Potential role of extracellular ATP released by bacteria in bladder infection and contractility

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**ABSTRACT**

Urgency urinary incontinence (UUI), the result of conditions such as overactive bladder (OAB), could potentially be influenced by both commensal and urinary tract infection-associated bacteria. The sensing of bladder filling involves interplay between various parts of the nervous system eventually resulting in contraction of the detrusor muscle during micturition. Here we model host responses to various urogenital bacteria, firstly by using urothelial bladder cell lines and then with myofibroblast contraction assays. To measure responses, we examined calcium influx, gene expression and alpha smooth muscle actin deposition assays. We found that organisms such as *Escherichia coli* IA2 and *Gardnerella vaginalis* ATCC 14018 strongly induced calcium influx and contraction, whereas, *Lactobacillus crispatus* ATCC 33820 and *L. gasseri* KC-1 did not induce this response. Additionally, supernatants from lactobacilli impeded influx and contraction induced by the uropathogens. Upon further investigation of factors associated with the purinergic signaling pathways, we found that influx and contraction of cells correlated to the amount of extracellular ATP produced by *G. vaginalis* ATCC 14018 and *E. coli* IA2. Certain lactobacilli appear to mitigate this response by utilizing extracellular ATP or producing inhibitory compounds which can act as a receptor agonist or calcium channel blocker. These findings suggest that members of the urinary microbiota may be influencing UUI.
Keywords

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Chapter 1: Introduction
1 Introduction

1.1 Focus of thesis

The focus of this thesis is to explore the mechanism used by *Lactobacillus crispatus* ATCC 33820 and *Lactobacillus gasseri* KC-1 to reduce the stimulation of calcium influx and myofibroblast contraction caused by uropathogens. This work relates to the regulation of bladder contractility and reducing the severity of urgency urinary incontinence (UUI).

1.2 Bladder physiology

Bladder physiology consists of two phases: a storage phase and a voiding phase. The storage phase is controlled by the sympathetic nervous system, where the norepinephrine-releasing hypogastric nerve as an adrenergic nerve causes relaxation of smooth muscle cells in the bladder wall and causes contraction of the internal sphincter\(^1\). The voiding phase is controlled by the parasympathetic nervous system where stimulation of the pelvic nerve as a cholinergic nerve results in the release of acetylcholine, which in turn invokes smooth muscle contraction\(^1\). These responses in the bladder are to eliminate urine in regular intervals. However, physical and psychological disorders accompanied by other factors can disrupt the normal physiology of the bladder and lead to disease\(^2\).

Beyond the cholinergic and adrenergic systems, the physiology of the bladder is controlled by various other neurotransmitters or neuroactive compounds which can exert different effects. Examples of these include adenosine triphosphate (ATP) and gamma-Aminobutyric acid (GABA), which have excitatory and inhibitory effects respectively. These can both be released by the nervous system, urothelial cells and potentially by different strains of bacteria, which all can have effects on bladder physiology\(^3-5\).
1.3 Bladder diseases

There are a number of diseases that can affect the bladder: cancer, overactive bladder (OAB) syndrome, urgency urinary incontinence (UUI: involuntary loss of urine associated with urgency), stress urinary incontinence (SUI: involuntary loss of urine on effort or physical exertion, or on sneezing or coughing), urinary tract infection (UTI), and asymptomatic bacteriuria (ABU). With the exception of ABU, some common symptoms and signs can be found, such as the loss of bladder control leading to the involuntary leakage of urine as well as an increased frequency or urgency to urinate. In addition, UTIs can be accompanied by suprapubic pain and fever. In contrast, although the urine of a patient with ABU contains the equivalent bacterial viable count as someone with a UTI, no symptoms and signs are expressed.

1.3.1 Urgency urinary incontinence

In patients that suffer from overactive bladder and UUI, the bladder feels fuller than it actually is. The frequent sensation to urinate is based on the contraction of smooth muscle cells in the bladder wall. Three large studies conducted in Europe and North America report prevalence of UUI from 1.8% to 9.3% in men and from 3.9% to 24.4% in women, accompanied by social, psychological, and economical burden.

The inability to properly control micturition is associated with depression, anxiety, social isolation, reduced functional status, increased risk of institutionalization and overall negative impact on quality of life. The economic burden of UUI in America is projected to be $82.6 billion by 2020. Despite the devastating impact of UUI, we still do not fully understand its underlying pathophysiology and this is a significant barrier to the development of targeted interventions aimed at eliminating or reducing symptoms. Several subtypes of urinary incontinence (UI) have been
defined by the International Continence Society, including UUI, SUI and mixed UI (MUI: involuntary loss of urine associated with urgency and with exertion, effort, sneezing or coughing)\textsuperscript{10,11}. Longitudinal studies and a systematic review of OAB and UUI suggest that these conditions are often chronic and symptom severity increases over time for many patients\textsuperscript{12,13}.

1.3.2 Frequent urination in patients with bacterial vaginosis

Bacterial vaginosis (BV) is a condition associated with bacterial dysbiosis with depletion of lactobacilli and overgrowth of bacteria such as \textit{Gardnerella vaginalis}. This organism is commonly found as a component of the urinary microbiota\textsuperscript{14}, likely through ascension from the vagina and urethra. Patients with BV often report the urgent need to urinate, and bladder contractions can increase after intercourse, potentially because of compounds released from \textit{G. vaginalis} into the bladder\textsuperscript{14}.

1.4 Bladder microbiome

The urinary tract above the urethra and including the bladder was long believed to be sterile. But, new methods such as 16S rRNA gene sequencing and advanced quantitative urine culture techniques have shown that numerous bacterial types are present and alive in this organ\textsuperscript{15-17}.

The genus \textit{Lactobacillus} has been found in urine more frequently in healthy subjects compared to patients with UUI (60% versus 43%), while \textit{Gardnerella} was more abundant in patients (26% versus 12% in controls)\textsuperscript{18}. Interestingly, \textit{L. gasseri} is considerably more prevalent in UUI patients than \textit{L. crispatus}, raising questions about how different species adapt to the bladder environment\textsuperscript{18}. 
1.4.1 The protective role of commensal bacteria

The discovery that bacteria permanently persist in the bladder may have a significant impact on health. Although the actual protective role of bacteria is still unknown, recent research has been able to find many links between dysbiosis in the urinary microbiota and bladder diseases\textsuperscript{19,20}. Lactobacilli are facultative anaerobic bacteria that produce antimicrobial substances such as lactic acid which decrease the pH of the vagina, contributing to protection against certain pathogens\textsuperscript{21,22}. They can also regulate and help maintain epithelial junctions, degrade toxic compounds released by pathogens, and reduce the ability of pathogens to adhere to the uroepithelium\textsuperscript{23}.

1.5 Uroepithelial barrier

The uroepithelium creates a line of separation between bacteria and underlying muscle and nerve tissues of the bladder. In addition to being a simple barrier, the urothelium acts as a sensory network to detect mechanical and chemical changes in the bladder\textsuperscript{24}. The uroepithelial cells express receptors for different neurotransmitters and release neurotransmitters into the sub-urethral space and lumen of the bladder. These neurotransmitters include ATP, nitric oxide (NO) and acetylcholine (Ach), which provide signals to the muscular and nervous networks to regulate bladder contraction\textsuperscript{25,26}.

Since the level of ATP released by urothelial cells fluctuates and as it depends on various bladder dysfunctions such as interstitial cystitis, OAB and painful bladder syndrome, ATP released into the voiding fluid can be measured as an indicator of these diseases\textsuperscript{27-30}. The uroepithelial cells shed, particularly during UTI, and exposure of inner layers to bacteria and their by-products can worsen the neurological disorders\textsuperscript{31,32}. This may explain urinary frequency accompanying UTI, as
well as loss of bladder control, since normal sensory and motor signaling pathways can be disrupted.\textsuperscript{33}

### 1.5.1 Urothelial receptors and channels

Transient receptor potential (TRP) plays a key role in nociceptive and mechano-sensory transduction in various organs. The bladder contains several TRP channels including TRPV1, TRPV2, TRPV4, TRPV8 and TRPA1 which are expressed in both urothelium and interstitial cells. Inflammation and injury in the urothelium can alter TRP channel expression.\textsuperscript{34-36}

The increased expression of TRPV1 indicates neuropathic pain and hyperalgesia.\textsuperscript{37} In addition, the number of TRPV1 on nerve fibers increases in neurogenic detrusor overactivity. The TRPV1 channel is considered as a new therapeutic target for development of painkillers.\textsuperscript{38} Stimulation of TRPV1 and TRPV4 can cause release of ATP and nitric oxide by the urothelium.\textsuperscript{39}

N-methyl-D-aspartate (NMDA) is an important receptor where the main subunit of this receptor is encoded by \textit{GRIN1}. Activation of this channel requires binding of glutamate to the epsilon subunit and binding of glycine to the zeta subunit. This channel is highly permeable to calcium ions and it can thereby regulate the level of Ca\textsuperscript{2+} influx. In addition, NMDA can mediate the release of neurotransmitters through the bladder urothelium.\textsuperscript{41} The activity of NMDA receptor in interstitial cystitis is increased.\textsuperscript{42} Blocking the NMDA receptor can reduce bladder hypertrophy during interstitial cystitis and reduce bladder contractility.\textsuperscript{43}

The purinergic receptor 2 (P2) families contain P2X and P2Y expressed in the urothelium as well as in sub-urothelial afferent nerves and myofibroblasts.\textsuperscript{44-47} In response to stretching, ATP can be released through the urothelium. The P2 receptor agonists can stimulate the urothelium to produce ATP.\textsuperscript{49} In addition, during the voiding phase, neuronal and non-neural (urothelial)
sources are forced to release Ach, which then stimulates muscarinic receptors on urothelial cell and smooth muscle cell, causing bladder contraction\textsuperscript{50}.

Many receptors mentioned above are expressed across various cell types including urothelial cells, sub-urethral tissues like myofibroblast, smooth muscle and the nervous tissue\textsuperscript{51,52,53}. Many receptors and ion channels are expressed in urothelial cells, which makes it possible to respond to different stimuli such as: Ach, ATP, noradrenaline, stretching during bladder filling, and even pH response\textsuperscript{54-62}.

1.5.2 Myofibroblast receptors and channels

The lamina propria lies under the urothelium and constitutes a layer of interstitial cells that are spindle-shaped with intracellular connections to myofibroblasts, connective tissue elements and neighboring sub-urethral nerves\textsuperscript{51,52,63}. Many receptors involved in smooth muscle contraction are also expressed in the myofibroblast, such as muscarinic, purinergic and TRPV1 receptors\textsuperscript{64}.

1.5.3 Detrusor smooth muscle cell receptors and channels

The detrusor muscle cells are located in the bladder wall. The detrusor muscle remains relaxed to allow the bladder to store urine, but it contracts to void the urine\textsuperscript{65}.

Many receptors such as muscarinic, purinergic and TRPV1 that are expressed in urothelial cell and sub-urethral tissue can also be expressed in smooth muscle cell. Some bacterial compounds released into the lumen of the bladder can pass through a compromised urothelium and after crossing the sub-urethral can reach smooth muscle cells and change bladder contractility\textsuperscript{51,52,63}.  
1.5.4 Urothelial enzymes

Monoamine oxidases are mitochondrial enzymes with high expression level in many organs. Enzymes such as MAOA and MAOB can degrade biogenic neuroactive chemicals, including dopamine, while MAOA degrades serotonin and norepinephrine, MAOB can degrade phenylethylamine. Reduction in the levels of these enzymes can lead to neurological disorders\textsuperscript{66, 67}.

1.6 ATP and micturition

ATP is an essential neuroactive molecule that is released in response to stretch and stimuli by neuronal sources such as parasympathetic nervous system and non-neuronal sources including the uroepithelium. It is an essential molecule for the onset of normal reflux of micturition and it acts on several different G-protein-coupled P2Y and ionotropic P2X receptors. The stimulation of P2X3 receptors causes an increase in afferent nerve activity, thereby increasing the sensation of bladder fullness and pain. In woman with bladder dysfunction the release of ATP is increased due to stretching\textsuperscript{68-73}.

In addition, ATP can increase the amount of intracellular $\text{Ca}^{2+}$ by two routes: through purinergic and TRPV1 channels from the plasma membrane, or through the endoplasmic reticulum (ER)\textsuperscript{74}.
1.6.1 The role of calcium in bladder function

Calcium has a vital role in the release of neurotransmitters by cells. Increasing the level of intracellular calcium can regulate important mechanisms in the cell, including gene transcription, proliferation and metabolism\textsuperscript{75}. In urothelial cells, many receptor types such as those associated with purinergic and TRP, pass calcium from outside to inside. Some of these receptors are sensitive to ATP and are also expressed in urothelial, sub-urethral tissues and smooth muscle cells. ATP, by increasing the level of intracellular calcium can lead urothelial cell to depolarization phase which is an excitatory phase and is essential for cell function. The depolarization phase can result in the release of compounds such as ATP and Ach into the sub-urethral space, which can alter the contractility of the bladder. In this regard, the continuous activation of these receptors, which causes the cytotoxic level of intracellular calcium, can force cells to mitochondrial dysfunction and apoptosis. Uropathogenic bacteria can stimulate these receptors and cause the influx of calcium and, by driving the cells into the depolarization phase, excitatory compounds such as ATP can be released\textsuperscript{76-80}.

1.7 The role of anion channel to balance anions and cations inside the cell

Although excess intracellular calcium can push the cell towards apoptosis. But, the normal level of intracellular calcium is essential for cell function and regulates important cell mechanisms\textsuperscript{81}. Therefore, the cell needs an inhibitory function or mechanism to control the level of intracellular calcium in order to balance anions and cations.

Anionic channels, such as volume-regulated anion channels (VRACs), GABA receptors, and glycine receptors, play key roles in the influx and efflux of anions to balance anions and cations.
within the cell. By increasing the level of anions like chloride inside of the cell, the voltage of the cell is decreased. When GABA and glycine stimulate their respective receptors, they can cause chloride influx and protect the cell against the side-effect of excess Ca$^{2+}$, as well as leading the cell to hyperpolarization, an inhibitory phase$^{82,83}$. Commensal bacteria in the intestine can release GABA and other inhibitory compounds$^{84}$, though this has yet to be shown for bacteria originating in the urinary tract. The GABA could protect cells from compounds produced by uropathogens, leading to stimulation of calcium influx and depolarization.

1.7.1 **GABA receptor agonists (GABAergic) and bladder function**

The pudendal afferent nerve can inhibit bladder contraction and increase bladder capacity when stimulated by GABAergic mechanisms$^{85}$. GABA confers noncholinergic and noradrenergic effects in the bladder that can inhibit the release of Ach from postganglionic cholinergic neurons through bicuculline-sensitive receptors. GABA may be associated with the chloride ion channel but not with the adrenergic inhibitory system, thereby allowing it to reduce bladder contractility$^{86}$. We know that purinergic and TRPV1 receptors expressed in urothelial and detrusor smooth muscle cells are sensitive to ATP and stimulate calcium influx and smooth muscle cell contraction. Thus, GABA, as an inhibitory neurotransmitter, could potentially stimulate chloride channels and reduce UUI symptoms caused by ATP.
1.8 The release of neurotransmitters by bacteria

The presence of a urinary microbiota raises the question of whether they can interact with the nervous system, given that the urinary tract is linked to the vagus nerve along with the gut. Studies have shown that the gut microbiota release neuroactive molecules such as GABA, serotonin, and norepinephrine, whose effects can be conveyed to the brain via the vagus nerve\textsuperscript{35, 36, 66}.

Some of these neuroactive molecules play signaling roles in the human body and demonstrate activity in the bladder. These neurotransmitters may bind to host receptors on uroepithelial cells or afferent nerves in the bladder and cause the release of host neurotransmitters or initiate detrusor muscle contraction\textsuperscript{87, 88}.

As an intracellular source of energy, ATP is used by eukaryotic and prokaryotic cells. It has been shown that certain bacterial species can release ATP, though the rate may differ between strains\textsuperscript{67, 40, 89, 90}. ATP can act on both ionotropic and metabotropic receptors that then activate the purinergic receptor which increases intracellular Ca\textsuperscript{2+} levels\textsuperscript{91}. Furthermore, ATP can induce Ca\textsuperscript{2+} influx through TRPV1 from the plasma membrane and stimulate Ca\textsuperscript{2+} influx through the ER\textsuperscript{92}.

Additional interactions are worthy of mention. For example, nitric oxide can induce the release of neurotransmitters such as GABA through Ca\textsuperscript{2+}-dependent exocytotic release mechanisms and Na\textsuperscript{+}-dependent mechanisms\textsuperscript{93}. Lactobacilli and bifidobacteria can generate nitric oxide in the presence of nitrate and nitrite\textsuperscript{94}. Glutamic acid produced by lactic acid bacteria and other acid-resistant bacteria can find its way into the extracellular medium. Glutamate decarboxylases (GAD) are involved in the production of GABA by decarboxylation of glutamate in the bacterial cell\textsuperscript{95, 96}. 
1.9 A proposed mechanism for the role of bacteria in inducing contraction or relaxation in the bladder

Given the information discussed about the production of molecules by bacteria and the effect that these molecules can have on muscle contractility, a mechanism can be proposed for the involvement of these organisms in bladder function. During UTI, uropathogenic bacteria could release neurotransmitters into the bladder lumen and, by stimulating related receptors, the urothelium releases its own neurotransmitters that could act on the nerves and subsequent muscles potentially causing involuntary bladder contraction\textsuperscript{97, 98}.

Bacteria could change bladder contractility by two methods. During UTIs, the invading uropathogens could stimulate uroepithelial receptors and cause calcium influx by releasing excitatory compounds like ATP [Fig 1]. Purinergic receptors, muscarinic receptors, and transient receptor potential channels are expressed in urothelial and sub-urothelial cells such as myofibroblasts, and smooth muscle cells and can function as calcium channels. By increasing the level of intracellular calcium, the voltage of the cell would increase, and this would lead to the depolarization phase essential for cellular function. This would drive the cell to release compounds in the sub-urethral space [Fig 1]. The released compounds, such as ATP and Ach by urothelial cell in sub-urethral space, could induce contraction of smooth muscle cells. The working hypothesis for this project is that bacterial compounds indirectly change bladder contractility. On the other hand, some of these compounds may be able to cross a compromised uroepithelium and, after crossing the sub-urethral space, can reach the smooth muscle cells and directly stimulate their receptors resulting in bladder contraction.
While the increase in intracellular calcium level is essential for cellular function, the continued activation of some receptors may result in mitochondrial dysfunction and ultimately lead to apoptosis.

By releasing inhibitory compounds like GABA, indigenous bacteria may stimulate receptors on urothelial cells and cause chloride influx. This could then cause an anion-cation equilibrium and, by decreasing intracellular voltage, lead the cell to hyperpolarization as an inhibitory phase. [Fig 1].
**Figure 1: GABA can inhibit the stimulation of calcium cause by ATP.** ATP as an excitatory compound can play an agonistic role for purinergic receptors and TRP channels (1). The calcium influx causes an increase in the intracellular voltage and leads the urothelial cell to the depolarization phase\textsuperscript{119} (2), resulting in the release of certain excitatory compounds such as ATP into the suburethral space (3). Inhibitory compounds such as GABA with agonist effects on chloride channels can stimulate GABA receptors (4), causing the chloride influx. By decreasing the intracellular voltage, these receptors try to balance cation and anion levels in the cell, and by driving the cell to hyperpolarization (5) can control the urothelial secretion and regulate bladder contractility.
1.10 Rational for the project

Bladder physiology is influenced beyond cholinergic and adrenergic systems, with several neurotransmitters and neuroactive compounds able to play stimulatory or inhibitory roles to influence different bladder phases\textsuperscript{63,64}. It is proposed that commensal bacteria play a major role in bladder homeostasis and this could have effects in patients with UTI and UUI\textsuperscript{99-101}.

By releasing compounds that have neurotransmitter activity, bacteria may induce calcium influx leading to contractility of smooth muscle cells, or inhibition of contractility which thereby allows the patient to have better control from UUI.

1.10.1 Hypothesis

It was hypothesized that ATP-releasing uropathogenic bacteria may change bladder contractility while some strains of Lactobacillus inhibit it by releasing neuroactive compounds or sequestering excitatory compounds.

1.10.2 Objectives:

A. Evaluate the ability of bacteria to release excitatory and inhibitory compounds that stimulate and inhibit calcium influx in urothelial cells.

B. Evaluate the ability of bacterial supernatants to induce contraction in myofibroblasts measured by collagen gel contraction assay.

C. Evaluate the ability of bacterial compounds in the bladder to alter uroepithelial gene expression.
Chapter 2: Methods and Materials
2 Materials and Methods

2.1 Bacterial supernatant preparation

*Escherichia coli* IA2 UPEC was maintained on Luria broth agar (LB, Difco, MD), *Lactobacillus gasseri* KC-1, *Lactobacillus crispatus* ATCC 33820, *Enterococcus faecalis* ATCC 33186, were maintained on de Man, Rogosa and Sharpe agar (MRS, Difco, MD), *Gardnerella vaginalis* ATCC 14018, *Lactobacillus vaginalis* NCFB 2810 were maintained on Columbia Blood Agar (CBA, Difco, MD) and *Gardnerella* Selective Agar (Difco. MD). For these studies, all strains of bacteria were grown in artificial urine\(^{102}\) which in preliminary experiments was shown not to stimulate the influx of calcium when in the presence of human cell lines.

Supernatants were collected from cultures grown overnight (24 hours) at 37°C after reaching stationary phase. Cultures were pelleted by centrifugation at 5000 rpm (Eppendorf Centrifuge 5804 R) for 15 minutes. The supernatant was pH adjusted to 7.0 with 0.1 M HCL or NaOH, filter sterilized with 0.22 um sterile syringe filter, and aliquoted and stored at -20 C° until use. In the case of *E. coli* IA2 and *E. faecalis* ATCC 33186, overnight cultures were diluted 1:100 with fresh artificial urine, returned to incubation at 37 °C and sampled at 1, 2, 3, 4, 5 and 24 hours for testing. For the experiments involving the addition of supernatants from *L. crispatus* ATCC 33820 or *L. gasseri* KC-1 to that from uropathogens, the urothelial cells were first treated with *L. crispatus* ATCC 33820 or *L. gasseri* KC-1 supernatant for one minute, then the uropathogenic supernatant was added. In the case of serial dilution, *L. crispatus* ATCC 33820 supernatant was diluted for 6-fold to the *E. coli* IA2 supernatant.

For investigating the sub-therapeutic concentration of ciprofloxacin, the *L. crispatus* ATCC 33820 was grown in deMan, Rogosa, Sharpe media (MRS, Difco, MD). Growth curves for
these bacteria were generated using a plate reader (Eon Biotek, VT) at OD600 and 37°C to determine exponential phase.

2.2 Cell culture

Bladder epithelial cells (5637 –ATCC HTB-9) were maintained in RPMI 1640 (Roswell-Park Memorial Institute media – Thermo Fisher Scientific, MA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Ma.) and 2 mM L-glutamine (Thermo Fisher Scientific, MA.) at 37°C and 5% CO2. The media was changed every 48 hours or more regularly if the cells were confluent (90%-100%), after washing by 1X PBS and trypsinization by 0.25% Trypsin-EDTA (1X) (Gibco), with the ratio of 1 to 10. Primary myofibroblast cells were extracted from the palmar fascia during surgery from normal tissue. Primary cultures were maintained in DMEM with 10% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA, USA), 1% L-glutamine (Life Technologies) and 1% antibiotic–antimycotic solution (Life Technologies) at 37°C in 5% CO2. All primary cell lines were used up to a maximum of four passages, after which they were discarded.

2.3 RNA isolation and qPCR from cell lines

RNA was isolated from the samples (200 ng/μL) using the Ambion by Life Technologies Purelink™ RNA mini kit (Thermo Fisher Scientific, MA), following the manufacturer’s instructions. cDNA was made following the instructions on the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Thermo-Fisher Scientific, MA) and PCR was conducted using a Master Cycler gradient PCR thermal cycler (Eppendorf, NY). Using GAPDH as the housekeeping gene, qPCR was set up with each sample being run on the plate in triplicate for each
of the conditions. A list of the primer sequences used can be found in Table 1. Power SYBR Green PCR Master Mix was used (Thermo Fisher Scientific, MA).

2.4 Intracellular calcium influx measured by fluorescent microscopy

The influx of calcium was measured using the Fluo-4 DirectTM Calcium Assay kit (InvitrogenTM, CA). Samples and reagents were prepared according to the protocol manual provided. Ninety-six well plates were seeded with 100 µl of 5637 cells at 1x10^5 cells/mL in supplemented RPMI and allowed to reach confluency, which occurred at about 48-72 hours. Cells were counted by using the Invitrogen Countess Automated Cell Counter (Thermo Fisher Scientific, MA.) per the manufacturers’ instructions. Fifty microliters of cell culture media were removed from the initial 100 µl and 50 µl of Fluo-4 DirectTM calcium reagent was added to each well. The plate was incubated at 37° C for 30 minutes at room temperature while protected from light. Controls included ionomycin (0.5 µM, Sigma ≥98% HPLC), ATP (0.5 µM, Sigma A1852), GABA (0.5 uM, Sigma BioXtra ≥99%) and LPS from E. coli (65 microgram/mL, Sigma L3755). The effect of treatments was assessed using a Nikon epifluorescence Ts2R scope at 10x magnification at 494 nm for excitation and 516 nm for emission for 60 seconds. The image intensity was calculated using ImageJ and is indicative of Ca^{2+} influx into the urothelial cell’s cytoplasmic space from either the extracellular environment or intracellular Ca^{2+} stores (here on out just referred to as Ca^{2+} influx).
2.5 Quantification of ATP

A luminescent assay kit (BacTiter-Glo™ Microbial Cell Viability Assay, G8230) was used to quantify the amount of extracellular ATP released by the bacteria into the supernatant and released by the cells into the cell media. A microplate reader (Synergy™ H4 Hybrid Multi-Mode) was used to quantify the amount of extracellular ATP.

2.6 Myofibroblast populated collagen contraction

A collagen matrix was set up using 1.8 mg/ml sterile collagen and a neutralization solution\textsuperscript{156}. The neutralization solution was made by mixing with three parts Waymouth Media (Sigma, W1625) and 2 parts 0.34M NaOH (Sigma, 221465). One-part neutralization mixture was then added to 4 parts collagen, mixed with $1 \times 10^5$ cells to a final volume of 500 $\mu$l and added to each well in a 24 well plate. After 45-minute incubation at 37\textdegree{}C, 1 mL 2% FBS was added to each well and the plate was incubated for an additional 72 hours at 37 \textdegree{}C. The media was then removed, fresh media and treatment was added, and the collagen matrix was released using a sterile spatula. The plate was scanned using a scanner (Canon PIXMA MP250) immediately after release and also at 1, 3, 5 and 24 hours. The size of the collagen matrix was measured using ImageJ and the percent contraction was calculated. To decrease any shock to the myofibroblast, all bacterial strains were grown in DMEM with 2\% FBS.

2.7 Immunocytochemistry

Myofibroblast cells were cultured in an 8 well $\mu$-Slide (ibidi, 80826) to become fully confluent (90\%-100\%). Cell cultures were washed with 1X PBS and fixed with 1 ml 4\% paraformaldehyde per well for 10 minutes at room temperature, then 1 ml 0.1-0.2\% Triton X-100
in PBS to each slide was added. After blocking the cells for non-specific staining with Background Sniper (Biocare Medical, BS966), they were incubated for 8 to 10 minutes, washed then incubated overnight at 4°C in 1% BSA in PBS containing Monoclonal Anti-Actin, α-Smooth Muscle (Sigma, A2547) diluted 1:200. The cells were washed, excess liquid aspirated, and secondary antibody solution was added (1-10μg/ml) (Alexa Fluor 488 Donkey anti-mouse IgG secondary antibody, ThermoFisher, A-21202) in 1% BSA in PBS. The cells were protected from light and incubated for 45 minutes at room temperature. After washing with PBS, samples were incubated with 100 μL DAPI (Diluted 1:10 000 in PBS) for 5 minutes in the dark. Confocal images were obtained with a Nikon Eclipse Ti2 (X60 objective lens, Nikon, Canada). Fluorescence intensity measurements were obtained from entire cells and analyzed with Image J software. Control specimens were identical to experimental specimens except they were exposed to irrelevant isotype matched antibody.

2.8 Myofibroblast populated collagen RNA extraction and qPCR

After incubation and aspiration of media, the collagen matrix was collected in microcentrifuge tubes for high-speed centrifugation for 5 minutes and then the supernatant was discarded. An aliquot of 100 μL pre-warmed 0.25 mg/ml collagenase was added to each well and incubated for 15 minutes at 37°C. RNA was isolated from the samples using the Direct-zol RNA Miniprep Kit (Zymo Research) following the manufacturer’s instructions, and Trizol reagent was used to lyse the samples. The RNA concentration was measured using nanodrop. cDNA was made following the instructions on the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Thermo-Fisher Scientific, MA) and PCR was conducted using a MasterCycler gradient PCR thermal cycler (Eppendorf, NY). Quantitative PCR was set up with each sample being run on the
plate in triplicate for each of the conditions, as described earlier. *GAPDH* was also used as the housekeeping gene. A list of the primers used can be found in Table 1.

## 2.9 Statistical Analysis

The data are expressed as mean ± SEM. Statistical significance was assessed using one-way ANOVA followed by Tukey’s test (GraphPad Prism 5). Significance was determined when P < 0.05.
Chapter 3: RESULTS
3 Results

3.1 Ca\textsuperscript{2+} influx of uroepithelial cell by bacterial supernatants

All strains of bacteria for calcium assay were cultured in artificial urine. The supernatant of \textit{E. coli} IA2 was able to induce the influx of Ca\textsuperscript{2+} into uroepithelial cells [Fig 2A]. Unlike a previous report\textsuperscript{103}, LPS from \textit{E. coli} did not stimulate the influx of calcium [Fig 2A]. Supernatants from \textit{E. coli} IA2 and \textit{G. vaginalis} ATCC 14018 increased the level of Ca\textsuperscript{2+} influx before plateauing at the 4-hour time point. In contrast, \textit{E. faecalis} ATCC 33186 supernatants were not able to increase the level of Ca\textsuperscript{2+} influx until the 4-hour time point [Fig 2B, C and D].
C. 

**G. vaginalis** ATCC 14018 supernatant added (hour)

D. 

**E. faecalis** ATCC 33186 supernatant added (hour)
Fig 2: Effects of *E. coli* IA2, *G. vaginalis* ATCC 14018 and *E. faecalis* ATCC 33186 on the stimulation of Ca\(^{2+}\) influx in 5637 uroepithelial cells. Supernatant from an overnight culture was added to the 5637 cells (A), supernatant from cultures of either *E. coli* IA2 (B) *G. vaginalis* ATCC 17018 (C), or *E. faecalis* ATCC 33186 (D) were taken hourly at 1,2,3,4,5 and 24 hours post addition and tested for their ability to induce Ca\(^{2+}\) influx in the 5637 cells. Forty μL of each treatment in artificial urine was added and fluorescence was measured using a Nikon epifluorescence Ts2R scope at 10x magnification at 494 nm for excitation and 516 nm for emission for 60 seconds. Image intensity was calculated using ImageJ. Each bar represents the total average image intensity 60 seconds after treatment of a duplicate sample. Statistical significance was determined using Tukey’s test, \(p \leq 0.05\).
3.2 *Lactobacillus crispatus* ATCC 33820 and *Lactobacillus gasseri* ATCC KC-1 supernatants reduce Ca\(^{2+}\) influx caused by uropathogenic bacteria

All strains of bacteria for calcium assay were cultured in artificial urine. The addition of *L. crispatus* ATCC 33820 and *L. gasseri* KC-1 supernatants not only could not stimulate the influx of calcium like *E. coli* IA2, but also mitigated the effects of calcium influx caused by *E. coli* IA2 supernatant by up to 50% [Fig 3A, B, C and D].
C

Fluorescent Image Intensity

Serial Dilution

E. coli S / Artificial Urine
L. crispatus S / E. coli S

D

E. coli S
L. crispatus S
50% E. coli S / 50% L. crispatus S
Fig 3: Effect of *L. crispatus* ATCC 33820 and *L. gasseri* KC-1 supernatant on Ca$^{2+}$ influx caused by uropathogenic supernatant. Treatments were added as either *E. coli* IA2 supernatant and AU or *L. crispatus* ATCC 33820 supernatant and AU, or *E. coli* IA2 supernatant+ *L. crispatus* ATCC 33820 supernatant (A), or *E. coli* IA2 supernatant and AU or *L. gasseri* KC-1 supernatant and AU, or *E. coli* IA2 supernatant+ *L. gasseri* KC-1 supernatant (B), or a six-fold serial dilutions of the *L. crispatus* ATCC 33820 supernatant in the *E. coli* IA2 supernatant (C), image examples of calcium influx caused by *E. coli* IA2, *L. crispatus* ATCC 33820 and a mixture of supernatants from the two bacteria (D). Fluorescence was measured using a Nikon epifluorescence Ts2R scope at 10x magnification at 494 nm for excitation and 516 nm for emission for 60 seconds. Image intensity was calculated using ImageJ. Each point represents the average image intensity of a time point done in duplicate. Statistical significance was determined using Tukey’s test, p≤0.05.
3.3 Quantification of bacterial extracellular ATP

All strains of bacteria for ATP assay were cultured in artificial urine. In order to quantify the amount of extracellular ATP released in bacterial supernatants, a luminescent assay was used. Supernatants obtained from *E. coli* IA2, *L. crispatus* ATCC 33820 and *L. gasseri* KC-1 overnight cultures contained [0.098 ± 0.008 μM], [0.024 ± 0.003 μM] and [0.024 ± 0.001 μM ATP], respectively, which was higher than AU [0.0067 ± 0.0011 μM]. In addition, supernatants from organisms that comprise the urinary microbiota, including *G. vaginalis* ATCC 14018 and *L. vaginalis* NCFB 2810 contained [1.30 ± 0.14 μM] and [0.314 ± 0.023 μM] ATP, respectively, which was greater than the AU control of [0.0067 ± 0.0011 μM].
**Fig 4: Release of extracellular ATP by bacteria and cells.** *E. coli* 1A2, *L. crispatus* ATCC 33820, *L. gasseri* KC-1, *G. vaginalis* ATCC 14018 and *L. vaginalis* NCFB 2810 supernatants were collected after overnight culture. The plate was incubated for 5 minutes and was read by a luminometer. A standard curve was generated using known concentrations of ATP to calculate the amount of ATP in each treatment sample. Each bar represents the average concentration of ATP in μM. Statistical significance was determined using Tukey’s test, p≤0.05.
3.4 ATP utilization by *L. crispatus* ATCC 33820

The amount of ATP remaining when *L. crispatus* ATCC 33820 was grown in AU supplemented with 0.1 mM ATP for 24 hours was $[21.67 \pm 1.51 \mu M]$, less than half the control $[51.56 \pm 5.06 \mu M]$ (P≤0.0001) [Fig 5A]. To determine the main cause of ATP reduction, *L. crispatus* ATCC 33820 was cultured in AU supplemented with different concentrations of ATP, as well as in AU supplemented with 50% *E. coli* IA2 supernatant, and 25% *G. vaginalis* ATCC 14018 supernatant individually as potential natural sources of ATP. The growth of *L. crispatus* ATCC 33820 was increased by increasing the ATP concentration, including ATP emanating from the *E. coli* IA2 and *G. vaginalis* ATCC 14018 supernatants [Fig 5B, C and D]. The *L. crispatus* ATCC 33820 also reduced the amount of ATP after overnight culture in AU supplemented with 25% of *E. coli* IA2 supernatant, and 25% of *G. vaginalis* ATCC 14018 supernatant individually [Fig 5E and F]. Supplementing *E. coli* IA2 with ATP had a somewhat inhibitory effect on its growth [Fig 5G]. In the presence of ATP or supernatant from *G. vaginalis* ATCC 14018, the pH of *L. crispatus*, ATCC 33820 became further reduced [Fig 5H].
A

$L.\ crispatu$s

- AU (0.1 mM ATP, 24 h) without bacteria
- AU (0.1 mM ATP, 24 h) with bacteria
- Artificial Urine (AU)
**D**

*L. crispatus*

- *L. crispatus* in *G. vaginalis* Supernatant
- *L. crispatus* in Artificial Urine

![Graph](image-url)
Fig 5: ATP utilization by *L. crispatus* ATCC 33820. *L. crispatus* ATCC 33820 was grown in AU supplemented with 0.1 mM of ATP. After overnight culture, the amount of ATP was evaluated by a luminometer (A). Growth curves of overnight culture were performed by *L. crispatus* ATCC 33820 and *E. coli* IA2 in the presence of different concentrations of ATP in AU (B and G), as well as the growth curve of *L. crispatus* ATCC 33820 in AU supplemented by 50% of *E. coli* and 25% of *G. vaginalis* ATCC 14018 supernatants (C and D). The ability of *L. crispatus* ATCC 33820 to reduce the amount of ATP in AU supplemented with 25% of *E. coli* IA2 supernatant, and 25% *G. vaginalis* ATCC 14018 supernatant individually, was also examined (E and F). The ability of 25% of *G. vaginalis* ATCC 14018 to induce *L. crispatus* ATCC 33820 to reduce pH was also examined (H). Each bar represents the average concentration of ATP in μM. Statistical significance was determined using Tukey’s test, p≤0.05.
3.5 The effect of sub-therapeutic concentrations of ciprofloxacin on the ability of *E. coli* IA2 to release ATP

After culturing bacteria with different concentrations of ciprofloxacin from 10 μg/mL to 0.031 μg/mL, the minimum inhibitory concentration (MIC) of the antibiotic against *E. coli* IA2 was found to be between 1 to 1.5 μg/mL. [Fig 6A]. Using a concentration below the MIC, such as 0.25, 0.125 or 0.0625 μg/mL caused *E. coli* IA2 to release more ATP up to 0.0247 ± 0.0015 μM [Fig 5B].
Fig 6: The effects of sub-therapeutic concentration of ciprofloxacin on *E. coli* IA2 to release ATP. Growth curves of an overnight culture of *E. coli* IA2 in the presence of different concentrations of ciprofloxacin in MRS were performed in order to determine the minimum inhibitory concentration of ciprofloxacin as well as subtherapeutic concentrations (A). *E. coli* IA2 supernatants were collected after overnight culture in MRS which supplemented with sub-therapeutic concentrations of ciprofloxacin. The plate was incubated for 5 minutes and was read by a luminometer (B). Each bar represents the average concentration of ATP in μM. Statistical significance was determined using Tukey’s test, p≤0.05.
3.6 Detection of ATP released by Urothelial cells

The urothelial cell media contained 0.0042 μM ATP, and after treatment with 0.009 μM ATP for 2 minutes, the ATP released by the urothelial cells increased to 9.237 ± 0.172 μM [Fig 7].
**Fig 7: Urothelial cells release ATP.** One hundred μl of RPMI was supplemented with 0.009 μM ATP and incubated for two minutes to analyze the amount of ATP in the medium after incubation. One hundred μl media from 1x10^6 urothelial cell were also collected and incubated for two minutes to analyze the amount of ATP in the cell media. 1 x 10^6 urothelial cells were treated with 0.009 μM ATP and incubated for 2 minutes. Then, the whole cell medium (100 μl) that was treated with 0.009 μM ATP was removed to analyze the amount of ATP released by the urothelial cells after incubation. Each bar represents the average concentration of ATP in μM. Statistical significance was determined using Tukey’s test, p≤0.05.
3.7 Expression of *MAOA* and *MAOB* in the 5637 cells exposed to bacterial supernatants

All strains of bacteria for the qPCR were cultured in RPMI 10% FBS. As ATP is capable of increasing the level of intracellular calcium, the influx of calcium can potentially cause mitochondrial dysfunction. Gene expression for mitochondrial enzymes, monoamine oxidase A and B was measured because of their potential ability to degrade neurotransmitters such as serotonin. The *E. coli* IA2 supernatant [0.78-fold change] caused downregulation in the level of *MAOA* gene expression, while *L. crispatus* ATCC 33820 supernatant [1.21-fold change] caused upregulation in the level of *MAOA* gene expression [Fig 8A]. The *E. coli* IA2 had no effect on *MAOB* gene expression, whereas, *L. crispatus* ATCC 33820 upregulated its expression by 44-fold [44.54-fold change] [Fig 8B].
A

MAO-A gene expression

- 15% *E. coli* S + 85% RPMI
- 15% *L. crispatus* S + 85% RPMI
- RPMI 10% FBS

Fold Change in Gene Expression Relative to GAPDH

5637 Treatment
Fig 8: Fold change of transcript expression of monoamine oxidases. Supernatants from both pure cultures of *E. coli* IA2 and *L. crispatus* ATCC 33820 were added to 5637 cell cultures for 3 hours. Expression of the genes encoding monamine oxidases (*MAOA*/ *MAOB*) were measured by quantitative PCR relative to *GAPDH* as a house keeping gene expression (A and B). Statistical significance was determined using Tukey’s test, *p*≤0.05. N=2.
3.8 The effect of GABA and the GABA receptor on Ca^{2+} influx caused by ATP and bacterial supernatant

The neurotransmitter γ-aminobutyric acid (GABA) was found to reduce the stimulation of calcium influx caused by ATP [Fig 9A] and inhibit the stimulation of calcium influx caused by *E. coli* IA2 supernatant [Fig 9B]. Inhibition of the GABA receptor by the GABA blocker reduced the ability of GABA to inhibit the stimulation of Ca^{2+} influx caused by ATP [Fig 9C].
**A**

Fluorescent Image Intensity

**B**

Fluorescent Image Intensity

---

**A**

- **0.5 uM ATP in Artificial Urine**
- **0.5 uM GABA in Artificial Urine**
- **0.5 uM ATP + 0.5 uM GABA in Artificial Urine**

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**B**

- **E. coli IA2 Supernatant**
- **0.5 uM GABA in Artificial Urine**
- **0.5 uM GABA + E. coli IA2 Supernatant**
C

Fluorescent Image Intensity

Cell Treatment

- 40 uM ATP in Artificial Urine
- 40 uM ATP + 40 uM GABA in Artificial Urine
- 100 uM GABA Blocker + (40 uM ATP + 40 uM GABA)
Fig 9: Effect of GABA and GABA receptor on Ca^{2+} influx in 5637 cells caused by ATP and *E. coli* IA2. To evaluate the ability of GABA to inhibit the stimulation of Ca^{2+} influx caused by ATP, AU containing 0.5 μM GABA was mixed with 0.5 μM ATP in AU (A). Similarly, to test the ability of GABA to reduce the stimulation of calcium influx caused by bacterial supernatant, GABA was mixed with *E. coli* IA2 supernatant (B). In addition, to evaluate the ability of GABA blocker to reduce the ability of GABA to inhibit the stimulation of calcium caused by ATP, urothelial cells were treated with 100 μM GABA blocker for 1 minute and then a mixture of ATP and GABA was added (C). Fluorescence was measured using a Nikon epifluorescence Ts2R scope at 10x magnification at 494 nm for excitation and 516 nm for emission for 60 seconds. Image intensity was calculated using ImageJ. Each point represents the average image intensity of a time point done in duplicate. Statistical significance was determined using Tukey’s test, p≤0.05.
3.9 The role of GABA receptors in neutralizing ionic imbalances within the cell

The GABA receptors facilitate the passing of anions such as chloride (Cl\(^{-}\)) to the interior of the cell, resulting in a decreased intracellular voltage and establishing an ionic balance. Blocking of the GABA receptors still allows the calcium channels to continue to function without restriction. It can be seen that the influx of calcium continues even after blocking the GABA receptor [Fig 10]. But, by blocking the purinergic and muscarinic receptors, the influx of Ca\(^{2+}\) has not changed. [Fig 10].
Fig 10: The role of GABA receptor in the balance between anion and cation in the cell. To evaluate the role of GABA, purinergic and muscarinic receptors in the Ca\(^{2+}\) influx, the urothelial cells were treated with 100 \(\mu\)M GABA blocker, 5 \(\mu\)M purinergic blocker and 5 \(\mu\)M muscarinic blocker. Fluorescence was measured using a Nikon epifluorescence Ts2R scope at 10x magnification at 494 nm for excitation and 516 nm for emission for 60 seconds. Image intensity was calculated using ImageJ. Each point represents the average image intensity of a time point done in duplicate. Statistical significance was determined using Tukey’s test, \(p \leq 0.05\).
3.10 The role of purinergic and muscarinic receptors to stimulate the influx of calcium

The stimulation of calcium influx caused by ATP after blocking the purinergic receptors decreased as expected [Fig 11 A]. However, while the calcium influx was reduced, it did not stop [Fig 11 A]. Ca\textsuperscript{2+} influx caused by ATP after blocking of muscarinic receptor was not diminished, indicating that the muscarinic receptor has no role in calcium transfer [Fig 11 B].
A

Fluorescent Image Intensity

Cell Treatment

40 uM ATP in Artificial Urine
5 uM Purinergic Blocker in Artificial Urine
5 uM Purinergic Blocker + 40 uM ATP

B

Fluorescent Image Intensity

Cell Treatment

40 uM ATP in Artificial Urine
5 uM Muscarinic Blocker in Artificial Urine
5 uM Muscarinic Blocker + 40 uM ATP

5637

ns

5637
Fig 11: The role of purinergic or muscarinic receptors on the influx of calcium.

To evaluate the role of purinergic, muscarinic receptor to stimulate the influx of calcium, the urothelial cells were treated with: 5 μM purinergic blocker for one minute, and then 40 μM ATP was added (A) and also 5 μM muscarinic blocker for one minute, and then 40 μM ATP was added (B). Fluorescence was measured using a Nikon epifluorescence Ts2R scope at 10x magnification at 494 nm for excitation and 516 nm for emission for 60 seconds. Image intensity was calculated using ImageJ. Each point represents the average image intensity of a time point done in duplicate. Statistical significance was determined using Tukey’s test, p≤0.05.
3.11 Myofibroblast contraction assay

All strains of bacteria for the collagen contraction assay were cultured in DMEM 2% FBS. The collagen contraction assay using primary myofibroblast cells seeded on top of a collagen matrix was tested against bacterial products. Supernatants from cultures of *E. coli* IA2 were able to induce the greatest degree of contraction [72.67% ± 0.87] in the myofibroblast cell line after 24 hours, and this was reduced when *L. crispatus* ATCC 33820 or *L. gasseri* KC-1 supernatants were added [48.56% ± 1.68, 29.82% ± 0.023, respectively] [Fig 12A, B and C]. Purified ATP caused the contraction of myofibroblasts in the first hour [30.30% ± 3.25] and continued for 24 hours [60.73 % ± 1.49] [Fig 12A and D]. While, GABA did not cause contraction in the myofibroblast assay, it inhibited contraction caused by *E. coli* IA2 [Fig 12A and E].

Previous reports suggest that the contraction caused by *E. coli* IA2 was due to LPS. However, after five hours of exposure of LPS to the myofibroblasts, contraction was half that induced by ATP [27.26% ± 1.05 versus 46.19 ± 1.78%] [Fig 12A and F].
A

E. coli

E. faecalis

L. crispatus

L. gasseri

GABA

ATP

LPS

E. coli/L. crispatus

E. coli/L. gasseri

E. coli/GABA

DMEM 2% FBS
Fig 12: Contraction of myofibroblast populated collagen matrix by bacterial supernatant, neurotransmitters and LPS. Bacterial supernatants from *E. coli* IA2, *L. crispatus* ATCC 33820 and *L. gasseri* KC-1 were added to a myofibroblast populated collagen matrix. In addition, GABA, ATP and LPS were included as controls (B-F). The final volume in each well was 500 uL and the collagen matrix was scanned at T= 1,3,5 and 24h and % contraction was determined using ImageJ. Each point represents the average % contraction of a duplicate sample.
3.12 Immunocytochemistry for intracellular alpha smooth muscle actin (α-SMA)

To further confirm that bacterial compounds impact myofibroblast contractive abilities, the effect on alpha smooth muscle actin was assessed. The *E. coli* IA2 supernatant did not increase the expression of the *ACTA2* [0.9798759-fold change] [Fig 13C]. *L. crispatus* ATCC 33820 also downregulated the level of *ACTA2* gene expression [0.5946411-fold change] [Fig 13C]. Thus, the ability of *E. coli* IA2 to increase the intracellular image intensity [Fig 13A and B] could potentially be based on alpha smooth muscle cells contraction, and the ability of *L. crispatus* ATCC 33820 to reduce the intracellular image intensity [Fig 13A and B] could potentially be based on alpha smooth muscle cells relaxation.
B. Myofibroblast

Fluorescent Image Intensity

Cell Treatment

15% *E. coli* S + 85% DMEM

15% *L. crispatus* S + 85% DMEM

DMEM 2% FBS

C. ACTA2 gene expression

Fold Change in Gene Expression Relative to beta-actin

Myofibroblast Treatment

15% *E. coli* S + 85% DMEM

15% *L. crispatus* S + 85% DMEM

15% *E. coli* S + 15% *L. crispatus* S + 70% DMEM

DMEM 2% FBS
**Fig 13: Confocal fluorescence microscopy analysis.** Bacterial supernatants from *E. coli* IA2, *L. crispatus* ATCC 33820 and in combination were co-cultured with myofibroblasts for 1 hour (A and B). The image intensity was measured by confocal microscopy with DAPI and FITC to show staining of alpha-smooth muscle actin. Bacteria supernatants identical to A, were added to the myofibroblasts that were grown in the collagen matrix and then incubated at 37°C with 5% CO₂ for 3 hours and tested for gene expression of alpha-smooth muscle actin (*ACTA2*) (C). Image intensity was calculated using ImageJ. Each point represents the average image intensity of three cells. Statistical significance was determined using Tukey’s test, \( p \leq 0.05 \). Expression of the gene encoding *ACTA2* was measured by quantitative PCR relative to beta-actin as a housekeeping gene expression. Statistical significance was determined using Tukey’s test, \( p \leq 0.05 \). N=2.
3.13 Evaluation of the bacterial supernatants to alter the tumor necrosis factor (TNF) gene expression in the myofibroblast assay

All strains of bacteria for the qPCR were cultured in DMEM 2% FBS. To determine if sustained activation of the calcium channel promoted apoptosis by bacterial components, TNF was measured as an indicator. The *E. coli* IA2 caused more than 700-fold upregulation \[714 \pm 19.91\] of TNF [Fig 13], whereas, exposure to *L. crispatus* ATCC 33820 only resulted in three-fold increase \[3.9 \pm 0.115\]. When *E. coli* IA2 and *L. crispatus* ATCC 33820 supernatants were mixed and applied to the assay, the expression of TNF induced by the *E. coli* IA2 was strongly mitigated \[52.38 \pm 3.98\] [Fig 13].
Fig 14: *Lactobacillus crispatus* ATCC 33820 can mitigate the expression of TNF caused by *E. coli* IA2. Bacterial supernatants, identical to the recipe of Fig 12, were added to the myofibroblasts that were grown in the collagen matrix and then incubated at 37 °C with 5% CO2 for 3 hours. Expression of the gene encoding TNF was measured by quantitative PCR relative to beta-Actin as a house keeping gene expression. Statistical significance was determined using Tukey’s test, p≤0.05. N=2.
Chapter 4: Discussion
4 Discussion

This study has shown that bacteria known to be present in the urinary microbiota may have the potential to influence bladder contractility. Evidence was acquired from uroepithelial and myofibroblast cell models. Uropathogenic bacteria by releasing extracellular excitatory compounds such as ATP induces Ca\(^{2+}\) influx. This along with release of ATP could potentially stimulate bladder contraction.

Prior to testing bacterial supernatants, the 5637 uroepithelial cells were shown to be capable of responding to agonists known to induce Ca\(^{2+}\) influx. Ionomycin, as well as ATP, were all able to induce Ca\(^{2+}\) influx as previously described in the literature\(^{104,105}\). Unsurprisingly, GABA as an inhibitory neurotransmitter was unable to stimulate Ca\(^{2+}\) influx\(^{106}\).

To evaluate the ability of bacteria to induce Ca\(^{2+}\) influx, it is important to use a cell culture medium in which all bacterial strains can grow well but cannot stimulate the influx of calcium. Interestingly, artificial urine did not stimulate calcium influx, and all strains of bacteria used in this study grew in this medium.

In a healthy person consuming regular fluids, the bladder empties urine at regular intervals. The period between voiding is shortened and uncontrolled in UUI patients. Thus, for uropathogenic bacteria to have a role, they must release compounds in the timeframe between voiding.

Supernatants from \textit{E. coli} IA2, \textit{G. vaginalis} ATCC 14018, and \textit{E. faecalis} ATCC 33186 cultures caused Ca\(^{2+}\) influx after 24 hours, but only \textit{E. coli} IA2 and \textit{G. vaginalis} ATCC 14018 consistently increased this influx and did so at the beginning of their log growth phase. The failure of \textit{E. faecalis} ATCC 33186 to cause the influx of calcium within the timeframe between voidings in UUI patients suggests it may play less of a role in this disease. This correlates with \textit{E. faecalis}
ATCC 33186 being associated more with ABU than symptomatic UTI\textsuperscript{107,108}. Since ABU-causing bacteria have longer doubling times and weaker adherence\textsuperscript{109-112}, the act of involuntary voiding thus prevents them from having sufficient time to produce agonists for receptors or channels responsible for Ca\textsuperscript{2+} transport.

Nevertheless, \textit{E. faecalis} ATCC 33186 and \textit{E. coli} IA2 released ATP into their extracellular environment, as noted by others\textsuperscript{113,114}. This may be one of the potential agonists for causing Ca\textsuperscript{2+} influx in the cells as demonstrated with in Figure 8A.

The results pertaining to \textit{G. vaginalis} ATCC 14018 were of interest because this organism is known to be associated with BV, and patients with this condition often report the urge to void urine. This species can ascend to the bladder through the urethra\textsuperscript{115,116}, and forms part of the urinary microbiota. Its high capacity to release ATP (1.30 ± 0.14 μM), may be sufficient for uroepithelial cells to release more ATP in the sub-urethral space, but in context of mitochondrial dysfunction could potentially be responsible for cellular apoptosis.

In contrast, \textit{L. crispatus} ATCC 33820 and \textit{L. gasseri} KC-1 were unable to release ATP to the same extent as \textit{E. coli} IA2, and interestingly both reduced the influx of Ca\textsuperscript{2+} caused by \textit{E. coli} IA2. In addition, serial dilution experiments indicate that small amounts of \textit{L. crispatus} ATCC 33820 compounds could cause this effect.

The attributes of \textit{L. crispatus} ATCC 33820 and \textit{L. gasseri} KC-1 are not associated with all lactobacilli. For example, \textit{L. vaginalis} NCFB 2810 is commonly found in the oral, vaginal and intestinal regions\textsuperscript{117} and it has been shown to release 0.314 ± 0.023 μM ATP, which is three times more than \textit{E. coli} IA2, and \textit{L. crispatus} ATCC 33820 and \textit{L. gasseri} KC-1 (0.024 ± 0.003 μM and 0.024 ± 0.001 μM ATP respectively) are significantly less than \textit{L. vaginalis} NCFB 2810.
Although the actual mechanism for a decrease in Ca\(^{2+}\) influx is unknown, it might be that *L. crispatus* ATCC 33820 and *L. gasseri* KC-1 release inhibitory neurotransmitters or antagonist compounds. One possible compound is GABA, with several *Lactobacillus* able to produce it using glutamate decarboxylase\(^{118}\). Three mechanisms are proposed to explain the lactobacilli countering *E. coli*-induced bladder contractility.

The first is based on the ability of the GABA receptor, glycine receptor and VRAC to control chloride influx. Many receptors like purinergic and TRP are expressed in urothelial cell, sub-urethral tissue and smooth muscle cells, and are sensitive to ATP and stimulation may cause calcium influx. By increasing the level of intracellular calcium, the intracellular voltage is increased and leads the cell to a depolarization phase, essential for cell function. This drives the cell to release ATP into the sub-urethral space and change bladder contractility. However, increasing the level of intracellular calcium can regulate many important mechanisms in the cell, though continuous activation of calcium channels leading to urothelial apoptosis. Therefore, urothelial cells need the inhibitory mechanisms to protect against abnormally high calcium levels. These mechanisms could potentially be based on the function of chloride channels. Many receptors like GABA, glycine and VRAC have the function of chloride channel. The stimulation of these receptors by chloride influx can decrease intracellular voltage and provide an equilibrium between intracellular cation and anion levels, leading to an inhibitory hyperpolarization phase [Figure 15].
Fig 15: How ATP released from uropathogenic bacteria induces urothelial cells to release more ATP into the sub-urethral. In UTI, ATP released by *E. coli* binds to TRP channels and purinergic receptors and causes calcium influx (1). Increasing the level of intracellular calcium is forced urothelial cells into ATP exocytosis. Some part of ATP in the sub-urethral binds to the receptors of the afferent sympathetic pelvic nerve and sends signals to the brain (2). On the other hand, the brain sends signals to the bladder through the parasympathetic efferent pelvic nerve releases Ach and ATP into the sub-urethral space. These excitatory neurotransmitters bind to corresponding receptors of smooth muscle cells and cause contraction (3). The other part of ATP can directly stimulate the corresponding receptors on smooth muscle cells and cause contraction. But, in the normal bladder, commensal GABA-releasing bacteria stimulate chloride channels that, by reducing intracellular voltage, drive urothelial cells into a hyperpolarization phase and regulate bladder contractility (4).
The experiments showed that GABA can reduce ATP-mediated calcium stimulation. But, when the GABA receptor is blocked by GABA receptor antagonist, it cannot reduce the influx of Ca\textsuperscript{2+} caused by ATP. This shows the importance of the GABA receptor in balancing anion and cation levels within the cells through chloride import. When the GABA receptor is blocked, one of the chloride channels is disrupted and the Ca\textsuperscript{2+} channels continue to import with lower inhibitory pressure. Notably, when urothelial cells were treated with purinergic and muscarinic blockers, there was no apparent stimulation of Ca\textsuperscript{2+} influx.

Another interesting result obtained pertaining to purinergic and muscarinic receptors demonstrated that they can import calcium. Due to the role of purinergic receptors in the Ca\textsuperscript{2+} pathway, the stimulation of calcium influx caused by ATP decreased following treatment with a purinergic. These results indicate that the muscarinic receptor has no role in passing Ca\textsuperscript{2+} from outside to inside urothelial cells.

One of the downstream effects of Ca\textsuperscript{2+} influx in cells is ATP release into the extracellular environment, including the sub-urethral space\textsuperscript{120}. In the urothelium, extracellular ATP serves as a key signaling molecule to tell the brain that the bladder is becoming full, which may lead to contraction of the detrusor bladder muscles\textsuperscript{120,121}.

As mentioned previously, the bladder uroepithelium is a barrier which acts as a sensor to inhibit the voiding reflux. Under certain conditions, it can release ATP via the connexin hemichannels, pannexin and several anion channels\textsuperscript{122}. The vesicular nucleotide transporter (VNUT) which can be expressed in uroepithelial cells is responsible for the ATP exocytosis. It is possible that stimulation of calcium influx in urothelial cells may cause increased expression of VNUT, with release of ATP into the sub-urethral space thereby affecting bladder contractility.
The second mechanism to consider is the role of *L. crispatus* ATCC 33820 releasing compounds that act as calcium channel antagonists by binding to the receptor site and blocking ATP binding. One such compound could be GABA. After blocking the GABA receptor, the ability of GABA to inhibit ATP-mediated calcium stimulation was diminished, but not eradicated. Even with GABA not bound to its receptor, it still reduced the stimulation of calcium caused by ATP, perhaps by acting as an antagonistic compound or calcium channel blocker.

The third mechanism is the ability of *L. crispatus* ATCC 33820 to utilize the excess ATP released by urogenital pathogens during UTI. This result indicates there is a possibility that *L. crispatus* ATCC 33820 can degrade the excess ATP. To investigate this, *L. crispatus* ATCC 33820 was cultured in artificial urine supplemented with various concentrations of ATP, as well as 50% of *E. coli* IA2, and 25% *G. vaginalis* ATCC 14018 supernatants. The results show that ATP and the pathogenic supernatants induce *L. crispatus* ATCC 33820 to grow further, and after overnight culture, *L. crispatus* ATCC 33820 reduced the amount of ATP in the bacterial supernatants.

In contrast, the culture of *E. coli* IA2 with different concentrations of ATP functioned as an inhibitory compound to reduce bacterial growth. In the presence of ATP or supernatant from *G. vaginalis* ATCC 14018, the pH of *L. crispatus* ATCC 33820 dropped even further. It appears that ATP increases *L. crispatus* ATCC 33820 metabolism, resulting in additional growth and subsequently increased lactic acid production.

In patients with UTI, antibiotics reduce the number of bacteria in the lumen, but those embedded in the uroepithelial cells or tissues, may resist the sub-therapeutic concentrations that reach them\(^{123}\). It is feasible that the subtherapeutic concentration of antibiotics could induce bacteria to release more ATP\(^{124}\), thereby worsening neurological feedback and increasing bladder contractions. Using sub-therapeutic concentrations of ciprofloxacin for *E. coli* IA2 (0.25, 0.125
and 0.0625 μg/mL), more ATP was released into the media. Sub-therapeutic concentrations of ciprofloxacin can potentially stimulate hydrogen channels and cause hydrogen influx. By passing the H⁺ of the hydrogen channel, sufficient energy is generated for the production of ATP from adenosine diphosphate (ADP). By increasing the level of ATP in the bacterial cell, ATP can be released through efflux pumps125.

Experiments showed that *E. coli* IA2 supernatant can affect MAOA and MAOB gene expression that can degrade biogenic amine neuroactive chemicals. Reducing the level of these enzymes can worsen the neurological disorders. The downregulation of MAOA expression, potentially based on the bacterial ATP release, increases the intracellular calcium causing enzymatic dysfunction of the mitochondria29. In contrast, *L. crispatus* ATCC 33820 upregulated MAOA/B expression, possibly based on the ability of these bacteria to release inhibitory compounds to establish an equilibrium between anions and cations, resulting in normal mitochondrial. Monoamine oxidase enzymes, MAOA and MAOB are found on the outer membrane of the mitochondria in eukaryotic cells and are critical for the breakdown of biogenic amine neuroactive chemicals such as tyramine, serotonin and dopamine. Decreased MAO enzyme activity has been linked to neurological disorder126.

Continuous activation of calcium channels and related receptors may result in abnormally high levels of calcium influx and lead the cell to its apoptotic phase. Apoptotic cells first undergo surface blebbing, then break into many membrane-bound apoptotic bodies121. Increased intracellular calcium can cause calcium overload in the mitochondria, leading to mitochondrial dysfunction. Under certain conditions, the mitochondrial permeability transition (MPT), a protein that forms in the inner membrane, allows the mitochondria to pass molecules smaller than 1500
daltons. Since ATP has a molecular weight of 551.2 daltons, it can cross the mitochondrial membrane into the cytosol, causing a depletion in mitochondrial ATP\textsuperscript{127}.

Serotonin, a substrate of these MAO enzymes, has been shown to induce contraction of the human detrusor muscle. Therefore, if serotonin is not being degraded as efficiently, it could stimulate the cholinergic neurons that innervate the detrusor and cause contraction\textsuperscript{128}. *Lactobacillus crispatus* ATCC 33820 increased production of MAO enzymes and, by breaking down certain neurotransmitters, could potentially reduce bladder contractility.

Fibroblasts play key roles during growth and healing after injury. They invade the wound and create a mature wound matrix. During the granulation phase, fibroblasts begin to obtain a new phenotype with an important contractile structure, expressing contractile proteins like alpha-SMA which are typical of SMA, particularly vascular smooth muscle cells\textsuperscript{129}. This provides support for use of myofibroblasts to examine contractility.

In fibroblasts, TNF induces apoptosis with the binding to TNF receptor-1 activating proteins associated with this receptor, leading to activation of the apoptotic cascade\textsuperscript{130-132}. Since bacterial compounds can pass through compromised uroepithelium and cross the sub-urethral space to reach smooth muscle cells, investigating the effects of bacterial compounds on TNF expression is important. The protective role of *L. crispatus* ATCC 33820 in reducing the expression of TNF induced by *E. coli* IA2 is worth pursuing, as is the ability of *L. crispatus* ATCC 33820 to release a calcium channel blocker.

An interesting finding with the collagen gel contraction assay is the ability of *L. crispatus* ATCC 33820 and *L. gasseri* KC-1 to not only cause contraction in myofibroblasts, but also to inhibit the contraction caused by compounds released by *E. coli* IA2. While the mechanism is unknown, it is proposed to involve the release of neuroactive molecules by the bacteria— ATP,
Ach as well as other stimulatory neurotransmitter in the case of uropathogens, and inhibitory neurotransmitters such as GABA and glycine in the case of *L. crispatus* ATCC 33820 and *L. gasseri* KC-1.

An important question that should be answered is: what is the main reason for myofibroblast contraction? The contraction of the collagen matrix has two main explanations, myofibroblast contraction and myofibroblast motion. By releasing an excitatory compound like ATP, *E. coli* IA2 can stimulate receptors like purinergic and TRP as calcium channels and cause the influx of calcium. By increasing the amount of intracellular calcium through the mechanism below, contraction of myofibroblasts and smooth muscle cells can be induced.

Actin and myosin interact according to sliding filament theory. The final trigger for contractions is a rise in intracellular Ca\(^{2+}\), which interacts with calmodulin and myosin light chain kinase to activate myosin. Calcium binds to calmodulin, activates it, then activates the kinase enzyme causing transfer of a phosphate group from ATP to myosin cross-bridges. Phosphorylated cross-bridges interact with actin to produce shortening, and therefore cellular contraction\(^{133}\).

Alpha-SMA in addition to being a marker of myofibroblast differentiation has been suggested to play a role in the production of contractile force during wound healing and fibro-constrictive diseases\(^{134}\). Here, a direct correlation was found between the contraction of α-SMA and the contraction of collagen gel caused by urogenital pathogens. There was also a direct correlation between α-SMA relaxation and relaxation of the collagen caused by *L. crispatus* ATCC 33820. Confocal microscopy showed that *E. coli* IA2 caused an increase in intracellular light intensity related to α-SMA. This has two possible explanations, an increase in the amount of intracellular α-SMA or the contraction of α-SMA. Studies using qPCR showed that *E. coli* IA2 compounds had no effect on *ACTA2* gene expression, suggesting that the image intensity could be
related to α-SMA contraction. On the other hand, since *L. crispatus* ATCC 33820 caused downregulation on ACTA2 expression, the ability of *L. crispatus* ATCC 33820 to reduce the α-SMA image intensity could be based on the ability of *L. crispatus* ATCC 33820 to downregulate ACTA2 gene expression.

**Conclusion**

These studies report the ability of urogenital pathogens to stimulate pathways associated with unintentional bladder contraction and UUI in an *in vitro* model, and the ability of indigenous members of the urinary microbiota, *L. crispatus* ATCC 33820 and *L. gasseri* KC-1, to mitigate the effects. These findings have implications for the approach to how UUI patients are managed, and potentially for the design of interventions that propagate lactobacilli in the bladder.
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References


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### Table 1: Primers

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### Abbreviations:

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<tr>
<td>UUI</td>
<td>Urgency Urinary Incontinence</td>
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<td>OAB</td>
<td>Overactive Bladder Syndrome</td>
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<td>UTIs</td>
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<td>ABU</td>
<td>Asymptomatic bacteriuria</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>SMC</td>
<td>Smooth Muscle Cell</td>
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<td>α-SMA</td>
<td>Alpha-Smooth Muscle actin</td>
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<td>ADP</td>
<td>Adenosine diphosphate</td>
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<td>GABA</td>
<td>Gamma Aminobutyric Acid</td>
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<td>Ach</td>
<td>Acetylcholine</td>
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<td>TRP</td>
<td>Transient receptor potential</td>
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<td>Purinergic</td>
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<td>Monoamine Oxidase</td>
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<td>GRIN1</td>
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<td>VRACs</td>
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<td>Ca^{2+}</td>
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CURRICULUM VITAE
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▪ A clinical trial to determine the extent to which probiotic therapy reduces the side effects of antibiotic prophylaxis therapy in pediatric patients with a history of recurrent urinary tract infections.

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1. Abbasian B. Potential role of extracellular ATP released by bacteria in bladder infection and contractility. Graduate Seminars, Department of Microbiology and Immunology, Schulich School of Medicine and Dentistry, Western University. January 14th, 2019. London, ON, Canada.

2. Abbasian B. The Role of Bacteria in Urgency Urinary Incontinence. Graduate Seminars, Department of Microbiology and Immunology, Schulich School of Medicine and Dentistry, Western University. April 16th, 2018. London, ON, Canada.


