overactive bladder (OAB), but little research has been done to understand the role of pathogenic bacteria in these conditions^{10,15,16,40-42}. The urogenital microbiota, ATP release and bacterial interaction has been overlooked by current UTI and OAB treatment methods. The fact that microbiota dysbiosis is associated with these conditions⁴³⁻⁴⁵ should factor into novel treatment approaches in the future.

Overall, bacterial eATP may significantly impact the host environment and neighbouring microorganisms; however, the mechanism of release, specific host impacts, and uptake mechanism have not been elucidated. Understanding which bacteria, both commensal and pathogenic, can produce or sequester eATP will contribute to a greater comprehension of the role eATP plays in bacterial growth and infection. In this chapter, uropathogenic and commensal bacteria isolated from the human urogenital tract and others will be screened to assess the release and sequestration of ATP. Additionally, the impact of ATP and bacterial ATP in supernatant on uropathogenic *P. mirabilis* 296 swarming will be investigated to understand if ATP can influence its growth pattern.

2.3 Materials and methods

All bacteria were maintained on agar: *E. coli* IA2 and *P. mirabilis* 296 on Lysogeny broth agar [LB; Difco, MD]; *Lactobacillus gasseri* KC-1, *L. crispatus* ATCC 33820 on de Man, Rogosa and Sharpe agar [MRS, Difco, MD]. All other bacteria were maintained on their preferred media (**Table 2-1**). All bacteria were grown at 37°C, and anaerobic species were grown in an anaerobic jar.

2.3.1 Evaluation of eATP production in liquid media from an overnight culture

To screen eATP release single colonies of bacteria were inoculated into media and incubated overnight aerobically and anaerobically (**Table 2-1**). Several media types were used; de Man, Rogosa and Sharpe [MRS; Difco, MD], lysogeny broth [LB; BD Difco, MD], brain heart and yeast extract [BHYE; BD Difco, MD], and vaginally defined media with peptone (VDMP)⁴⁶; as each contains different levels of ATP which could impact the study. For example, MRS has high levels of ATP, which impact screening and bacterial uptake. Bacteria were grown in 3 mL of media aerobically and anaerobically at 37°C overnight. Drop plates were used to calculate colony forming units (CFU) of each bacterium. The liquid culture was centrifuged at $6000 \times g$ for 10 minutes, then 100 μL of cell-free supernatant was collected and placed in an opaque white 96-well plate. If needed, the plates were frozen at -80°C , then thawed to room temperature for 45 minutes before ATP quantification. The established BacTiter-Glo microbial cell viability assay [Promega, WI] was used according to the protocol to quantify ATP contents with a luminometer [Biotek, VT]. With each screening, a media-only control and media-specific ATP standard curve were used to normalize ATP levels. All ATP concentrations were then related to bacterial CFU for comparisons between strains.

Table 2-1. Bacterial strains screened for eATP production after 18 hours of growth

Bacterial Strain	Medium	Origin or Relevant Characteristics
<i>E. coli</i> UTI89	LB	Patient with an acute bladder infection
<i>E. coli</i> DSM 103539	LB	Asymptomatic bacteriuria isolate
<i>E. coli</i> Nissle 1917	LB	Feces of German soldier in 1917
<i>E. coli</i> IA2	LB/VDMP	Patient with a urinary tract infection
<i>E. coli</i> K-12	LB	Patient with diphtheria in 1922
<i>E. coli</i> CFT073	LB	Patient with pyelonephritis
<i>P. mirabilis</i> 296	LB	Patient with a urinary tract infection
<i>S. marcescens</i> db11	LB	Insect isolate
<i>E. faecalis</i> ATCC 33816	LB	Type strain
<i>B. subtilis</i> 168	LB	Mutagenized <i>B. subtilis</i> Marburg
<i>S. anginosus</i> T-29	BHYE	Group F <i>Streptococcus</i>
<i>S. pyogenes</i> FF22	BHYE	Group A <i>Streptococcus</i>
<i>S. uberis</i> ATCC 27958	BHYE	Type strain, a bovine udder infection
<i>S. pyogenes</i> 71-679	BHYE	Group A <i>Streptococcus</i>
<i>L. lactis</i> subsp. <i>lactis</i> T-21	BHYE	Group N <i>Streptococcus</i>
<i>S. pyogenes</i> 71-698	BHYE	Group A <i>Streptococcus</i>
<i>S. pyogenes</i> W-1	BHYE	Group A <i>Streptococcus</i>
<i>S. dysgalactiae</i> subsp. <i>equisimilis</i> T-148	BHYE	Group C <i>Streptococcus</i>
<i>S. pyogenes</i> MGAS8232	BHYE	Group A <i>Streptococcus</i>
<i>S. pyogenes</i> NGAS979	BHYE	Group A <i>Streptococcus</i>
<i>L. crispatus</i> ATCC 33820	MRS/VDMP	Type strain, eye isolate
<i>Limosilactobacillus reuteri</i> RC-14	MRS	Vagina of a healthy individual
<i>Lactocaseibacillus rhamnosus</i> GR-1	MRS	The urethra of a healthy patient
<i>Lactocaseibacillus rhamnosus</i> GG	MRS	Feces of a healthy patient
<i>Lactiplantibacillus plantarum</i> LP39	MRS	Type strain, fermented food
<i>Lactocaseibacillus paracasei</i> ATCC 25302	MRS	Type strain
<i>Lactocaseibacillus casei</i> 393	MRS	Type strain, dairy products

2.3.2 ATP quantification

Following the manufacture's protocol, a luminescent assay kit (BacTiter-Glo microbial cell viability assay [Promega, WI, G8230]) was used to quantify eATP released by the bacteria into the supernatant. BacTiter-Glo microbial cell viability assay has already successfully been used to assess various bacterial strains, including *L. crispatus* ATCC 33820 and *E. coli* IA2, in a variety of media¹⁰. Luminescence was measured with a microplate reader (Synergy™ H4 Hybrid Multi-Mode [BioTek, USA]) from which ATP could be quantified.

2.3.3 Evaluation of eATP from *E. coli* IA2 and *L. crispatus* ATCC 33820 over 24 hours

Both *E. coli* IA2 and *L. crispatus* ATCC 33820 were grown in VDMP anaerobically at 37°C for 24 hours. Samples were collected every 30 minutes for the first six hours and every hour proceeding for 24 hours to measure longitudinal changes in supernatant eATP concentrations. A 150 µL aliquot was removed from the culture at each time point, drop plates with dilutions were created, and then the sample was pelleted by centrifugation at 5000 rpm (Eppendorf Centrifuge 5804 R) for 10 minutes. From the cell-free supernatant, 100 µL was added to an opaque white 96-well plate for eATP assessment. Plates were frozen at -80°C after collection, then thawed for 45 minutes before screening. BacTiter-Glo microbial cell viability assay, which uses luminescence-based quantification, was employed to determine the concentration of ATP in the supernatant. A VDMP-ATP standard curve was created and used to quantify luminescence, then correlated to CFU. A growth curve was run concurrently with samples from the cultures using the plate reader [Eon Biotek, VT] at OD600 and 37°C to determine growth phases.

2.3.4 *Proteus mirabilis* 296 directional growth assays

Overnight liquid cultures of *E. coli* IA2 and *P. mirabilis* 296 were grown in LB aerobically overnight at 37°C; *L. gasseri* KC-1 was grown in MRS anaerobically at 37°C overnight. After growth *E. coli* IA2 and *L. gasseri* KC-1 were centrifuged at 5000 rpm (Eppendorf Centrifuge 5804 R) for 10 minutes, then the supernatant was filter sterilized with a 0.22 µm sterile syringe filter. To determine what impacts directional growth, L-shaped patterns were drawn along LB agar aseptically of 30 µL of 10 µM ATP [Sigma, St. Louis, MI], ddH₂O, *E.*

coli IA2 or *L. gasseri* KE-1 supernatant. The ATP concentration was determined based on infective bladder levels of eATP. The hinge of the L allows for the observation of a directional change in bacterial swarming. After applying the treatment, plates were allowed to dry at room temperature completely, then 10 µl of *P. mirabilis* 296 overnight culture was applied at the start of the pattern (**Figure 2-1**). Dried plates were then incubated at 37°C aerobically and photographed every 2-hours with AlphaImager (15/30 second Reflective-White) between 6 and 14 hours of growth to track directional growth.

2.3.5 Statistical analysis

All statistical comparisons were performed using GraphPad Prism 9.1 software. Anaerobic and aerobic data were statistically compared with a two-way analysis of variance (ANOVA), complemented with Sidak's multiple comparisons test. CFU and eATP concentrations were correlated with a two-tailed Pearson's test, and a simple linear regression was fit to the data.

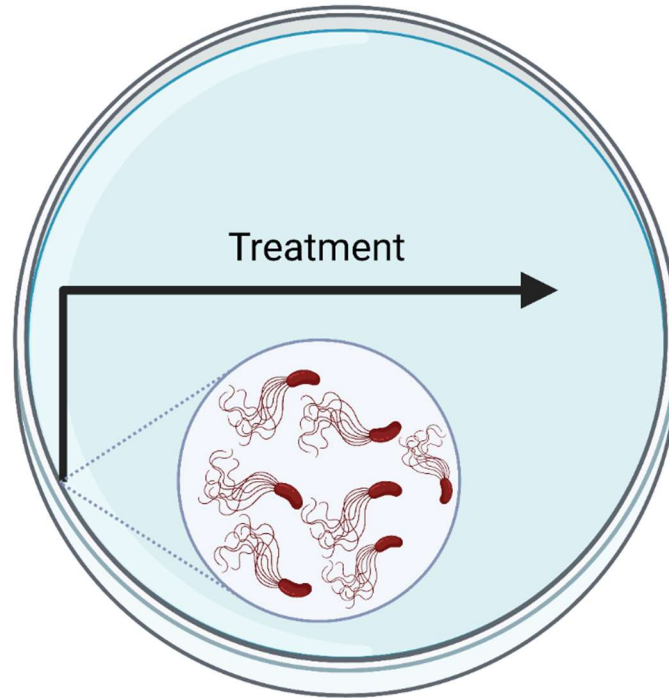


Figure 2-1. *Proteus mirabilis* 296 directional growth assay. Application of 30 μL of 10 μM ATP, ddH₂O, *E. coli* IA2 or *L. gasseri* KE-1 supernatant along treatment line followed by inoculation of *P. mirabilis* 296 at the base. Growth was monitored over 24 hours. Created using biorender.com.

2.4 Results

2.4.1 Bacterial release of ATP during growth

Bacterial release of ATP was quantified after anaerobic growth for 24-hours by luminescence (**Figure 2-1**). Secretion of ATP is not species-specific; there were significant differences between bacterial strains of the same species (two-way ANOVA, $P < 0.001$, **Figure 2-1A-D**). The highest producers were *Streptococcus pyogenes* NGAS979, *S. pyogenes* MGAS8232, *S. equisimilis* T-148, and *S. anginosus* T-29. The lowest producers were *Enterococcus faecalis* ATCC 33816, *Bacillus subtilis* 168, and *Limosilactobacillus reuteri* RC-14. *S. pyogenes* NGAS979 released the most ATP (10.675 μM to 14.875 μM during growth); *L. reuteri* RC-14 was the lowest producer, consuming ATP from the MRS media while it grew. Beneficial LAB produced lower amounts of eATP under 0.2 μM (**Figure 2-1A**). Pathogenic and commensal *E. coli* strains produced a greater range of eATP, up to 0.4 μM (**Figure 2-1B**). *Streptococcus* produced more eATP than all other examined bacteria, up to 14 μM , significantly higher than all other species (two-way ANOVA, $P < 0.001$) (**Figure 2-1C**). Other pathogens and uropathogens had lower eATP levels (under 0.15 μM) than some commensal species (**Figure 2-1D**).

Since cellular respiration may be linked to the bacterial release of ATP, a comparison was made between aerobic and anaerobic growth (**Figure 2-2**). Uropathogenic and commensal bacteria were grown in the same conditions with and without oxygen. There were differences found for ATP release in anaerobic conditions (two-way ANOVA). The urogenital environment is anaerobic, so these values represent what might be present during an infection in the urinary tract. Under anaerobic conditions, uropathogenic *E. coli* CFT073 had a six-fold increase in ATP released (two-way ANOVA, $P < 0.001$), *E. coli* K-12 has a three-fold increase (two-way ANOVA, $P < 0.001$), and commensal *E. coli* Nissle 1917 had a nine-fold increase (two-way ANOVA, $P = 0.0015$). There was no significant difference observed in the other bacteria.

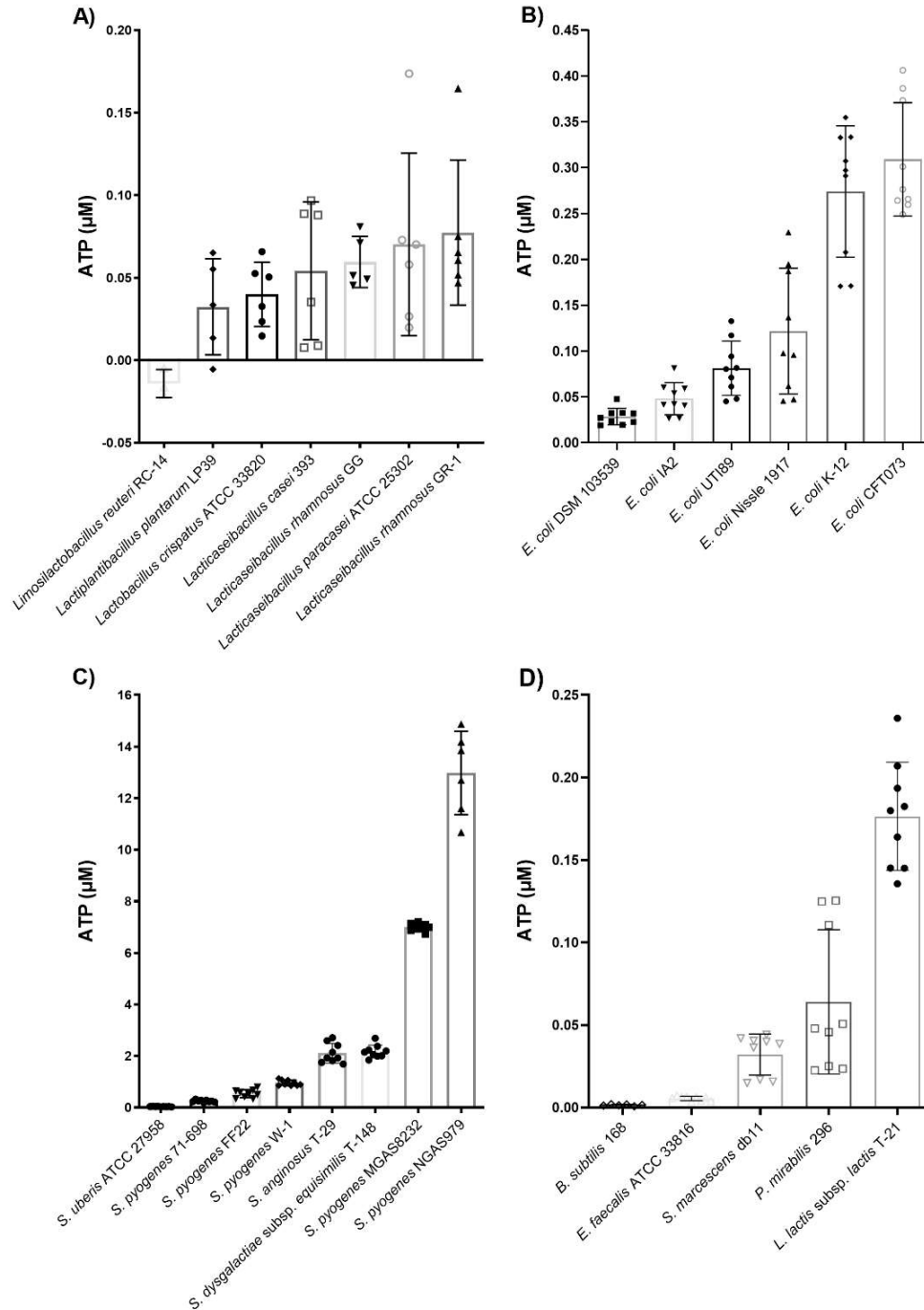


Figure 2-2. Bacteria release ATP during growth. Bacterial eATP (μM) in culture supernatant, normalized to CFU and a media specific ATP standard curve, following 24-hour anaerobic incubation. **(A)** LAB, previously all *Lactobacillus*. **(B)** *E. coli* pathogenic and commensal. **(C)** Various *Streptococcus*, *S. pyogenes* (Groups A, N and C). **(D)** Other bacteria of interest. Data are displayed as mean \pm SD (n=2-3 biological replicates with 3 technical replicates (*L. reuteri* RC-14 n=3 no technical replicates)).

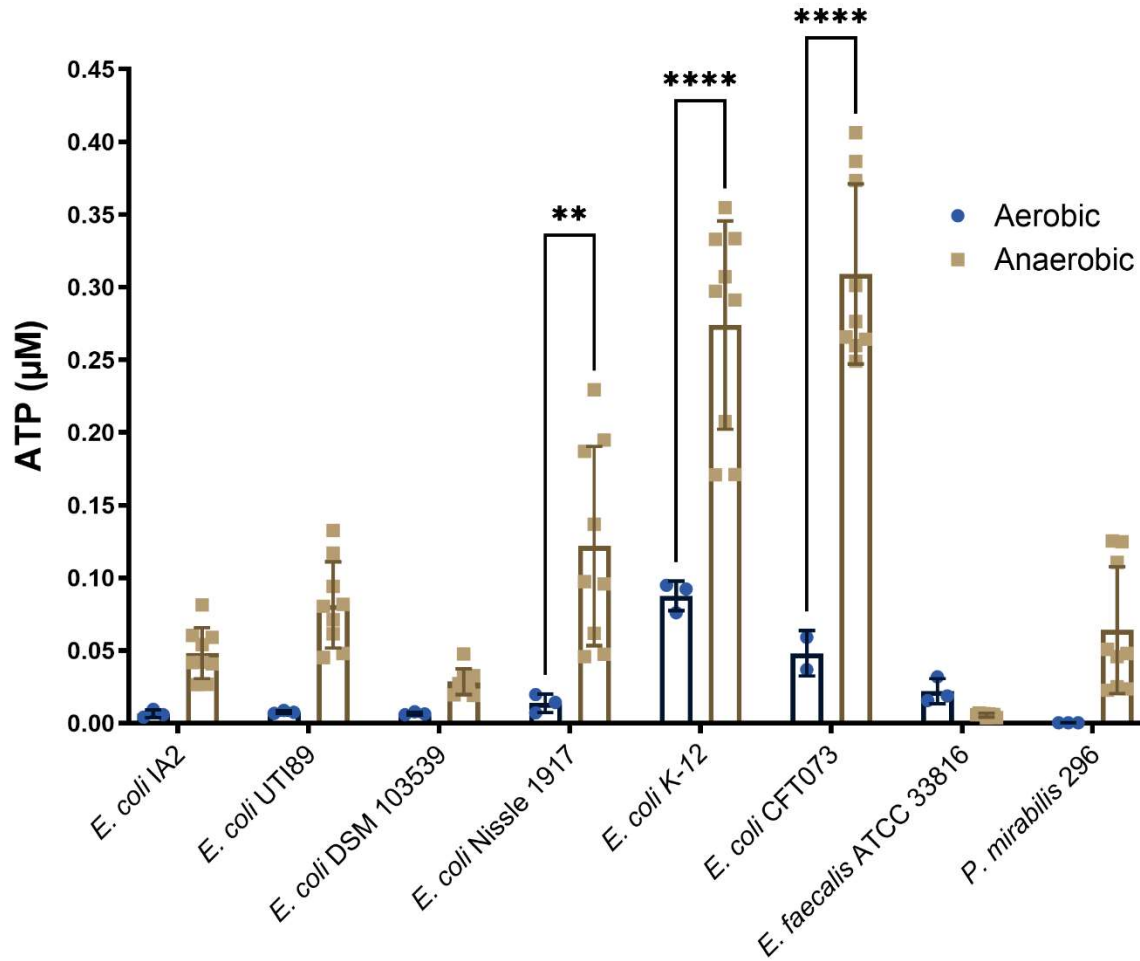


Figure 2-3. Anaerobic respiration can affect ATP release in some bacteria. Bacterial eATP (μM) in culture supernatant, correlated to CFU, following 24-hour aerobic or anaerobic incubation. Data represent mean \pm SD (two-way ANOVA) ($n=2-3$, anaerobic values have 3 technical replicates). ** $p<0.01$, **** $p<0.0001$, all others are not significant.

2.4.2 Extracellular ATP fluctuations during growth of *E. coli* IA2 and *L. crispatus* ATCC 33820

To determine if eATP levels fluctuate with bacterial growth phase, *E. coli* IA2 and *L. crispatus* ATCC 33820 eATP concentrations were monitored over 24 hours, and the results were compared to a growth curve and normalized to CFU (**Figure 2-3**). Anaerobic growth conditions and VDMP were used to mimic the urogenital environment. *E. coli* IA2 eATP levels peaked at the start of log-phase growth, then reduced rapidly during growth and stationary phase (**Figure 2-3A, Top**). There was a correlation between *E. coli* IA2 CFU and eATP (Pearson correlation, two-tailed, $r = -0.1665$, $r^2 = 0.02773$, $P = 0.3706$); linear regression is shown with the fitted line, which shows the proportional decrease of eATP with increased bacterial counts (**Figure 2-3A, Bottom**). *L. crispatus* ATCC 33820 eATP levels increased during log-phase growth, peaking near the end, then reduced rapidly during stationary phase growth with a slight increase in the last four hours (**Figure 2-3B, Top**). There was no correlation between *L. crispatus* ATCC 33820 CFU and eATP (Pearson correlation, two-tailed, $r = 0.0581$, $r^2 = 0.003381$, $P = 0.7560$); linear regression shows that as eATP and bacterial counts tend to increase and decrease together (**Figure 2-3B, Bottom**).

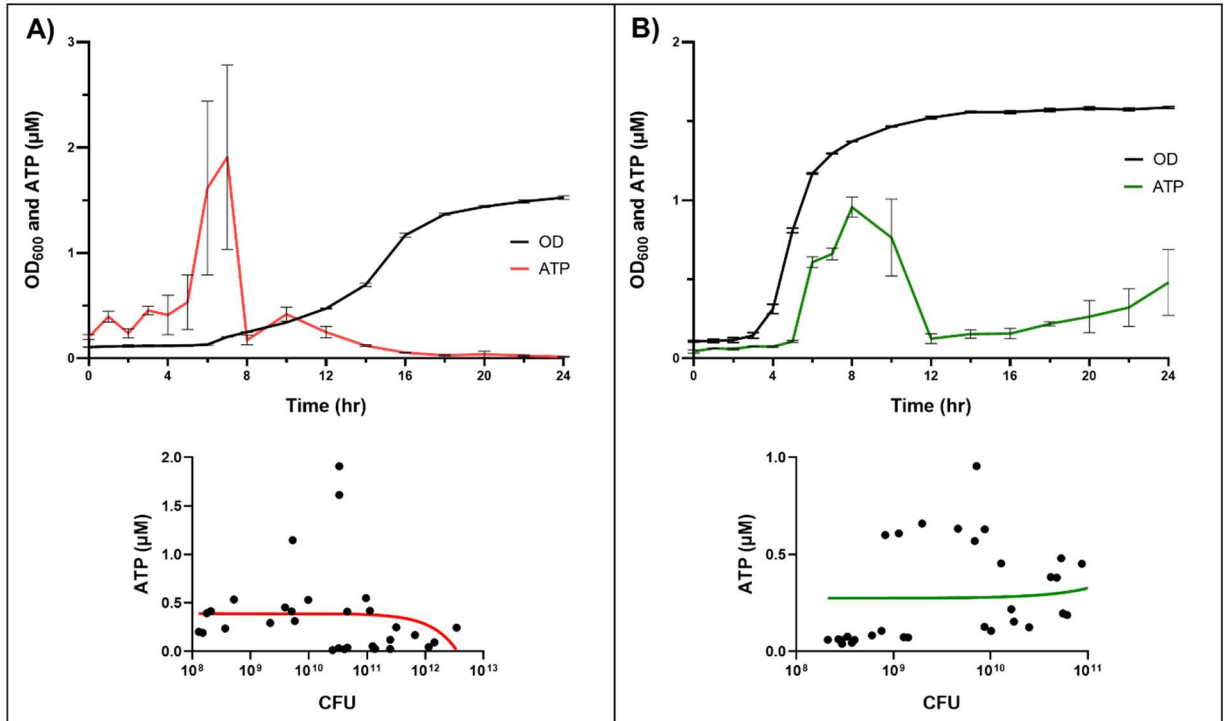


Figure 2-4. eATP concentrations fluctuate with anaerobic bacterial growth of *E. coli* IA2 and *L. crispatus* ATCC 33820. (A) *E. coli* IA2 growth curve and eATP in the culture supernatant (VDMP), top. Correlation of *E. coli* IA2 CFU and eATP, with linear regression, bottom. Data points are mean \pm SD of 3 biological replicates. (B) *L. crispatus* ATCC 33820 growth curve and eATP in the culture supernatant (VDMP), top. Correlation of *L. crispatus* ATCC 33820 CFU and eATP, with linear regression, bottom. Data points are mean \pm SD of 3 biological replicates (Two-tailed Pearson correlation and simple linear regression).

2.4.3 Influence of eATP on *Proteus mirabilis* directional swarming

To determine if eATP can influence the directional swarming abilities of uropathogenic *P. mirabilis* 296 bacterial supernatants with high eATP concentrations and a 10 μM ATP solution were added in an “L” pattern to agar plates; then swarming directionality was qualitatively assessed (**Figure 2-4**). Cell-free supernatant from *E. coli* IA2 ($\sim 0.05 \mu\text{M}$ ATP) and *L. gasseri* KC-1 ($\sim 0.025 \mu\text{M}$ ATP) were applied to assess if swarming could follow bacterial ATP released during growth of other urogenital bacteria. Non-directional swarming (expansion from the point of inoculation in a ring fashion) is seen with the ddH₂O control and the *L. gasseri* KC-1 supernatant. With both the ATP and *E. coli* IA2 supernatant treatments, there is a similar directional influence observed. In early-phase growth (consolidation), there is an observable direction change at the crux of the “L”, and in late-phase growth (swarming), the rings stretch along the treatment line. Comparison of the two groups shows that higher eATP levels can influence the swarming pattern causing a directional shift.

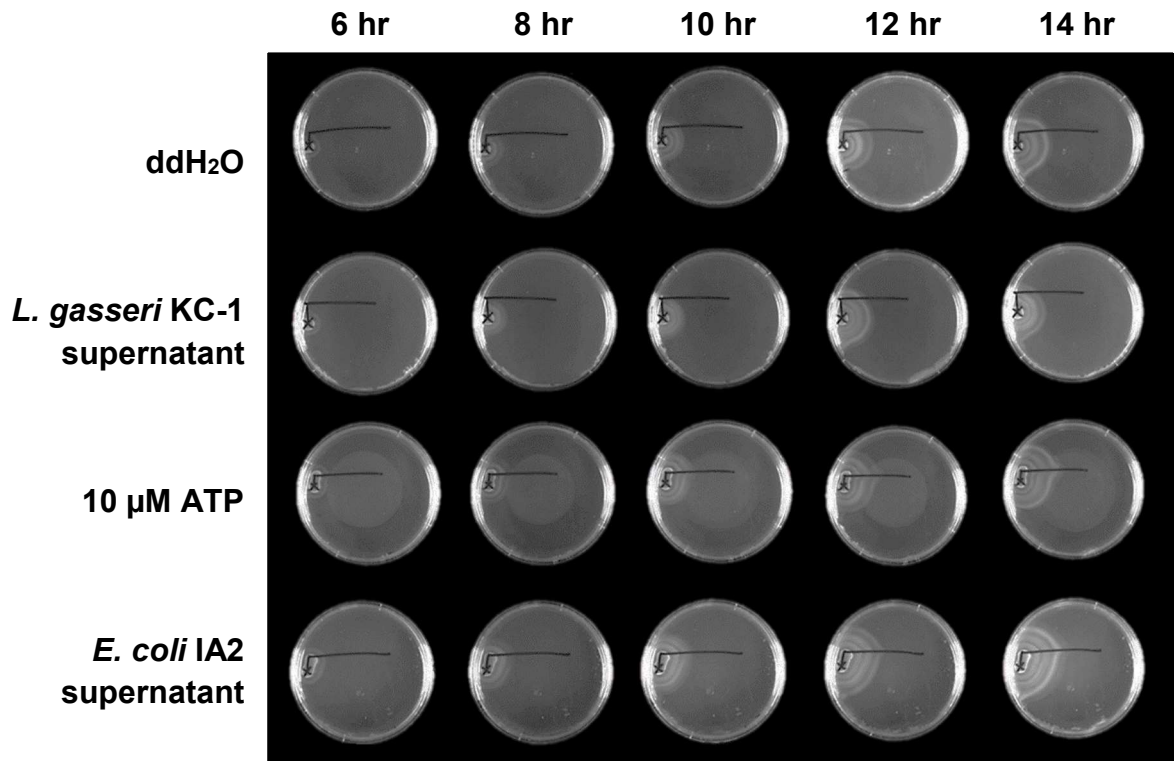


Figure 2-5. High levels of eATP influence *Proteus mirabilis* 296 swarming patterns. Time series photos of *P. mirabilis* 296 swarming influenced by eATP (10 μ M) and bacterial supernatant. Each treatment was inoculated along the sharpie line, then *P. mirabilis* 296 was added at the “X”. Plates were incubated anaerobically at 37°C and photographed with AlphaImager (15/30 second Reflective-White) every 2 hours between 6- and 14-hours post-inoculation (n=3, only one plate pictured which is representative of the general observed trends).

2.5 Discussion

This study investigated the bacterial release of ATP during growth and the impact eATP has on uropathogenic *P. mirabilis* 296 swarming. A range of pathogenic and commensal bacteria (Gram-positive and Gram-negative) were selected, and their ATP release was quantified after 24 hours of growth. There was a significant variance in eATP concentrations between pathogenic and commensal bacteria. Previously, lactobacilli were shown to produce less eATP ($\sim 0.025 \mu\text{M}$) than pathogenic bacteria ($0.1\text{-}1 \mu\text{M}$)^{3,10,26}, and this study's findings further support that claim. In addition, strains of the same species had significantly different eATP levels, suggesting that the release of eATP is species and strain specific.

Commensal lactic acid bacteria had a lower range of ATP release than the other observed bacteria, ranging from $0.014 \mu\text{M}$ by *L. reuteri* RC-14 to $0.077 \mu\text{M}$ by *Lacticaseibacillus rhamnosus* GG. The lower eATP concentrations observed in *Lacticaseibacillus Lactiplantibacillus* and *Limosilactobacillus* spp., align with previously observed low eATP levels, compared explicitly to uropathogenic species¹⁰. The eATP concentration for *L. crispatus* ATCC 33820 was $0.039 \mu\text{M}$ after 24 hours of growth which is higher than previously observed¹⁰ but correlates with observed increases in late phase growth. Specifically, eATP levels decreased in early stationary phase then increased as growth approached 24 hours, suggesting a link to nutrients availability. The probiotic *L. reuteri* RC-14⁴⁷⁻⁴⁹ was the only strain to reduce eATP from the medium (MRS). All other bacteria released ATP and potentially consumed it during growth. Studies have shown that *L. reuteri* RC-14 can confer health benefit to the vagina and these findings raise the possibility that its consumption of eATP could be a factor in conveying the host benefit in the urogenital system⁴⁷⁻⁴⁹.

There was significant variation in *E. coli* ATP release between strains and generally significantly higher concentrations of ATP compared to commensal species. Probiotic *E. coli* Nissle 1917 had significantly higher ATP release ($0.12 \mu\text{M}$) compared to several commensal species, which might be related to a generally higher ATP release in *E. coli*. The most significant producer was *E. coli* CFT073, a known uropathogen; the lowest producer was *E. coli* DSM 103539, isolated from a case of asymptomatic bacteriuria⁵⁰. The lack of symptoms in an infection caused by *E. coli* DSM 103539 could be related to its lower ATP release;

potentially, it is less pathogenic due to lower eATP. Both *P. mirabilis* and *E. faecalis* are associated with recurrent, catheter, and asymptomatic UTIs⁵¹⁻⁵³. The increased symptomatic presentation associated with higher ATP suggests a correlation; infections with decreased symptoms have eATP concentrations comparable to commensal strains. Reduced ATP could play a role in the asymptomatic presentation of infections. With reduced bacterial eATP, there could be a reduction in purinergic host signaling, leading to less bladder epithelium interaction, host immune interaction, pain, and reduced contractions^{15,19}. *E. coli* IA2 was isolated from a patient with symptomatic UTI and has lower levels of eATP measured at 24 hours. During growth, *E. coli* IA2 released 1.9 μM before log phase, which is sufficient to cause contractions through purinergic signaling¹⁰.

The release of ATP has been linked to biofilm formation³³; however, this has not been studied in the bladder environment. Increased biofilm formation is seen with ATP supplementation in *E. coli* K-12³³, which released 0.31 μM of ATP, relatively higher than other *E. coli*. A common cause of recurrent and catheter-associated UTI, *P. mirabilis*^{53,54}, can swarm using the same genetic pathways as biofilm formation⁵⁵ and could be influenced by ATP. Biofilm formation plays a crucial role in *P. mirabilis* virulence^{32,35} and could reduce the need for environmental modulation by eATP, as *P. mirabilis* 296 released 0.07 μM of ATP. Release of ATP by other uropathogens, the host, or both could promote *P. mirabilis*⁴⁸ to seek the site where a primary infection is occurring, which could lead to co-infection with other uropathogens to worsen the condition.

As part of our wider evaluation of ATP releasing organisms, we tested other less typically common urogenital tract isolates and other human disease-associated bacteria, including *S. pyogenes*. One of the most common sources of invasive infection in humans, *S. pyogenes* (group A, GAS), causes 700 million infections per year, some with serious sequelae⁵⁶. Interestingly, the *Streptococcus* genus includes many species that colonize human mucosal membranes (>100 species), and they are primarily opportunistic pathogens causing infections in response to weak immune systems⁵⁶. Human-colonizing and other *Streptococcus* were screened, both known pathogenic and infrequent opportunistic infectors. These had the most significant variation of all screened genera, with a range of 0.03 μM by *S. uberis* ATCC 27958 to 13 μM by *S. pyogenes* NGAS979 (a 433-fold difference). The *S. pyogenes* NGAS979 was one of the strains responsible for the 2015 Canada-wide invasive infection

outbreak⁵⁷ and released significantly more ATP than all other species screened. These finding supports the idea that eATP plays a role in virulence, and higher levels could contribute to modulating the site of infection on the skin as they do in the bladder. Perhaps, in a host with a weakened immune system, ATP release can aid in the switch from colonizer to pathogen. On the skin, ATP has been found to play a similar role in neurotransmission through purinergic receptors to trigger pain^{15,58}.

Previously, bacterial ATP release has been linked to cellular respiration efficiency and nutrients availability^{6,59}. Comparing aerobic and anaerobic growth conditions, we observed a significant increase in ATP release in three species and generally higher ATP levels in anaerobic growth. Mempin *et al.*³ observed reduced eATP in cytochrome *bd* oxidase *E. coli* K-12 knockouts grown aerobically. We observed a significantly higher eATP level by *E. coli* K-12 with anaerobic growth, and though both conditions lead to a reduction in cellular respiration, they may not be comparable. A possible reason for the discrepancy between our results and theirs is using a knockout versus modifying the growth environment. As the ATP release mechanism is unknown, it might not be linked to cytochrome *bd* oxidase, and the *E. coli* K-12 knockout released less ATP due to loss of cellular respiration. Although cellular respiration reduces ATP in anaerobic conditions, the pathway is not impaired, and a bacterium could still release high concentrations of ATP. Many of the bacteria we screened are uropathogenic species and infect in an anaerobic environment. Similar growth conditions to the infective environment resulting in higher eATP could further support release being linked to virulence.

In the *E. coli* IA2 and *L. crispatus* ATCC 33820 cultures, the growth phase was shown to regulate eATP levels. The most significant extracellular concentrations of ATP in both strains were observed during log-phase growth followed by a decrease in the stationary phase; *E. coli* IA2 eATP peaked at 7 hours around the start of log-phase growth reaching 1.9 μ M, followed by rapid depletion during log-phase growth, which continued in the stationary phase until the eATP was almost entirely depleted. Mempin *et al.*³ observed peak eATP around late log phase, which was about 14 nM; similarly, we have a secondary peak of 14 nM in later log phase. The differences in eATP peak concentrations here are potentially due to fewer time points observed by Mempin *et al.*³. We sampled every 30 minutes (0-6 hours) and every hour after, whereas they sampled at 0, 3, 6, 9, and 24 hours; the reduced time

points may have resulted in a missed peak concentration. The *L. crispatus* ATCC 33820 eATP peaked at 8 hours near the end of log-phase growth and depleted rapidly for 5 hours into stationary phase before increasing slightly. The release and depletion of ATP during growth suggests a relation to the growth phase that is bacteria species-specific and has been previously observed in *Lactobacillus* and *E. coli*^{3,10}.

High concentrations of ATP and *E. coli* IA2 supernatant influenced uropathogenic *P. mirabilis* 296 directional swarming patterns. Classic *P. mirabilis* growth on agar can be broken into two phases, consolidating (the thicker white ring) and swarming (the clear ring)⁶⁰. The consolidation phase initiates growth and is a resting phase during which there is an upregulation of nutrient uptake systems, central metabolism, and respiration⁶⁰. This swarming motility could initiate a catheter-associated UTI by allowing migration from the periurethral region to the bladder³⁷. Due to the similarity between biofilm formation and swarming^{33,55}, we investigated if eATP could have a similar effect on swarming as it does on biofilm formation. Using both ATP and bacterial supernatant, the same impact on *P. mirabilis* 296 growth was found, indicating that *E. coli* IA2 eATP can influence the growth of another uropathogenic organism. On the contrary, *L. gasseri* KC-1 supernatant had no effect on swarming direction, suggesting the lower levels of ATP in commensal species may not influence their growth. During the consolidation phase, there was growth along the line which diverges from the standard ring to preferentially travel along the supplemented path. In the bladder, when there are increased ATP concentrations, infected or not, *P. mirabilis* 296 could respond similarly with directed swarming up a catheter, urethra or inside the bladder, accelerating infection onset.

We detected eATP from a wide range of bacterial species, supporting the idea that there may be an un-deciphered bacterial-environmental-ATP link. The release mechanism is not yet identified, nor is the impact bacterial ATP has on the host fully understood. The observation of higher ATP levels is generally associated with known pathogenic species, which supports the connection to virulence and the establishment of infection. The bacterial release of ATP may play a role as a nutrient molecule or signaling molecule to the bacterial community. The reduction and release of ATP in a bacterial growth-phase-dependent manner could be bacteria using the environment to store excess energy to use later in growth when nutrient levels are decreased. Once released, ATP could similarly provide nutrients for other species

as well as communicate proximity and directionality. The role of eATP release in bacteria needs further characterization; however, this study furthers the idea that it does play a role in bacterial growth, communication and the potential impact on the environment.

2.5.1 Conclusion

The release of ATP was demonstrated during the growth of pathogenic and commensal Gram-negative and Gram-positive bacteria. During growth, eATP levels fluctuate, increasing during log-phase then depleting in stationary phase, suggesting release is growth-phase dependent. Bacterial and supplemented ATP can regulate and change the growth patterns of swarming *P. mirabilis* 296, a uropathogenic species that infects an ATP-saturated environment. The fluctuations in eATP and variation between species suggest that bacterial eATP has hitherto undiscovered functions related to the releasing bacteria, the bacterial community and the host environment.

2.6 References

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Chapter 3

3 The potential role of lactobacilli to modulate extracellular ATP

This chapter focused on the role lactobacilli potentially play in mitigating extracellular ATP (eATP) in the urogenital environment. It also examined if prebiotic substances can enhance lactobacilli and, in turn, reduce ATP *in vitro*, potentially leading to a novel treatment path for urinary tract infection (UTI), overactive bladder (OAB) and urgency urinary incontinence (UUI).

3.1 Abstract

Urogenital microbiota disruption and divergence from lactobacilli dominance is often associated with UTI, OAB and UUI. Increased levels of eATP observed in these conditions are unaddressed by current treatments. Prebiotic substances have been shown to bolster indigenous lactobacilli in the intestine and vagina. We hypothesized that certain prebiotic compounds could stimulate growth of urinary lactobacilli and reduce urinary ATP levels to those associated with good health. *Lactobacillus crispatus* ATCC 33820, *Lactobacillus gasseri* KC-1 and uropathogenic *Escherichia coli* IA2 were tested for their ability to utilize ten prebiotic candidates. Of those, D-mannose, raffinose and sodium acetate were found to enhance lactobacilli growth but not that of *E. coli* IA2. In addition, when cultured with urothelial cells, exposure to D-mannose and raffinose enhanced lactobacilli growth in a co-culture with *E. coli* IA2 and increase eATP reduction from culture. The interaction between eATP and bacteriocin regulation was also investigated in this chapter, as it was postulated that it could be a mechanism to acquire intracellular ATP from other bacteria. In deferred bacteriocin assays, the inhibitory activity decreased in the presence of eATP, indicating that bacteriocin production could be related to ATP availability. These *in vitro* culture-based studies demonstrated that lactobacilli could modulate eATP.

3.2 Introduction

A healthy female urogenital tract has a microbiota dominated by lactobacilli^{1,2}. These commensal microbes modulate the environment by producing lactic acid, bacteriocin-like-molecules, hydrogen peroxide, and biosurfactants, limiting pathogenic adherence^{3,4}. In urogenital diseases, including UTI and OAB, there is often a decline in *Lactobacillus* species^{5,6}. Antibiotics and lifestyle modification are often used successfully to treat complicated and uncomplicated UTIs⁷. Lifestyle modifications such as pelvic floor training, timed fluid consumption and micturition, and bladder training are the primary treatments for OAB⁸. There are also several OAB drug treatments which relax the bladder to relieve urgency⁹. Despite the occurrence and awareness of OAB, most treatments are unsatisfactory according to patients⁸⁻¹⁰.

Urogenital microbiota changes, sometimes broadly defined as dysbiosis, are commonly found in UTI and OAB patients and are unaddressed by interventions. It is known that antibiotic use can disrupt the urogenital microbiota for months following treatment of an infection¹¹. However, studies have shown that a lactobacilli-dominated microbiota can be recovered with the consumption of probiotic *Limosilactobacillus reuteri* RC-14 and *Lacticaseibacillus rhamnosus* GR-1^{11,12}. Though not a primary therapy for UTI or OAB, probiotics could help manage these conditions by restoring the microbiota¹³⁻¹⁶.

Another potential treatment option still in its relative infancy for OAB and UTI is the use of prebiotics, defined as, "a substrate that is selectively utilized by host microorganisms conferring a health benefit"¹⁷. Prebiotics mainly used in humans are typically complex carbohydrates including fructans (fructooligosaccharides (FOS) and inulin), galactans (galactooligosaccharides (GOS)), and human milk oligosaccharides (HMO). These molecules are thought to promote bifidobacteria and lactobacilli but not pathogenic microorganisms, such as *E. coli*¹⁸⁻²⁰. There could also be other compounds with prebiotic potential, including sugar alcohols (maltitol, lactitol, and xylitol)²¹, lactulose (disaccharide)²², raffinose (trisaccharide)²³, and short-chain fatty acids (acetate, butyrate)²⁴. More complex substrates, such as lyophilized skim milk, have also been used as a prebiotic to supplement *L. rhamnosus* GR-1 in capsules to address lactobacilli loss in women with recurrent UTIs and urogenital infections^{25,26}. In human and *in vitro* studies, lactobacilli growth is enhanced by

GOS^{27,28}, FOS^{28,29}, and HMO³⁰⁻³². Collins et al.³³ identified lactulose as a vaginal prebiotic candidate that can selectively enhance the growth of lactobacilli while antagonizing vaginal pathogens. D-mannose is also a potential urogenital prebiotic because it can effectively reduce the risk of recurrent UTIs and limit *E. coli* adhesion to bladder cells enabling better clearance^{34,35}. Prebiotics could be used to increase native lactobacilli presence or combined with probiotics to disrupt an infectious microbiota by shifting it towards one associated with health. The increased commensal presence could reduce the uropathogenic microbial load and impact.

Promoting a urogenital microbiota predominant in *Lactobacillus* could, in turn, reduce eATP and its potentially adverse effects on the bladder. In the bladder, one function of ATP is to signal bladder fullness through purinergic receptors in the epithelium³⁶. In patients with UUI, lower urine volumes have ATP concentrations equivalent to a full bladder in a healthy patient, thereby causing the urgency feeling^{37,38}. The ATP released by uropathogenic *E. coli* IA2 impacts host Ca²⁺ influx and smooth muscle concentrations; however, *L. crispatus* ATCC 33820 and *L. gasseri* KC-1 can reduce this effect^{39,40}. While some lactobacilli have already been shown to positively impact the bladder environment, the relationship between ATP and *Lactobacillus* is still not well understood.

In considering how lactobacilli might influence other organisms and host responses, it is worth examining bacteriocins. These are proteinaceous antimicrobial agents produced by bacteria, including lactobacilli. Some bacteriocins are cytolytic in nature against their bacterial targets and cause release of ATP into the environment⁴¹. Therefore, production of bacteriocins may be a means by which bacteria further attain ATP from competitive microbes. Several lactobacilli express bacteriocins or bacteriocin-like molecules, including *L. crispatus* ATCC 33820, which has been shown to have a cytotoxic effect on *E. coli* *in vitro*^{42,43}. A vaginal isolate from a healthy woman, *Lacticaseibacillus rhamnosus* 160 produces bacteriocin lactocin 160, which can inhibit vaginal pathogens, including *Gardnerella vaginalis*⁴⁴. Vaginal isolates *L. salivarius* subsp. *salivarius* CRL 1328 and *L. acidophilus* XH1 and probiotic strain *L. acidophilus* KS400 produce a bacteriocin that is active against urogenital pathogens⁴⁵⁻⁴⁷. In preliminary studies, we have shown that in the presence of excess eATP, bacteriocin activity is decreased, suggesting these substances may play a role in ATP release. We hypothesize bacteriocin expression could increase to facilitate

the acquisition of ATP from other bacteria when nutritional availability is diminished and vice versa when nutrition is restored. Thus, ATP release could be adding to uropathogen virulence by decreasing bacteriocin production and limiting environmental competition.

In this study, the overall goal was to investigate the ability of *Lactobacillus* spp. to limit the effects of eATP release by uropathogenic species. Specifically this study aimed to identify potential prebiotics for use in a bladder environment to enhance lactobacilli reduction of eATP. Additionally, the relationship between ATP and bacteriocin production was investigated using standard producers and an indicator strain⁴⁸.

3.3 Methods and Materials

3.3.1 Strains and growth conditions

Escherichia coli IA2 was maintained on lysogeny broth agar [LB; Difco, MD] and grown in LB broth aerobically at 37°C. *L. gasseri* KC-1 and *L. crispatus* ATCC 33820 were maintained on de Man, Rogosa and Sharpe agar [MRS, Difco, MD] and grown in MRS broth in an anaerobic jar at 37°C. All *Streptococcus* were maintained on brain heart infusion agar with 0.005% added yeast extract [BHYE, BD Bacto] or Todd-Hewitt agar [TH, Sigma-Aldrich, St. Louis, MO] grown in those broths aerobically at 37°C and 5% CO₂. The standard bacteriocin indicator strain *Micrococcus luteus* T-18⁴⁸ was maintained and grown in BHYE and TH at 37°C and 5% CO₂.

3.3.2 Prebiotic impact on bacterial growth

Ten different candidate substances with potential prebiotic properties were examined to assess their impact on commensal and uropathogenic bacterial growth. Selected prebiotic candidates were; lactulose [Alfa Aesar], D-(+)-mannose [Sigma-Aldrich, MO], 2'-O-fucosyllactose [Glycom, DK], lacto-n-neotetraose [Glycom, DK], lacto-n-tetraose [Glycom], 6'-O-sialyllactose [Glycom], D-(+)-raffinose [Sigma-Aldrich], sodium acetate [Fisher Scientific, MA], ATP [Sigma-Aldrich] and arabinogalactan [Sigma-Aldrich]. Growth curves were used to investigate the impact on uropathogenic *E. coli* IA2, commensal *L. crispatus* ATCC 33820 and *L. gasseri* KC-1. Several minimal media were used to elucidate prebiotic effect and mimic the urogenital environment: minimal salt media (MSM) without yeast

extract⁴⁹, dextrose free vaginally defined medium with peptone (dfVDMP)⁵⁰, carbohydrate-free vaginally defined medium with peptone (cfVDMP)⁵⁰, carbohydrate-free MRS (cfMRS), artificial urine⁵¹ with and without tryptic soy broth [TSB, Difco], and 10X diluted MRS and LB. Prebiotics were supplemented into media at concentrations of 0.5%, 1%, 5%, and 10% (wt/vol) then media were sterilized by 0.22 μ M filtration. Bacteria were grown overnight and then diluted (10^{-2}) in a minimal medium for prebiotic screening. Growth curves at 37°C were created using a plate reader for 20-24 hours with reads at optical density 600 nm (OD600) every 30 minutes after a 15-second shake [Eon Biotek, VT].

3.3.3 ATP quantification

A luminescent assay kit (BacTiter-Glo Microbial Cell Viability Assay [Promega, WI, G8230]) was used to quantify eATP released by the bacteria and urothelial cells in cell-free supernatant. The manufacturer's protocol was followed for sample and reagent preparation. A standard ATP curve was prepared according to the manufacturer's protocol in the media used for each screening. Luminescence was measured with a microplate reader (Synergy™ H4 Hybrid Multi-Mode [BioTek, USA]) from which ATP could be quantified.

3.3.4 ATP depletion assay

Reduction of ATP from media with and without a prebiotic was assessed with *L. crispatus* ATCC 33820 and *L. gasseri* KC-1 in dfVDMP. Three treatments were tested: medium only, 0.1 mM ATP supplemented medium and 0.1 mM ATP with 0.5% (wt/vol) prebiotic supplemented medium. For each treatment and time point, 1.5 mL of medium was added to a microfuge tube (n=3), then 10 μ L of bacteria from an overnight culture was added, and tubes were incubated at 37°C. At 0, 3.5, 4.5, 5.5, 6.5 and 24 hours, three tubes were removed to enumerate CFU and ATP. Drop plates were created from dilutions on MRS agar and grown overnight anaerobically. The remaining sample was pelleted by centrifugation at $14,000 \times g$ for 10 minutes, then 100 μ L cell-free supernatant was added to an opaque white 96-well plate. Biological replicates were measured in technical duplicate, and an ATP standard curve with dfVDMP was added to the plate. All plates were frozen at -80°C and thawed for 45 minutes before screening with BacTiter-Glo Microbial Cell Viability Assay. The luminescence-based results were then correlated to the standard curve and CFU.

3.3.5 Bacteriocin deferred growth assay

Bacteriocin producers and an indicator strain (*M. luteus* T-18) were grown overnight at 37°C and under 5% CO₂ in TH or BHYE broth. From the overnight culture, 100 µL of each bacteriocin-producing strain (**Supplementary, Table 3-2**) was inoculated down an agar plate (TH or BHYE) tilted at 90° to create a single line of growth then air dried (n=3-4). The producing strain was exposed to 1 µg/10 µL of ATP in sterile water on a disk during growth or 10 µL of sterilized ddH₂O. Time trials were completed to determine the ideal growth time for each producer for observation of a zone of inhibition (ZOI). Plates were then incubated at 37°C and 5% CO₂ for 12-24 hours, depending on the bacteriocin under investigation. After incubation, the producing strain was cleaned off the plate using a sterile cotton swab dipped in ddH₂O and disks were removed. The plate was placed over a filter paper disk covered in chloroform for 45 minutes to surface sterilize, then dried in a fume hood for an additional 20 minutes. A lawn of indicator strain *M. luteus* T-18 was added to the plate and incubated for 24-48 hours at 37°C, under 5% CO₂. Plates were imaged using white light at 24, 36 and 48 hours with an AlphaImager. The ZOI and growth into the ZOI were qualified using ImageJ, with threshold quantification used to quantify growth into the ZOI. (**Figure 3-1**).

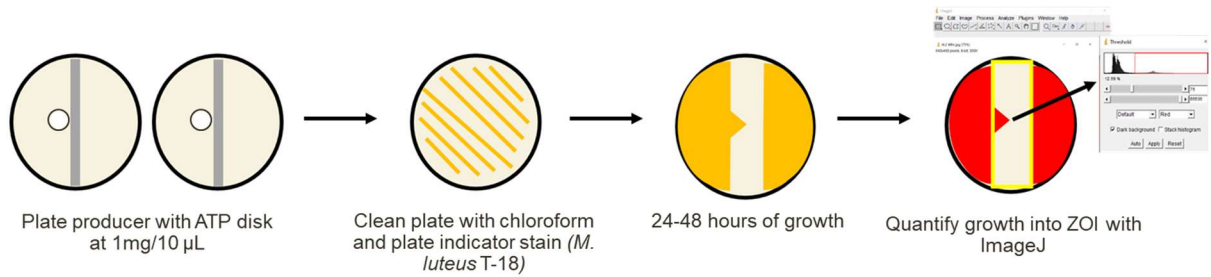


Figure 3-1. Bacteriocin deferred growth assay. Producing strains were grown overnight, then 100 μ L was dripped down the plate to create a growth line. Next, a blank disk with ATP at 1 mg/ 10 μ L or ddH₂O was added beside the producer line. After 12-24 hours of growth, the producer strain was cleaned from the plate re-sterilized with chloroform. After re-sterilization, a lawn of *M. luteus* T-18 was added, and the plates were re-incubated for 24-48 hours. Finally, plates were imaged, and ImageJ was used to quantify growth into ZOI.

3.3.6 RNA extraction and reverse transcription

Streptococcus uberis 42 was grown overnight in BHYE broth at 37°C and 5% CO₂. From culture, 100 µL was dripped down a BHYE agar plate, as in the deferred growth assay. An ATP disk with 1 µg/10 µL or ddH₂O was placed beside the culture line before incubation (n=6). Plates were incubated for 18 hours at 37°C and 5% CO₂ to ensure bacteriocins and other virulence factors were produced. RNA extraction was completed using TRIzol® Reagent with the PureLink® RNA Mini Kit, the manufacturer's protocol was followed for sample and reagent preparation with the following modifications. First, sterile loops were used to transfer a 1 cm² area of bacteria adjacent to the disk to 500 µL of TRIzol reagent [Ambion, TX] and 0.1 mm zirconia beads. Samples were then bead beaten twice for 30 seconds at 4,800 rpm with a BeadBug™ 6 microtube homogenizer [Sigma-Aldrich, St. Louis, MO]. Next, 100 µL of chloroform was added, and two chloroform washes were done to reduce phenol contamination; the second was equal volume to the aqueous upper layer. Next, the On-column PureLink® DNase treatment was used to ensure the RNA was DNA-free. RNA was eluted in 30 µL of 56°C nuclease-free H₂O, then evaluated using a DeNovix DS-11 Spectrophotometer and determined to have A260/280 and A260/230 ratios between 1.7–2.2 and 1.8–2.4 respectively. High-Capacity cDNA Reverse Transcription Kit was used to make cDNA following the manufacturer's instructions [Applied Biosystems, CA].

3.3.7 Quantitative PCR analysis

PowerTrack SYBR Green Master Mix [Applied Biosystems, CA] was used for quantitative PCR (qPCR). Primers from Lasagno et al.⁵² were used to identify ubericin A (*ubaA*), ubericin A immunity protein (*ubaI*), uberolysin A (*ubIA*), and Lantibiotic nisin-U (*nsuA* and *nsuB*) (**Supplementary, Table 3-3**). For gene expression analysis, *gyrB* was used as the endogenous reference gene, an identified stably expressed reference gene⁵³. The total reaction was 10 µL, including 4.5 µL of cDNA (diluted 6× in nuclease-free H₂O), 5 µL of SYBR (2×), and 0.5 µL of mixed forward and reverse primers (500 nM final concentration). QuantStudio 5 Real-Time PCR System [Thermo Fisher Scientific] was used to run qPCR; reaction conditions were 50°C for 2 minutes, then 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Final analysis was completed with QuantStudio 5 Real-Time PCR System [Thermo Fisher Scientific] and associated

QuantStudio Design and Analysis Software v1.5.1 [Thermo Fisher Scientific]. Fold change was used to calculate gene expression ($2^{-\Delta\Delta Ct}$), and statistics were performed on the $-\Delta\Delta Ct$ values⁵⁴.

3.3.8 Cell and bacteria co-culture experiments

Bladder epithelial cells [5637, ATCC HTB-9] passage number 4-7 were maintained in RPMI 1640 [Roswell-Park Memorial Institute media 1640, Thermo Fisher Scientific, MA] supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine at 37°C and 5% CO₂. The medium was refreshed as needed once the cells reached confluency (90-100%, about every 48 hours). Next, the spent medium was removed, and cells were washed with phosphate-buffered saline (PBS), then trypsinized by 0.25% Trypsin-EDTA [Gibco, MA]. Cell co-culture experiments were run after cells had reached confluency. All cells were used for up to a maximum of four passages (P8-11) before being discarded.

Cell co-culture experiments were undertaken with bladder epithelial cells (5637) and bacteria to compare eATP levels with and without prebiotics. Uroepithelial cells were seeded (~0.3 million/well) into 24-well plates and grown to 90% confluency in 2 mL RPMI 1640. A nontoxic dose of 0.5% (wt/vol) prebiotic was added to the cell culture medium without FBS. Prebiotics were selected based on efficacy from growth curves earlier in the study, lactulose, D-(+)-mannose, and D-(+)-raffinose. In addition, a co-culture of *E. coli* IA2 and *L. crispatus* ATCC 33820 or *L. gasseri* KC-1 was added at a multiplicity of infection of fifty. The plate was incubated at 37°C and 5% CO₂ for six to eight hours. After growth was complete, cell viability (using Invitrogen Countess Automated Cell Counter [Thermo Fisher Scientific]) and morphology were determined.

During growth, samples were collected every 30 minutes; 150 µL was removed from each well aseptically to create CFU drop plates and screen eATP. Drop plates were done on LB and MRS agar grown overnight aerobically or in an anaerobic jar, respectively at 37°C. The remaining sample was pelleted by centrifugation at 14,000 × g for 10 minutes, then 100 µL of cell-free supernatant was added to an opaque white 96-well plate for ATP screening. Plates were frozen at -80°C after collection, then thawed for 45 minutes before screening. BacTiter-Glo Microbial Cell Viability Assay was used to determine the concentration of ATP

in the supernatant. An ATP standard curve was created in RMPI-1640 and used to normalize luminescence.

3.3.9 Statistical analysis

All statistical comparisons were performed using GraphPad Prism 9.1.1 software. Non-linear regression (K) and area under the curve (AUC) were compared using ordinary one-way analysis of variance (ANOVA). Data were tested for normality using D'Agostino and Pearson test. A two-way ANOVA was used to statistically compare experiments with two factors, complemented with Tukey's multiple comparisons test. Normally distributed data were compared with an unpaired, two-tailed t-test, with Welch's correction.

3.4 Results

3.4.1 Prebiotic impact on *Lactobacillus* and *E. coli* growth

Bacterial growth curves were compared to identify ideal prebiotics. The media used mimicked the urogenital environment. Growth curves were initially compared based on exponential growth rate then by the area under the curve as it was a more standard value. Of the ten tested potential prebiotic substances, only lactulose, D-mannose, raffinose, sodium acetate, and 6'-O-sialyllactose impacted bacterial growth (**Table 3-1**).

An initial screening found *L. crispatus* ATCC 33820 growth was enhanced by lactulose (1%), D-mannose (5%) and LNT (1%) based on K and Y-max (**Supplementary Table 3-2, Supplementary Figure 3-7**). In minimal TSB-free artificial urine *L. crispatus* ATCC 33820 growth was enhanced by lactulose (5%) (K, one-way ANOVA, $p=0.0319$), and D-mannose (1% and 5%) (K, one-way ANOVA, $p=0.0195$, $p=0.0076$) (**Supplementary Table 3-2, Supplementary Figure 3-8**). In artificial urine with 0.5% prebiotic, *L. crispatus* ATCC 33820 growth was significantly enhanced by lactulose (K, one-way ANOVA, $p<0.0001$), D-mannose (K, one-way ANOVA, $p<0.0001$), and raffinose (K, one-way ANOVA, $p<0.0001$) (**Supplementary Table 3-2, Supplementary Figure 3-9**). A prebiotic concentration of 0.5% had no impact on *L. crispatus* ATCC 33820 in VDMP (**Supplementary Table 3-2, Supplementary Figure 3-9**). In minimal medium cfVDMP, cfMRS, and MSM supplemented with 1% prebiotics, *L. crispatus* ATCC 33820 growth was significantly

enhanced by D-mannose (AUC, one-way ANOVA, $p < 0.0001$) (**Supplementary Table 3-2, Supplementary Figure 3-10**). Lactulose (1%) significantly inhibited growth in *L. crispatus* ATCC 33820 in cfVDMP (AUC, one-way ANOVA, $p < 0.0001$); growth was significantly enhanced by lactulose (1%) in cfMRS (AUC, one-way ANOVA, $p < 0.0001$) (**Supplementary Table 3-2, Supplementary Figure 3-10**). Raffinose (1%) significantly inhibited the growth of *L. crispatus* ATCC 33820 in cfVDMP (AUC, one-way ANOVA, $p < 0.0001$) (**Supplementary Table 3-2, Supplementary Figure 3-10**). There was significant reduction in *L. crispatus* ATCC 33820 growth with sodium acetate (1%, 2.5% and 3%) in 10× diluted MRS (AUC, one-way ANOVA, $p < 0.0001$) and VDMP (AUC, one-way ANOVA, $p = 0.0002$, $p = 0.0001$, $p = 0.0002$) (**Supplementary Table 3-2, Supplementary Figure 3-12, 13**).

In minimal TSB-free artificial urine *E. coli* IA2 growth was significantly enhanced by D-mannose (1% and 5%) (K, one-way ANOVA, $p = 0.0495$, $p < 0.0001$), and raffinose (5%) (K, one-way ANOVA, $p = 0.0173$) (**Supplementary Table 3-2, Supplementary Figure 3-8**). In artificial urine with 0.5% prebiotic, *E. coli* IA2 growth was significantly enhanced by raffinose (K, one-way ANOVA, $p = 0.0352$) (**Supplementary Table 3-2, Supplementary Figure 3-9**). In VDMP with 0.5% prebiotic concentration, there was no impact on *E. coli* IA2 growth (**Supplementary Table 3-2, Supplementary Figure 3-9**). In minimal media cfVDMP, cfMRS, and MSM supplemented with 1% D-mannose, *E. coli* IA2 growth was significantly enhanced (AUC, one-way ANOVA, $p = 0.0091$, $p < 0.0001$, $p < 0.0001$) (**Supplementary Table 3-2, Supplementary Figure 3-10**). Lactulose (1%) significantly inhibited growth of *E. coli* IA2 in cfVDMP (AUC, one-way ANOVA, $p = 0.0001$) (**Supplementary Table 3-2, Supplementary Figure 3-10**). Raffinose (1%) significantly inhibited the growth of prebiotic *E. coli* IA2 in cfVDMP (AUC, one-way ANOVA, $p < 0.0001$) and significantly enhanced growth in cfMRS (AUC, one-way ANOVA, $p = 0.0014$) and MSM (AUC, one-way ANOVA, $p = 0.0013$) (**Supplementary Table 3-2, Supplementary Figure 3-10**). *E. coli* IA2 growth was significantly reduced by sodium acetate (2.5% and 3%) in 10× diluted LB (AUC, one-way ANOVA, $p = 0.0426$, $p = 0.046$); there was no significant impact in VDMP (**Supplementary Table 3-2, Supplementary Figure 3-12, 13**).

Raffinose (0.5% and 1%) significantly enhanced *L. gasseri* KC-1 growth (AUC, one-way ANOVA, $p < 0.0001$) in VDMP (**Supplementary Table 3-2, Supplementary Figure 3-11**). In 10× diluted MRS, lactulose (1%), D-mannose (0.5% and 1%), and raffinose (1%) significantly reduced *L. gasseri* KC-1 growth (AUC, one-way ANOVA, $p < 0.0001$, $p < 0.0001$, $p = 0.0280$, $p < 0.0001$) (**Supplementary Table 3-2, Supplementary Figure 3-11**). Prebiotic supplementation had no significant effect on *L. gasseri* KC-1 growth in artificial urine (**Supplementary Table 3-2, Supplementary Figure 3-11**). Growth of *L. gasseri* KC-1 was significantly enhanced by sodium acetate (1%, 2.5% and 3%) in 10× diluted MRS (AUC, one-way ANOVA, $p < 0.0001$) and VDMP (AUC, one-way ANOVA, $p < 0.0001$) (**Supplementary Table 3-2, Supplementary Figure 3-12, 13**).

Table 3-1. Average fold change in bacterial growth between control and prebiotic supplementation

Prebiotic substance (wt/vol)	<i>L. crispatus</i> ATCC 33820	<i>E. coli</i> IA2	<i>L. gasseri</i> KC-1
Lactulose (0.5%)	++	-	+
Lactulose (1%)	+	+++	-
Lactulose (5%)	--	--	NA
D-mannose (0.5%)	++++	--	-
D-mannose (1%)	+++++	+++++	-
D-mannose (5%)	+	+++	NA
Raffinose (0.5%)	+++	-	++++
Raffinose (1%)	-	+++++	--
Raffinose (5%)	+	---	NA
Acetate (3%)	---	--	+
Acetate (2%)	---	--	++
Acetate (1%)	---	-	+++++
Arabinogalactan (5%)	+	+	NA
6'-O-sialyllactose (5%)	-	+	NA
LNT (5%)	-	-	NA
LNnT (5%)	-	-	NA
2-O-Fucosyllactose (5%)	-	-	NA

Fold change average: 1 and up + + + + +; 0.76 to 1 + + + +; 0.51 to 0.75 + + +; 0.26 to 0.5 + +; 0.001 to 0.25 +; -0.001 to -0.25 -; -0.26 to -0.5 - -; -0.51 to -0.75 - - -; -0.76 to -1 - - - -

3.4.2 Prebiotic impact on supplemented ATP uptake by *L. crispatus* ATCC 33820 and *L. gasseri* KC-1

To assess the prebiotic impact on lactobacilli uptake of ATP, *L. crispatus* ATCC 33820 and *L. gasseri* KC-1 were grown with 1 μ M ATP in dfVDMP with and without prebiotic at 0.5% (D-mannose and raffinose, respectively). The eATP concentrations were monitored over 24 hours, normalized to CFU/ mL, and a reduction rate was calculated (ATP μ M/hr) (**Figure 3-2**). Anaerobic conditions and dfVDMP were used to mimic the urogenital environment.

Initially, *L. crispatus* ATCC 33820 ATP-D-mannose had significantly higher eATP concentrations than ATP-alone (one-way ANOVA, $p=0.0006$); however, by 3.5 hours, they were not significantly different (**Figure 3-2A, Top**). The rate of ATP reduction with and without D-mannose was similar, only significantly different at two time points (one-way ANOVA, $p=0.001$, $p=0.0176$) (**Figure 3-2A, Bottom**). The addition of raffinose resulted in significantly higher eATP concentrations than ATP-alone for the first 6.5 hours in *L. gasseri* KC-1 (one-way ANOVA, $p=0.0051$, $p<0.0001$) (**Figure 3-2B, Top**). The rate of ATP reduction with and without raffinose was similar, only significantly different at time point 6.5 (one-way ANOVA, $p=0.0002$) (**Figure 3-2B, Bottom**). Both, *L. crispatus* ATCC 33820 and *L. gasseri* KC-1 entirely reduced the supplemented ATP from media after growth (**Figure 3-2**).

Supplementation with ATP significantly impacted growth by 24 hours, *L. crispatus* ATCC 33820 and *L. gasseri* KC-1 had significantly higher CFU/ mL compared to media alone (two-tailed t-test, Tukey's, $p<0.0001$) (**Supplementary Figure 3-14**). Prebiotic and ATP supplemented, *L. crispatus* ATCC 33820, and *L. gasseri* KC-1 had significantly higher CFU compared to media alone by 24 hours (two-tailed t-test, Tukey's, $p<0.0001$, $p=0.0412$) (**Supplementary Figure 3-14**).

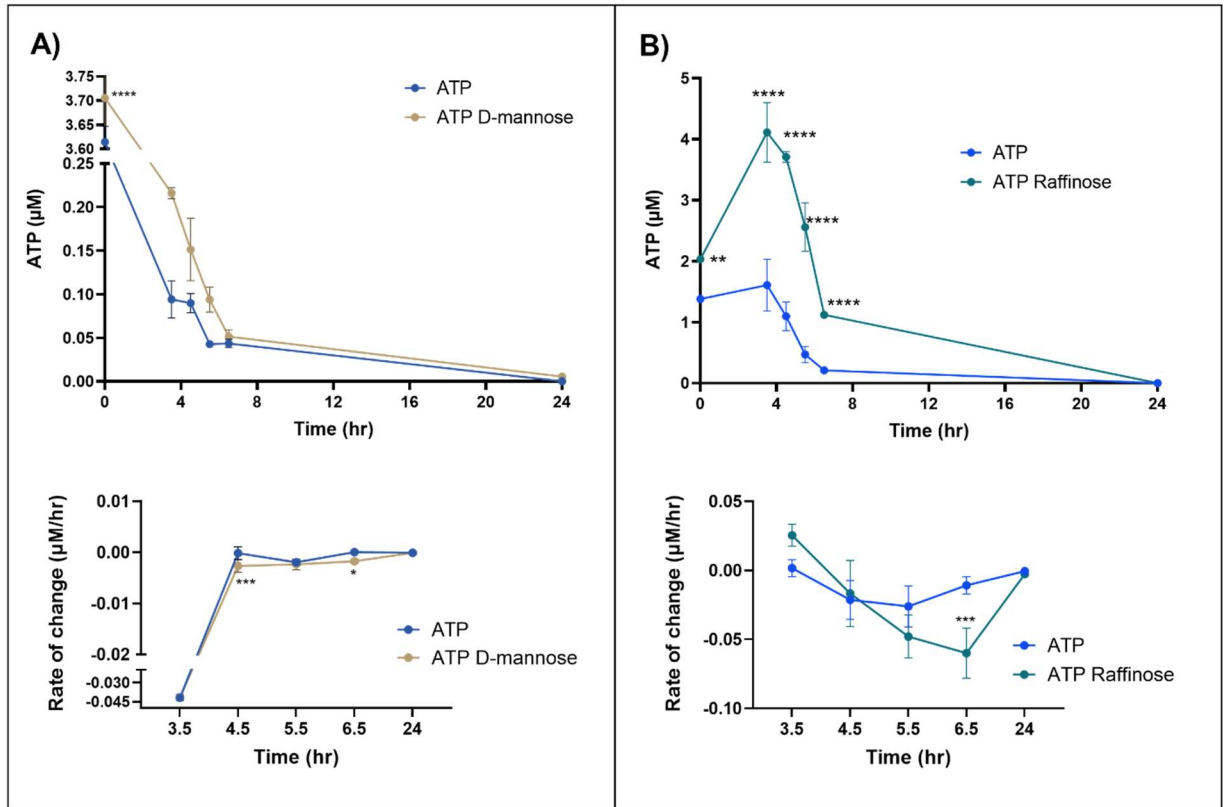


Figure 3-2. ATP reduction in media by *L. crispatus* ATCC 33820 and *L. gasseri* KC-1. Bacterial eATP (μM) reduction in culture supernatant, normalized to CFU/ mL, with and without prebiotic 0.5% (wt/vol) over 24 hours. **(A)** *L. crispatus* ATCC 33820 supplemented with D-mannose and rate of change ($\mu\text{M/hr}$). **(B)** *L. gasseri* KC-1 supplemented with raffinose and rate of change (ATP $\mu\text{M/hr}$). Data are displayed as mean \pm SD (n=3 biological replicates with 2 technical replicates). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, all others are not significant.

3.4.3 Bacteriocin production and gene regulation in the presence of ATP

Deferred growth assays and qPCR were used to determine the change in bacteriocin production in response to ATP exposure. Several known bacteriocin producers and the standard indicator *M. luteus* T-18 were used to assess bacteriocin production (**Supplemental Table 3-2**). Growth into the ZOI by *M. luteus* T-18 when the producer was grown with ATP was significant with *Enterococcus faecalis* PB4 (unpaired, two-tailed t-test, $p=0.0141$), *Streptococcus uberis* ATCC 27958 (unpaired, two-tailed t-test, $p=0.0006$), *Streptococcus uberis* 42 (unpaired, two-tailed t-test, $p=0.0264$), *Streptococcus rattus* BHT (unpaired, two-tailed t-test, $p=0.0212$), *Streptococcus salivarius* M18 (unpaired, two-tailed t-test, $p=0.0074$), and *Streptococcus salivarius* K12 (unpaired, two-tailed t-test, $p=0.0006$) (**Figure 3-3**). Several bacteriocins did not inhibit *M. luteus* T-18 growth after 24 hours; therefore, the effect was not observable (*Streptococcus pyogenes* 71-679, *Streptococcus pyogenes* FF22, *Streptococcus pyogenes* 71-724, *Streptococcus anginosus* T-29, *Pseudomonas aeruginosa* P1, *Streptococcus zooepidermicus* 4881, and *Streptococcus agalactiae* 74-628). *Streptococcus lactis* A5 bacteriocin production was too robust, and *M. luteus* T-18 could not grow, which affected the analysis. In response to ATP supplementation, bacteriocin gene production was not significantly decreased (unpaired two-tailed t-test done with $\Delta\Delta C_t$ values) (**Figure 3-4**).

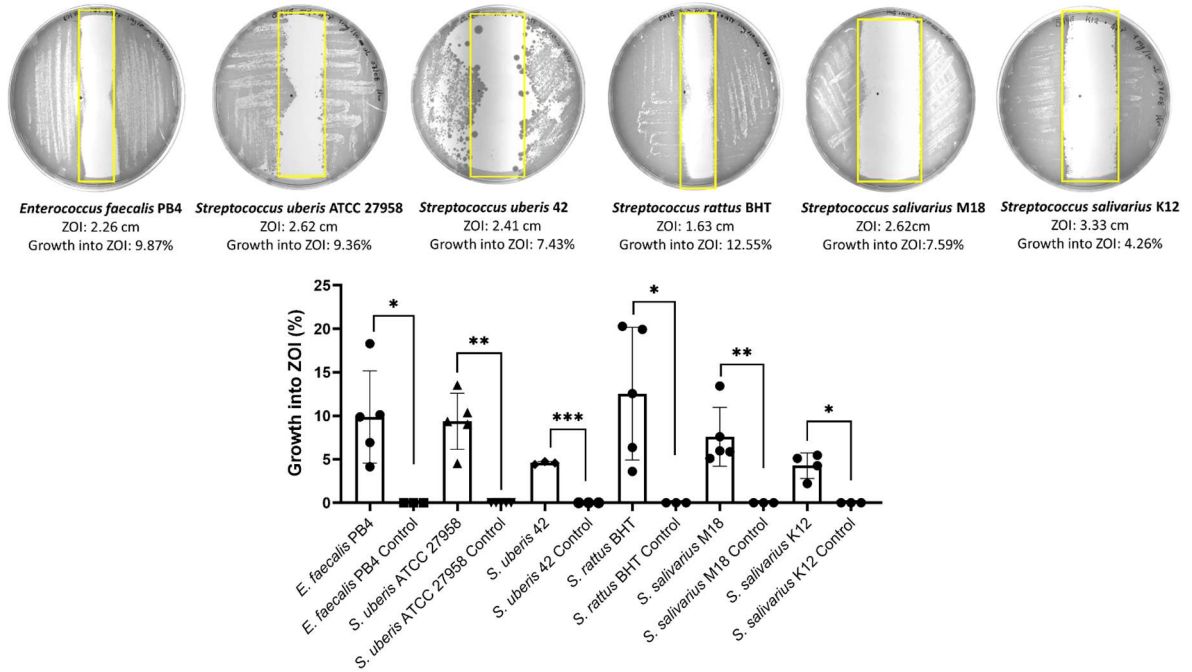


Figure 3-3. ATP impacts bacteriocin production. Bacteriocin producers were grown with a disk of ATP (1 $\mu\text{g}/10 \mu\text{L}$) after growth plates were cleaned, disks removed and used for differed growth assays. Growth of *M. luteus* T-18 into ZOI after bacteriocin producer growth quantified in ImageJ, plates pictured are representative of each bacteria. Data displayed as growth into ZOI (%) \pm SD (n=3-5) (unpaired two-tailed t-test). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

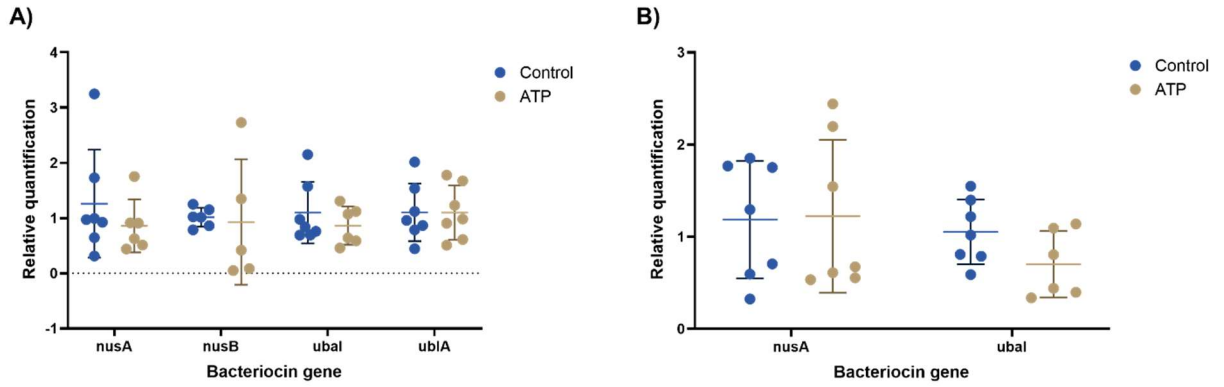


Figure 3-4. ATP impact on bacteriocin genes in *S. uberis* 42 and *S. uberis* ATCC 27958. Bacteriocin gene quantification with qPCR after growth with ATP (1 μ g/10 μ L). (A) *Streptococcus uberis* 42 (B) *Streptococcus uberis* ATCC 27958 bacteriocin genes quantified using qPCR. Data are displayed as Relative quantification ($2^{-\Delta\Delta C_t}$) \pm SD (n=6) (unpaired two-tailed t-test done with $\Delta\Delta C_t$ values). All values are non-significant.

3.4.4 Prebiotic and bacterial cell co-culture to examine eATP levels

Urothelial cells 5637 were cultured with bacteria and prebiotic compounds to assess the impact of the prebiotic-probiotic combination on eATP. Supplementation of lactulose (1%) caused significant ATP release from the urothelial cells (two-tailed t-test, $p=0.0014$, $n=2$). There was no significant difference between the co-culture with or without lactulose (**Supplemental Figure 3-15**).

D-mannose (0.5%) significantly impacted eATP uptake by *L. crispatus* ATCC 33820 in a co-culture with *E. coli* IA2 and urothelial cells 5637 (**Figure 3-5 and Supplemental Figure 3-16**). D-mannose did not induce significant urothelial 5637 cell release of ATP, nor did *L. crispatus* ATCC 33820 release or induce urothelial 5637 release of ATP, compared to 5637 cells alone (two-way ANOVA, $n=4$). There was no significant difference in eATP levels between all treatments for the first 4 hours (**Supplemental Figure 3-16**). Consistently, *L. crispatus* ATCC 33820 eATP levels were significantly lower than *E. coli* IA2 and the co-culture (two-way ANOVA, $p<0.0001$) (**Figure 3-5A**). The co-culture with D-mannose eATP level was significantly reduced compared to *E. coli* IA2 at 4.5, 5, and 5.5 hours (two-way ANOVA, $p<0.0001$, $p=0.0004$, $p<0.0001$) (**Figure 3-5A**). The eATP levels in the co-culture vs co-culture with D-mannose were significantly greater at 5 and 5.5 hours (two-way ANOVA, $p=0.0191$, $p=0.0101$) (**Figure 3-5A**). *L. crispatus* ATCC 33820 growth was significantly promoted in co-culture with and without D-mannose compared to alone (unpaired, two-tailed t-test, $p=0.0046$, $p=0.0035$) (**Figure 3-5B**).

Raffinose (0.5%) significantly impacted eATP uptake by *L. gasseri* KC-1 in a co-culture with *E. coli* IA2 and urothelial cells (two-way ANOVA, $p<0.0001$) (**Figure 3-6, Supplementary Figure 3-17**). Raffinose did not induce significant urothelial 5637 cell release of ATP nor did *L. gasseri* KC-1 release or induce urothelial 5637 release of ATP compared to 5637 cells alone (two-way ANOVA, $n=4$). The eATP level in *L. gasseri* KC-1 was significantly lower than all other groups' from 2-6 hours (two-way ANOVA, $p<0.0001$) (**Supplemental Figure 3-17, Figure 3-6**). Release of ATP by *L. gasseri* KC-1 was lower compared to other treatments at all time points except 6.5 hours compared to co-culture with raffinose (two-way ANOVA, $p<0.0001$) (**Supplemental Figure 3-17, Figure 3-6**). Co-culture has significantly less eATP compared to *E. coli* IA2 at 5 and 6 hours (two-way

ANOVA, $p=0.0285$, $p=0.0002$); the two groups were otherwise comparable (**Figure 3-6A**). The co-culture with raffinose had significantly reduced eATP compared to *E. coli* IA2 alone at 5-6.5 hours (two-way ANOVA, $p=0.0285$, $p=0.0048$, $p<0.0001$, $p<0.0001$) (**Figure 3-6A**). The eATP levels in the co-culture vs co-culture with raffinose were significantly lower at 5 hours (two-way ANOVA, $p<0.0001$) and significantly greater at 5.5, and 6.5 hours (two-way ANOVA, $p=0.0103$, $p<0.0001$) (**Figure 3-6A**). By 4 hours of growth, *L. gasseri* KC-1 growth was significantly promoted in co-culture with raffinose compared to alone (two-way ANOVA) (**Figure 3-6B**). Co-culture with raffinose significantly promoted growth of *L. gasseri* KC-1 compared to co-culture alone at 6 hours (two-way ANOVA, $p=0.0112$) (**Figure 3-6B**). Compared to *E. coli* IA2 alone, *E. coli* IA2 growth was significantly promoted in co-culture with raffinose at 5 and 6 hours (two-way ANOVA, $p<0.0001$, $p=0.0069$) and at 5 hours compared to co-culture (two-way ANOVA, $p=0.0054$) (**Figure 3-6B**). Co-culture growth was only significantly greater than *E. coli* IA2 alone at 5 hours (two-way ANOVA, $p=0.0285$) (**Figure 3-6B**).

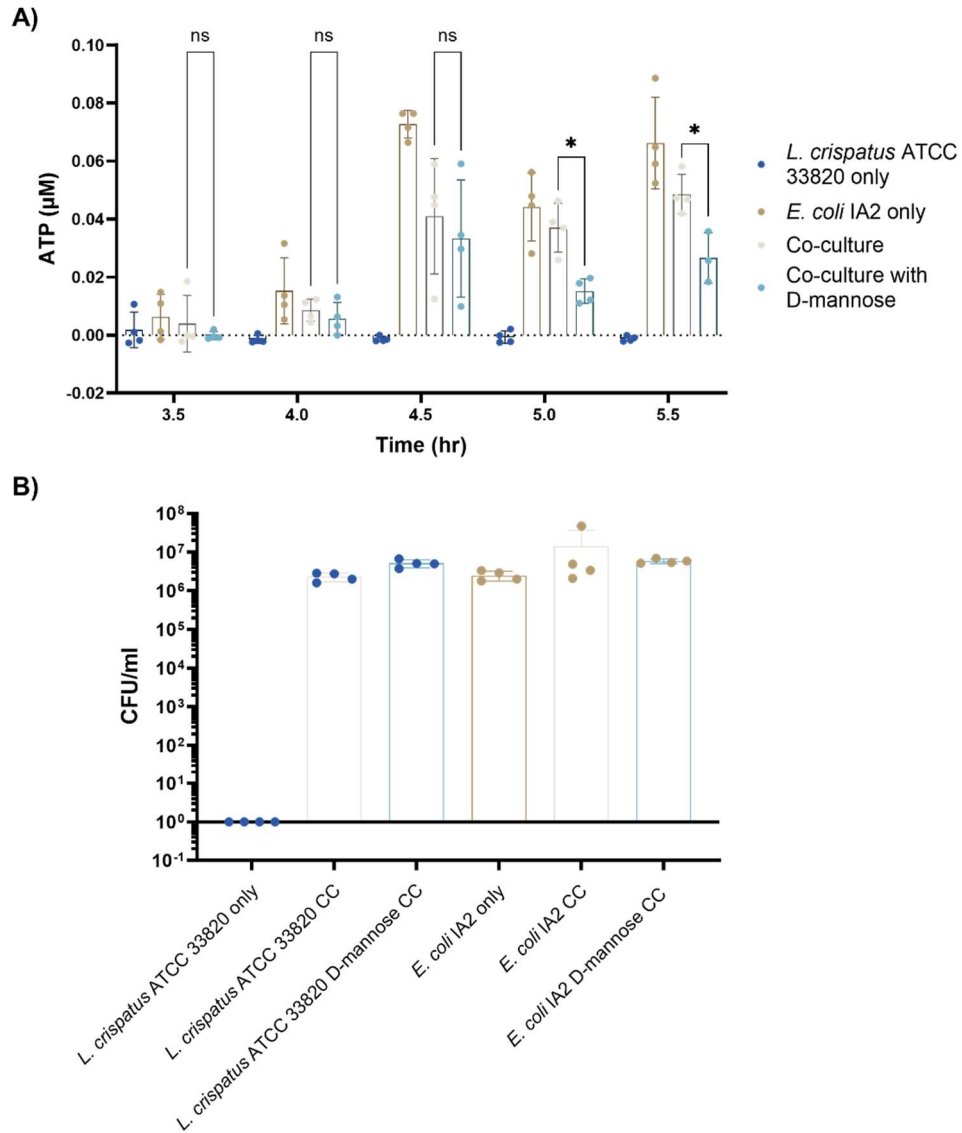


Figure 3-5. D-mannose increases *L. crispatus* ATCC 33820 eATP reduction in co-culture with *E. coli* IA2 and urothelial cells. *L. crispatus* ATCC 33820 co-cultured with *E. coli* IA2 and urothelial 5637 cells, with and without D-mannose; each bacterium was also cultured alone with urothelial 5637 cells. eATP (μM) levels in minimal cell media, correlated to a standard curve and normalized to CFU. **(A)** Complete screening of eATP (μM) levels in minimal cell media correlated to the standard curve. **(B)** The final CFU enumeration was taken at 5.5 hours. Data displayed as mean \pm SD (two-way ANOVA with Tukey's) (n=4). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns= not significant.

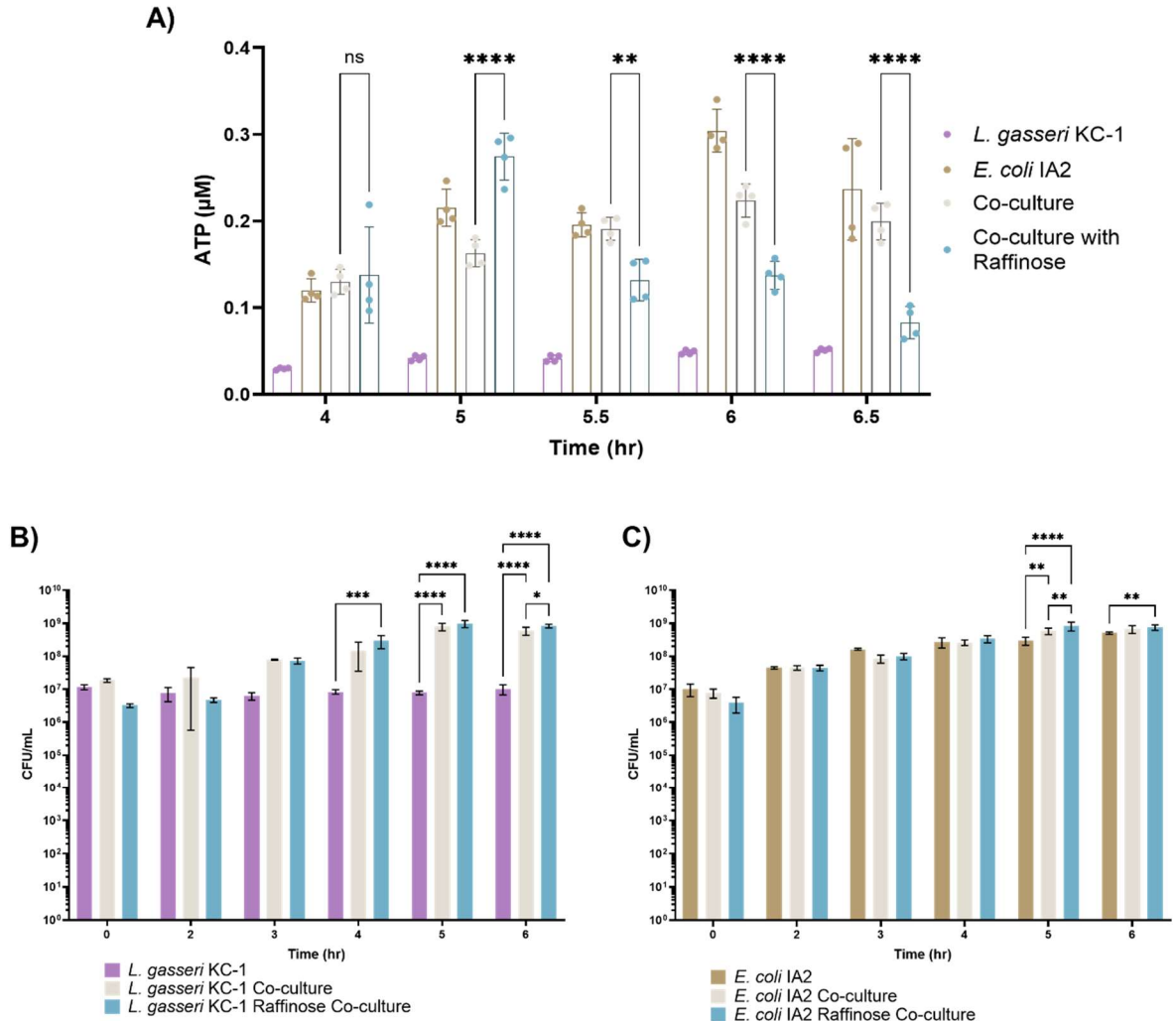


Figure 3-6. Raffinose increases *L. gasseri* KC-1 eATP reduction in co-culture with *E. coli* IA2 and urothelial cells. *L. gasseri* KC-1 was co-cultured with *E. coli* IA2 and urothelial 5637 cells, with and without raffinose; each bacterium was also cultured alone with urothelial 5637 cells. (A) eATP (μM) levels in minimal cell media, normalized to the ATP standard curve. (B) CFU enumeration was taken every hour after the first two hours. Data displayed as mean \pm SD (two-way ANOVA with Tukey's) (n=4). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.5 Discussion

This study has characterized several prebiotic candidates that promote *L. crispatus* ATCC 33820 and *L. gasseri* KC-1 growth and can impact the eATP levels produced by uroepithelial cells and uropathogenic *E. coli* IA2. This dynamic relationship has previously been undocumented and could be used to further our understanding of the management of eATP in bladder conditions.

Lactulose and D-mannose supplementation stimulated *L. crispatus* ATCC 33820 growth. Though D-mannose can enhance uropathogenic *E. coli* IA2 growth at higher concentrations, it has no impact at the lower and equally effective concentration used to enhance *L. crispatus* ATCC 33820 growth. Raffinose and sodium acetate supplementation enhanced *L. gasseri* KC-1 and antagonized *E. coli* IA2.

The urogenital environment can be challenging to replicate, leading to the testing of various media to ensure that the effect was observable with varied nutrient availability. Previous work in our lab has identified lactulose and raffinose as potential prebiotic candidates to address bacterial vaginosis by promoting a lactobacilli dominant vaginal microbiota³³. D-mannose has been considered for years as a potential treatment of complicated and uncomplicated UTIs, reducing *E. coli* adhesion to the epithelium^{34,35,55}. Promotion of a lactobacilli dominated microbiota could be a novel way for D-mannose consumption to contribute to reduced eATP in the bladder during infection. Sodium acetate consumption can alkalinize the urine^{56,57}, potentially leading to *L. gasseri* promotion, since several lactobacilli²⁴, including *L. gasseri*⁵⁸, exhibit alkaline tolerance. Sodium acetate has never been used as a prebiotic or for treatment of bladder conditions; however, it is used via intravenous injection to alkalinize urine and serum in patients with drug intoxication⁵⁶. Oral consumption of lactulose and D-mannose and intravenous injection of raffinose leads to accumulation of the compounds in urine^{55,59-62}, impacting the microbiota. Thus, these prebiotics could help modulate the bladder environment during an infection or prevent through the promotion of lactobacilli growth.

Reduction of eATP from the environment through uptake promotes *L. crispatus* ATCC 33820 and *L. gasseri* KC-1 growth. When cultured with ATP, both lactobacilli could

completely reduce the eATP after 24 hours of growth and had an associated growth benefit. There was equal ATP reduction in media by lactobacilli with prebiotic supplementation. Similarly, prebiotic and ATP supplementation promoted the growth of lactobacilli, suggesting that ATP could act to a certain extent like a prebiotic in nature. D-mannose supplementation to *L. crispatus* ATCC 33820 resulted in a consistent rate of ATP reduction over time. The reduction rate varied with ATP supplementation only and was significantly lower than the D-mannose supplemented at several points despite similar cell counts. Raffinose supplementation of *L. gasseri* KC-1 led to an initial increase in ATP; however, the accelerated reduction rate accounted for this by 24 hours of growth. Raffinose supplementation led to greater ATP reduction by *L. gasseri* KC-1; the rate of ATP reduction in the first 6 hours increased with raffinose while it slowed without. In terms of clinical relevance, when eATP levels increase in the bladder during an infection, the lactobacilli present in the microbiota might be enhanced by the addition of a prebiotic, resulting in more efficient pathogen clearance and decreased eATP associated symptoms. The ability of lactobacilli to reduce higher concentrations of ATP during growth could make them particularly suited to address bladder conditions where higher eATP plays a role in urgency and frequency.

Bacteriocin production is a trait of many probiotic strains, improving the organism's competitiveness in a microbiota environment, including the urogenital tract. Utilizing known producers, a reduction in bacteriocin effectiveness was demonstrated when an excess of ATP was available. This phenomenon was observable in all classes of bacteriocins and suggests that the production of these compounds could be related to ATP scavenging. Corroborating this, pore-forming and lytic bacteriocins cause leakage of intracellular material into the environment⁴¹, which the producing strain can then utilize for growth. Specifically, lantibiotics have been shown to induce intracellular ATP release through bacteriocin-formed pores into the extracellular environment^{63,64}. Nisin U, a lantibiotic produced by *S. uberis* ATCC 27958 and *S. uberis* 42, had a decreased production in the deferred growth assay. Though significant bacteriocin gene changes were not found in either strain, there was a trend for decreased expression of nisin U and uberin A genes in the ATP supplemented group. Further investigation is needed to understand this interaction fully. Overall, the results suggest that an additional role of pathogen-released ATP could be the downregulation of

bacteriocin production by bacteria already present in the environment, allowing for easier infection establishment. Reduction of ATP from the bladder environment by lactobacilli could help reduce this effect.

The ability of lactobacilli to reduce eATP from an *in vitro* infective bladder model with urothelial cells and an *E. coli* IA2 co-culture was examined. When cultured with bladder epithelial cells, *E. coli* IA2 released higher ATP levels, triggering urothelial cell release of Ca^{2+} through purinergic signalling³⁹. Lactulose, D-mannose and raffinose were tested based on their promotion of *Lactobacillus* growth. Lactulose proved to be less effective as it induced high ATP release from the urothelial cells that was not seen with the other two prebiotics. Additionally, there was no eATP reduction by *L. crispatus* ATCC 33820 with lactulose compared to co-culture alone. The supernatant from cells with *L. crispatus* ATCC 33820 independently had significantly lower eATP levels than supernatant with *E. coli* IA2 and the co-culture; there was no significant release of ATP by urothelial cells or *L. crispatus* ATCC 33820 when cultured together. This suggests that *L. crispatus* ATCC 33820 could have a limited impact on the bladder, inducing no further eATP disruption. On the other hand, the reduced eATP levels in co-culture suggest that *L. crispatus* ATCC 33820 can take up eATP released by *E. coli* IA2 and any urothelial-triggered ATP release. Further research is needed to verify this finding.

D-mannose supplementation did not stimulate *L. crispatus* ATCC 33820 growth but enhanced eATP uptake in the co-culture, and raffinose similarly affected *L. gasseri* KC-1 eATP reduction. Growth of *L. gasseri* KC-1 and *E. coli* IA2 was increased by raffinose towards the end of the experimental time period. As with *L. crispatus* ATCC 33820, *L. gasseri* KC-1 released ATP concentrations comparable to 5637 cells alone. An increase in ATP was seen in the co-culture with raffinose which was also observed in the prebiotic supplemented ATP reduction experiment; the increased ATP reduction rate accounted for this rise within two hours. With raffinose supplementation in the co-culture, *L. gasseri* KC-1 reduced the *E. coli* IA2 eATP level entirely; the final level was comparable to *L. gasseri* KC-1 alone. D-mannose and raffinose are promising potential prebiotics that can be used in conjunction with *L. crispatus* ATCC 33820 or *L. gasseri* KC-1 to address and reduce eATP from the bladder environment.

3.5.1 Conclusion

We have demonstrated that D-mannose and raffinose have prebiotic potential; they stimulate lactobacilli with minimal impact on uropathogenic *E. coli* IA2 growth and enhance eATP reduction. The use of a urothelial cell model in this study was suitable for investigating eATP in the bladder environment. This work implies that symptoms commonly associated with bladder conditions such as UTI and OBA, which may be triggered by bacterial ATP, could potentially be reduced by a prebiotic or a prebiotic-probiotic combination. Furthermore, as the prebiotics can be found in the urine, their ingestion may enhance an individual's inherent microbiota to help maintain a healthy state.

3.6 References

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3.8 Supplementary

3.8.1 Impact of Covid-19 on research

The Covid-19 pandemic had a significant impact on this research. There was limited to no access to the research facility for five months, putting the project on hold. I had planned to complete additional cell co-culture assays, examine Ca^{2+} influx impact, repeat the ATP reduction assay, repeat the differed growth assays with a control at pH 3.7, and compare bacteriocin gene production in several producers. The global plastic shortage and use of qPCR plates in covid-19 testing kits also limited my experimental options.

3.8.2 Figures and tables

Table 3-2. Fold change in bacterial growth between control and prebiotic supplementation

Media	Prebiotic	<i>L. crispatus</i> ATCC 33820	<i>E. coli</i> IA2	<i>L. gasseri</i> KC- 1
TSB-Free Artificial Urine	Lactulose (1%)	-0.004299	-0.2993	-
	Lactulose (5%)	-0.2547*	-0.4663	-
	D-mannose (1%)	0.1678*	0.3398*	-
	D-mannose (5%)	0.1899****	0.7353****	-
	Raffinose (5%)	0.001107	-0.6575	-
	Arabinogalactan (5%)	0.1117	0.009927	-
	6'-O-sialyllactose (5%)	-0.08363	0.02273	-
	LNT (5%)	-0.06656	-0.01402	-
	LNnT (5%)	-0.03029	-0.09222	-
	2-O-Fucosyllactose (5%)	-0.01566	-0.2226	-
Artificial Urine	Lactulose (0.5%)	0.8358****	-0.08014	-
	D-mannose (0.5%)	1.0945****	-0.01723	-
	Raffinose (0.5%)	0.7616****	-0.1336*	-
VDMP	Lactulose (0.5%)	0.04621	-0.04655	-
	D-mannose (0.5%)	0.06669	-0.05616	-
	Raffinose (0.5%)	-0.04316	0.01325	-
cfVDMP	Lactulose (0.5%)	-	-	0.05723
	D-mannose (0.5%)	-	-	-0.07247
	Raffinose (0.5%)	-	-	1.0382****
	Lactulose (1%)	-0.5710****	-0.1197**	-0.2002
	D-mannose (1%)	2.0909****	0.07493**	-0.09539
	Raffinose (1%)	-0.5217****	-0.1734****	0.8954****
cfMRS	Lactulose (1%)	0.1984****	0.02204	-
	D-mannose (1%)	0.3103****	0.08274****	-
	Raffinose (1%)	-0.005305	0.04491**	-
MSM	Lactulose (1%)	-0.01305	0.7501	-
	D-mannose (1%)	1.132****	38.58****	-
	Raffinose (1%)	0.05176	4.919**	-
×10 diluted MRS	Lactulose (0.5%)	-	-	-0.02036
	D-mannose (0.5%)	-	-	-0.06010*
	Raffinose (0.5%)	-	-	-0.048013
	Lactulose (1%)	-	-	-0.1510****
	D-mannose (1%)	-	-	-0.2419****
	Raffinose (1%)	-	-	-0.2755****
×10 diluted MRS/LB	Acetate (3%)	-0.8668****	-0.3042	0.1480****
	Acetate (2.5%)	-0.6812****	-0.2998*	0.3639****
	Acetate (1%)	-0.5871****	-0.2045*	1.100****

VDMP	Acetate (3%)	-0.5715***	-0.3872	-0.03391****
	Acetate (2.5%)	-0.5721***	-0.3075	0.2612****
	Acetate (1%)	-0.5552***	-0.2419	1.612****

*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, all others non-significant.

Table 3-3. Bacteriocin producing stains, their bacteriocin(s) and bacteriocin classification

Producer	Bacteriocin	Class
<i>Streptococcus uberis</i> ATCC 27958	Nisin U and Ubercin A	Lantibiotic and Class IIa ^{65,66}
<i>Streptococcus pyogenes</i> 71-679	Salivaricin A	Lantibiotic ⁶⁷
<i>Streptococcus lactis</i> A5	Nisin A	Lantibiotic ⁶⁸
<i>Streptococcus pyogenes</i> FF22	Streptococcin A-FF22	Lantibiotic ⁶⁹
<i>Streptococcus pyogenes</i> 71-724	Salivaricin A	Lantibiotic ⁷⁰
<i>Streptococcus uberis</i> 42	Nisin U and Ubercin A	Lantibiotic and Class IIa ^{65,66,71}
<i>Streptococcus rattus</i> BHT	BHT-A and BHT-B	Lantibiotic and Unknown class II ⁶⁹
<i>Enterococcus faecalis</i> PB4	1. Bacteriocin 31	1. Unclassified
	2. Enterocin 1071A	2. Unclassified
	3. Enterocin Se-K4	3. Class IIa
	4. Enterolysin A	4. Class III
	5. Enterocin AS-48	5. Unclassified
	6. Enterocin EJ97	6. Unclassified
	7. Enterocin W alfa	7. Class IIb
	8. Enterocin W beta	8. Class IIb
	9. Enterocin 7B	9. Unclassified ⁷²
<i>Streptococcus anginosus</i> T-29	Stellalysin	Lytic ⁶⁸
<i>Pseudomonas aeruginosa</i> P1	Pyocin S1 and S2	S-type bacteriocins ⁷³
<i>Streptococcus zooepidermicus</i> 4881	Zoocin A	Bacteriocin-like peptide ⁷⁴
<i>Streptococcus salivarius</i> K12	Salivaricins A and B	Bacteriocin-like inhibitory substance ⁶⁸
<i>Streptococcus salivarius</i> M18	Salivaricins A2, 9 and MPS	Bacteriocin-like inhibitory substance ⁷⁵
<i>Streptococcus agalactiae</i> 74-628	Unknown	Unknown ⁷⁶

Table 3-4. qPCR primers used in this study

Target Gene	Primer Sequence 5'-3'	Accession Number	Amplicon size (bp)
<i>ubaA</i>	F: ATCGGTGGCAAAACTGTAAA R: GCCCGTTCATGATGGAATTA	EF203953	115
<i>ubaI</i>	F: CTTTGCATGCTCAAGGGAAT R: CATAGCGGATATTGGAAATCG	EF203953	216
<i>ublA</i>	F: GGGATAGCCTCAGGTACTGC R: AGCTGAGGCTGAAACTGCTC	DQ650653	129
<i>nsuA</i>	F: TGAAGATTTTAATTTGGATCTCATCA R: TGACAACCACAGGTTGCAGT	DQ146939	150
<i>nsuB</i>	F: TCCCATATGATCTGGCAAT R: CTGATTATCAACCCGCGAAT	DQ146939	374
<i>gyrB</i>	F: AATACCCATGAGGGTGGGAC R: GCTGTCAATCCTTCACGAACA	AB238638.1	143

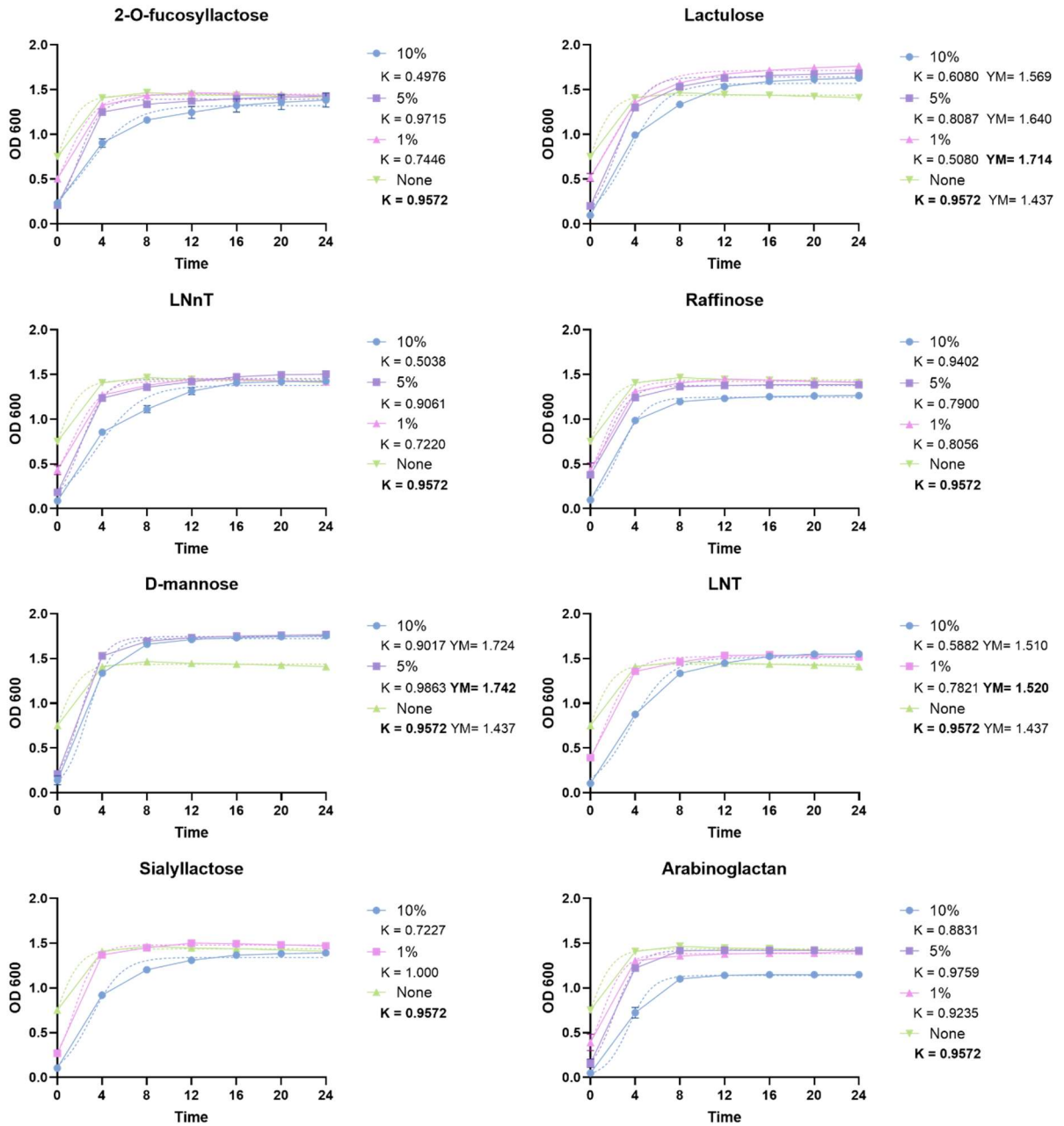


Figure 3-7. *L. crispatus* ATCC 33820 with prebiotic supplementation in artificial urine.

24-hour growth curves of *L. crispatus* ATCC 33820 supplemented with 10%, 5%, 1%, prebiotic (wt/vol) in artificial urine. Data are displayed as mean OD₆₀₀ of two biological replicates (solid line) and non-linear fit (dashed line). K and Y-max are included to indicate greater growth rates, bold values.

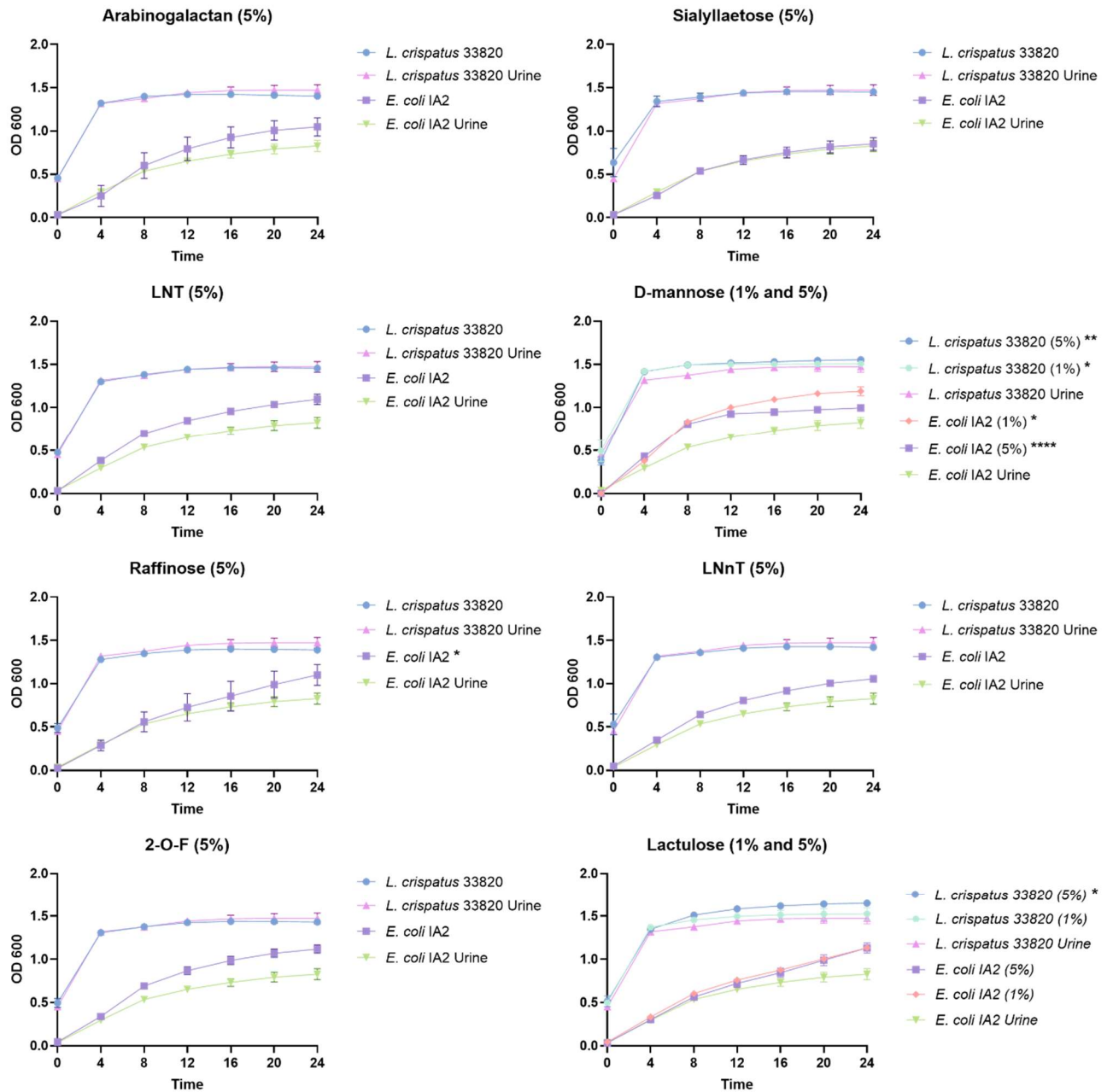


Figure 3-8. *L. crispatus* ATCC 33820 and *E. coli* IA2 with prebiotic supplementation in TSB-free artificial urine. 24-hour growth curves of *L. crispatus* ATCC 33820 and *E. coli* IA2 supplemented with prebiotic in TSB-free artificial urine. Data are displayed as mean OD₆₀₀ ± SD of three-four biological replicates. Exponential growth rate constant 'K' was compared using ordinary one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

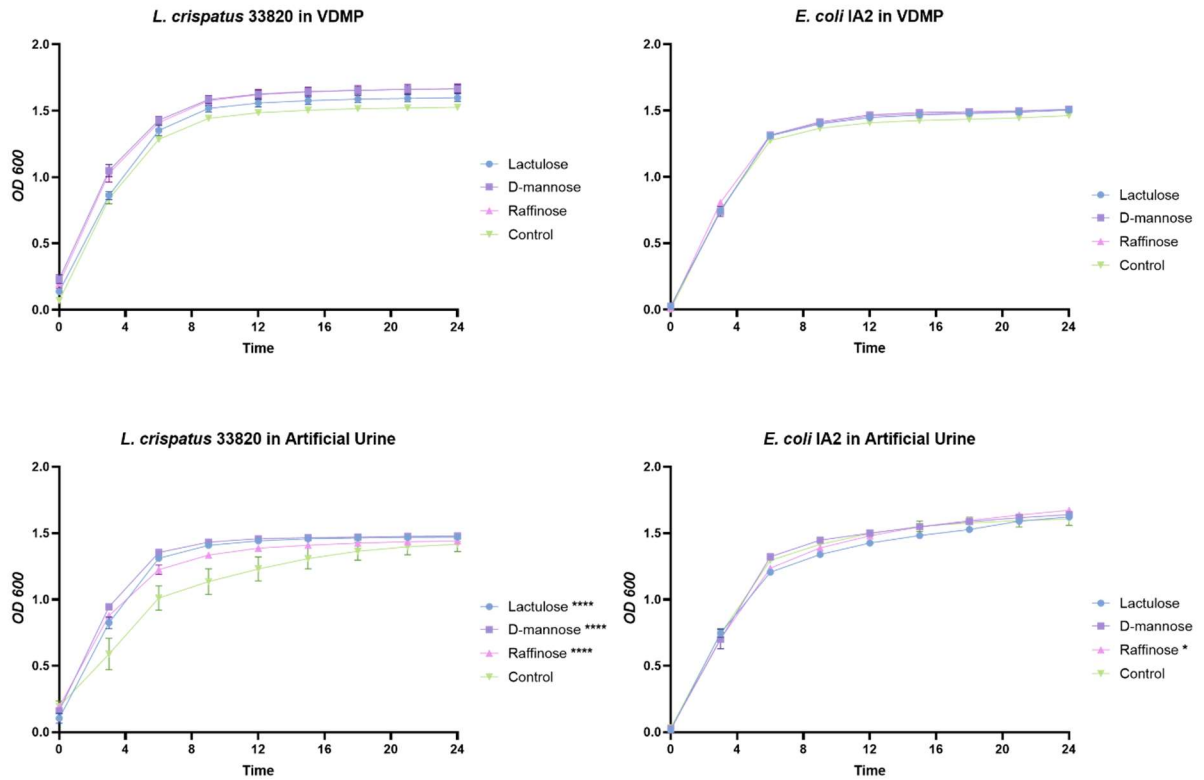


Figure 3-9. *L. crispatus* ATCC 33820 and *E. coli* IA2 with 0.5% (wt/vol) prebiotic supplementation in artificial urine and VDMP. 24-hour growth curves of *L. crispatus* ATCC 33820 and *E. coli* IA2 supplemented with 0.5% (wt/vol) prebiotic in VDMP and artificial urine. Data are displayed as mean OD₆₀₀ ± SD of three biological replicates. Exponential growth rate constant 'K' was compared using ordinary one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

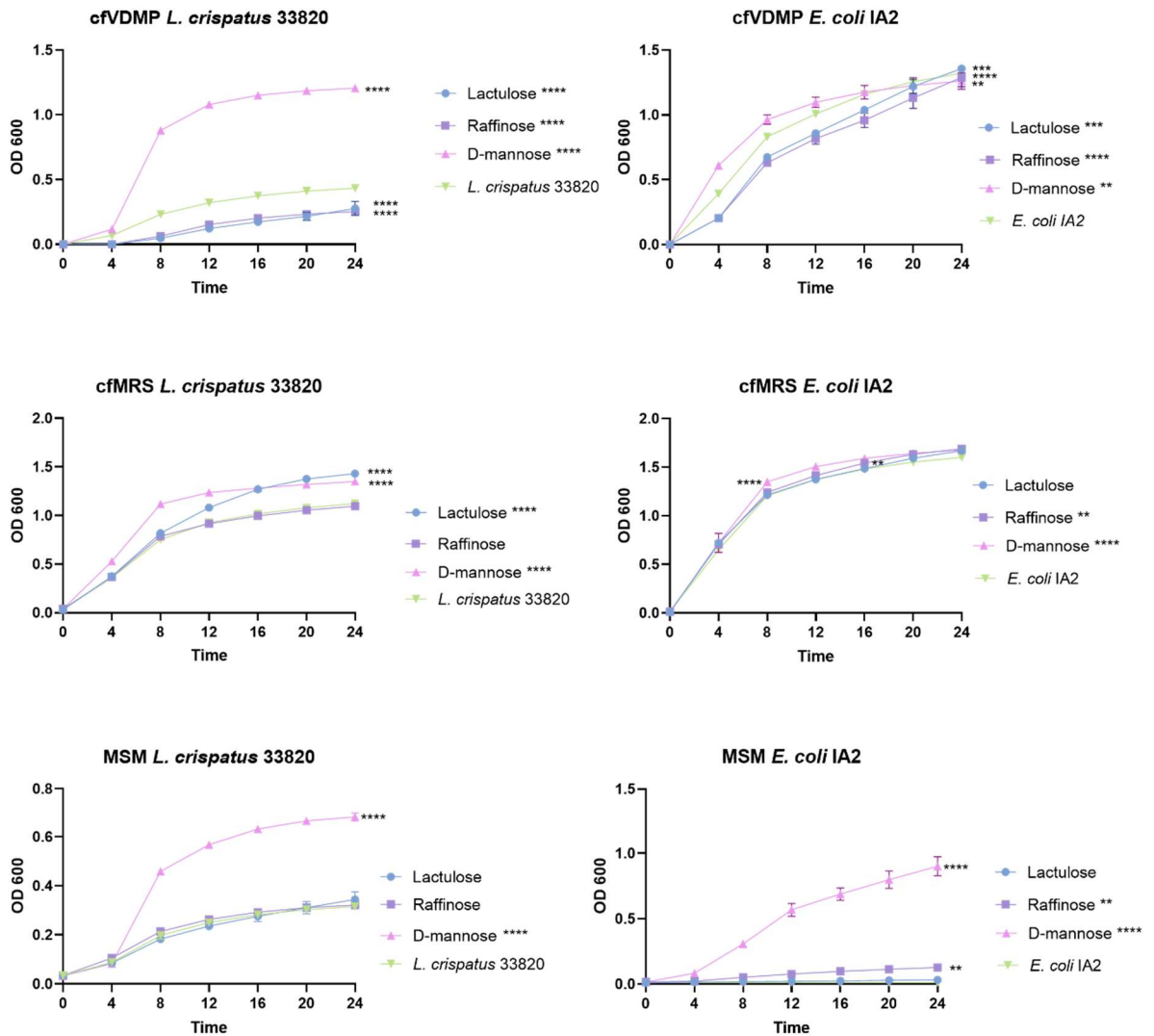


Figure 3-10. *L. crispatus* ATCC 33820 and *E. coli* IA2 with 1% (wt/vol) prebiotic supplementation in VDMP, cfMRS, and MSM. 24-hour growth curves of *L. crispatus* ATCC 33820 and *E. coli* IA2 supplemented with 1% (wt/vol) prebiotic in VDMP, cfMRS, and MSM. Data are displayed as mean OD₆₀₀ ± SD of three biological replicates. The AUC was compared using ordinary one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

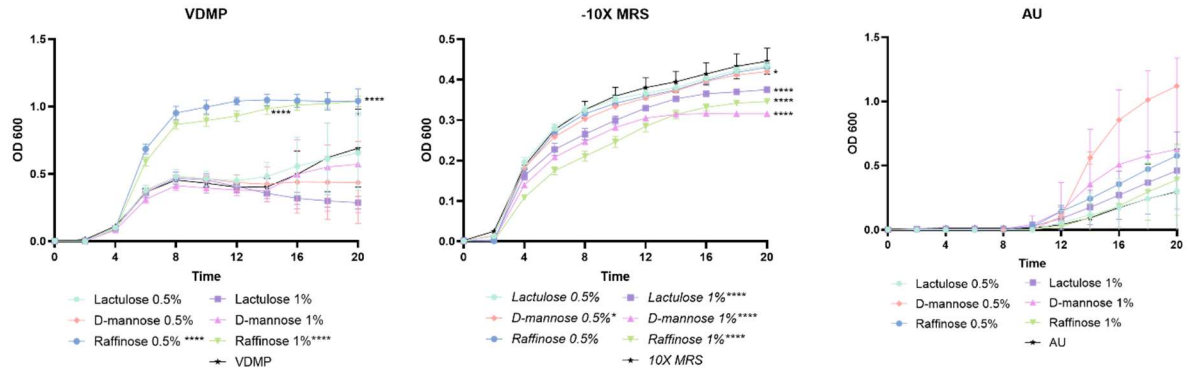


Figure 3-11. *L. gasseri* KC-1 with prebiotic supplementation in VDMP, 10× diluted MRS, and AU. 20-hour growth curves of *L. gasseri* KC-1 with 0.5% and 1% (wt/vol) prebiotic in VDMP, 10× diluted MRS, and artificial urine. Data are displayed as mean OD-600 ± SD of three biological replicates. The AUC was compared using ordinary one-way ANOVA. *p<0.05, **p<0.01, *p<0.001, ****p<0.0001.**

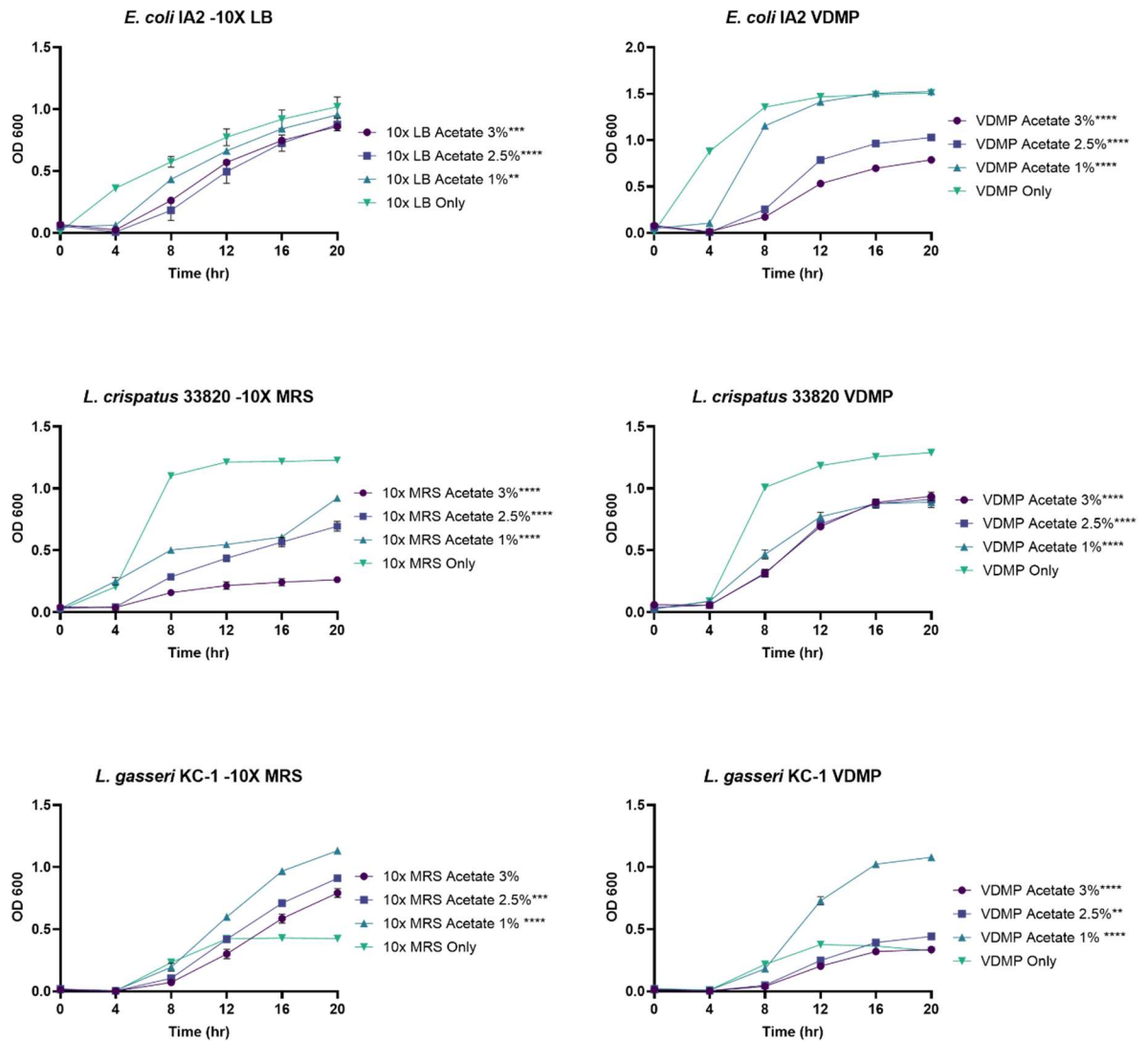


Figure 3-12. *E. coli* IA2, *L. crispatus* ATCC 33820, and *L. gasseri* KC-1 with prebiotic supplementation in 10× diluted LB or MRS, and VDMP. 20-hour growth curves of *L. gasseri* KC-1 with 0.5% and 1% (wt/vol) prebiotic in VDMP, 10× diluted MRS, and artificial urine. Data are displayed as mean OD-600 ± SD of three biological replicates. The AUC was compared using ordinary one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

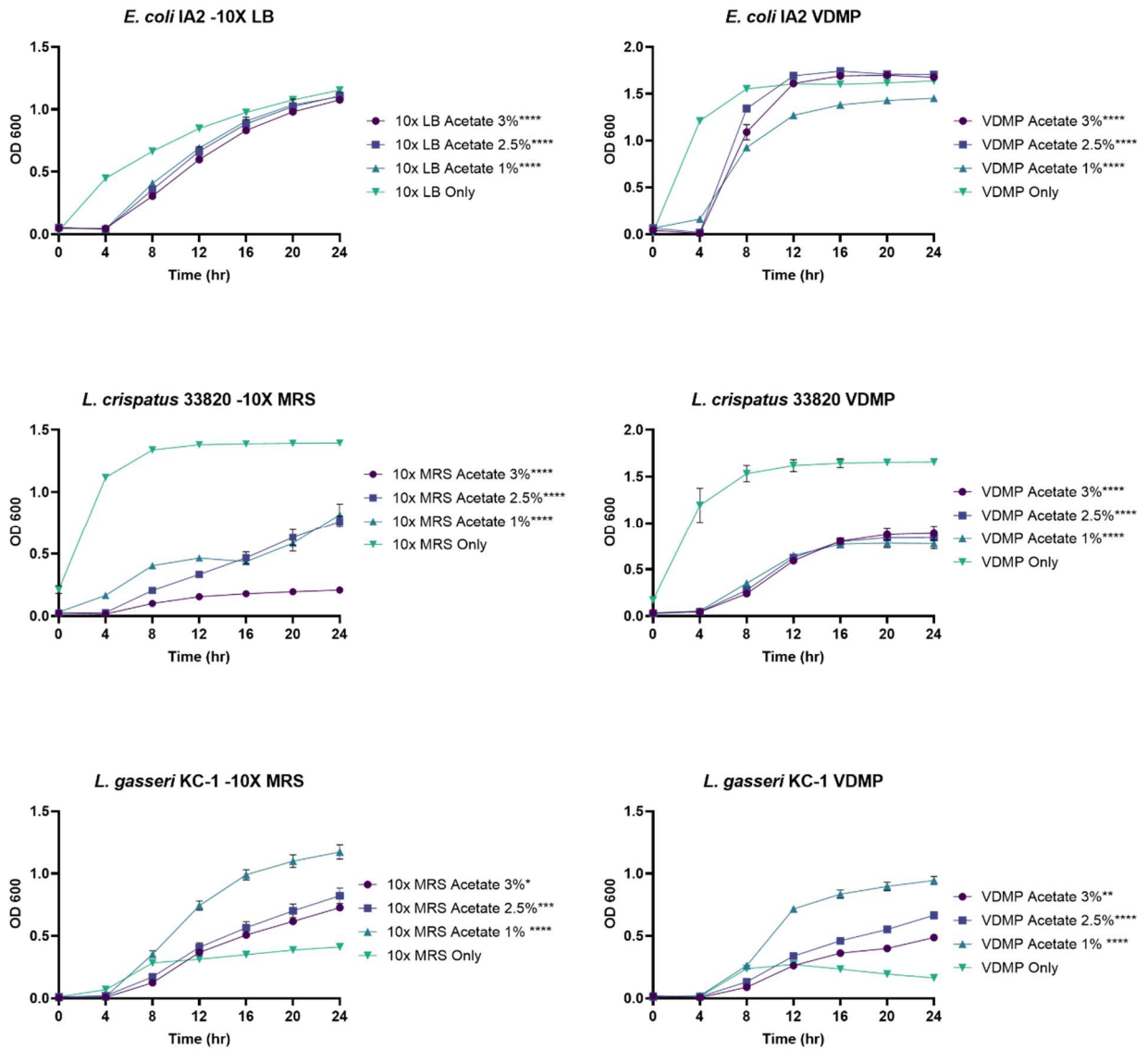


Figure 3-13. *E. coli* IA2, *L. crispatus* ATCC 33820, and *L. gasseri* KC-1 with prebiotic supplementation in 10× diluted LB or MRS, and VDMP. 24-hour growth curves of *L. gasseri* KC-1 with 0.5% and 1% (wt/vol) prebiotic in VDMP, 10× diluted MRS, and artificial urine. Data are displayed as mean OD₆₀₀ ± SD of three biological replicates. The AUC was compared using ordinary one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

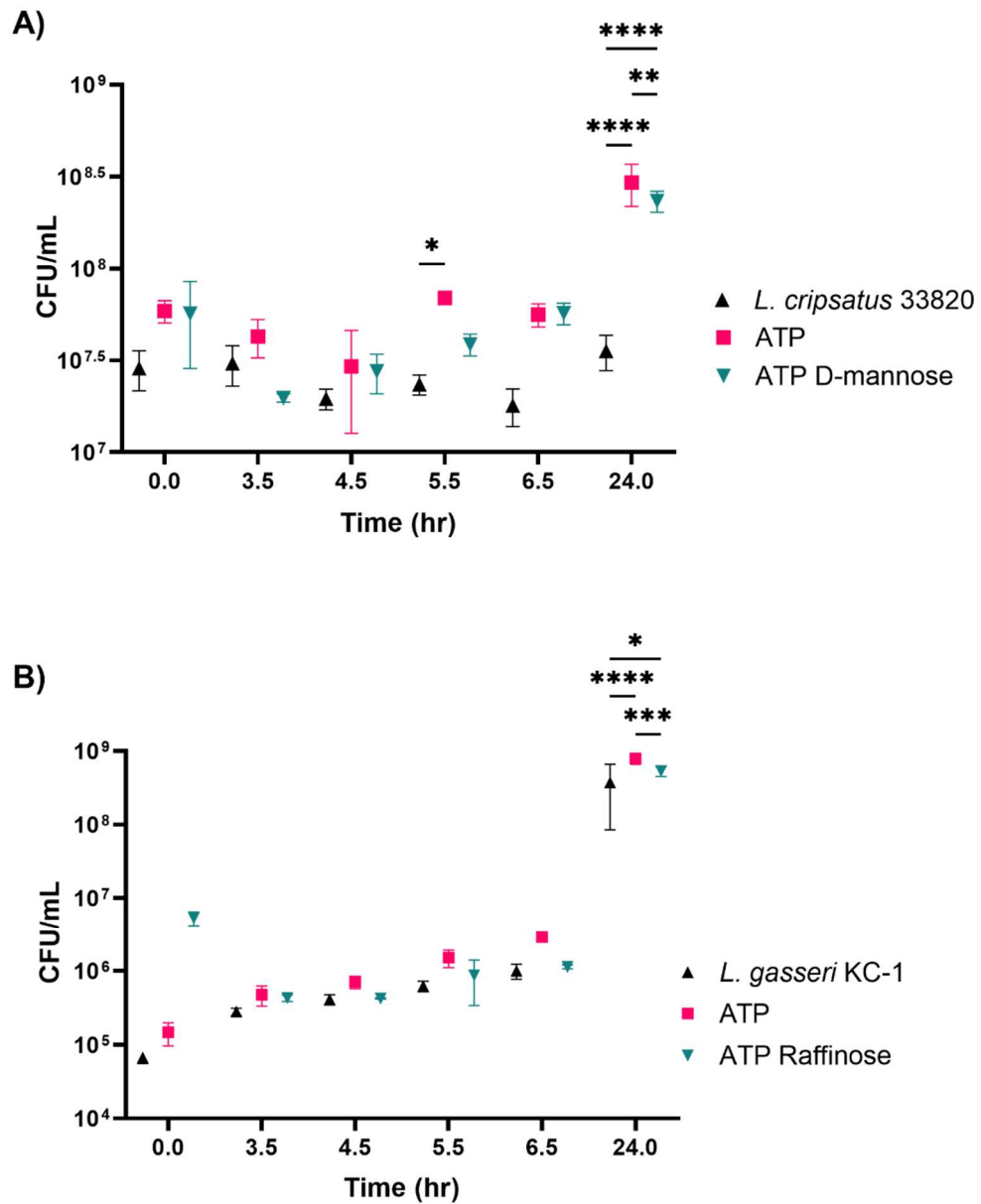


Figure 3-14. CFU enumeration of *L. crispatus* ATCC 33820 and *L. gasseri* KC-1. Time course CFU enumeration of (A) *L. crispatus* ATCC 33820 (B) *L. gasseri* KC-1 grown with prebiotic 0.5% and 1 μ M ATP, 1 μ M ATP only, and media only. Data displayed as mean CFU per \pm SD (two-way ANOVA) at each time point of 3 biological replicates. * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001.

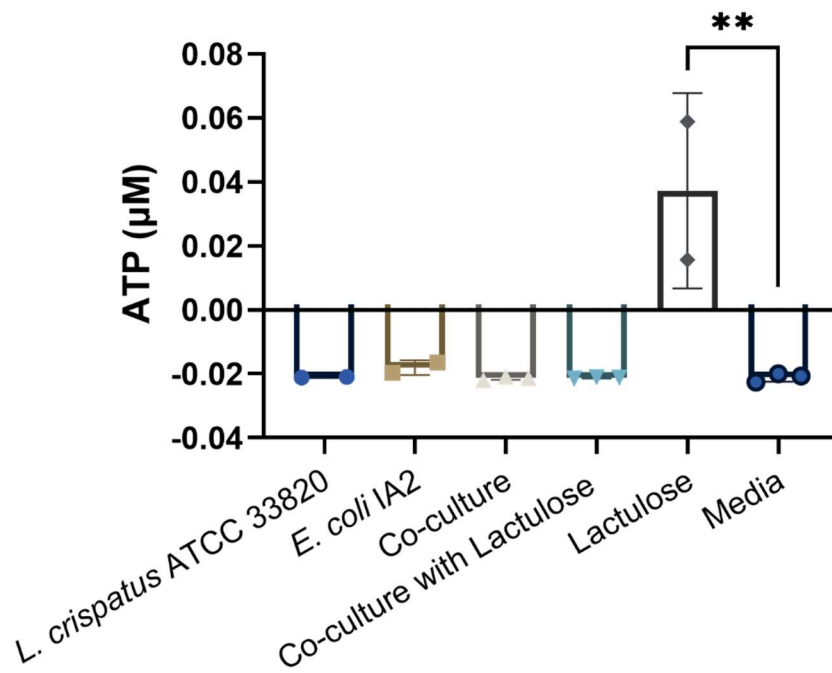


Figure 3-15. Lactulose effect on urothelial 5637 cells. *L. crispatus* ATCC 33820 co-cultured with *E. coli* IA2 and urothelial 5637 cells, with and without lactulose; each bacteria was also cultured alone with urothelial 5637 cells. Complete screening of eATP (μM) levels in minimal cell media normalized to the ATP standard curve. Data displayed as mean \pm SD (two-way ANOVA with Tukey's) (n=2-3).

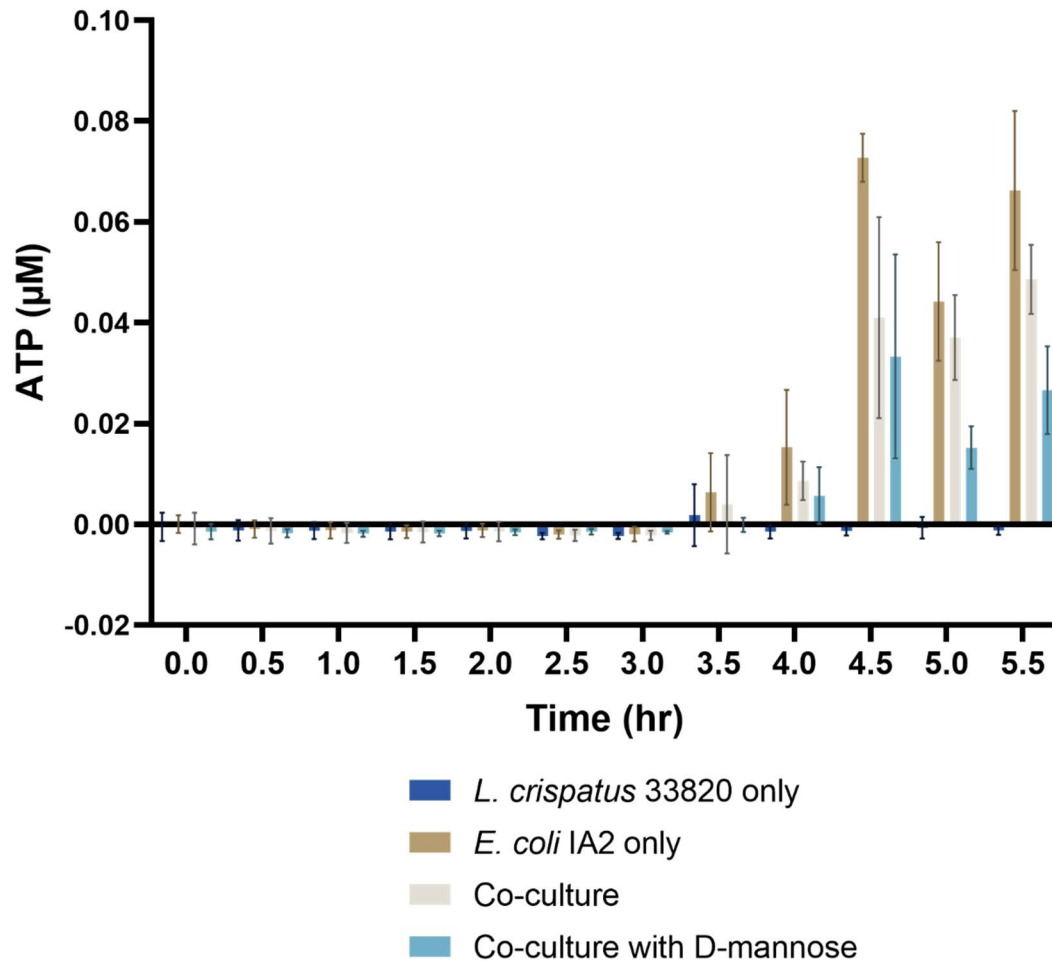


Figure 3-16. D-mannose impact on *L. crispatus* ATCC 33820 reduction of eATP from cell culture. *L. crispatus* ATCC 33820 co-cultured with *E. coli* IA2 and urothelial 5637 cells, with and without D-mannose; each bacterium was also cultured alone with urothelial 5637 cells. Complete screening of eATP (μM) levels in minimal cell media normalized to the ATP standard curve. Data displayed as mean \pm SD (two-way ANOVA with Tukey's) ($n=4$).

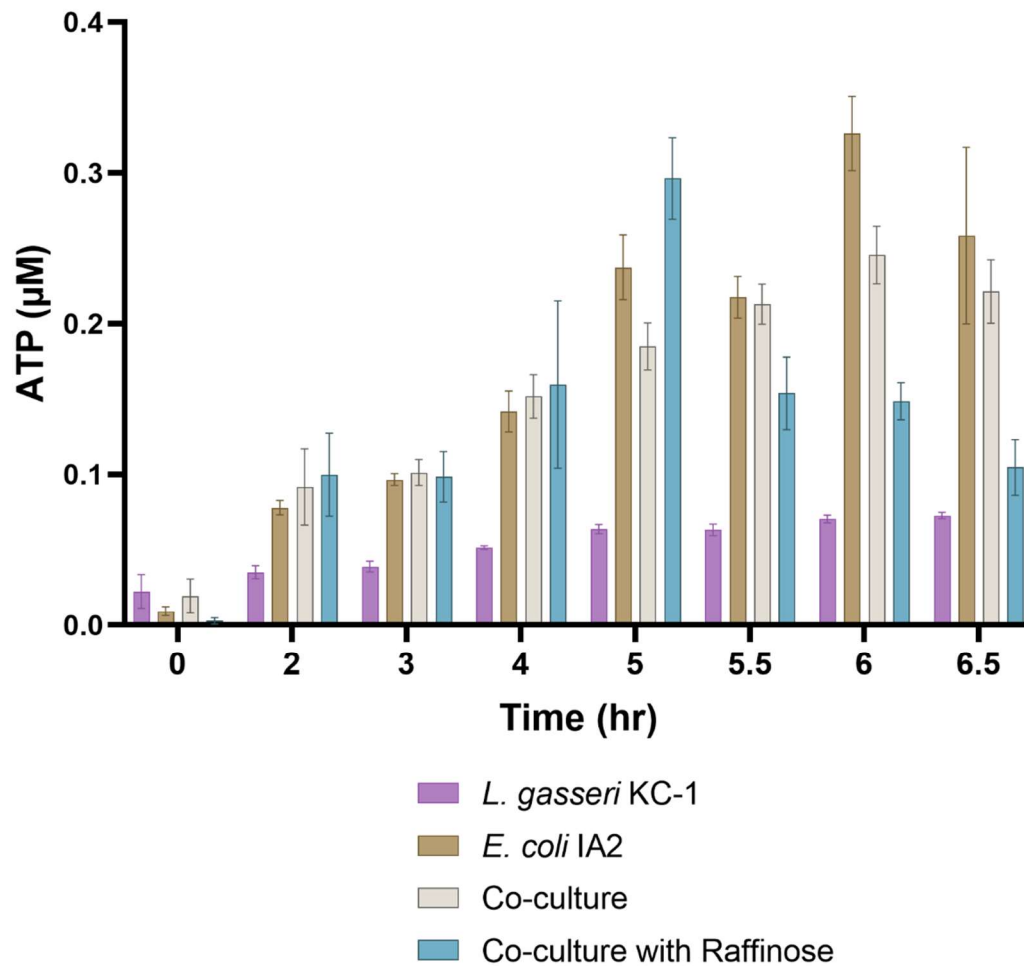


Figure 3-17. Raffinose increases *L. gasseri* KC-1 eATP reduction in co-culture with *E. coli* IA2 and urothelial cells. *L. gasseri* KC-1 was co-cultured with *E. coli* IA2 and urothelial 5637 cells, with and without raffinose; each bacterium was also cultured alone with urothelial 5637 cells. Full screening of eATP (μM) levels in minimal cell media, normalized to the ATP standard curve. Data displayed as mean \pm SD (two-way ANOVA with Tukey's) (n=4). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Chapter 4

4 General discussion

The work undertaken in relation to this project supports our hypothesis that lactobacilli could be further implemented in the bladder environment to reduce extracellular adenosine triphosphate (eATP). This is important, as high levels of eATP are thought to play a negative role in bladder conditions, especially those involving urinary urgency and infection. By applying specific prebiotics that have been identified in this thesis, it is feasible to promote lactobacilli with strong ATP depleting activity and may be possible to reduce urinary urgency.

4.1 Understanding the impact of ATP release and uptake by bacteria

There is a known association between high urinary adenosine triphosphate (ATP) concentrations and conditions, including; urinary tract infection (UTI), overactive bladder (OAB), dysuria, urinary urgency incontinence (UUI) and bladder muscle contractions¹⁻⁴. Increased urinary ATP has been used as a biomarker to diagnose UTI since at least 1971⁵; recent studies suggest that it might be used as an indicator for OAB, lower urinary symptoms, and dysfunction^{1,6}. Our lab has shown that uropathogenic and pathobiont bacteria release ATP. In the bladder, this could influence smooth muscle contraction associated with urinary contraction through purinergic signaling pathways⁷. Uropathogens release ATP, triggering purinergic host receptors to release urothelial ATP, leading to an increased ATP concentration. Repetitive purinergic signaling contributes to the high ATP concentrations in the bladder during these conditions. A urinary microbiome of a healthy individual may contain ATP-reducing species that limit bacterial triggered ATP release. Further studies will determine the extent to which reduction of bacterial ATP influences purinergic signaling in the bladder and regulation of urgency symptoms.

It is known is that many bacteria release ATP during growth and thereby can potentially impact purinergic signaling pathways upon the host⁸⁻¹¹. Although the mechanisms of ATP

release and uptake are yet unknown, it is observable across bacteria, suggesting it may benefit the organism. However, releasing cellular energy from bacterial cells in the form of ATP during growth seems illogical, as we would predict a conservative approach to fuel cellular processes. Physiologically, the release is only logical if there is a direct benefit, such as bacterial or host communication, an energy reserve, bioenergetic molecule sharing between microorganisms or between them and the host. The change in eATP concentrations during growth^{10,12} and in response to the host^{8,9,13}, environmental stress^{10,14,15}, and nutrient availability¹⁶, suggest ATP release could be a regulatable mechanism controlled by the bacteria throughout growth. The influence on host neurotransmission and bacterial-host communication benefits of eATP also suggest that release is purposeful and valuable^{11,13}. Additionally, the bacterial ATP triggered purinergic signaling, causing host release of ATP⁷ could then act as a nutritional source for the organisms. Release and uptake of ATP support the idea that bacteria could be using the environment to store ATP for later consumption or to share with other key symbionts.

In Chapter 2, a range of commensal and pathogenic bacteria were shown to release ATP. During *Lactobacillus crispatus* ATCC 33820 and *Escherichia coli* IA2 growth, eATP concentrations fluctuated, rising to peak during log-phase growth then depleting in stationary phase. In the stationary phase, growth has ceased, but cells remain metabolically active, and bacteria are faced with nutrient deprivation, as well as physical and chemical stresses¹⁷. The cells address these conditions with a reduction in size and an increase in the DNA/protein ratio, and they adapt to survive conditions of stress and starvation¹⁷. If ATP is available extracellularly, this may be a means to senescence survival without active replication. Bacteriocin production typically occurs in the late-log or stationary phase^{18,19}, giving the producing strain an advantage in the competitive environment for niche and potentially nutrients by liberating ATP from target cells. The fluctuation in eATP concentrations could be related to the stress associated with stationary phase nutrient deprivation and competition. Stationary phase growth is similar to the consolidation phase in *Proteus mirabilis* swarming; it is a resting state during which there is an upregulation of nutrient uptake systems, central metabolism, and respiration²⁰. We observed a tendency to migrate towards eATP during *P. mirabilis* consolidation, suggesting that the ATP may motivate directional growth through nutrient availability, signaling, or another undiscovered method. While bacteria are growing

and producing an abundance of ATP, the cells could release excess into the environment for storage and reduce nutrient deprivation after exponential growth.

Following the same idea, bacteriocins contribute to the release of nutrients that can supplement late phase survival¹⁸. It could be beneficial for bacteria to release excess nutrients or ATP in a hostile environment if this reduces bacteriocin production. Streptococci are prolific producers of bacteriocin²¹ and eATP, as we observed. Bacteriocin production contributes to community development in streptococci through interspecies signaling, limiting the outgrowth of a signal streptococcal strain in a co-colony²². It is possible that ATP acts as a communication signal between species and that higher release is related to stable colonization of several organisms. We found decreased bacteriocin production with supplemented ATP, suggesting that ATP could limit bacteriocin production within a microbiota. If the ATP producers are supplemented in late phase growth, the environment might be more hospitable and able to support the survival of many species (**Figure 4-1**).

Bacteria could use ATP as a signaling molecule to communicate with neighbouring bacteria and the host, influencing the environment. There are purinergic receptors found on almost all mammalian tissues. Purinergic signals control many physiological and pathological processes in humans, including cell proliferation, cell motility and death, development and regeneration of body systems, neurotransmission, neuromodulation and secretion of ions or other small molecules^{3,23-28}. Short- and long-term trophic events can be triggered by ATP^{23,28}. Bacterial ATP can influence the immune system, induce smooth muscle contractions, stimulate host cell release of ATP, and utilize host ATP^{7-9,13}. Purinergic receptors on the urothelium triggered by bacterial ATP release host cellular ATP creating an ATP excess in the bladder. Abundant urinary ATP induces Ca²⁺ influx triggering smooth muscle contraction and is associated with urgency^{2,7,29,30}. The present study showed that ATP in *E. coli* IA2 supernatant could influence the directional growth of uropathogenic *P. mirabilis*. These results suggest that *E. coli* IA2 could be influencing other pathogens, in addition to modulating the environment⁷. For example, increased urinary ATP during UTI⁶ and OAB¹ could promote *P. mirabilis* growth. Taken together, these findings support the idea that bacterial ATP could allow host and bacterial communication and influence the bladder environment.

Generally, bacteria considered more pathogenic, or those that lead to symptomatic presentation in the host (*E. coli*, *Streptococcus*, *Gardnerella vaginalis*) release higher ATP concentrations than those considered commensal in the bladder. The most significant producer in this study was group A *Streptococcus pyogenes* NGAS979, a particularly virulent skin and mucosal infector³¹. Streptococci are common skin and oral cavity colonizers and opportunistic pathogens, for which their release of ATP may be an additional virulence factor. When ATP is applied to the epidermis, it elicits a pain response³² which results in inflammation²³. With pain and inflammation causing agitation, an opportunistic pathogen could become more virulent. However, this response can be reversed with a reduction of ATP^{33,34}. In addition, the burning associated with ATP release could lead to disruption of the epithelial barrier allowing an infection under the skin. Significant release of ATP by *S. pyogenes* NGAS979 could be contributing to virulence in one of these ways. Further research is needed to understand why relatively large amounts of ATP are released by this strain compared to other bacteria.

Some bacteria not considered pathogenic have been found to release more ATP than other commensals: specifically, *G. vaginalis* 14018 and *Limosilactobacillus vaginalis* NCFB 2810⁷ (reclassified from *Lactobacillus vaginalis* NCFB 2810). These are bacteria commonly found in the reproductive and urogenital tract; *G. vaginalis* is associated with a disordered microbiota and bacterial vaginosis; *L. vaginalis* is a commensal vaginal isolate⁷. Identifying common urogenital bacteria, which could have a similar impact on the bladder to that of *E. coli* IA2, supports the idea that UII is related to bacterial ATP release and may not be easily predictable by compositionally based 16S rRNA gene sequencing analysis. Often OAB is defined as urgency without active infection. If the microbiota of an individual has native species which release high ATP concentrations, this could promote pathogen growth and cause infection. Further investigation is required into whether certain urinary microbiota species may be a part of the disease process through this mechanism.

Ultimately the purpose behind the bacterial release of ATP is still unknown. However, this study and others provide strong support for the idea that ATP exchange between bacteria, the environment, and the host occurs. Bacterial-host interactions can be impacted through purinergic signaling, with eATP acting as a neurotransmitter. Bacteria may utilize the environment to store ATP for use in later phase growth. In addition, ATP may enable inter-

bacterial communication and help establish colonization, promote and direct growth (**Figure 4-1**). With an increasing understanding of bacterial-ATP and its interaction with the host, a more comprehensive role of eATP can be generated.

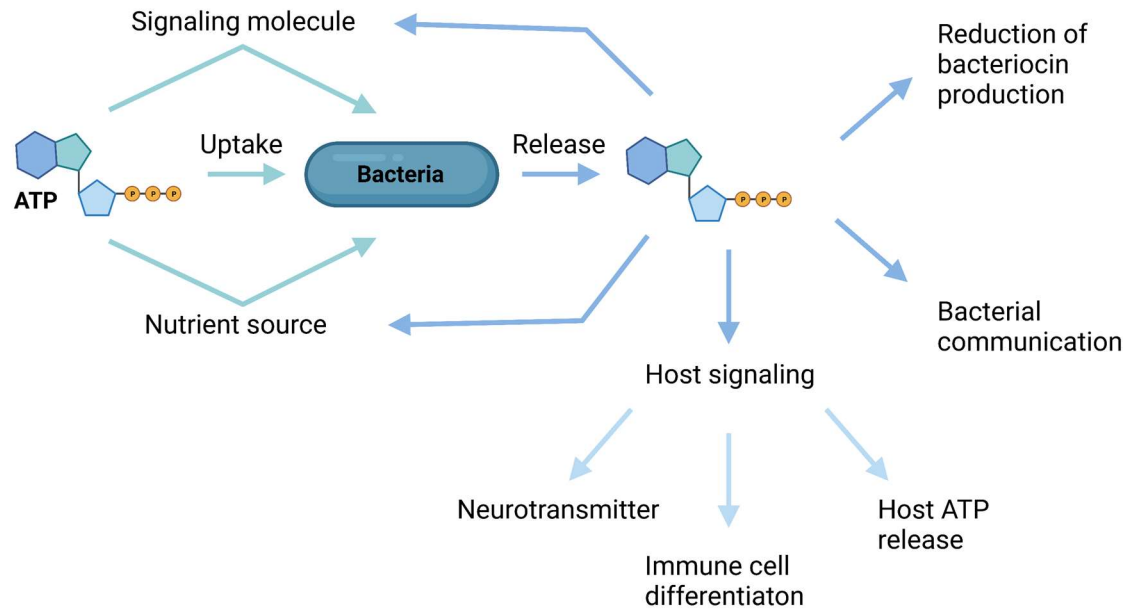


Figure 4-1. The potential impact of ATP secretion and uptake on the bacteria, the microbiome and the host. Excess ATP taken up by bacteria could be a source of energy in late phase growth and a chemotaxis signaling molecule. Bacterial release of ATP could influence bacteriocin production, intraspecies communication, and host signaling. In humans, bacterial ATP is a neurotransmitter targeting purinergic receptors, triggering immune differentiation, and inducing ATP release. Created using biorender.com.

4.2 A proposed method for eATP reduction in the bladder

The stretch feeling associated with a full bladder is controlled by a system where ATP plays a key role: as the bladder fills, urinary ATP increases and triggers Ca^{2+} influx and smooth muscle contractions^{29,30,35}. When urgency is felt at lower bladder volumes, there are ATP concentrations similar to those seen in a healthy full bladder². Conversely, with a reduction of ATP signaling there is reduced urgency³⁶.

In Chapter 3, we examined the ability of lactobacilli to reduce ATP *in vitro* and the impact prebiotics could have on this process. Both *L. crispatus* ATCC 33820 and *Lactobacillus gasseri* KC-1 released significantly lower amounts of ATP compared to uropathogens and were able to consume more ATP during growth than was released by any uropathogen tested. Using media to mimic the urogenital environment, lactulose, D-mannose, raffinose, and sodium acetate were efficacious prebiotic candidates that enhanced lactobacilli growth and led to reduced ATP levels.

D-mannose and raffinose accumulated in the urine after administration³⁷⁻³⁹ and had no impact on urothelial cells *in vitro*. These prebiotics stimulated *L. crispatus* ATCC 33820 and *L. gasseri* KC-1 leading to an increased ATP reduction rate. Further studies are needed to improve this experimental model and assess whether oral or topical application of a prebiotic can have the same effect. A prebiotic and probiotic blend could be lyophilized for administration orally, as a vaginal suppository, cream, or gel⁴⁰. Lactulose had an adverse urothelial cell interaction resulting in greater ATP release. However, this prebiotic could still increase lactobacilli present in the vaginal tract⁴¹, potentially enhancing urinary tract lactobacilli due to the interconnected microbiomes⁴². Sodium acetate showed prebiotic potential for *L. gasseri* KC-1 with suppression of *E. coli* IA2 growth. Based on another study, this prebiotic could be used in conjunction with antibiotic erythromycin to enhance its antibacterial effects⁴³.

Growth of *L. crispatus* ATCC 33820 and *L. gasseri* KC-1 supplemented with ATP was similarly enhanced as with prebiotic supplementation. However, adding a prebiotic to ATP supplemented media had no greater impact on growth than ATP alone. In co-culture with *E. coli* IA2, both *L. crispatus* ATCC 33820 and *L. gasseri* KC-1 growth was enhanced, which

suggests the ATP release from *E. coli* IA2 was a stimulant. Thus, increased eATP concentrations during infection may foster lactobacilli growth and consumption (ATP) in individuals with abundant native lactobacilli. This interaction could contribute to UTI resolution without antibiotic intervention, as occurs in some patients⁴⁴.

When applied to urothelial cells, lactobacilli supernatant reduced *E. coli* IA2 impact on Ca²⁺ influx and muscle contractions *in vitro* through purinergic receptor binding with GABA⁷. In co-culture with urothelial cells and *E. coli* IA2, lactobacilli can reduce ATP through uptake. Lactobacilli uptake of ATP is enhanced with prebiotic supplementation, resulting in lower eATP concentrations than co-culture alone. Increased ATP reduction is not caused by a greater bacterial load. In the minimal RPMI 1640 with *E. coli* IA2, lactobacilli might limit eATP uptake to ensure nutrient availability in the stationary phase; the addition of a prebiotic provides more nutrients allowing further ATP reduction. Bacteriocin production is also related to nutrients and ATP availability. Late phase antimicrobial molecules and bacteriocins that promote lactobacilli growth and could inhibit surrounding organisms could be decreased in response to surplus ATP.

Secretion of ATP by *L. crispatus* ATCC 33820 increased up to exponential growth, while *E. coli* IA2 eATP peaked before the exponential phase. This may be explained by a study that showed that reduction in cellular respiration lowers ATP output¹². Lactobacilli glycolytic and fermentative respiration produces less ATP, which could relate to their relatively low secretion. During exponential growth, lactobacilli could benefit from ATP uptake, as it might reduce the cellular respiration requirements. These are significant findings that require further investigation to elucidate fully.

These *in vitro* studies appear to be a basis for ATP-related UUI regulation in a bladder by adding lactobacilli through the application of probiotics or stimulation of indigenous strains using prebiotics. We have demonstrated that pathogenic bacteria secrete ATP during growth, lactobacilli can reduce ATP from culture and *in vitro* bladder model, and prebiotic supplementation can enhance ATP reduction. This concept is worth pursuing clinically to manage OAB and uncomplicated UTI. Current therapeutic options fail to address ATP-related urgency, and this is an antibiotic-free intervention that could relieve patient symptoms in a novel way. (**Figure 4-2**)

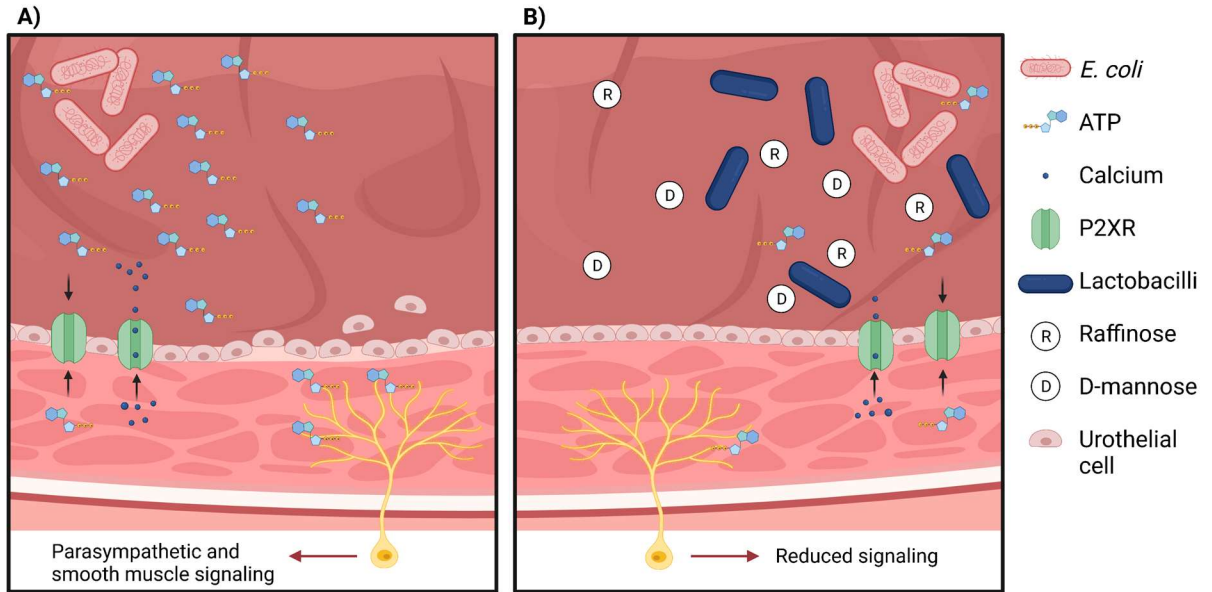


Figure 4-2. Potential impact of lactobacilli on ATP in the bladder. (A) With a UTI, *E. coli* IA2 secretes ATP, which binds to host purinergic receptors leading to calcium and ATP influx. Increased neurotransmitters in the sub-urethral space, ATP, can stimulate the parasympathetic pelvic nerve leading to pain and smooth muscle contractions. (B) With the introduction of prebiotics, there might be an increase in lactobacilli and a decrease in eATP. Thus, with the decreased ATP load, there could be reduced purinergic and parasympathetic signaling. Additionally, lactobacilli presence reduces calcium influx through GABA stimulation of ion channels to reduce intracellular voltage and smooth muscle contractions. Created using biorender.com.

4.3 Future directions

Future work should validate the findings and identify the bacterial mechanism of ATP regulation and how it is secreted, transported across membranes, and if this is an active process. A set of experiments with labelled ATP could be used to visualize the release, uptake and transport through the bacterial cell⁴⁵. Luciferase could be used to visualize and track ATP movement in real-time. Plate imaging with luciferase could be used to track ATP release or consumption by bacteriocin producers⁴⁵. Imaging in conjunction with bacteriocin detection via differed growth assay could elucidate the ATP-bacteriocin interaction. Further studies could also interrogate how eATP operates as a signaling molecule between bacteria and the host.

Another limitation of this study is the introduction of lactobacilli to the urinary tract. Studies have examined probiotic and prebiotic applications to the vaginal tract^{21,41,46,47}, with some positive reductions in recurrent UTI reduction and increased lactobacilli abundance. Lactobacilli could be introduced orally, topically or with a catheter; studies on urinary microbial abundance after introduction could be used to confirm effectivity and potential engraftment. Application to sanitary and incontinence pads could also be examined to see if the lactobacilli numbers increase. A synbiotic cream or gel⁴⁰ could also be assessed. Prebiotics tested in this study are known to accumulate in the urine. Investigating an individual's urinary microbiota before and after consumption of the prebiotics would be worth testing. The effect of prebiotics on eATP reduction could be tested in a human urine model whereby urine from UTI or OAB patients and healthy controls could be supplemented with prebiotics, then lactobacilli and eATP concentrations monitored over 24 hours.

The interaction between bacterial ATP and host purinergic signaling is being investigated and addressed beyond the bladder. For example, Scott et al.⁴⁸ have developed a yeast-based probiotic that responds to increased bacterial ATP in the gut by expressing purinergic receptors to neutralize it, leading to decreased inflammation in IBD mouse models. Understanding the dynamics between host-bacteria-ATP will lead to novel therapeutic avenues such as those outlined in this thesis.

4.4 Concluding statement

This study found that a range of pathogenic and commensal bacteria release and uptake ATP during growth. This ATP can influence the directional growth of uropathogenic *P. mirabilis*, which could have implications in recurrent UTIs. In an *in vitro* environment, lactobacilli were found to reduce ATP with minimal impact on urothelial cells. Thus, the addition of a prebiotic could provide a nutrient source fostering lactobacilli growth and lead to further reduction of ATP. During a UTI, introducing a prebiotic into a urogenital microbiota containing lactobacilli might be sufficient to observe this effect and its benefits in an individual suffering from UUI.

Our understanding of the role that eATP plays in bladder conditions is in its infancy. This study has opened up new ideas aimed at improving the management of UUI in UTI and OAB. This thesis has shown the potential for eATP reduction in the bladder by lactobacilli when combined with prebiotic D-mannose or raffinose.

4.5 References

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