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Metabolic origins of urogenital malodour in women and their relationship with their microbiota: can probiotics help?

Scarlett Puebla Barragan, *The University of Western Ontario*

Supervisor: Reid, Gregor, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Microbiology and Immunology

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Abstract

The objectives of this thesis were to characterize the biochemical origins of malodour in the female bladder, elucidate how vaginal *Lactobacillus* species interact with malodorous compounds and assess the potential to deliver probiotic strains topically.

Metabolomic tools were used to explore differences between the urine of healthy women and those positive for *Escherichia coli* urinary tract infection (UTI). Positive samples had increased concentrations of the fishy-smelling biogenic amine (BA) trimethylamine (TMA). The BAs cadaverine, putrescine, and tyramine, causatives of malodour in bacterial vaginosis (BV) were also quantified. Putrescine was elevated in BV samples. *In vitro*, the capacity of five uropathogenic *E. coli* strains to produce the four BAs of interest was tested. When grown in human urine, *E. coli* was shown to produce putrescine and TMA, and also biosynthesize cadaverine which, might be produced under specific conditions in some patients. This confirmed that there is an overlap in the malodorous compounds present in the urogenital tract of women with BV and UTI, two highly prevalent conditions driven by dysbiosis.

The same metabolomic approaches were used to characterize clinical strains of *Lactobacillus crispatus*, an abundant species in the healthy vagina. There were no differences in terms of BA profiles between those isolated from *Lactobacillus* dominated microbiotas and those from a dysbiotic vagina. The latter had higher inhibitory activity towards common uropathogens, potentially due to metabolic adaptation.

The amine-degradation capacity of *L. crispatus* was further characterized and previous exposure to BAs led to higher tolerance. Tests with cell-free extracts revealed that bacterial metabolites alone can reduce the amount BAs. Some strains were able to degrade BAs, while others were found produce them.

In order to assess the feasibility that malodour-reducing lactobacilli could be placed in a topical preparation for delivery to the perineal and vulval skin, four different oils were assessed. Coconut oil and petroleum jelly emerged as the best candidates for retention of viability.

It is our hope that these findings will help to develop probiotic-based products that not only restore homeostasis, but also treat the malodour that impairs a woman's quality of life.

Keywords

Probiotics, *Lactobacillus*, metabolomics, dysbiosis, urinary tract infections, bacterial vaginosis, putrescine, cadaverine, tyramine, trimethylamine, biogenic amines.

Summary for Lay Audience

Yearly, millions of women seek medical advice due to vaginal and urinary foul-smell. The most common causes are bacterial vaginosis (BV) and urinary tract infection (UTI). Antibiotics are prescribed but can take several days to provide relief from the odour. This is a major burden for patients and takes a toll on their life-quality.

A healthy vagina has its own collection of microbes with the most abundant being lactobacilli. They use the nutrients within the vagina and, in exchange, provide protection to the host primarily by not allowing the establishment of harmful microbes. When lactobacilli reserves are reduced there are increased chances of infection.

Some probiotics ('live bacteria that, when taken in adequate amounts, confer a health benefit on the host'), such as *Lactocaseibacillus rhamnosus* GR-1 and *Limosilactobacillus reuteri* RC-14, are used to help maintain equilibrium within the vagina, thus, preventing recurrence of infection. We propose that certain probiotics could be used, alone or in combination with antibiotics, to target malodour, thereby providing relief sooner. However, not all probiotics are the same and thorough screening must be carried out to select ones with specific malodour reducing characteristics.

Here, we investigated compounds known as biogenic amines, which are the culprits of fishy vaginal and urinary smell. We found that patients with UTI have large quantities of these chemicals in their urine. We also identified that certain lactobacilli that are common in the vagina can degrade these compounds.

We suggest the direct application of probiotics through a cream or ointment, such as petroleum jelly or coconut oil, could allow women to self-manage malodour.

Overall, the findings compiled in this thesis provide a basis for the probiotic industry to develop products that can combat a major reason for women to seek medical care.

Co-Authorship Statement

The experiments and data analyses within this thesis were primarily carried out by Scarlett Puebla Barragan with supervision from Gregor Reid. The manuscripts presented within were primarily written by Scarlett Puebla Barragan. Exceptions are listed below:

Chapter 2: Biogenic amines and malodour in *Escherichia coli*-caused urinary tract infections—a metabolomics approach

Scarlett Puebla Barragan and Gregor Reid conceived the experiment with input from Mark Sumarah and Justin Renaud. Scarlett Puebla Barragan, Justin Renaud, and Mark Sumarah performed the LC-MS/MS analyses. Scarlett Puebla Barragan did the data analysis with input from Justin Renaud. Scarlett Puebla Barragan interpreted the results with input from Gregor Reid, Justin Renaud, and Mark Sumarah.

Chapter 3: Interstrain variability of human vaginal *Lactobacillus crispatus* for metabolism of biogenic amines and antimicrobial activity against urogenital pathogens

Scarlett Puebla Barragan, Emiley Watson, Charlotte van der Veer, Remco Kort, and Gregor Reid conceived the experiment with input from Jeremy Burton. Charlotte van der Veer and Remco Kort coordinated the collection of human samples. Emiley Watson and Charlotte van der Veer performed the antimicrobial experiments. Emiley Watson and Mark Sumarah carried out the LC-MS/MS analyses. Scarlett Puebla Barragan, John Chmiel, and Charles Carr performed the data analyses. Scarlett Puebla Barragan interpreted the results with input from Gregor Reid.

Chapter 4: Probiotics to reduce biogenic amines that cause urogenital malodour

Scarlett Puebla Barragan and Gregor Reid conceived the experiment with input from Jeremy Burton. Scarlett Puebla and Gregor Reid conceived the experiment. Charlotte van der Veer and Remco Kort coordinated the collection of human samples. Scarlett Puebla Barragan, Justin Renaud, and Mark Sumarah performed the LC-MS/MS analyses. Scarlett Puebla Barragan optimized and carried out the HPLC-UV method. Scarlett Puebla Barragan and Paul Akouris carried out the growth and degradation experiments. Stephanie Collins and Shannon Seney organized the collection of vaginal swabs. Kaitlyn Al and Charles Carr prepared the samples for 16S rRNA gene sequencing. David Carter from LRGC performed the sequencing. Sequencing results were analysed by Kaytlin Al, Charles Carr, and Scarlett Puebla Barragan using custom

code and code contributed from Gregory Gloor. Scarlett Puebla Barragan analysed the rest of the data and interpreted the results with input from Gregor Reid.

Chapter 5: Topical probiotics for women's urogenital health: selection of the best oil-based vehicle

Scarlett Puebla and Gregor Reid conceived the experiment. Scarlett Puebla Barragan, Serenah Jafelice, and Britney Lamb performed the experiments. Scarlett Puebla Barragan analysed and interpreted the results with input from Gregor Reid.

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Ana Bertha Barragán Birrueta

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Chapter 1

1 The focus of the thesis

The female urogenital environment of healthy women is populated mostly by bacteria from the *Lactobacillus* genus. These organisms play a key role in maintaining homeostasis. When equilibrium is disrupted a state of dysbiosis arises, in which pathogens dominate and infection can occur. The most common conditions resulting from this disruption in the urogenital microbiome are bacterial vaginosis (BV) and urinary tract infection (UTI). Each has its own etiology and symptomatic presentation, but both are accompanied by malodour and both adversely affect quality of life.

Since the early 1980s, administration of lactobacilli has been proposed to help restore and maintain urogenital health. This is now referred to as a probiotic application. Much is now known about how lactobacilli can reduce the incidence of BV and UTI but to date this has not considered effects against malodour (Puebla-Barragan & Reid, 2019).

In this thesis, the relationship between lactobacilli and the compounds that cause malodour is investigated. In addition, this characteristic of strains was examined with a view of selecting candidate probiotics. The long-term hope is that lactobacilli can be applied to rapidly eliminated malodour, even before antibiotics have eradicated the causative organisms. This would provide a holistic approach for women to manage their urogenital health.

1.1 The urogenital anatomy and microbiome of women

The female urogenital tract comprises the vulvar region, vagina, cervix, fallopian tubes, uterus, urethra, bladder, ureters and kidneys (Figure 1.1B). The vulva entails the structures of the female external genitalia (i.e. mons pubis, labia majora, labia minora, clitoris, vestibule, Bartholin's glands, and Skene's glands) (Figure 1.1A) (Lau et al., 2021; Nguyen & Duong, 2020; Weinstock, 2012), while the lower urinary tract is formed by the bladder and the urethra (Mangera et al., 2013). We are studying these areas as a whole due to their interconnection and anatomic proximity.

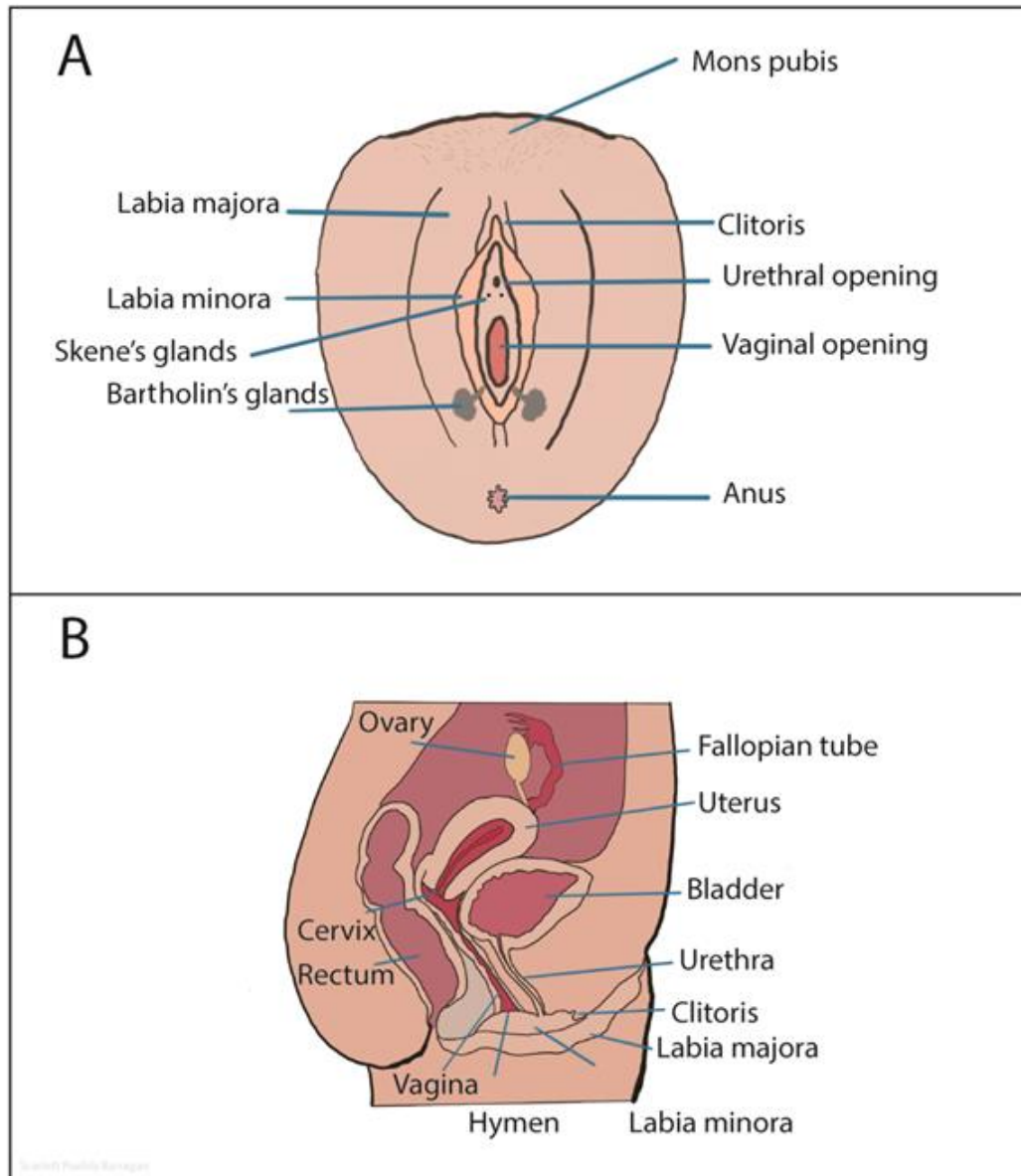


Figure 1.1 The anatomy of the female urogenital tract

The human body is colonized by communities of microorganisms, which are specific to each anatomical environment. This collection of organisms is known as the human microbiota (Weinstock, 2012); while the microbiome term takes into account biotic and

abiotic factors, including microorganism genomes (Cho & Blaser, 2012). An association has been identified between an increasing number of disease conditions and the human microbiome (Lau et al., 2021; Weinstock, 2012).

The urogenital tract in women is composed of diverse bacterial communities which live in a mutualistic relationship where the host provides nutrients to support bacterial growth and replenishment, and bacteria play key roles in maintaining homeostasis within the environment. For instance, urine provides a constant influx of nutrients that support its microbiota, as it is rich in electrolytes, amino acids, and carbohydrates (Bouatra et al., 2013; Neugent et al., 2020; Parsons et al., 1990). Similarly, the vaginal epithelium and the cervico-vaginal are rich in mucins, glycogen, and glycoproteins, which provide nutrients to the vaginal microbiota (Fuochi et al., 2017; Godha et al., 2017; Mirmonsef et al., 2014). Meanwhile, indigenous *Lactobacillus* spp. have a protective function by inhibiting potentially pathogenic organisms from inducing infection (Ma et al., 2012). Although the vaginal microbiota consists of a range of aerobic and anaerobic microorganisms, lactobacilli are the most common and numerous (Borges et al., 2014).

The disruption of the vaginal microbiota is known as ‘dysbiosis’, and it is often associated with clinical conditions such as BV, UTI, infection by human immunodeficiency virus (HIV), desquamative inflammatory vaginitis, atrophic vaginitis, vulvovaginal candidiasis (VVC), and trichomoniasis. Vaginal dysbiosis has also been correlated with adverse pregnancy outcomes such as preterm birth and maternal and neonatal infections (Van de Wijgert & Jaspers, 2017). Urinary tract infection and BV are the most common reasons for women to visit the doctor. Antibiotics are often prescribed for these conditions and it is not surprising that the indigenous microbiota are significantly impacted by these therapies. While prescribed antibiotics are necessary to treat symptomatic infection, their use as prophylactic agents makes less sense given the side effects and the fact that no agent is specific for the causative pathogen or primary symptoms – pain and frequency for UTI, itch for VVC and malodour for BV.

Given the correlation between lactobacilli dominance and health, it makes sense to consider applying these organisms to the urogenital region as a means to maintain and

restore well-being. These could be effective as prophylactic measures or as adjunct to antimicrobial therapy.

1.2 Role of lactobacilli in the urogenital environment

This section contains segments adapted and reproduced with permission (Appendix A) from: Puebla-Barragan, S., & Reid, G. (2019). Forty-five-year evolution of probiotic therapy. Microbial cell, 6, 184–196. Supplemental material available for download at: <http://microbialcell.com/researcharticles/2019a-puebla-barragan-microbial-cell/>

Efforts to characterize the vaginal microbiota using 16S rRNA gene sequencing, led to the proposal of five different community types. They were based on *Lactobacillus crispatus*, *L. iners*, *L. gasseri*, or *L. jensenii* dominance, plus a group representing a dysbiotic state (Ravel et al., 2011). However, this approach is problematic for many reasons. It uses statistical associations to categorize different microbiota profiles, without considering the health implications of each. It was based on North American subjects, whereas those from other regions can have different dominant *Lactobacillus* species. It also does not aid in identifying which patients require therapeutic intervention (Reid, 2019). Instead, a focus on the function of specific bacterial species and the presence of specific metabolites such as gamma-hydroxybutyrate or biogenic amines would be more appropriate (Ma et al., 2012; McMillan et al., 2015; Reid, 2018, 2019).

The long-held belief that beyond the distal urinary tract used was sterile, was proven incorrect when sequencing techniques identified a urinary microbiota. Similar to the vagina, the bladder is dominated by *Lactobacillus* species, along with *Gardnerella*, *Streptococcus*, *Corynebacteria*, *Escherichia*, *Aerococcus* and *Staphylococcus*, which are less frequent and normally present in lower abundance (Brubaker & Wolfe, 2016, 2015; Pearce et al., 2014).

Lactobacillus species are not simply bystanders but play an active role in maintaining the health of the urogenital environment. They physically compete with pathogens for sites of adhesion to the urothelium, while also producing antimicrobial compounds such as

bacteriocins, surfactants, and lactic acid (D'Alessandro et al., 2021; G et al., 2011; Karaoğlu et al., 2003; Tachedjian et al., 2017)

Lactic acid is the main organic acid produced by lactobacilli. It helps maintain an acid pH in the vagina which is ideal for lactobacilli maintenance while inhibiting the growth of pathogens. It is also hypothesized to elicit immunomodulatory effects by increasing anti-inflammatory cytokines in cervicovaginal cells and inhibiting pro-inflammatory mediators associated with HIV (Hearps et al., 2017; Tachedjian et al., 2017).

1.2.1 Bacterial vaginosis

Bacterial vaginosis is one of the most common conditions associated with vaginal dysbiosis. Its prevalence ranges from 23% to 29% in women across world regions, and the global economic burden of treating it is estimated to be around US \$4.8 billion (Peebles et al., 2019). It occurs when the abundance of *Lactobacillus* spp. is diminished, thereby reducing the concentration of lactic acid and other metabolites, resulting in an increase in pH and overgrowth of anaerobic pathogenic bacteria. The condition increases the risk for miscarriage, preterm birth, as well as for acquiring and transmitting HIV (Eastment & McClelland, 2018; Gustin et al., 2021). It is polymicrobial by nature with the most common genera being *Gardnerella*, *Prevotella*, *Atopobium*, *Megasphaera*, *Sneathia*, and *Dialister* (McMillan et al., 2015; Ravel et al., 2011).

The Amsel criteria has long been used to diagnose BV using at the presence of at least three of four criteria: vaginal pH higher than 4.5; presence of epithelial cells covered in bacteria (clue cells); a positive amine or 'whiff' test (fishy odour in vaginal discharge, before or after 10% KOH is added); and white discharge adherent to the vaginal walls (Amsel et al., 1983; Spiegel et al., 1983). A second diagnostic method is often used, albeit this was never its intention. Termed the Nugent test after its author, it consists of a scoring system based on Gram-stained vaginal smears (Figure 1.2). Depending on the proportion of Gram positive rods presumed to be lactobacilli versus dense biofilms of Gram negative rods and Gram positive cocci and smaller rods like *Atopobium* sp., a score is designated from 1-10 (Nugent et al., 1991; Spiegel et al., 1983). However, this approach requires observer training and objectivity (Redelinghuys et al., 2020) and its

usefulness is being questioned. Since cases of BV can be asymptomatic, the finding of a Nugent score of 10 does not necessarily mean that antibiotics will be prescribed; the clinical signs and symptoms must be present. This in itself is a dilemma for physicians as it is not clear why symptoms would not occur when the microbial milieu is so pathogenic in abundance. On the other hand, since the antibiotics for BV, namely metronidazole and clindamycin, are not effective at penetrating the biofilms typically present (on clue cells), it makes sense not to prescribe drug treatment when the patient has no presenting signs or symptoms. Furthermore, no effective treatment for malodour exists per se.

Recurrence of BV (and UTI) is common (Foxman, 2014; Ma et al., 2012), debilitating and with a significant bearing on quality of life. One line of investigation that began in 2003 was to administer a *L. crispatus* strain (CTV-05) to the vagina in the hope that it would colonize and prevent BV and UTI recurrences (Antonio & Hillier, 2003; Cohen et al., 2020; Hemmerling et al., 2009, 2010). In clinical studies, this showed promise for preventing UTI but not sufficiently effective against BV (Czaja et al., 2007). This is particularly interesting as the hypothesis was that using a species commonly found in the vagina would be preferable. Notably, Reid and Bruce took a different approach, believing that the inhibitory capabilities of the lactobacilli were more important than the species, especially since probiotic organisms do not colonize (Bruce & Reid, 1988; Hill et al., 2014).

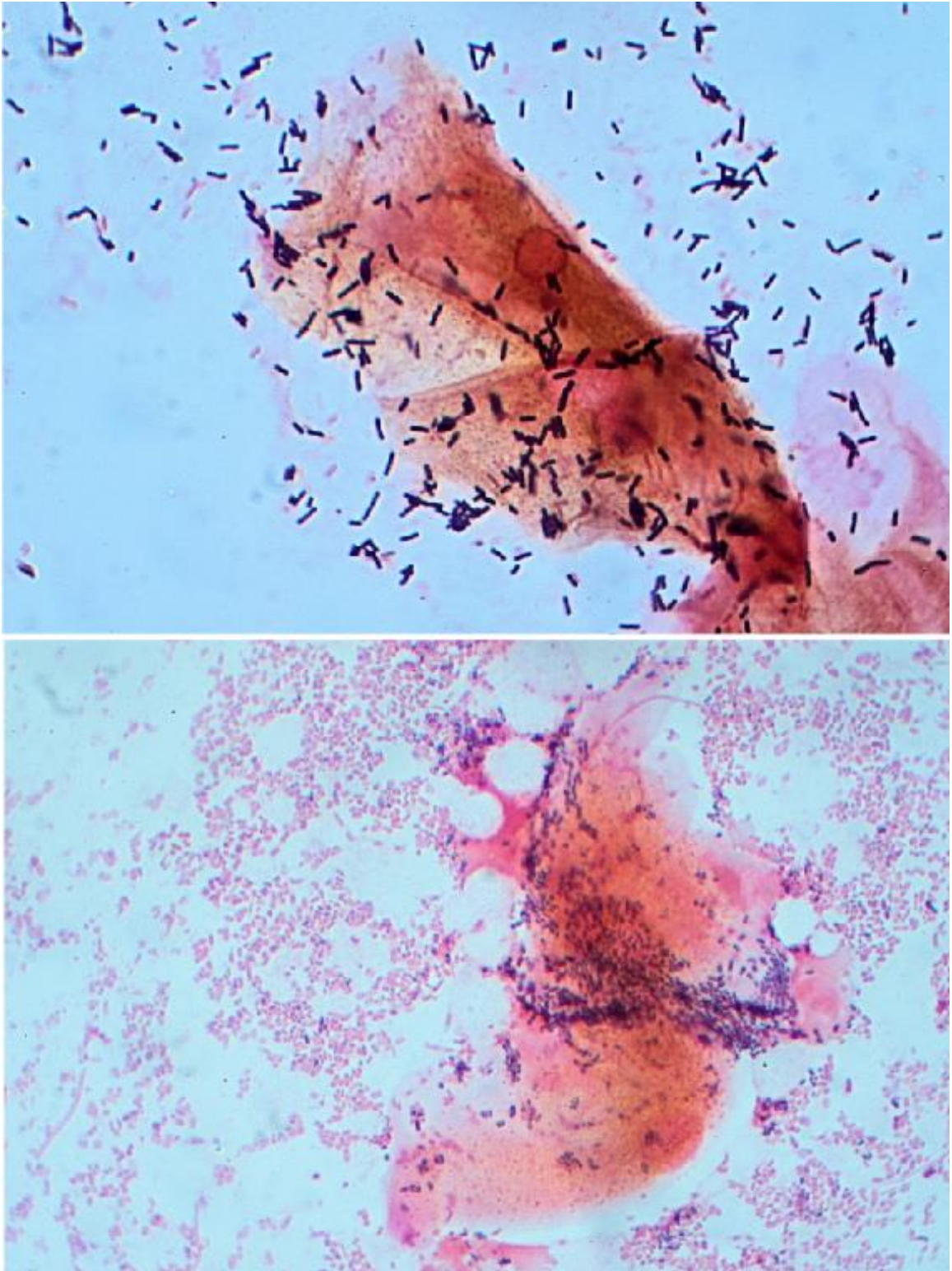


Figure 1.2 Gram-stained smear of secretions in normal vaginal fluid (top) vs bacterial vaginosis (bottom) (Beards, 2021)

1.2.2 Urinary tract infections

The perineum acts as an anatomical barrier between the anus and the urethra. But this is only approximately 4cm and therefore uropathogens can cross from the anus to the urethra, bladder and vagina (Stapleton, 2016). This makes it all the more important for the indigenous strains to be able to out-compete the pathogens as they arrive. Indeed, the perineum and urethra contain microorganisms which help prevent the ascension of pathogens into the urinary tract (Bruce et al. 1973; Dong et al., 2011). The majority of uncomplicated UTIs (80-90%) are caused by *Escherichia coli*, originating from the gastrointestinal tract (Salvatore et al., 2011) via the faecal-vaginal-periurethral route (Moreno et al., 2008). Infections of the lower urinary tract in patients with no predispositions (structural, metabolic or immunological) are considered uncomplicated UTIs (Chiu, 2013).

Before a UTI is diagnosed, significant bacteriuria ($\geq 10^5$ CFUs/mL of bacteria in a fresh urine specimen (Chiu, 2013)), as well as symptoms and signs must be present. These signs and symptoms may vary, but the more common are: dysuria, urgency, increased frequency of urination, abdominal pain, and malodour (Chiu, 2013; Sj et al., 2004). It has been estimated that UTI occurs in over a third of the female population during their lifetime (Salvatore et al., 2011). Moreover, due to anatomical differences and easier access of pathogens from the anus, UTIs are more common in females than in males due to anatomical differences (i.e. the urethra is much shorter in females) (Chiu, 2013).

The urethra and the bladder form the lower urinary tract in women (Mangera et al., 2013), while the upper tract is composed of the kidneys and ureters which drain to the bladder (Saint-Elie et al., 2010). The anatomical location of infection as well as its severity are the basis of the classification of UTIs (Chiu, 2013). Lower UTIs include cystitis and acute urethral syndrome typically treated with oral antibiotics (Salvatore et al., 2011).

Upper UTI (also known as pyelonephritis) occurs due to an invasive infection of the renal parenchyma. It is characterized by fever, renal angle tenderness, nausea, and vomiting; in some cases, symptoms of dysuria may be present. Complications include urosepsis,

kidney damage and renal failure. Patients with pyelonephritis are treated with intravenous antibiotics. All urinary infections that involve the upper tract, as well as those occurring in patients with predispositions, such as indwelling catheters, cancer, calculi, anatomical abnormalities, spinal cord injury, are considered complicated UTIs (Chiu, 2013).

Recurrent UTI is when a secondary infection follows another one which was clinically resolved; it can be caused by the same bacteria as in the initial infection (relapse) or by a different one (reinfection) (Salvatore et al., 2011). There are three approaches to management: long-term, low dose antibiotics; a single antibiotic post-intercourse; or three-day antibiotics in which the prescription has already been issued and the patient self-diagnoses, starts the antibiotic and sends a urine sample for confirmation.

For symptomatic UTI, the first line treatment is an antibiotic (for example, nitrofurantoin, fosfomycin, trimethoprim-sulfamethaxole, trimethoprim alone or a fluoroquinolone) (Chu & Lowder, 2018), signs and symptoms tend to persist for at least three days (Frimodt-Moller, 2002).

Some have considered targeting the symptoms, for example the pain by treating with an analgesic such as ibuprofen. Clinical studies have shown that this approach can be as effective as antibiotic treatment in uncomplicated UTIs (Bleidorn et al., 2010, 2016; Franco, 2005), although it is not clear why a pain killer would interfere with uropathogenesis as ibuprofen and its metabolites do not appear to have direct antimicrobial activity (Whiteside et al., 2019). The main advantage is through providing symptomatic relief within one day. This illustrates how approaches that target specific signs and symptoms can play a role in disease management.

1.3 Malodour in the urogenital tract

Volatile compounds are produced as a part of the regular metabolism of humans, plants, fungi and bacteria. Microbial associated diseases tend to be identified by specific smells which, in many cases, are caused by volatile organic compounds (Thorn & Greenman, 2012). These compounds are the result of microbial metabolism, and they can include fatty

acids, terpenoids, aromatic compounds, nitrogen containing compounds (e.g. amines), and volatile sulfur compounds (Schulz & Dickschat, 2007).

Biogenic amines are basic nitrogenous compounds that have been correlated with BV, particularly, cadaverine, putrescine, tyramine, trimethylamine (TMA). These compounds are known causes of malodour (Borgogna et al., 2021; McMillan et al., 2015; Nelson et al., 2015). Until now, the cause of malodour during a UTI was not been well established. With the exception of TMA, none of the biogenic amines previously described for BV had been correlated with UTI (Lam et al., 2014). Nonetheless, since both conditions are a direct result of dysbiosis of the urogenital tract, we considered it likely that the malodorous metabolites found in BV would also present during a UTI.

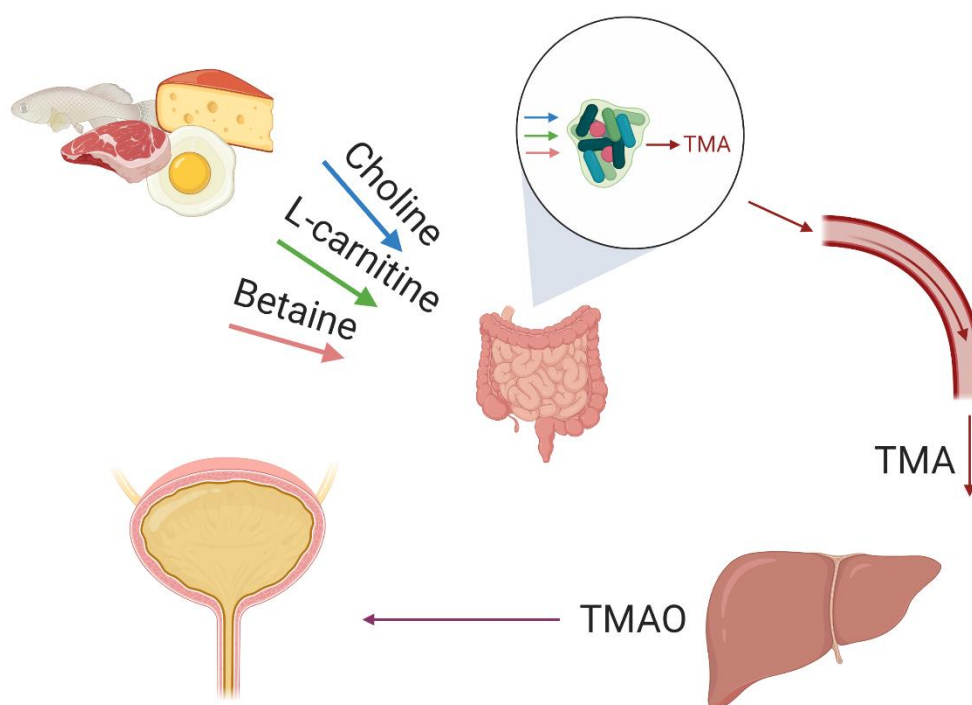


Figure 1.3 Biosynthesis of TMA and TMAO

One compound, TMA, which has a characteristic fishy smell, is a mammalian/microbial co-metabolite that is produced by bacterial reduction of the mammalian-derived trimethylamine N-oxide (Figure 1.3; Lam et al., 2014, 2015). Tyramine is produced via

the decarboxylation of tyrosine by uropathogens such as *Enterococcus faecalis* (Whiteside, 2018). Cadaverine is mostly produced by bacteria, such as *E. coli*, via the decarboxylation of lysine (Watson et al., 1992). Putrescine is produced by bacteria and human cells through the metabolism of ornithine or agmatine (Tabor & Tabor, 1985; Tofalo et al., 2019).

Increased concentrations of tyramine, putrescine, and tyramine are associated with a higher risk of transition from a *L. crispatus*-dominated vaginal microbiota to one with lower abundance of this species. The mechanism appears to be biosynthesis that consumes protons, and thereby increases the pH of the environment. Furthermore, biogenic amines have been found to reduce the production of lactic acid and to slow down the growth of several vaginal *Lactobacillus* species. This suggests that these amines play a direct role in diminishing the *Lactobacillus*-mediated protection and makes the environment more favourable for pathogens (Borgogna et al., 2021; Nelson et al., 2015).

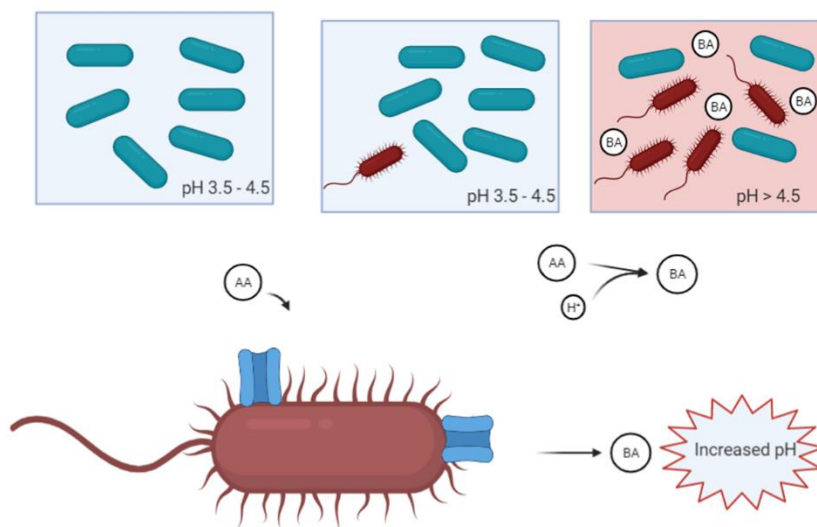


Figure 1.4 Biogenic amine biosynthesis increases the pH of the vaginal environment (adapted from Nelson et al., 2015)

1.4 Probiotics for female urogenital health

The emergence of a new field of science is exciting, yet invariably faces challenges to its validity and acceptance of its scope of influence. This is certainly the case for probiotics, defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”(FAO/WHO Working Group, 2002; Hill et al., 2014).

An early observation of the role that beneficial bacteria play in health was made by Elie Metchnikoff in 1905. He proposed that the reason behind increased longevity in the Bulgarian population was due to the lactobacilli used to produce a yogurt commonly consumed in that region, and not the product itself as it was previously believed (Metchnikoff, 1908). Nonetheless, although these findings set the grounds for research on potential beneficial microorganisms, it was not until many decades later that formal research of probiotics began.

Prior to the early 2000’s, most microbiology studies related to humans were focused on pathogenic organisms. After 2001, there was a significant increase in the endogenous microbes of the human body and a belief that they could be determinants of health (Relman, 2002; Relman & Falkow, 2001). When the Human Genome Project was being carried out, it was deemed that for it to be sufficiently relevant, there had to be an understanding of the relationship between humans and their microorganisms (Davies, 2001). This was a precursor for the Human Microbiome Project (HMP) and Metahit Consortium which would compile an inventory of microbial organisms and their genomes in the mouth, gut, vagina, and skin (but not bladder) of a healthy human cohort (Peterson et al., 2009; Qin et al., 2010).

The introduction of a definition of probiotics was followed a year later by the establishment of the International Scientific Association for Probiotics and Prebiotics (ISAPP) in 2002 (Reid et al., 2003). This was a major factor in stimulating and focusing research in this area and emphasizing the importance of scientific rigour and production standards for probiotics. This has been reflected in peer-reviewed publications which have increased substantially on the scientific database PubMed from just over 1,000 in 2002 to over 33,000 today (July, 2021). The breadth of the definition was intentional to

allow capture of a range of host benefits. Subsequently, other terms have emerged such as psychobiotics, next-generation probiotics, baby-biotics, but none adequately defined or sufficiently different that they would fall outside the existing probiotic definition. These terms seem to group probiotics in very specific clusters, with very definite uses, when in reality most of the probiotic strains available will have more than one targeted benefit on the host. Therefore, such terminology is confusing to healthcare providers, producers and consumers.

The increase in products termed ‘probiotic’ on the market does not necessarily equate with a reason to celebrate successful translation of science to commerce and consumers. Too many products fail to comply with the characteristics required to be called probiotic. Many false claims and rampant misuse of the term has resulted in mainstream consumer channels providing incorrect information to consumers.

Probiotics are not inside us, not in fermented food, not necessarily better if there are more species or a higher viable count. Too many formulations are being concocted not based on research evidence, but on marketing and what might appeal to consumers. For example, products are being composed supposedly to improve vaginal health using strains not documented to compete with urogenital pathogens, improve immunity or do anything that can restore homeostasis to that region of the body. In other words, there are no data to support their selection. Yet, the internet, the use of words to reach the first page of search engines and use of pseudo-experts for promotion provide a means for these products to be highly rated and appear to be the best clinically documented for preventing or curing bacterial or yeast infections in the vagina.

The net result is misleading and confusing to consumers as well as making healthcare professionals leery of the whole field of probiotics. To counter this, we need to re-state important facts.

For a product to be considered probiotic, it must comply with three core characteristics: (1). The strain(s) must be characterized, including genetically and phenotypically and a rationale given based on documented experiments published in peer-reviewed papers, for their inclusion for the intended use. (2). The product must contain sufficient live

microorganisms at time of use that are equivalent to when the product was shown in clinical studies to confer a benefit to the desired target site. (3). The delivery method, dosage and length of use should be based on scientific evidence in humans if they are the intended recipient.

It makes it difficult for consumers if the product label does not state strain designations because it becomes impossible to track the research performed on the contents. Dosages are rarely stated on labels, and some products only contain filtered extracts or ferments or lysed bacteria, meaning that no live microorganisms are present: thus, the product is not probiotic, and the term should not have been used.

The lack of effective treatment options for urogenital conditions such as BV and UTI has increased the interest in probiotic research as a tool to address this problem (Bilardi et al., 2013; Tandogdu & Wagenlehner, 2016). Clinical observations by urologist Andrew Bruce in 1973, set the wheels in motion for considering lactobacilli as probiotics for the urogenital tract of women (Bruce et al., 1973; Bruce & Reid, 1988). While the rest of the field was trying to develop vaccines and therapies against uropathogenic *E. coli*, none of which have so far borne fruit, he believed that replenishment of lactobacilli into the vagina where *E. coli* were dominant after repeated UTIs and antibiotic treatments, might restore homeostasis and protect the host.

Probiotics can be used to replenish organisms that are naturally in each niche but whose numbers have been depleted and illness has occurred. For example, administering *Lactobacillus crispatus* into the vagina to counter BV or ascension of uropathogenic *E. coli* into the bladder (Stapleton et al., 2011). But optimally, probiotic strains should be selected for their properties not their origin. These can include the ability to counter pathogens/conditions causing illness, with the aim being to restore health and ideally allowing the indigenous beneficial microbes to return. The example would be to orally administer *Lacticaseibacillus* (formerly *Lactobacillus*) *rhamnosus* GR-1 (LGR-1) and *Limosilactobacillus* (formerly *Lactobacillus*) *reuteri* RC-14 (LRC-14), which are not species highly prevalent in the vagina, but whose administration results in recovery from infection and the return of indigenous *L. crispatus* and *L. iners* (Macklaim et al., 2015).

Inspired by the LGR-1/LRC-14 studies, a number of probiotic strains have been developed to prevent urogenital infections, *Lactobacillus acidophilus* A-212, *Lacticaseibacillus rhamnosus* A-119, with *Streptococcus thermophilus* A-336; *L. rhamnosus* PBO1 with *Lactobacillus gasseri* EN-153471 (EB01); and *L. rhamnosus* Lcr35 in vaginal ovules. Strains LGR-1 and LRC-14 are the only ones approved for oral use in Canada and the United States. The positive early clinical studies performed with LGR-1 and LRC-14 showing improved vaginal microbiota and reduced infection recurrence (Reid, 2017), have been confirmed by others (Beerepoot et al., 2012; Petricevic et al., 2008; Vujic et al., 2013). The mechanisms of action include an increased ascension of probiotic and indigenous lactobacilli from rectal skin to the vagina, reduced pathogen ascension, localized inhibition and displacement of pathogens, and priming of antimicrobial defences (Karlsson et al., 2012; Reid et al., 2011). Anti-fungal effects have also been reported coinciding with improved curing of VVC (Köhler et al., 2012; Martinez et al., 2009).

The vaginal administration of probiotic *Lactobacillus* began in the late 1980s (Bruce & Reid, 1988), and has since led to other strains being tested for urogenital health including *L. crispatus* CTV05 to prevent recurrence of UTI (Stapleton et al., 2011), *L. rhamnosus* IMC 501 with , *L. paracasei* IMC 502 (Verdenelli et al., 2016b) for vaginal health, *L. rhamnosus* Lcr35 to aid in the management of BV and VVC (Petricevic & Witt, 2008; Rossi et al., 2010), and *L. gasseri* EN-153471 (EB01) to help treat BV in conjunction with antibiotics (Larsson et al., 2011).

A study of the genome of LGR-1 showed that it is better adapted to the vagina than the most commonly used intestinally-derived probiotic *L. rhamnosus* GG, by having a unique cluster for exopolysaccharide production, metabolizing lactose and maltose, and to better withstand oxidative stress (Petrova et al., 2018).

Probiotic strains have been shown to reduce oral malodorous compounds. For example, *Streptococcus salivarius* K-12, administered after an antimicrobial mouthwash, was shown to reduce levels of volatile compounds, as measured by a halimeter and changes in the bacterial composition of the participants' saliva (Burton et al., 2006). Results showed

a decrease of more than 100 ppb in the concentration of malodourous compounds after 7 days. Moreover, *S. salivarius* K12 was still detected after a week of instillation, and the growth of halitosis causing bacteria was suppressed. A similar approach could be used for the reduction of malodorous compounds in the female urogenital tract.

Previous studies have reported that certain lactobacilli strains can degrade biogenic amines. Although these results correspond mainly to strains relevant to the food industry and not to human health, they prove that there are *Lactobacillus* strains with this capacity (Niu et al., 2019; Zhang et al., 2021).

Lactobacillus-mediated reduction of biogenic amines can be a result of inhibiting the bacterial species that produce them, or it can be mediated by amine oxidase activity. These are enzymes that can be divided in two subfamilies depending on their cofactors. The amine oxidases family that contain copper as their cofactor is divided into three subclasses: diamine oxidase, primary-amine oxidase, and diamine oxidase. These enzymes catalyze the oxidative deamination of biogenic amines (Cona et al., 2006; Grimsby et al., 1991; Levering et al., 1981; Niu et al., 2019; Tipton et al., 2012). Unfortunately, the specific pathways and enzymes used by lactobacilli to degrade amines are still not well characterized, but their potential for this use is clear.

Vaginal administration of probiotic *Lactobacillus* using suppositories has already been tested for efficacy and safety. A clinical study where healthy women received the commercially available probiotic suppositories SYN BIO® *gin* (containing *L. rhamnosus* IMC 501 and *Lacticaseibacillus paracasei* IMC 502), demonstrated that the treatment was well tolerated and there were no reports of side effects. Furthermore, an increase in the lactobacilli level of the participants was observed (Verdenelli et al., 2016a). The fact that probiotics have been successfully and safely applied in a direct manner, indicates that their topical application could be an effective mean of delivery in the treatment of urogenital tract infections in women.

1.4.1 Topical use of probiotics for urogenital applications

This section contains segments adapted and reproduced with permission (Appendix B) from: Puebla-Barragan S, Reid G. Probiotics in cosmetic and personal care products: trends and challenges (2021). Molecules, 26,1249. Supplemental material available for download at: <https://www.mdpi.com/1420-3049/26/5/1249>

The increased interest in microbes colonizing the human body, not simply those infecting it, has led to many studies attempting to manipulate the microbiome in a given niche, in favour of health. The use of beneficial microbes for this purpose has seen the field of probiotics grow substantially. This includes personal care applications, where the market for probiotics is projected to grow at a 12% rate in the next ten years, with North America the driver (FactMR, 2020).

The topical delivery of probiotics is an emerging area principally used for the treatment of skin disorders. Specifically, several lactobacilli strains have been shown to have the capability of efficiently adhering to the keratin on the skin, where they express antimicrobial capabilities against pathogens and prevent biofilm formation (Lopes et al., 2017). Of importance, some topical formulations have been able to preserve the viability and probiotic capabilities of the bacteria (Mehdi-Alamdarloo et al., 2016).

As previously stated, a healthy vaginal environment is in most cases populated by an abundance of lactobacilli. Various triggers, from use of douches and antibiotics to multiple sexual partners and influx of pathogens into the area, can disrupt the homeostasis giving rise to BV, UTI, VVC and other inflammatory and irritant conditions. This provided a rationale 48 years ago to supplement the urogenital tract with lactobacilli to restore a healthy state (Bruce et al., 1973; Puebla-Barragan & Reid, 2019). Since then, the vaginal administration of probiotic strains of *Lactobacillus* through suppositories or ovules has been explored (Bruce & Reid, 1988).

Given the significant negative impact of antimicrobial therapy on the urogenital microbiota and failure to restore homeostasis, probiotic strains have been used in combination to help with recovery. These include *L. gasseri* EN-153471 (EB01) for the

management of BV (Larsson et al., 2011) and LGR-1 plus LRC-14 in combination with antibiotics or anti-fungals (Anukam et al., 2006; Martinez et al., 2009; Vujic et al., 2013). Additional strains have become available in the American market with minimal clinical and scientific documentation (Skokovic-Sunjic, 2021). The supplements are believed to function through ascension from the rectal skin to the vagina, where they reduce pathogen ascension and inhibit and displace pathogens while also conferring antimicrobial defences through the production of bioactive compounds such as lactic acid and bacteriocins.

Therefore, these are essentially cosmetic in action because the skin is the surface of interaction. These products are regulated as drugs or natural health products with functional and structural or even disease risk reduction claims. The application of strains directly into the vagina using suppositories is approved in Canada. Elsewhere, products are being delivered through coating tampons and pomades (Handalishy et al., 2014; Sauperl et al., 2020), but further evidence is required to confirm they are probiotic and benefit the host. The development of topical gel containing probiotic lactobacilli is being pursued, with promising preliminary results for treating VVC (Donders et al., 2020).

The potential for probiotic strains to reduce urogenital malodour has not been well investigated (Bilardi et al., 2013). Many non-probiotic products such as vaginal douches, vinegar rinses, and fragrances claim to help reduce malodour. However, their efficacy is dubious and they can increase the risk of infection, including sexually acquired through disruption of the beneficial microbes (Brotman et al., 2008; Fashemi et al., 2013). An advantage of an effective probiotic would come from its ability to grow and produce metabolites that degrade or neutralize malodorous compounds (McMillan et al., 2015; Puebla-Barragan et al., 2020). Depending on the nature of such a product, it may have to be registered as an over-the-counter (OTC) drug and not as a cosmetic or personal care product.

1.5 Knowledge gaps

Urogenital dysbiosis is a complex phenomenon which is not yet fully understood.

Likewise, the role of microbial metabolites and their effects in disrupting or maintaining

homeostasis require more investigation. This thesis aims to fill some of these gaps and make a connection between the metabolome and the microbiota.

To date, most of the focus has been in characterising the communities in the vaginal microbiome, without enough emphasis on why they are there and what are they doing. Long-held unproven concepts are being questioned, for example with the finding that few lactobacilli strains can break down glycogen to use it as a nutrient source (van der Veer et al., 2019).

Current diagnosis of BV and UTI leading to the instigation of treatments has not changed for over forty years. In essence, this comprises broad spectrum antibiotics to try and eradicate certain pathogens. It is far from ideal due to non-specific killing of commensals, drug resistance development by pathogens and failure to treat specific patient complaints, such as malodour. Prior to the beginning of this doctoral project, the causes of malodour during UTI were understudied, and their correlation with vaginal malodour was overlooked.

In order to understand pathogenesis and health, studies are needed to examine the strains present, those being administered as probiotics, and the products they produce. This requires metabolomic applications that will be described below.

1.6 Approaches for filling the knowledge gaps

1.6.1 Use of metabolomics and multivariate statistics

The low molecular weight products of cellular processes are known as metabolites. Organisms produce them due to genetic or environmental changes. The set of metabolites synthesized by a biological system is its metabolome (Fiehn, 2002). Metabolomics is the comprehensive identification and quantification of all metabolites in a biological system produced as a response to biological processes (Dettmer et al., 2007). Metabolomics studies look at ‘which’ metabolic pathways are active and functional in an organism. Different ‘omics’ techniques complement each other by answering different questions. While genomics looks at what can happen, transcriptomics at what appears to be

happening, and proteomics at what makes it happen, metabolomics links genotypes and phenotypes by investigating what has happened (Dettmer et al., 2007; Fiehn, 2002).

Targeted metabolomics looks for specific compounds and uses authentic standards to confirm the identity of the analytes. Therefore, the sample preparation protocol has to be designed to specifically retain the metabolites of interest (Dettmer et al., 2007; Fiehn, 2002). This allows to measure specific responses of organisms. This approach can be quantitative and isotopically labelled standards can facilitate metabolite detection. Nonetheless, coverage of the metabolome is limited, increasing the odds of overlooking the metabolomic response of interest (Ribbenstedt et al., 2018).

Untargeted metabolomics aims to detect as many metabolites as possible and, relying on multivariate statistics, it can identify novel biomarkers and global differences between groups. Since untargeted metabolomics looks for global differences, sample preparation is simpler and is done by only removing salts or large peptides and proteins that could interfere with the analysis (Dettmer et al., 2007; Fiehn, 2002). One of the main limitations of untargeted analyses is the detection of unknown compounds (Sindelar & Patti, 2020). Although methods are being developed to improve this, it remains a major bottleneck of the workflow (Roberts et al., 2012; Wang et al., 2019; Zhou et al., 2020). Furthermore, since it is not a quantitative approach, the identification of baseline metabolite levels is more complicated than in targeted metabolomics (Ribbenstedt et al., 2018).

Ultimately, the selection of an approach will depend on the research question. In many cases, both approaches are used to complement each other and yield a more comprehensive overview of the diverse metabolic processes taking place in a given niche – in our case, the urogenital tract.

Metabolomic analyses are commonly performed to identify differences in metabolite abundances through comparative experiments (Fiehn, 2002). Since analytes have diverse physicochemical properties and are present at different concentrations, each protocol must be designed to properly identify the metabolites of interest. As such, designing an appropriate methodology that suits the analytes of interest and that objectively answers

the research question is critical. Ideally large sample sizes increase statistical power and detect biologically relevant sample clustering, but this is not always feasible. After experimental design, the workflow generally begins with the sample collection, followed by storage and pre-processing samples in a way that minimizes the formation or degradation of metabolites due to enzymatic or oxidation processes (Dettmer et al., 2007; Fiehn, 2002).

The next stage is sample preparation, which might include the addition of an internal standard as well as the extraction of the analytes of interest. When working with complex biological matrices (i.e. serum, plasma, urine, whole blood, saliva, or culture media) it is important to bring them into a form compatible to the analytical technique, while also removing matrix components that could cause interference. (Dettmer et al., 2007; Fiehn, 2002).

Sample preparation is followed by a separation step, which is normally carried out using either liquid (LC) or gas (GC) chromatography, although the former is the most common technique used in metabolomics and was the one used throughout this thesis (Ribbenstedt et al., 2018). In liquid chromatography, separation is commonly performed via high performance liquid chromatography (HPLC), which is based on the separation of compounds based on their size and polarity. For this purpose, the sample is injected into a column which will act as the stationary phase. Often C18 columns are used to perform reverse-phase chromatography, in which the stationary phase is hydrophobic, and the mobile phase is hydrophilic. In practical terms, this means that hydrophobic or less polar compounds will be retained by the column and the mobile phase will cause their elution. The opposite is known as normal-phase chromatography, in which a hydrophilic stationary phase is used: such as a hydrophilic interaction chromatography (HILIC). In this case, polar compounds are retained due to higher affinity to the column, and a non-polar mobile phase is used for their elution. This is ideal for the separation of highly polar molecules such as amines and amino acids (Dettmer et al., 2007; Roberts et al., 2012; Sindelar & Patti, 2020; Theodoridis et al., 2013).

Next, sample analysis is carried out, mostly by specialized detecting technologies such as nuclear magnetic resonance (NMR) or tandem mass spectrometry (MS/MS), although the former requires more specific technical skills and can be more expensive (Fiehn, 2002; Forcisi et al., 2013; Theodoridis et al., 2013).

Most HPLC instruments are equipped with an UV detector, which uses absorbance to detect the eluting molecules. However, the applications for this technique are limited and normally used for simpler analyses or as a complement to other metabolomic methods (Bélanger et al., 1997).

In targeted approaches, analysis and quantification are usually carried out with specialized software provided by the manufacturers of the analytical instrument, or with open source platforms (Wenig & Odermatt, 2010). Given the high-throughput nature of untargeted metabolomics, multivariate analyses are required. Amongst the most used are principal component analysis (PCA) and partial least squares projection to latent structures (PLS).

Multivariate analyses allow projecting the data in more than two dimensions, allowing identification of similarities and clustering between samples. These tools help to differentiate between classes in complex data sets (Long, 2013; Worley & Powers, 2013).

The final step is the identification of relevant metabolites, which can then be used for further targeted analyses (Fiehn, 2002; Roberts et al., 2012; Theodoridis et al., 2013). The output will depend on the instrument used. For instance, when working with MS/MS the variables used to identify each compound are the retention time (RT), the exact mass (m/z), and the fragmentation pattern of the molecule. These outputs are then matched with authentic standards (in targeted studies) or with the use of databases, such as METLIN, that compiles theoretical and experimental data on the spectra of the most common metabolites (Smith et al., 2005). A summary of a typical metabolomic study workflow is shown in Figure 1.4.

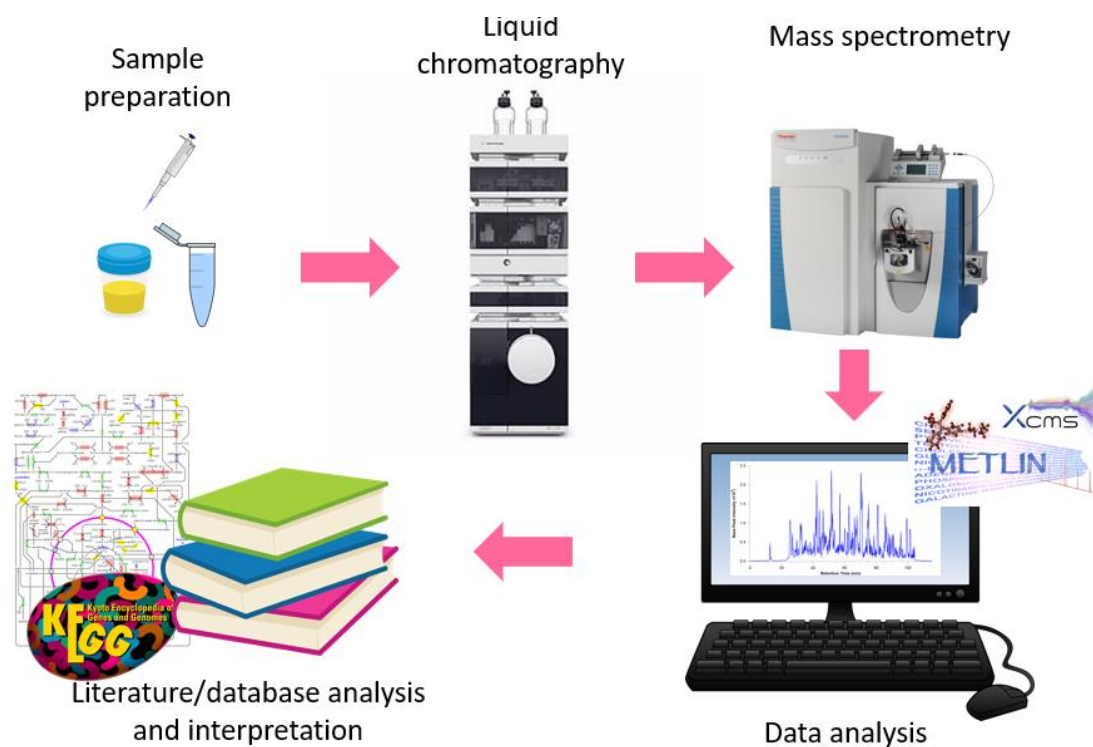


Figure 1.4 Workflow of a metabolomics study

1.6.2 Identifying lactobacilli that degrade biogenic amines

As mentioned above, it is critical to select probiotic strains based on the characteristics required and intended use. Unfortunately, the rationale is simply whether a species is commonly found.

We recently collaborated with a Dutch group to investigate *L.crispatus* strains from healthy women and those with a history of BV. The hypothesis was that the strains from healthy women would be different genetically and phenotypically from those isolated from women who had suffered from infection, because presumably these strains were not protective. However, the results did not support the hypothesis and subject of origin was not predictive of their properties, emphasizing that candidate probiotic strains cannot simply be by isolating ones from healthy subjects.

With access to these strains, we were presented with the opportunity to examine the metabolic profiles and investigate whether these or other strains could degrade biogenic amines.

1.7 Project scope and relevance

Hypothesis: Pathogenic bacteria in the urogenital tract produce biogenic amines, and lactobacilli can reduce their concentration.

Objectives:

- (i) **Identify malodorous compounds formed during UTIs.** In Chapter 2 we used untargeted and targeted metabolomics to evaluate urine samples from women with an active UTI. We also explored the *in vitro* ability of uropathogenic strains of *E. coli* to produce biogenic amines in female urine. We identified that the malodorous amines trimethylamine, cadaverine, and putrescine are elevated in UTI conditions. This approach was novel given that malodour in UTIs is a sign that has been often overlooked.
- (ii) **Identify biochemical characteristics in *Lactobacillus crispatus* strains that make them good probiotic candidates.** Chapter 3 focuses on the metabolomic profiling of clinical isolates of *L. crispatus*. We confirmed that strains sourced from healthy patients did not have a specific metabolomic or genomic profiles that differentiate them from those isolated from dysbiotic vaginal environments. We also identified that strains from dysbiotic environments showed higher antimicrobial activity towards common urogenital environments, suggesting that *L. crispatus* adapt to their environment by developing traits that might aid them survive stress exposure.
- (iii) **Identify lactobacilli strains with malodour-degrading activity and characterize their interaction with biogenic amines.** In Chapter 4 we tested if commensal and/or probiotic bacteria can degrade biogenic amines. We successfully characterised several strains with this ability. We also identified that cell-free supernatants could retain this activity. These findings open major opportunities for the development of odour-degrading products for the urogenital tract either through the use of probiotics or with the use of their metabolites.
- (iv) **Evaluate probiotic bacteria compatibility with oils safe for human use.** With a view to developing a practical treatment for women, oils safe for human use (i.e. coconut oil, olive oil, petroleum jelly, and mineral oil) were used in Chapter 5 to

test survival rates of a freeze dried commercial blend of the probiotics LGR-1 and LRC-14. Such vehicle could be used to deliver different strains and by-products to provide the recipient with rapid relief from malodour while also aiding in restoring urogenital homeostasis. Specifically, we identified coconut oil and petroleum jelly as ideal candidates for this purpose. Our findings provide valuable information on the potential of malodour-degrading lactobacilli to be implemented in a topical treatment.

Both UTI and BV are highly prevalent health problems. Major discomfort is caused by the malodour that these conditions cause, which has a significant impact in the quality of life of women. This project will help to better understand the probiotic capabilities of lactobacilli in the female urogenital tract. Specifically, how they can be used to target malodour. This is a valuable approach in terms of conjoint treatment (along with antibiotics and/or pain-relieving agents) of women with BV and UTI. These observations are of great significance, given that the current line of treatment, with antibiotics, is not efficient. Therefore, if probiotics could be used to target specific symptoms, and in this way, improve the way these conditions are managed, the impact on the quality of life of patients would be very significant.

The proof-of-concept in terms of their use in an oil-based formulation for topical delivery will provide the basis for further studies in which novel formulas optimize their viability and retain their activity. This approach also has many potential applications by combining different microorganisms in one chamber and different substances in the other. This concept can be of great value as it is an approach that can be applied to alleviate other microbiome-influenced conditions that can be treated topically (e.g. disorders caused by skin dysbiosis).

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Chapter 2

2 Biogenic amines and malodour in *Escherichia coli*-caused urinary tract infections—a metabolomics approach

This chapter is reproduced with permission (Appendix C) from: Puebla-Barragan, S., Renaud, J., Sumarah, M., & Reid, G. (2020). Malodorous biogenic amines in Escherichia coli-caused urinary tract infections in women—a metabolomics approach. Scientific Reports, 10, 9703.

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2.1 Abstract

Many women suffer from urinary tract infections (UTIs). In addition to pain and increased urgency to urinate, malodour is a major issue for these patients. The specific factors causing this malodour are unclear, and there are no targeted treatment options to counteract it effectively. We used a metabolomics approach to compare the chemical composition of metabolites in the urine of women with *E. coli* UTIs ($n = 15$) and those who are healthy ($n = 10$). The biogenic amines trimethylamine and putrescine, which cause malodour in other urogenital conditions, were significantly increased in UTI patients. Conversely, the precursor of trimethylamine, trimethylamine N-oxide, was lower. To further confirm the source of the malodorous compounds, *in vitro* experiments were conducted by incubating strains of uropathogenic *E. coli* in sterilized urine from healthy women. All tested strains accumulated trimethylamine and putrescine. Notably, cadaverine was also produced by *E. coli* strains *in vitro*; however, it was not significantly different between both groups. This study identifies the origin of urogenital malodour in women with UTIs.

2.2 Introduction

Urinary tract infections (UTIs) are a highly prevalent global health problem, with over 50% of women expected to experience at least one in their lifetime (Foxman, 2003; Tandogdu & Wagenlehner, 2016). Lower UTIs are more common than those infecting the kidney, and *Escherichia coli* (UPEC) strains are responsible for approximately 80% of the cases. These uropathogens originate from the gastrointestinal tract (Salvatore et al., 2011) and enter the bladder via the fecal-vaginal-periurethral route (Moreno et al., 2008). These infections are present with dysuria, increased frequency of urination, abdominal pain, and malodour (Sj et al., 2004). However, besides ammonia, which is a known cause for foul-smelling urine (Forsgren-Brusk et al., 2017; Norberg et al., 1984), other sources of malodour during infection and the source of their production require further characterization. None of the currently available treatment regimens target malodour. A better understanding of the chemical origins of malodour during this condition would enable the creation of more targeted and efficient therapies.

Biogenic amines are basic nitrogenous compounds with well-established organoleptic characteristics (Table 2.1) associated with malodour (Nelson et al., 2015). Most of them are biosynthesized from amino acids, as shown in Figure 2.1. Trimethylamine (TMA), tyramine, cadaverine, and putrescine are of interest since they are known causes of urogenital malodour in conditions such as bacterial vaginosis (BV) (McMillan et al., 2015; Nelson et al., 2015). Until now, these compounds have not been associated with urinary malodour, except for trimethylamine (TMA), which has a characteristic fishy odour and was previously proposed as a candidate biomarker for *E. coli*-associated UTI (Lam et al., 2014). It is a mammalian/microbial co-metabolite since the production of TMA by bacteria depends on the reduction of mammalian-derived trimethylamine N-oxide (TMAO) (Lam et al., 2014, 2015).

Tyramine is synthesized via decarboxylation of tyrosine, and it is produced by uropathogens such as *Enterococcus faecalis* (Whiteside, 2018), but it has not been reported in UPEC strains. Cadaverine, which is primarily a bacterial metabolite (Tofalo et al., 2019), is the product of the decarboxylation of lysine, and it is thought to be synthesized by *E. coli* in anaerobic and low pH conditions (Watson et al., 1992).

Putrescine, produced both by human and bacterial cells (Tofalo et al., 2019), is a product of amino acid catabolism (Tabor & Tabor, 1985), either from ornithine or agmatine, the latter being a product of the decarboxylation of arginine (Figure 2.1).

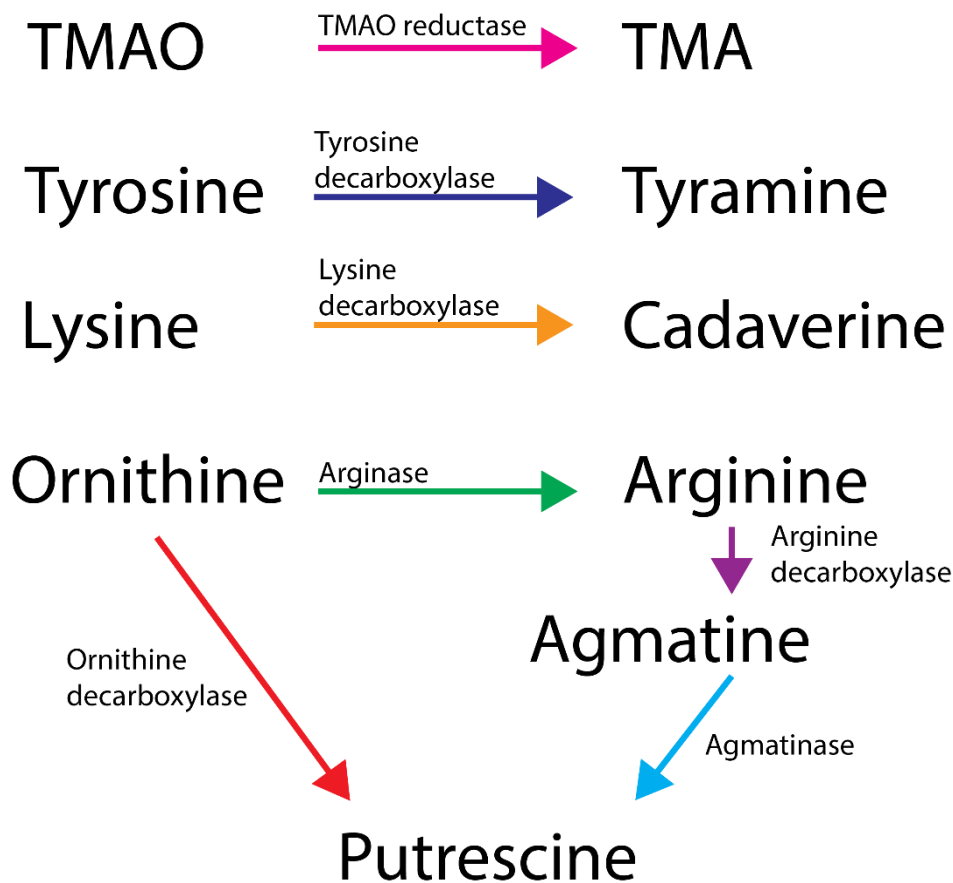


Figure 2.1 Biosynthetic origin of biogenic amines

Each arrow represents a single reaction catalyzed by the described enzyme.

Table 2.1 Odour characteristics of biogenic amines

COMPOUND	ODOUR DESCRIPTION	ODOUR DETECTION THRESHOLD	ODOUR STRENGTH
Trimethylamine	Fishy/oily/rancid/sweaty/fruity(The Good Scents Company, n.d.-a)	0.000032 ppm (Leonardos et al., 1969)	Very high (The Good Scents Company, n.d.-a)
Tyramine	Mild/meaty/dirty/cooked/phenolic/rubbery (The Good Scents Company, 2006)	NA	Moderate (The Good Scents Company, 2006)
Cadaverine	Sperm/dead-animal/animalic (The Good Scents Company, n.d.-b)	190 ppm (Wang et al., 1975)	NA
Putrescine	Animal/rotting/fish (The Good Scents Company, n.d.-c)	22 ppm (Wang et al., 1975)	NA

It has long been hypothesized that some of these compounds play an important role in pathogenesis. For instance, entero-invasive strains of *E. coli* are unable to produce cadaverine, a compound that interferes with the invasive process (Maurelli et al., 1998). While putrescine enhances survival within oxidative environments (Campilongo et al., 2014) and augments cell growth and proliferation (Igarashi & Kashiwagi, 2018). Consistent with these observations, Satink et al. showed that putrescine levels increased in patients with UTI before antibiotic therapy, then decreased as a result of the treatment, presumably because the producers were eradicated (Satink et al., 1989). However, no differences were detected for cadaverine in either case, likely because urinary cadaverine is primarily derived from the gastrointestinal microbiota and dietary sources (Tofalo et al., 2019).

It has been reported that cadaverine production increases when UPEC strains are grown under nitrosative stress, potentially conditioning them for enhanced colonization of the host (Bower et al., 2009; Bower & Mulvey, 2006). This is of relevance since, during a UTI, there is a significant formation of nitric oxide and reactive nitrogen intermediates (Carlsson et al., 2001; Wheeler et al., 1997). Nevertheless, the specific role of cadaverine in resistance to nitrosative stress is unclear. Potentially, during an infection UPEC strains produce a significant amount of cadaverine, which would, in turn, cause malodorous urine. Yet, previous work has shown no change in cadaverine levels during UTI (Satink et al., 1989).

The present study aimed to take a metabolomics approach to identify molecules responsible for urinary malodour and their biosynthetic origins.

2.3 Materials and Methods

2.3.1 Urine samples collection and processing

For *in vitro* testing purposes, mid-stream urine samples were collected from three healthy pre-menopausal women (age 20-40). These were centrifuged at 4500 x *g* for 10 min to remove any solid particles, followed by filter-sterilization using 0.22 µm syringe filters. To ensure asepsis, 10µL of urine were plated on LB plates. To determine hydration levels, and for normalization purposes, creatinine concentration was quantified using the Creatinine (urinary) Colorimetric Assay Kit (Cayman Chemical Co., Ann Arbor, MI, USA) as described by the manufacturer. Hydration levels were determined according to a validated 8-scale urine colour chart (Armstrong et al., 1994; Rolker et al., 2017).

Creatinine levels were used to adjust the concentrations of the metabolites present in the sample as it is an indicator of the hydration levels of the patient, hence allowing to adjust for potential variability in the overall dilution of the urine output.

Thirty-one urine samples from women with UTI were provided by Dr. Ana Cabrera from the Division of Microbiology at the Pathology and Laboratory Medicine Centre of London Health Sciences Centre (London, Ontario, Canada). None of the patients were identified to the study group. These samples included 10 UTI-negative controls and 21

samples from patients clinically diagnosed with a UTI, from which 15 were positive for *E. coli*, 3 for *Staphylococcus aureus*, 2 for *Klebsiella pneumoniae*, and 1 for *Pseudomonas aeruginosa*. Diagnosis criteria were based on the presence of signs and symptoms and significant bacteriuria (bacterial growth $\geq 10^5$ colony-forming units (CFU)/mL). To further characterize the samples, pH was measured using test strips and creatinine was quantified. Figure 2.2 shows a linear correlation between both methods used to determine hydration levels.

All urine samples were obtained in accordance to article 2.4 of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans – TCPS 2 (2018).

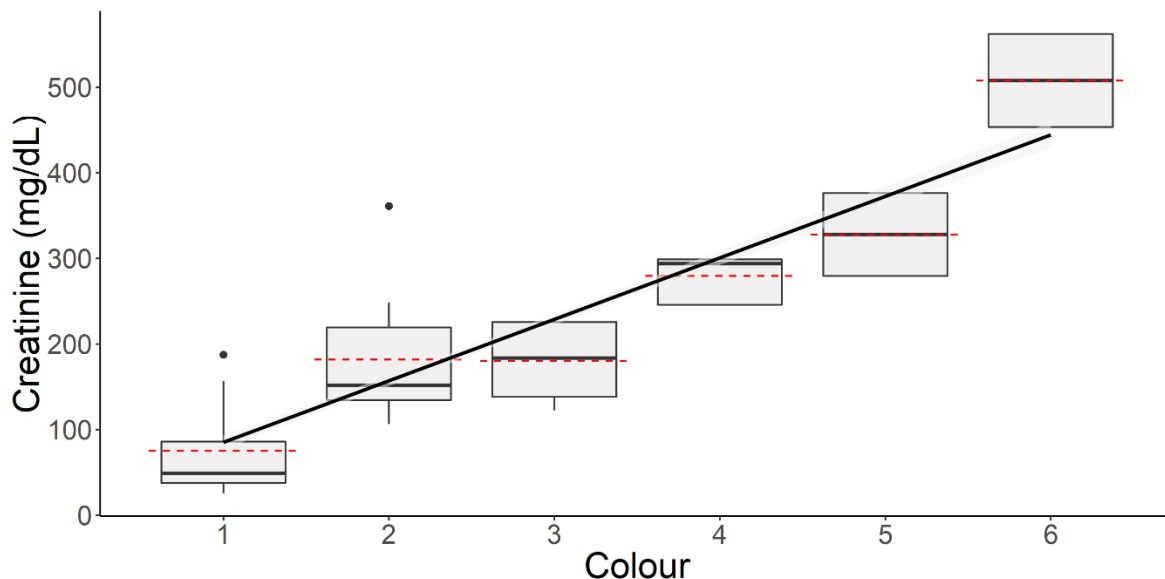


Figure 2.2 Validation of the methods used to measure hydration levels

The dashed red line indicates the mean creatinine level and the black line represents the median. There is a positive correlation between the two methods ($R^2 = 0.7103$).

2.3.2 LC-MS analysis of urine samples

Samples were prepared based on the methodology proposed by Spagou et al., 2011. Briefly, aliquots of 250 μ L were taken of each urine culture and diluted with pure methanol (1:3 for clinical samples and 1:6 for the *in vitro* analysis), vortexed, incubated on ice for 30 min, and centrifuged at $\times 16000g$ for 10 min. Supernatants were then filtered using 0.22 μ m PTFE syringe filters and added to vials for analysis. Samples were

analyzed using a Q-Exactive Quadrupole Orbitrap MS, coupled to an Agilent 1290 HPLC system with an Agilent HILIC-Z (2.1 × 100 mm, 2.7 μm; Agilent) column. Compounds were resolved with mobile phases of 20 mM ammonium formate in water (A) and 20 mM ammonium formate in 90% acetonitrile (B) operating with the following gradient: 0 min, 100% B; 0.5 min, 100% B; 5.3 min, 80% B; 9.5 min, 30% B; 13.5 min, 30% B, 14.5 min 100% B and 16.5 min, 100% B. The following conditions were used for heated electrospray ionization (HESI): HESI(+) capillary voltage, capillary voltage, 3.5 kV; capillary temperature, 250 °C; sheath gas, 30.00 units; auxiliary gas, 8.00 units; probe heater temperature, 450 °C; S-Lens RF level, 60.00. The LC-MS was operated using a top 3 data-dependent acquisition (DDA) experiment that involved a full MS scan in the mass range of m/z 58-870 at 35,000 resolution, followed by MS/MS scans at 17,500 resolution, isolation window of m/z 1.2 and collision energy of 28. The biogenic amines and amino acids quantified in the full MS scans were: cadaverine, putrescine, tyramine, spermine, spermidine, histamine, TMA, phenylalanine, leucine, tryptophan, isoleucine, methionine, valine, tyrosine, proline, alanine, threonine, glutamine, serine, asparagine, glutamic acid, aspartic acid, histidine, arginine, cystine, lysine, ornithine, glycine, and TMAO. The standards used for calibration curves were acquired from Sigma Aldrich (St. Louis MO). TMAO was analyzed semi-quantitatively using the log transformation of its peak area (Figure S7).

2.3.3 Uropathogenic *Escherichia coli* cultures

A time-course analysis was performed using clinical UPEC strains GR-12, IA2, 536 and J96, to assess whether there is a significant difference in polyamine production amongst strains over time. All the strains used were clinical isolates from patients with acute UTI (Clegg, 1982; Edén et al., 1982; Hull et al., 1981; Müller et al., 1983). Samples were prepared by inoculating pooled urine (from three healthy females) with UPEC and incubated aerobically for 24 h at 37° C; aliquots were taken at 0h, 3h, 6h, 9h, 12h, and 24h, and analyzed through HILIC-MS, post-acquisition recalibration was performed to the results and the concentrations of the metabolites of interest was quantified. Growth was monitored measuring optic density at a wavelength of 600 nm to ensure uniform growth of the strains.

2.3.4 LC-MS Data analysis

Proteowizard (Chambers et al., 2012) was used to convert the Thermo .raw files to.mzml format, with peak peaking filter applied. Features were detected using the XCMS package (Benton et al., 2010; Smith et al., 2006; Tautenhahn et al., 2008) with the centWave (Tautenhahn et al., 2008) method (ppm tolerance 1.0). The signal to noise threshold was set to 5, the noise was set to 3×10^6 and pre-filter was set to six scans with a minimum 5,000 intensity. Retention time correction was conducted using the obiwarp (Prince & Marcotte, 2006) method. Grouping of features was set to those present in at least 25% of all samples (retention time deviation 10 s; m/z width, 0.015). The 'fillPeaks' function with default settings. Remaining zeros values were imputed with two thirds the minimum value on a per mass basis. Large mass salt clusters and ionization artifacts were filtered using the McMillan correction (McMillan et al., 2016). For the targeted analysis, Xcalibur (Thermo Scientific, Waltham, MA) was used to quantify the metabolites of interest. Compounds were identified by accurate mass, comparison of retention times to authentic standards or by accurate mass and comparison of fragmentation patterns to MS/MS databases (Smith et al., 2005).

The data generated were submitted to the EMBL-EBI MetaboLights database with the identifier MTBLS1294 (<https://www.ebi.ac.uk/metabolights/MTBLS1294>) (Haug et al., 2013).

2.3.5 Statistical analysis

RStudio version 1.2.1335 was used for all statistical analyses. Plots were generated with the ggplot2 package (Wickham, 2009). For the untargeted metabolomics analysis, the FactoMineR package with Pareto scaling was used to perform a PCA (McMillan et al., 2016). Targeted metabolomics comparisons in the clinical samples were analyzed using a two-sample two-tailed t-test analysis and defined a p-value of ≤ 0.05 as statistically significant. Time-course trials were analyzed using a linear mixed-effect model with Tukey's test for multiple comparisons and to control for type I error. Log transformation was used to correct the distribution and heteroscedasticity of the data (Di Guida et al., 2016).

2.4 Results

2.4.1 Clinical sample characterization

In total, 31 human urine samples were obtained, comprising both of clinically defined UTI-negative ($n = 10$) and UTI-positive ($n = 21$) samples. Of the latter, *E. coli* was the dominant pathogen in 15 individuals, while *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* were dominant in 3, 2 and 1 individuals, respectively. There were no significant associations between UTI and urinary pH or creatinine concentration (Supplementary Material, Figures S1, S2). Only samples positive for *E. coli* were discussed in the subsequent results. Analyses of samples infected by other pathogens can be found in the Supplementary section.

2.4.2 Metabolomics of UTI-positive clinical samples

The metabolomic profiles of the human urine samples were analyzed by high-resolution LC-MS. Biogenic amines and other small polar compounds are not well suited for chromatographic separation using a C18 reverse-phase column. Therefore, a hydrophilic interaction chromatography (HILIC) approach was used (Figure 2.3).

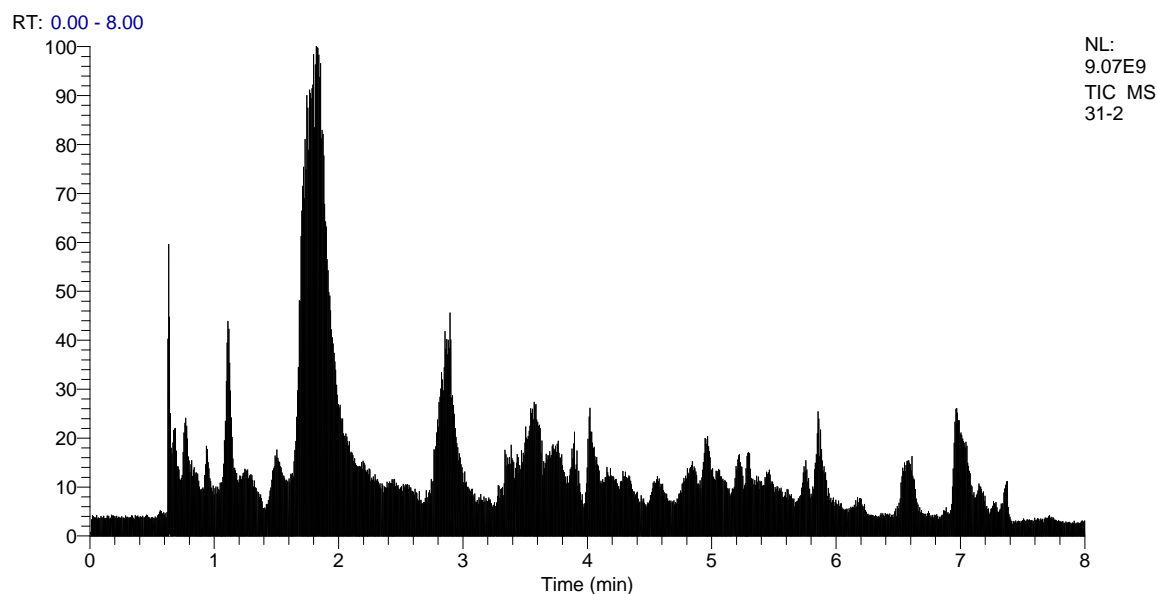


Figure 2.3 Chromatogram of an UTI positive sample

Separation carried out using HILIC chromatography, ideally suited for polar compounds (e.g. biogenic amines and amino acids).

The features extracted by XCMS were analyzed using a principal components' analysis (PCA) model. No distinct grouping of samples could be observed in the 1st dimension. However, separation of both UTI and healthy groups was observed in the 2nd and 4th (27.2% total explained variance). The main compounds responsible for the separation of these groups were TMA and TMAO (Figure 2.4).

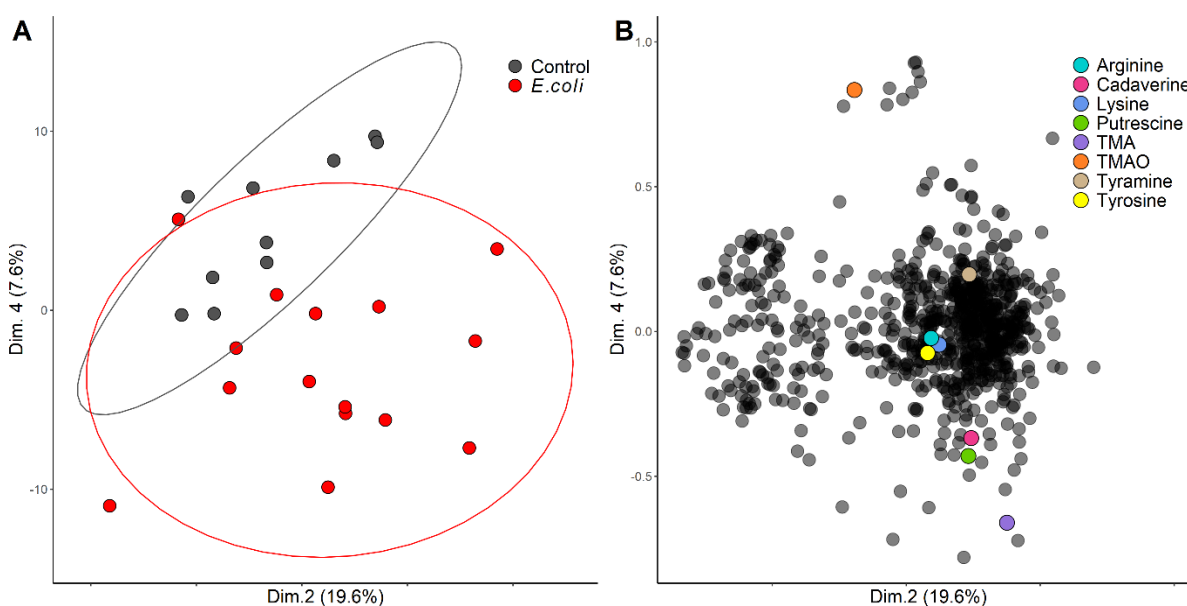


Figure 2.4 TMA and its precursor TMAO are driving the separation between UTI-positive and negative samples in multivariate analysis

(A) Principal components analysis (PCA) score plot of Healthy (black, $n=10$) vs UTI-positive samples (red, $n=15$), where each point represents a single sample from a single woman. The location of each point displays differences in the metabolome, with samples closer to each other being similar. Ellipses represent the 95% confidence intervals; (B) PCA loadings, which show the weights of each metabolite in the principal component cartesian plane. Each point represents a single metabolite. Biogenic amines, as well as their precursors, are colour coded. Biogenic amines, as well as some standard amino acids, were also quantified in the clinical urine samples. Following normalization with urinary creatinine concentrations, both TMA and putrescine were significantly elevated between groups, whereas cadaverine and tyramine were not (Figure 2.5).

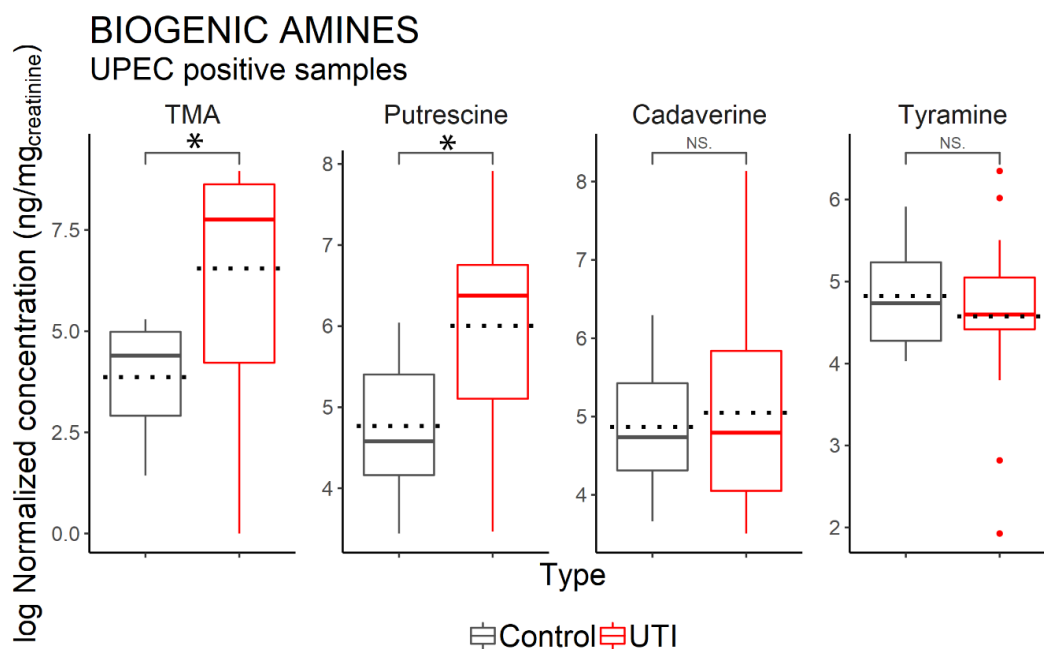


Figure 2.5 Comparison of the concentration of biogenic amines in Healthy vs UTI positive patients

Control consists of the urine of healthy patients (n=10). TMA and putrescine levels are higher in patients with UPEC caused UTI (n=15). Only the samples positive for UPEC were included in this analysis. Black lines indicate the median and the red dashed lines represent the mean. Significant differences were determined on log-transformed values using a two-sample t-test ($*p \leq 0.05$).

The concentrations of the precursors of these amines are shown in Figure 2.6; only TMAO—which yields TMA—was significantly lower in UTI patients. On the amino acid analysis, aspartic acid and glutamic acid were significantly increased in the UTI positive group, whereas serine and asparagine were decreased (Figure 2.7).

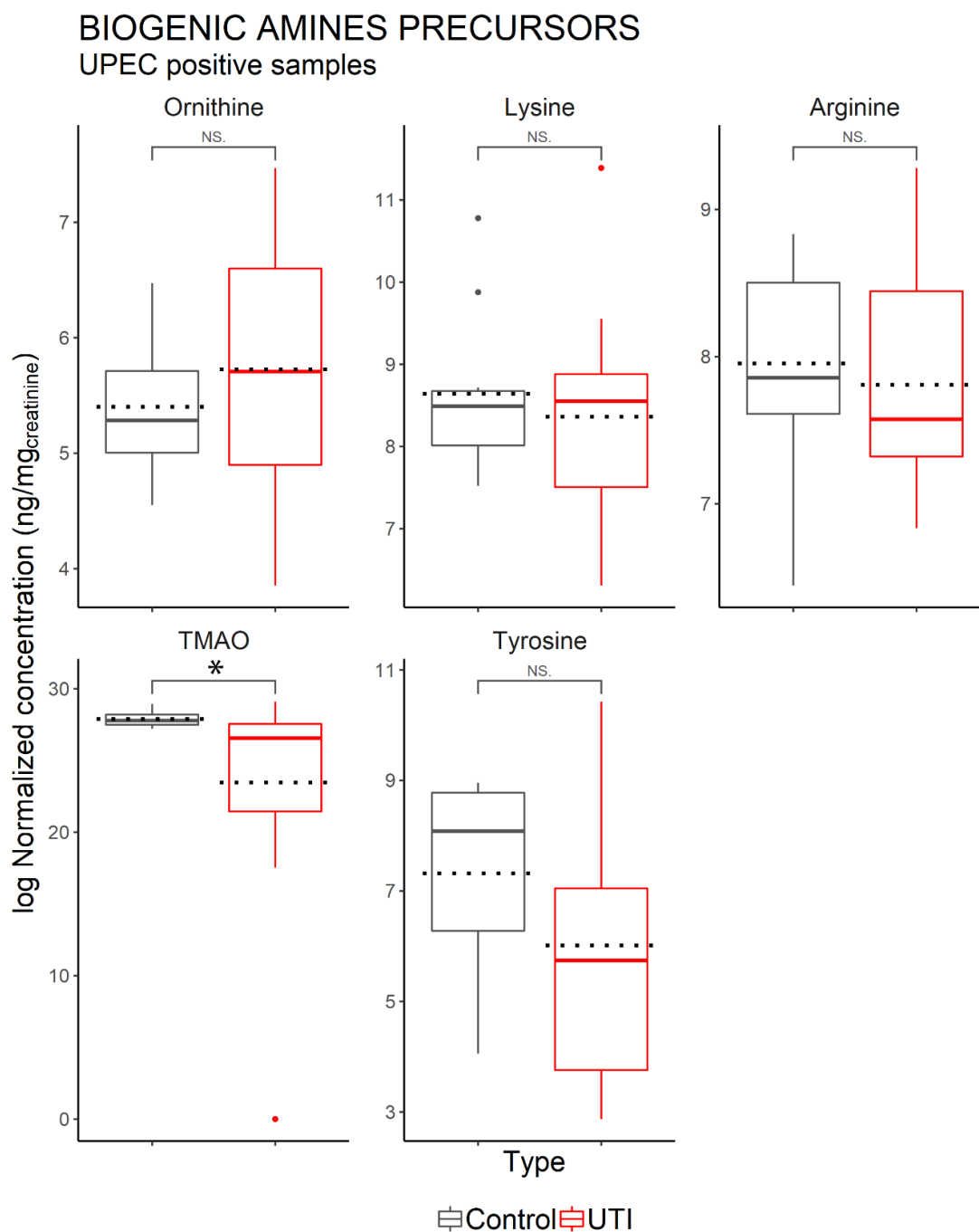


Figure 2.6 Comparison of the concentration of the precursors of biogenic amines in Healthy vs UTI positive patients

Control consists of the urine of healthy patients ($n=10$). TMAO, which is the precursor of TMA, was significantly decreased. Only the samples positive for UPEC were included in this analysis ($n=15$). Black lines indicate the median and the red dashed lines represent the mean. Significant differences were determined on log-transformed values using a two-sample t-test ($*p \leq 0.05$).

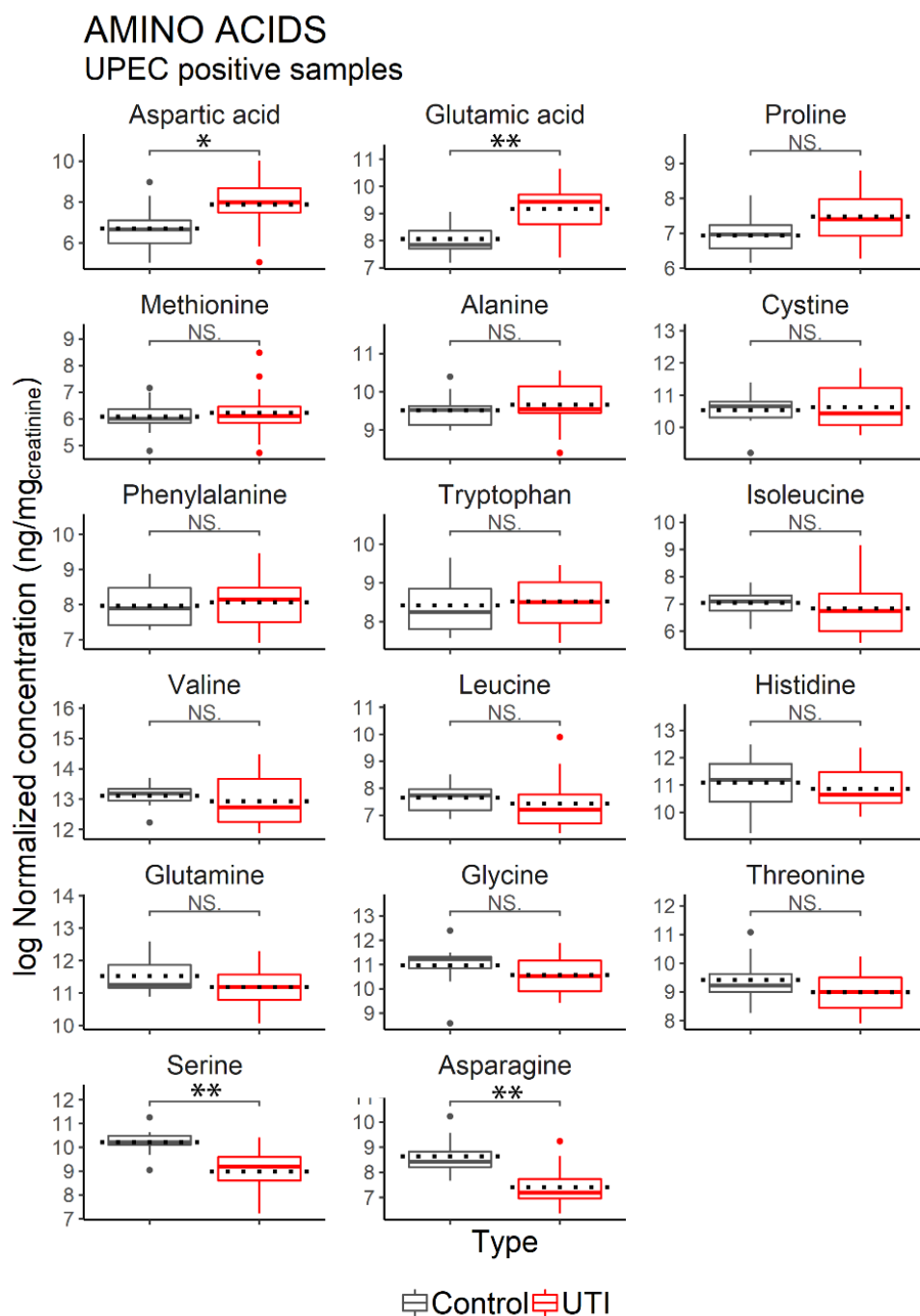


Figure 2.7 Comparison of the concentration of amino acids in Healthy vs UTI positive patients

Control consists of the urine of healthy patients ($n=10$). Glutamic acid and aspartic acid are significantly elevated in UTI patients, while serine and asparagine are significantly lower. Only the samples positive for UPEC were included in this analysis ($n=15$). Significant differences were determined on log-transformed values using a two-sample t-test ($*p \leq 0.05$, $**p \leq 0.005$).

2.4.3 *In vitro* analysis of UPEC strains in sterile urine

To better understand the metabolic dynamics involved in the production of biogenic amines by UPEC, the presence of metabolites from four different clinical UPEC strains grown in sterile female urine was assessed over 24 hours. Many of the biogenic amines identified in clinical samples were also found *in vitro* and showed significant changes within 3 hours (Figure 2.8 and 2.9).

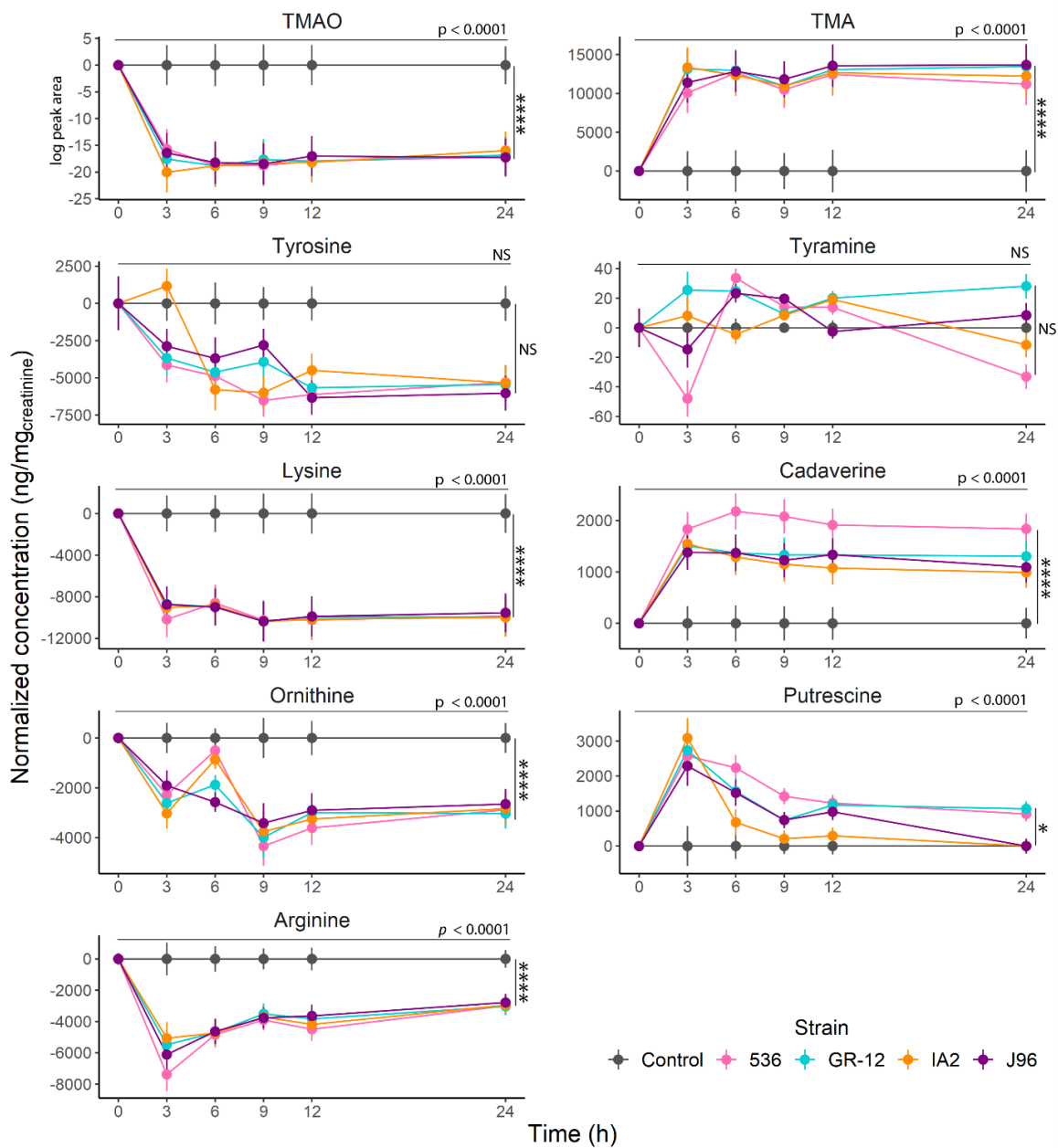


Figure 2.8 Time course analysis on the formation and uptake of biogenic amines and their precursors by different UPEC strains

Precursors on the left column and biogenic amines on the right. TMAO was analyzed semi-quantitatively based on the log of its peak area. Metabolite levels standardized using the control as a baseline, which consisted of sterile urine spiked with LB media. A linear mixed-effect model with repeated measures was used to calculate statistical significance. Tukey test was used to determine individual differences and to control for type I error (Tables 2.2 and 2.3). The horizontal significance line and p-value correspond to the main effect of Time over the concentration of each metabolite after 24h. Vertical significance corresponds to the comparison of each strain vs the control. Data are presented as means of 3 independent experiments \pm SEM. (* $p \leq 0.05$, *** $p \leq 0.0001$).

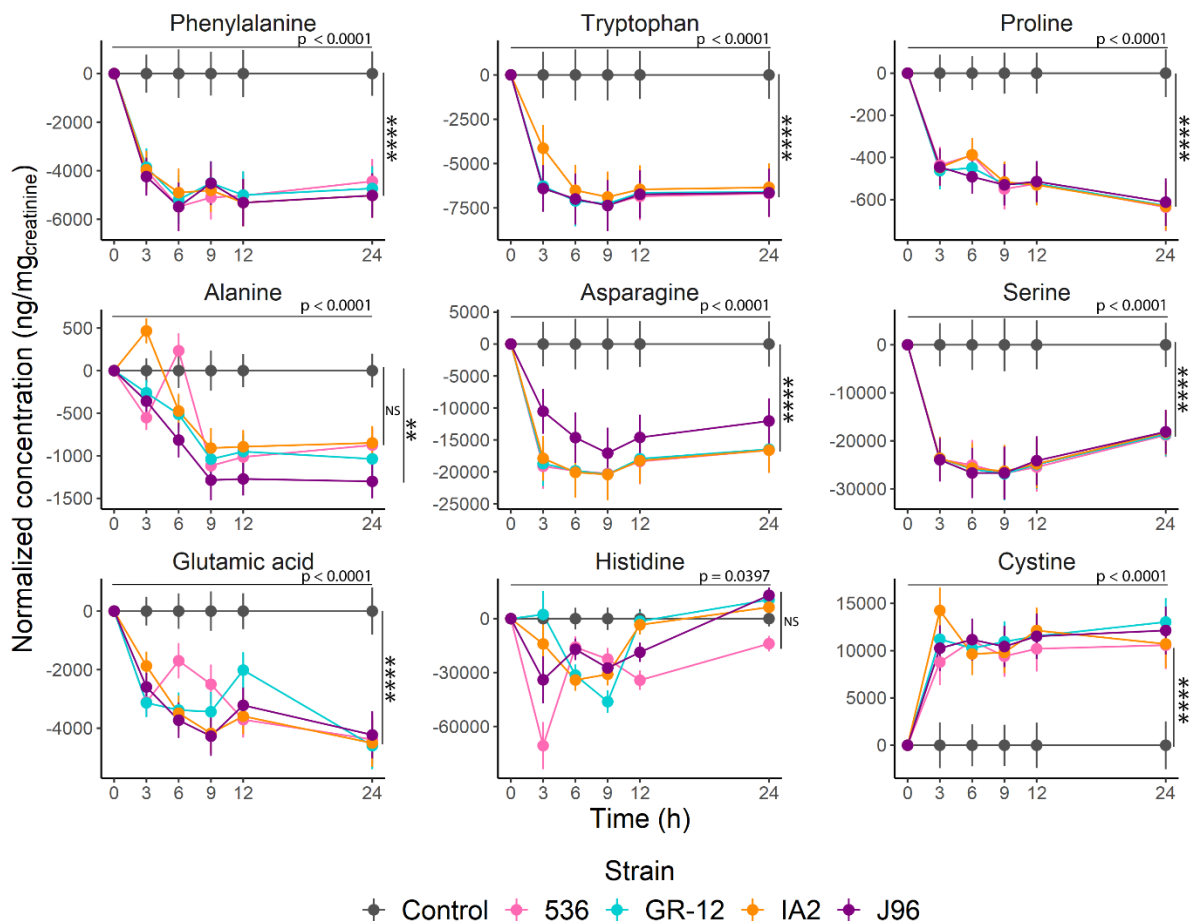


Figure 2.9 Time-course analysis of the formation and uptake of amino acids by different UPEC strains

Metabolite levels standardized using the control as a baseline, which consisted of sterile urine spiked with LB media. A linear mixed-effect model with repeated measures was used to calculate statistical significance. Tukey post-hoc test was used to determine individual differences and to control for type I error (Tables 2.2 and 2.3). The horizontal significance line and p-value correspond to the main effect of Time over the concentration of each metabolite. Vertical significance corresponds to the comparison of each strain vs the control after 24h. Data are presented as means of 3 independent experiments \pm SEM. (** $p \leq 0.01$, **** $p \leq 0.0001$).

Similar trends in terms of metabolite changes were observed between all five tested UPEC strains (Tables 2.2 and 2.3), showing an accumulation of cadaverine and TMA, whereas a maximal amount of putrescine was produced after 3h and diminished thereafter. Except for cystine, where an accumulation over time was observed, most amino acids were reduced in concentration by the UPEC strains during the incubation. Alanine showed significant spikes at hours 3 and 6 by strains IA2 and 536 respectively

and was degraded thereafter; after 24h only strain J96 was significantly different from the control. Histidine and tyramine did not show a major change in amounts relative to their starting concentrations. The amino acids: aspartic acid, methionine, isoleucine, valine, leucine, glutamine, glycine, and threonine were not included in this analysis as they were below the detection limit of the method.

Table 2.2 Multiple comparisons between strains of the concentration of biogenic amines across time

Only significant values are shown, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

Strain 1	Metabolite	Time (h)	Strain 2	p value	Significance level	
536	Putrescine	6	IA2	≤ 0.0001	****	
		12	IA2	0.039	*	
		24	IA2	0.0449	*	
			J96	0.0449	*	
	Cadaverine	3	GR-12	≤ 0.0001	****	
			GR-12	≤ 0.0001	****	
			IA2	≤ 0.0001	****	
			J96	≤ 0.0001	****	
		6	GR-12	≤ 0.0001	****	
			IA2	≤ 0.0001	****	
			9	J96	≤ 0.0001	****

			GR-12	≤ 0.0001	****
			IA2	≤ 0.0001	****
		12	J96	≤ 0.0001	****
			GR-12	≤ 0.0001	****
			IA2	≤ 0.0001	****
		24	J96	≤ 0.0001	****
	TMA		GR-12	≤ 0.0001	****
	TMA	3	IA2	≤ 0.0001	****
	TMA	6	IA2	≤ 0.0001	****
			IA2	0.0131	*
	Putrescine	24	J96	0.0131	*
		9	IA2	0.0432	*
		12	IA2	0.0013	**
			IA2	0.0001	****
	Cadaverine	24	J96	0.0113	*
GR-12	TMA	3	J96	0.0293	*
IA2	TMA	3	IA2	0.0109	**

Table 2.3 Multiple comparisons between strains of the concentration of amino acids and/or biogenic amines precursors across time

Only significant values are shown, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

Strain 1	Metabolite	Time (h)	Strain 2	p value	Significance level
536	TMAO	3	IA2	≤ 0.0001	****
	Ornithine	6	GR-12	0.0058	**
			J96	≤ 0.0001	****
	Arginine	3	GR-12	0.0166	*
			IA2	0.002	**
	Proline	6	J96	0.0014	**
	Alanine	3	IA2	0.0355	*
		6	J96	0.0282	*
	Glutamic acid	3	IA2	0.0331	*
			GR-12	0.0013	**
			IA2	0.0006	***
			J96	0.0001	****
		9	IA2	0.0014	**
			J96	0.0007	***
		12	GR-12	0.0013	**

	Histidine	3	GR-12	0.0303	*
	Cystine	6	IA2	0.0002	***
GR-12	TMAO	3	IA2	0.0187	*
	Tryptophan	3	IA2	≤0.0001	****
	Glutamic acid	3	IA2	0.0283	*
			IA2	0.0031	**
12		J96	0.0406	*	
IA2		3	J96	0.0385	*
		6	J96	0.0003	***
	Tryptophan	3	IA2	≤0.0001	****
	Proline	6	J96	0.001	***
	Cystine	6	IA2	0.0115	*
J96	Asparagine	3	536	≤0.0001	****
			GR-12	≤0.0001	****
			J96	≤0.0001	****
		6	536	≤0.0001	****
			GR-12	≤0.0001	****
			IA2	≤0.0001	****
		9	536	0.0242	*
			GR-12	0.0177	*

		IA2	0.016	*
	12	536	0.005	**
		GR-12	0.0163	*
		IA2	0.0074	**
	24	536	0.0004	***
		GR-12	0.0006	***
		IA2	0.0004	***

2.5 Discussion

Here, we present a comprehensive profile of the production of odorous compounds during UPEC-UTI. Only the biogenic amines TMA and putrescine were present at high concentrations in patients with UTIs compared to controls. This contrasts with results in patients with BV, where cadaverine and tyramine are the most elevated malodorous compounds (McMillan et al., 2015; Nelson et al., 2015).

Using an *in vitro* model, cadaverine was found to be significantly increased, perhaps due to nitrosative stress (Carlsson et al., 2001; Keay et al., 2014) which is higher in a steady-state culture than in the human bladder, allowing for its accumulation. The *in vitro* model provides a static system that allows for better appreciation of the rates of consumption and production of specific metabolites, while *in vivo*, there is a continuous flow of urine into the bladder from the kidneys that replenishes substrates and makes it difficult to identify the changes in metabolite concentration.

Notably, differences were also found in cadaverine levels between strains. Following reports that cadaverine production can interfere with the invasive process of UPEC, we hypothesize that its production is reduced during colonization of the bladder as a means to enhance infectivity (Maurelli et al., 1998). Thus, the environment of the bladder may

influence the expression of the genes that regulate cadaverine production and perhaps explain why previous reports have found contrasting results.

Putrescine levels were increased *in vivo* and *in vitro* in agreement with findings in patients with an overactive bladder (Keay et al., 2014). This is associated with an increase in ornithine decarboxylase, which has been shown to block the calcium potassium channel. Since putrescine levels can modulate intracellular calcium levels in urothelial cells, it suggests a correlation between UTIs and overactive bladder (Tang & Hazen, 2014) and a role for putrescine in urinary urgency. This was further supported by the finding that ornithine, a major precursor of putrescine, showed a marked decrease in the *in vitro* samples. More studies are warranted to investigate this result.

TMA was one of the metabolites that exhibited a highly significant increase in the present study. The precursor of TMA, TMAO, was significantly different between the clinical samples. It is worth investigating whether the increase of TMA during a UTI could affect other symptoms and signs. Acetylcholine present in urine can be converted into choline via the enzyme AChE, allowing TMA to be produced from choline through choline TMA lyase activity. The cholinergic system has an important role in voiding the bladder (Tang & Hazen, 2014), with acetylcholine from parasympathetic nerves and non-neuronal cells within the urothelium, affecting, directly and indirectly, muscle contraction (Tang & Hazen, 2014).

Except for cystine, most of the amino acids analyzed in our time-course were degraded over time. Previous reports have shown that when UPEC strains are grown in urine, several genes responsible for amino acid catabolism are upregulated (Snyder et al., 2004). It is thought that this is an important ability of UPEC to colonize the bladder (Alteri & Mobley, 2015). Notably, serine was significantly reduced both *in vivo* and *in vitro*, suggesting it is being used as a substrate by UPEC. This is consistent with previous reports showing upregulation of genes responsible for amino acid catabolism, specifically D-serine deaminase (*dsdA*) when UPEC were grown in human urine (Mann et al., 2017; Snyder et al., 2004). Both D- and L-serine are gluconeogenic amino acids that can be degraded to produce oxaloacetate or pyruvate, which can enter the TCA cycle, therefore,

they can be used as a carbon source (Alteri & Mobley, 2015). Furthermore, the catabolism of D-serine in the urinary tract has been identified as a key signaling mechanism for virulence gene expression (Anfora et al., 2007; Haugen et al., 2007; Roesch et al., 2003).

A significant decrease in asparagine levels was also found. Asparagine metabolism by *E. coli* has not been extensively studied, but it is thought that the organism can utilize this amino acid by conversion to aspartic acid using an asparaginase enzyme (Willis & Woolfolk, 1975). This is in agreement with our results, where asparagine was significantly reduced while aspartic acid increased.

Overall, this study revealed that the odorous compounds TMA and putrescine are present at high concentrations during a UTI and are produced by UPEC strains. It is important to note that these findings do not necessarily mean that higher concentrations of biogenic amines cause an increase in malodour, and further sensory studies with trained analytical panelists are required. Nonetheless, our findings open the door to the development of new ways to reduce urinary malodour, a problem that is particularly prevalent in urinary incontinence patients and takes days to resolve when antibiotics are administered.

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Chapter 3

3 Interstrain variability of human vaginal *Lactobacillus crispatus* for metabolism of biogenic amines and antimicrobial activity against urogenital pathogens

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3.1 Abstract

Lactobacillus crispatus is the dominant species in the vagina of many women. With the potential for strains of this species to be used as a probiotic to help prevent and treat dysbiosis, we investigated isolates from vaginal swabs with *Lactobacillus*-dominated and a dysbiotic microbiota. A comparative genome analysis led to the identification of metabolic pathways for synthesis and degradation of three major biogenic amines in most strains. However, targeted metabolomic analysis of the production and degradation of biogenic amines showed that certain strains have either the ability to produce or to degrade these compounds. Notably, six strains produced cadaverine, one produced putrescine, and two produced tyramine. These biogenic amines are known to raise vaginal pH, cause malodour, and make the environment more favourable to vaginal pathogens. *In vitro* experiments confirmed that strains isolated from women with a dysbiotic vaginal microbiota have higher antimicrobial effects against the common urogenital pathogens *Escherichia coli* and *Enterococcus faecium*. The results indicate that not all *L. crispatus* vaginal strains appear suitable for probiotic application and the basis for selection should not be only the overall composition of the vaginal microbiota of the host from which they came, but also specific biochemical and genetic traits.

3.2 Introduction

As the dominant organism in the vagina of many healthy women, *Lactobacillus crispatus* is thought to be an important contributor to reproductive health (Petrova et al., 2015). For this reason, the species has been postulated to be an excellent candidate for probiotic use to restore and maintain vaginal health (Barrons & Tassone, 2008). Given the high incidence of conditions that result from vaginal dysbiosis (McMillan et al., 2015; Reid, 2018; Schlabritz-Loutsevitch et al., 2016), including urinary tract infections (UTIs) and bacterial vaginosis (BV), as well as an increased risk of sexually-transmitted diseases and preterm labour (Reid, 2018), vaginal administration of *Lactobacillus crispatus* has been shown to be an effective approach to improving women's health (Cohen et al., 2020). The species' ability to adhere to vaginal epithelial cells, block the adherence of pathogenic bacteria, are believed to be important mechanistic contributors (Andreu et al., 1995), as well as a broad-spectrum inhibitory activity against a range of Gram-positive and Gram-negative bacteria (Kim & Rajagopal, 2001). This is in part due to the production of lactic acid, but also other molecules, including peptide-based antimicrobial bacteriocins may be involved.

Another relevant marker of female urogenital health is the presence or absence of large amounts of biogenic amines such as putrescine, cadaverine, and tyramine (Nelson et al., 2015). These compounds, commonly produced by urogenital pathogens, are elevated in patients with BV (McMillan et al., 2015) and UTI (Puebla-Barragan et al., 2020), and are known causes of malodour. Beyond that, large amounts of these compounds increase the odds of vaginal dysbiosis (Borgogna et al., 2021). Therefore, a high abundance of strains that reduce the amounts of biogenic amines are desirable in the vaginal environment. Since *L. crispatus* is highly abundant in healthy vaginas it is likely that this species has the ability to regulate the levels of biogenic amines, however, this property has not been evaluated yet.

However, it is not clear if these properties and the prevalence of this *L. crispatus* species are sufficient to select candidate probiotic strains. As bacterial metabolites play a role in host health and disease, the present study was designed to examine the by-products of strains of *L. crispatus* isolated from healthy women and those with dysbiosis (van der Veer et al., 2019). Liquid chromatography-mass spectrometry (LC-MS) was used, given its ability to differentiate by-products of vaginal microbiota strains (McMillan et al., 2015).

3.3 Materials and methods

3.3.1 Bacterial strains used in this study

The strains, listed in Table S1 (Appendix D), were collected as outlined by van der Veer *et al.* (2019) with all material approved by each subject with written consent and prior approval obtained from the Institutional Review Board (IRB) (document reference number W12_086 # 12.17.0104).

In brief, swabs were obtained from the Sexually Transmitted Infections clinic in Amsterdam, the Netherlands. Subjects RL01, RL03, RL04, RL05, RL06, RL08, RL09, RL10, RL11, RL12, RL16, RL21, RL22, RL26, RL27, and RL32 were healthy women whose vaginal swabs were dominated by lactobacilli (LVM) as determined by a Nugent score 0-3; the other subjects had a dysbiotic vaginal microbiota (DVM) with Nugent score 7-10. The swabs were plated on modified Trypticase Soy Agar and incubated anaerobically at 37°C for 24h. Following this, single colonies underwent 16S sequencing for identification purposes. The strains were then cryopreserved at -80°C in vaginally defined medium plus peptone (VDMP) (Geshnizgani & Onderdonk, 1992). A total of 28 strains were genome sequenced using Illumina MiSeq to generate FASTQ workflow. The genomes were assembled, reordered, and annotated and deposited at DDBJ/ENA/GenBank (Appendix D).

The 28 strains along with an additional four strains (RL01, RL04, RL12, RL22) were brought to Canada for antimicrobial analysis but unfortunately 13 did not

survive (Table S2 in Appendix D). One strain, RL33, then did not grow sufficiently well for metabolomic analysis.

For antimicrobial testing, *Enterococcus faecalis* ATCC 19433 (Nilsen et al., 2003), an oral isolate of *Lactobacillus helveticus* (Fremaux & Klaenhammer, 1994), and a beer isolate of *Pediococcus pentosaceus* (Jiang et al., 2021), were used as positive controls for the production of bacteriocins as known producers. Strains from uropathogenic species: *Enterococcus faecium* ATCC 19434 (Agudelo Higueta & Huycke, 2014), *Escherichia coli* UTI 89 (Mysorekar & Hultgren, 2006), *Enterococcus faecalis* ATCC 19433 (Agudelo Higueta & Huycke, 2014), *Gardnerella vaginalis* ATCC 14018 (Anukam & Reid, 2008), *Prevotella bivia* ATCC 29303 (Atassi et al., 2006), and *Candida albicans* TIMM 1768 (Panthee et al., 2018), were used as indicator strains.

3.3.2 Phylogenetic and functional genomics analyses

To determine if there was a correlation between the health status of the source of the strains and their genomic profiles, available *L. crispatus* genomic assemblies from BioProject PRJNA390079 were downloaded (NCBI; April 2021). All genomes were assessed for quality using QUAST v5.0.2 (Gurevich et al., 2013) and completeness using CheckM 1.1.3 (Parks et al., 2015). Genomic assemblies with N50 <10 kb and completeness of less than 95% were excluded from further analysis. All 28 genomic assemblies passed the quality control and were annotated using Prokka v1.14.5 (Seemann, 2014). The --gram pos and --mincontiglen 200 (bp) were specified. The pangenome was determined using Roary v3.13.0 with the assumptions that a core gene is defined as gene found in all but one of the isolates (>95%) and a minimum percentage identify for blastp of 99% (Page et al., 2015). The core gene phylogenetic tree was constructed using the core gene alignment from Roary in RAxML v8.2.12 (Stamatakis, 2014) with the flags, -f a, -# autoMRE, and -m GTRGAMMA, and visualized using the R package ggtree v2.4.1 (Yu et al., 2017). Functional capacity of the genomes was analyzed using eggNOG v5.0 and eggNOG-mapper v2 using the Lactobacillaceae database (Huerta-Cepas et al., 2017, 2019). Distance matrices for functional category abundance

was calculated using the R package *vegan* v2.5-7 (Oksanen et al., 2020) following an established method (Petrova et al., 2018; Wuyts et al., 2017), and plotted using the R package *ggplot2* v3.3.3 (Wickham, 2016).

3.3.3 LC-MS protocol

Three individual colonies were selected from each strain as independent biological replicates. Next, they were grown anaerobically for 24 hours in VDMP media at 37°C. Following the addition of methanol in a 1:1 ratio, supernatants were collected after centrifugation for 10 minutes at $10,000 \times g$, filtered using 0.45 μ m PTFE syringe filters and deposited into HPLC vials. The samples were separated with an Agilent HILIC-Z column (2.1 \times 100 mm, 2.7 μ M; Agilent) in an Agilent 1290 HPLC system coupled to a Q-Exactive Quadrupole Orbitrap MS. Mobile phases consisted of 20 mM ammonium formate in water (A) and 20 mM ammonium formate in 90% acetonitrile (B). Gradient conditions were as follows: 0 min, 100% B; 0.5 min, 100% B; 5.3 min, 80% B; 9.5 min, 30% B; 13.5 min, 30% B, 14.5 min 100% B and 16.5 min, 100% B. Heated electrospray ionization (HESI) was operated in positive mode at a capillary voltage of 3.4 kV. capillary temperature, 250 °C; sheath gas, 30.00 units; auxiliary gas, 8.00 units; probe heater temperature, 450 °C; S-Lens RF level, 60.00. Full MS scans were obtained in the mass range of m/z 58–870 at 35,000 resolution, MS/MS scans set at 17,500 resolution, isolation window of m/z 1.2, and collision energy of 28.

3.3.4 Metabolite identification

The raw files were converted into .MZML format using ProteoWizard (Chambers et al., 2012) and chromatogram alignment and deconvolution was completed using the XCMS package in R (Smith et al., 2006). Features were detected with the centWave method (Tautenhahn et al., 2008) at a 1 ppm tolerance and the prefilter was set to 3 to 5,000, noise to 1000, and a signal-to-noise threshold of 5. The *obiwarp* (Prince & Marcotte, 2006) method was used to correct retention times. Features present in at least 25% of the samples were grouped. Two-thirds of the

minimum value of each feature was used to replace zeros. The McMillan correction was utilized to remove large mass salt clusters and ionization artifacts (McMillan et al., 2016). Principal component analysis was completed using the FactoMineR package in R (Lê et al., 2008) and the data was exported and analyzed. Score plots were generated using the R package ggplot2 v3.3.3 (Wickham, 2016). Biogenic amines were identified by exact mass and fragmentation patterns.

3.3.5 Biogenic amines pathway analysis

Amino acid sequences relevant to the biosynthetic and biocatalytic pathways of putrescine, cadaverine, and tyramine were downloaded (NCBI; May 2021). FASTA files containing the available *L. crispatus* coding sequences were analysed using blastp. A graphical summary indicating presence or absence of specific enzymes was elaborated using the R package ggplot2 v3.3.3 (Wickham, 2016). Pathways' diagrams were plotted based on the information available at the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database (Kanehisa, 2000, Kanehisa, 2019, Kanehisa et al., 2021).

3.3.6 Agar well diffusion assays

A series of agar well diffusion assays, a standard method used to detect bacteriocins and other antimicrobial compounds, were performed to identify the inhibitory profile of the strains (Holder & Boyce, 1994; Tahara et al., 1996). *L. crispatus* strains were grown as a lawn across the surface for 48 hours on Columbia blood agar (CBA) plates anaerobically in a BD GasPak™ EZ container systems at 37°C. To obtain extracellular anti-microbial molecules in a crude fraction, plates were then frozen at -80°C for 1 hour and thawed at room temperature for 1 hour while inverted to allow the liquid portion to be released from the agar matrix. Following thawing the resulting supernatant was collected from the lid of the plate, the volumes were normalized, the samples were neutralized using NaOH or HCl, and then all samples were filter sterilized (0.22 µm). To establish the inhibitory profile, indicator strains were plated on M17 agar (10% lactose). Next, wells of 1

cm in diameter were bored into the agar using the base of a 1000 μ L pipette tip and 50 μ L of each individual supernatant sample was deposited into the wells. Following an incubation period of 48 hours at 37°C the plates were imaged with a scale and the zones of inhibition were measured using ImageJ software (Schneider et al., 2012).

Included in all assays were pH neutralized *E. faecalis* ATCC 19433, *L. helveticus*, and *P. pentosaceus*, *L. crispatus* supernatants, and a CBA supernatant brought to pH 5 using lactic acid as positive controls for bacteriocin production and a CBA supernatant sample that was neutralized, unaltered, and one that was brought to pH 5 with lactic acid and then neutralized as negative controls. The assays were repeated using indicator strains *E. faecium* ATCC 19434, *E. coli* UTI 89, *E. faecalis* ATCC 19433, *G. vaginalis* ATCC 14018, *P. bivia* ATCC 29303, and *C. albicans* TIMM 1768 (N = 4 biological replicates).

To verify the presence of an inhibitory protein within the supernatants, an agar well diffusion assay with *E. faecium* ATCC 19434 as an indicator strain was repeated in two parts with a small subset of strains, one with supernatants that were heated to 85°C for 45 minutes prior to filter sterilization and a second where the supernatants were treated as described above. The remaining supernatants from this experiment were also subjected to a protein-degrading trypsin, a protease, using 1 mg/mL final concentration and an agar well diffusion assay was conducted.

3.3.7 Statistical analysis

Statistical analyses were completed using RStudio version 1.2.1335. Differences in inhibition areas between groups were evaluated using a linear mixed effects model to control for individual strain effects; the following R packages were used: rstatix v0.7.0 (Kassambara, 2021), emmeans v1.6.0 (Lenth, 2021), and FSA v0.8.32 (Ogle et al., 2021). For differences of the amount of biogenic amines between health-status groups a T-test was performed using the R package ggsignif v0.6.1 (Ahlmann-Eltze, 2021). Differences in biogenic amine amounts between strains were calculated using a one-way analysis of variance (ANOVA) with the Dunnett's

test, to correct heteroscedasticity marginal means were used and the matrix of covariance was adjusted, this was performed using the R packages rstatix v0.7.0 (Kassambara, 2021), emmeans v1.6.0 (Lenth, 2021), and sandwich v3.0-1 (Zeileis, 2004).

3.4 Results

3.4.1 Untargeted metabolomics and functional genomics

PCA analysis of the metabolites identified through LC-MS did not find any clustering between the *L. crispatus* strains as shown in Figure 3.1. Similarly, neither the phylogenetic nor the functional analyses revealed any specific grouping according microbiota status (Figure 3.1 and S1 (Appendix D)).

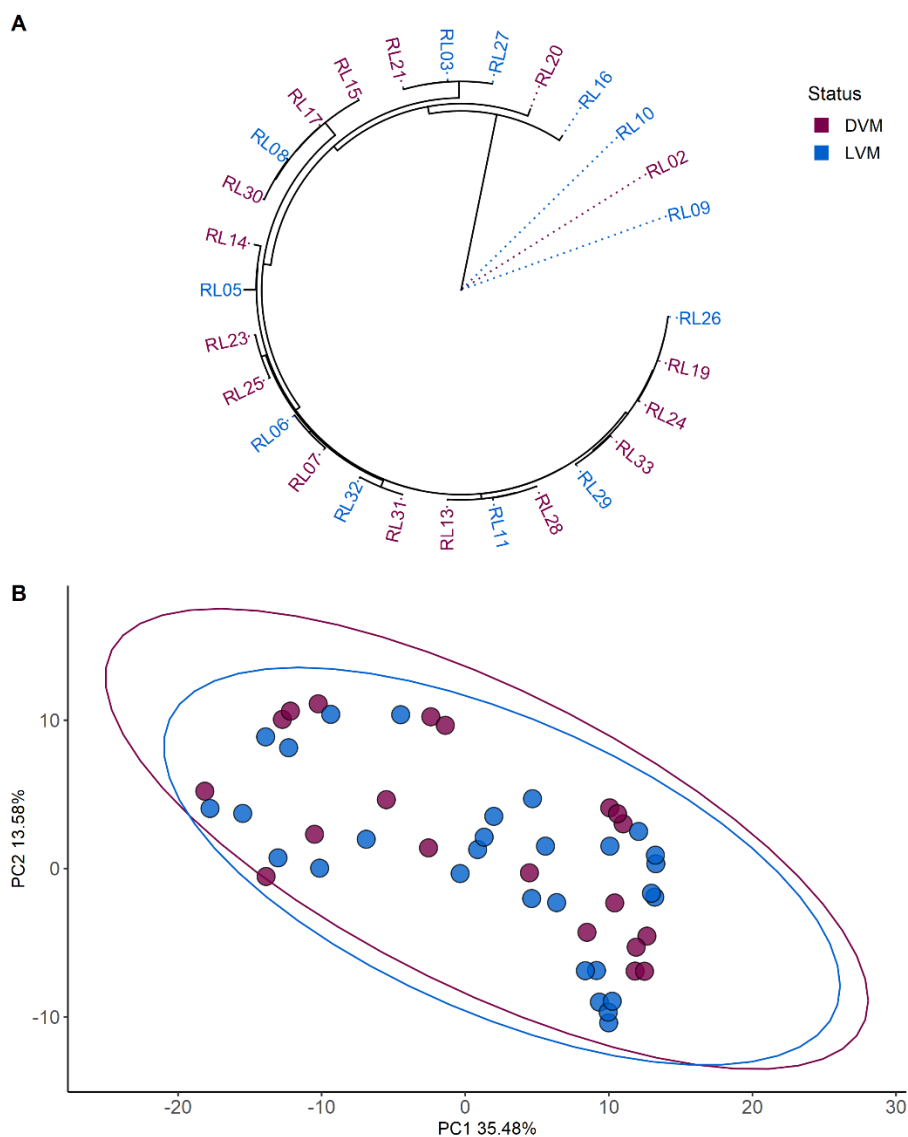


Figure 3.1 Phylogenetic and metabolomic clustering analyses

(A) Core gene phylogenetic tree. (B) Untargeted metabolomics. Ellipses represent the 95% confidence intervals. Colour coding represents grouping. Burgundy represents strains isolated from dysbiotic vaginal microbiota (DVM) and blue represents strains isolated from *Lactobacillus* dominated vaginal microbiota (LVM).

3.4.2 Targeted metabolomics

Between isolated strains from *Lactobacillus*-dominated and dysbiotic vaginal microbiota groups, no significant differences were identified in the amounts of putrescine, cadaverine, and tyramine (Figure 3.2). Semi-quantitative analysis was based on the areas

under the curve of the features corresponding to the amines already present in the media, or those produced after incubation.

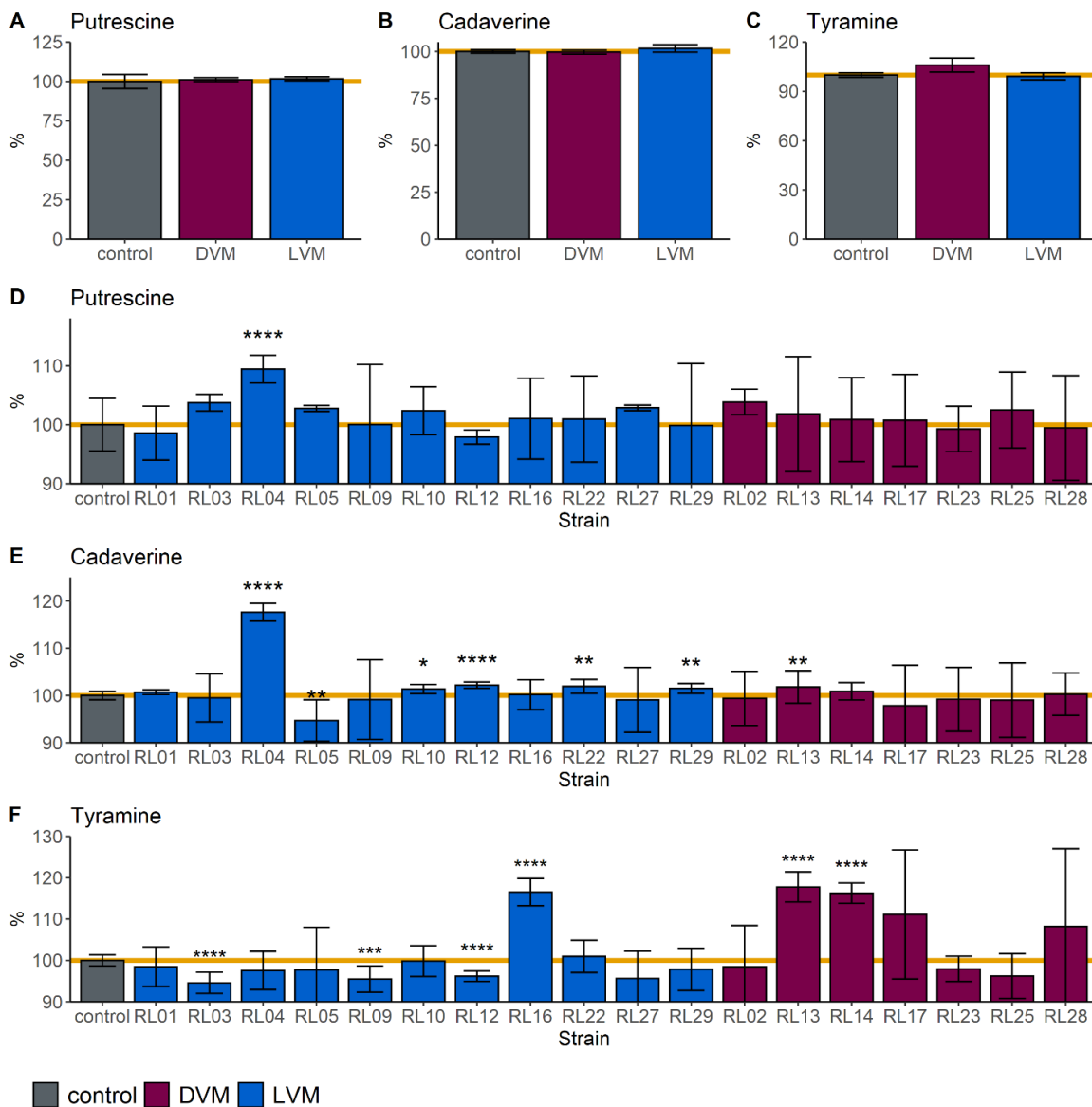


Figure 3.2 Production and degradation of biogenic amines

Semi-quantitative metabolomics analysis of the differences in putrescine (A, D), cadaverine (B, E), and tyramine (C, D), between the control (VDMP media only) and spent media by microbiota status (A-C) and by strain (D-E). Burgundy represents strains isolated from dysbiotic vaginal microbiota (DVM) and blue represents strains isolated from *Lactobacillus* dominated vaginal microbiota (LVM). Data are represented as means of three independent experiments per strain tested (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

3.4.3 Biogenic amines' pathway analysis

The analysis of the biosynthetic and biocatalytic pathways for putrescine, tyramine, and cadaverine (Figure 3.3 and Figure 3.4) revealed that all strains possess the gene that encodes for the enzyme lysine racemase, which catalyzes the bidirectional reaction from L-lysine to D-lysine. The gene encoding for lysine decarboxylase was absent from all strains; this enzyme is responsible for the decarboxylation of L-lysine. However, all strains appear to have the gene that encodes for the enzyme D-ornithine/D-lysine which decarboxylates D-lysine into cadaverine and D-ornithine into putrescine. Only six strains have the encoding sequence for agmatine deiminase (RL03, RL14, RL16, RL20, RL31, RL32), however none have the gene encoding N-carbamoylputrescine amidase, which is required in addition to produce putrescine from agmatine. All strains have the genes that encode for arginine decarboxylase and arginine racemase which convert L- and D-arginine into putrescine, respectively. Arginine racemase also catalyzes the two-way reaction between L- and D- ornithine. All strains also possess the gene for ornithine decarboxylase which synthesizes putrescine from L-ornithine. Only strain 20 has the gene encoding for tyrosine decarboxylase, which converts L-tyrosine into tyramine.

Of the degrading enzymes, all strains except RL09, RL15, RL17, RL20, and RL30 have the gene for the enzyme diamine oxidase, which converts putrescine into 4-aminobutanal. Strain RL14 also has the gene for putrescine aminotransferase, which also converts putrescine into 4-aminobutanal. Additionally, all strains have genes encoding multicopper oxidases. A summary of these of where the genes encoding for these enzymes are found in known pathways findings can be found in Figure 3.5.

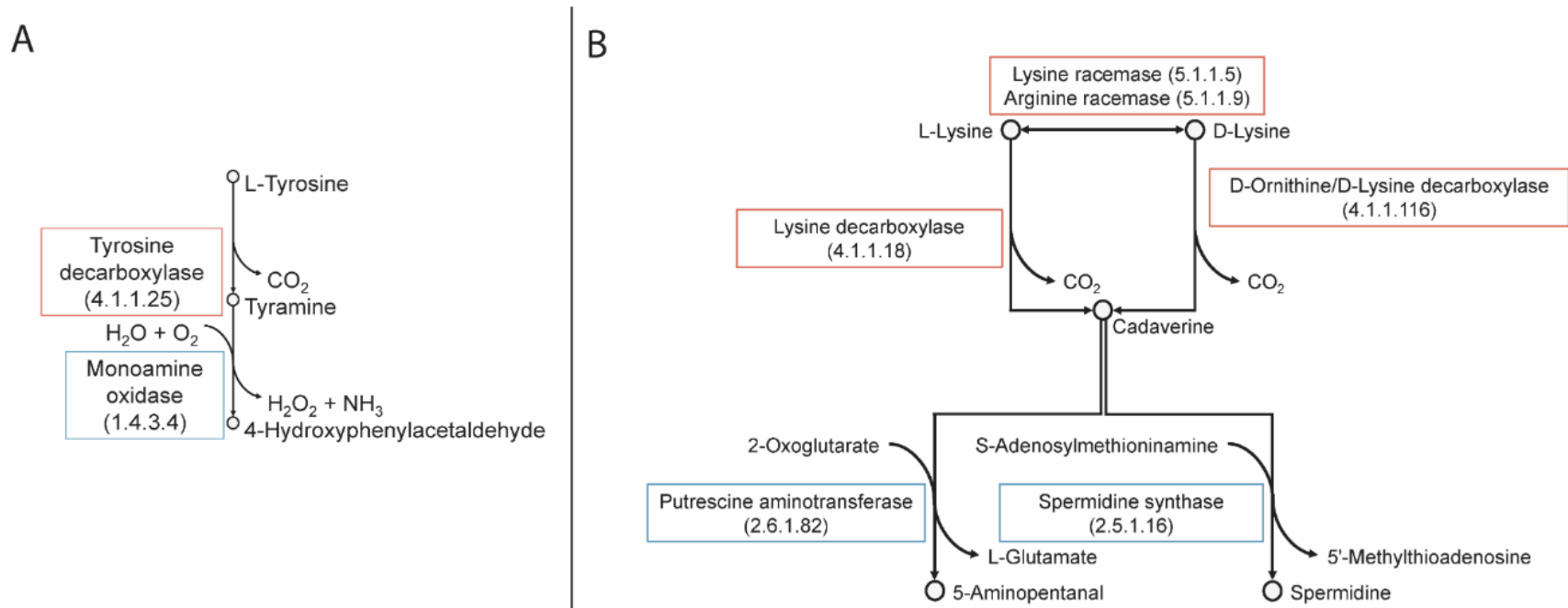


Figure 3.3 Metabolic pathways for the synthesis and degradation of tyramine (A) and cadaverine (B)

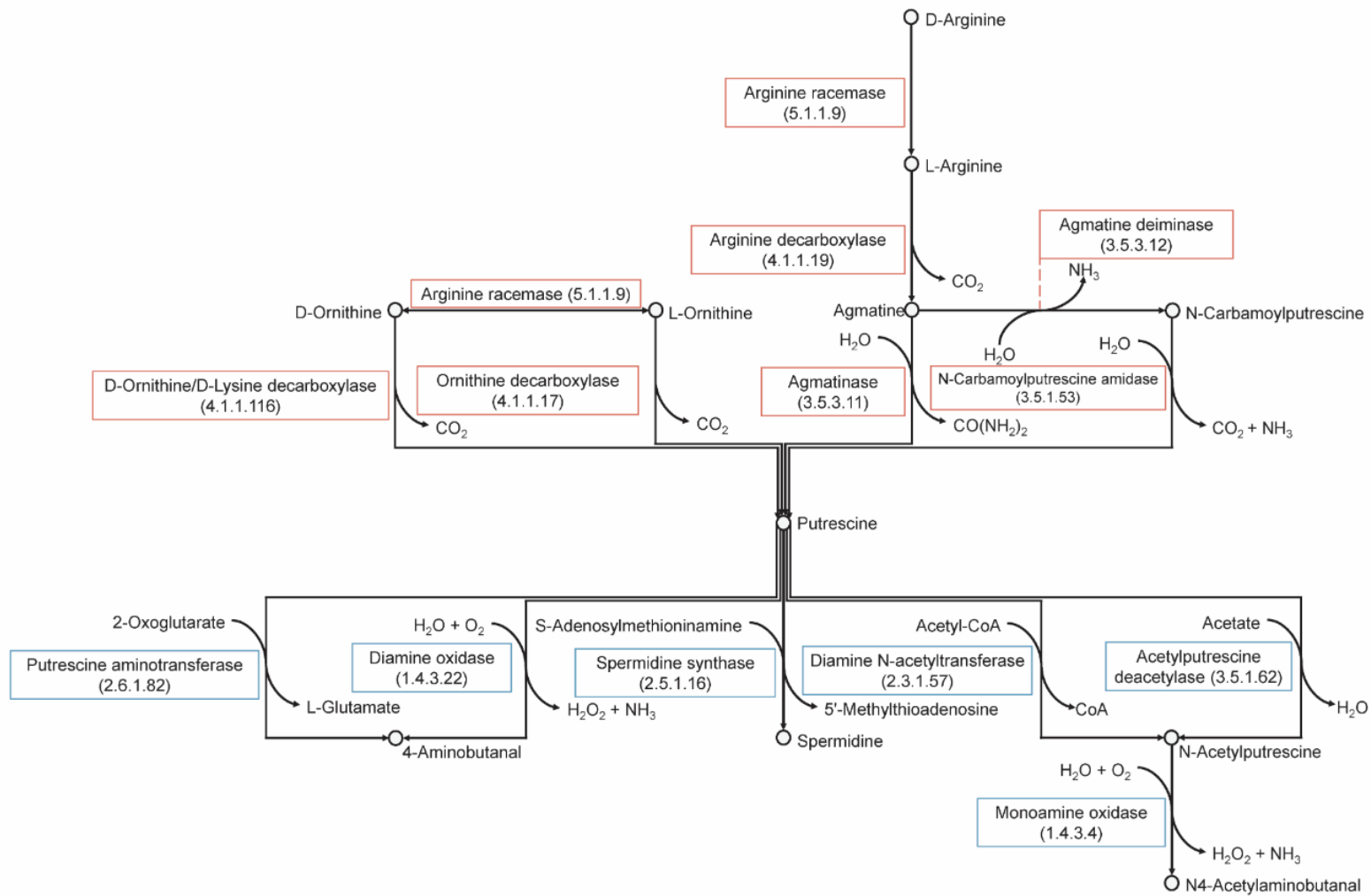


Figure 3.4 Metabolic pathways for the synthesis and degradation of putrescine

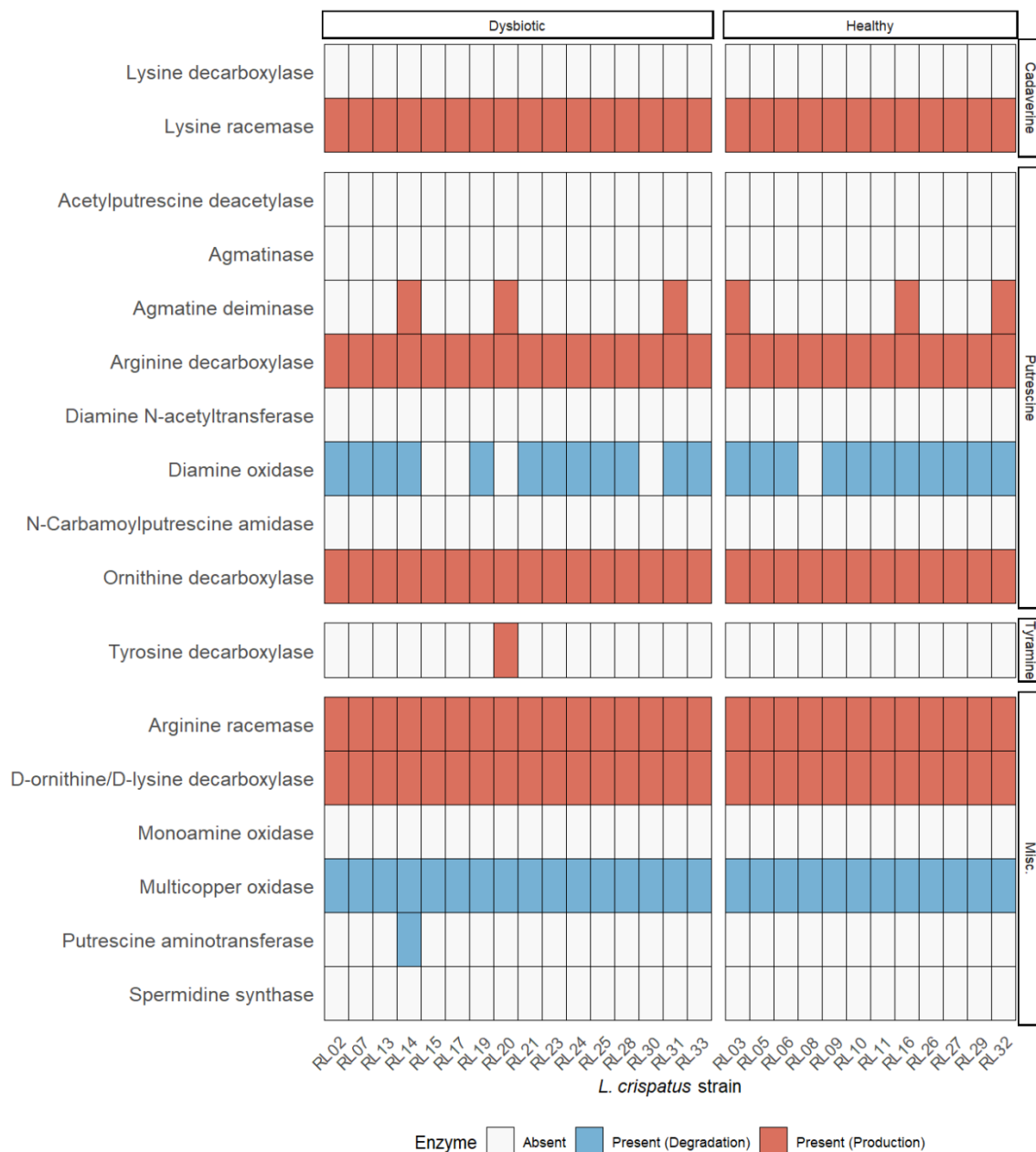


Figure 3.5 Key enzymes in the metabolism of biogenic amines

Summary of the presence or absence of genes encoding for the enzymes involved in the biosynthesis (red) or degradation (blue) of biogenic amines. All available genomes were used for this analysis.

3.4.4 Antibacterial activity

Agar well diffusion assays were conducted using 19 of the *L. crispatus* strains, against *E. faecium*, *E. coli*, *G. vaginalis*, *P. bivia*, and *C. albicans* as indicator strains. Results showed that strains isolated from dysbiotic patients were more effective in inhibiting *E. faecium* and *E. coli* than those isolated from healthy patients (Figure 3.6). No significant differences were observed in the inhibition of *G. vaginalis*, *C. albicans*, and *P. bivia*.

The supernatants and treatments with heat and trypsin showed no inhibition of *E. faecium* (Supplementary Figure S3, Appendix D).

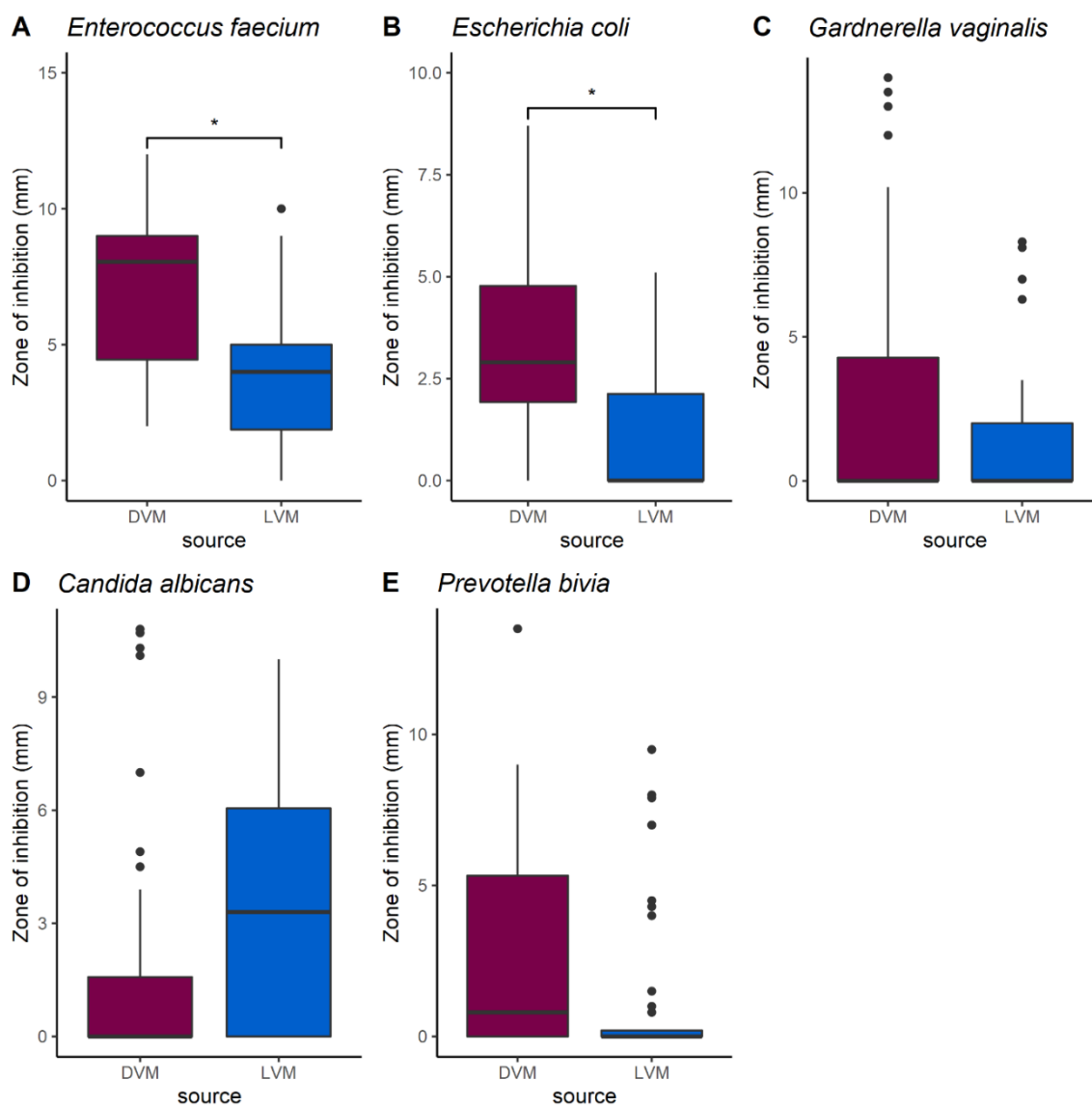


Figure 3.6 Differences between the antimicrobial capacity of *L. crispatus* strains isolated from dysbiotic and *Lactobacillus* dominated vaginal environments.

Burgundy represents strains isolated from dysbiotic vaginal microbiota (DVM; N = 8) and blue represents strains isolated from *Lactobacillus* dominated vaginal microbiota (LVM; N = 11). A linear mixed effects model to control for individual strain effects was used to evaluate differences between the groups ($*p \leq 0.05$).

3.5 Discussion

Although the *L. crispatus* species has been proposed as an excellent candidate for probiotic use to restore and maintain vaginal health because of its preponderance in healthy women, the present study showed that not every strain necessarily has the

ideal properties. Untargeted metabolomic analysis of 18 freshly isolated vaginal strains of *Lactobacillus crispatus* did not show any distinguishable groupings fitting healthy versus dysbiotic origins. Similarly, genomic and functional analyses of all available genomes did not reveal any specific pattern to suggest that the health status of the source host plays a significant role in the specific characteristics of the strains. Altogether, the results showed that all the strains are highly similar both in terms of genes and metabolism.

However, when a more specifically focused targeted metabolomic analysis was performed of the production and degradation of biogenic amines it showed that strains have either the ability to produce or to degrade these compounds. In addition, the evaluation of specific amino acid sequences and metabolic pathways showed that all strains have the genetic potential to produce and to degrade cadaverine and putrescine, while only strain RL20 can produce tyrosine decarboxylase which produces tyramine. Nonetheless, data obtained *in vitro* showed that other two strains (i.e. RL16 and RL14) can produce tyramine when grown in conditions that simulate the vaginal environment (unfortunately strain RL20 was not available for this analysis). These findings have implications for products using *L. crispatus* ostensibly for vaginal health, as potentially some of these strains may actually produce malodorous compounds. Altogether, these results also suggest that targeted approaches are better suited for the identification of probiotic candidates than those that provide a global overview.

In a study of another lactic acid bacterium, *E. faecium* E17, the tyrosine decarboxylation pathway was found to be a key survival mechanism in low acid and nutrient-depleted conditions (Pereira et al., 2009). Within enterococci, the functioning of this pathway also contributes to the binding and immunomodulation of enterocytes. Another species, *Enterococcus durans* IPLA655, uses this ability as a survival and colonization mechanism that enhances adhesion to the intestinal epithelium (Gómez et al., 2010). The capacity of *L. crispatus* to utilize this pathway could be a method of removing the tyrosine for use by enterococci, similar

to the iron sequestering abilities of *L. crispatus* during menstruation (Elli et al., 2000).

The production of biogenic amines, within the vagina is linked to an increase in pH which promotes colonization by BV-associated bacteria. Although all strains showed the genetic potential to produce cadaverine and putrescine, only six strains (i.e. RL04, RL10, RL12, RL13, RL22, and RL29) produced cadaverine *in vitro* and one (i.e. RL04) produced putrescine. This could be a result of an adaptation of these strains to conditions of higher pH, and under stressful *in vitro* conditions they reacted by raising pH. Unfortunately, this makes the environment more favourable to pathogens, plus these compounds emit malodour. Such characteristics are not ideal for vaginal probiotics.

One strain showed *in vitro* capacity to degrade cadaverine (RL05), and three could reduce the amount of tyramine (RL03, RL09, RL12, RL13, RL14, and RL26). Based on our analysis of metabolic pathways, degradation of putrescine is most likely being converted to 4-aminobutanal through diamine oxidase, while tyramine are most likely to be degraded by a multicopper oxidase (Li et al., 2021; Pištěková et al., 2020). Interestingly, there are strains, such as RL01 and RL04 that both produce and degrade biogenic amines. This suggests an adaptation ability of the species depending on what compounds are in the surroundings. The required enzymes for degrading activities are present in the genomes, so it might require certain conditions and larger concentrations of amines in the media to activate them. This capacity makes them good probiotic candidates. A potential *L. crispatus* based probiotic that could degrade malodorous compounds efficiently would be a welcome addition to the current treatments of conditions such as BV.

Admittedly, only one strain was isolated from each host (van der Veer et al., 2019), which makes it possible that several *L. crispatus* strains co-exist in the vaginal environment creating a symbiotic relationship where some produce biogenic amines and others can degrade them. More studies are required to better characterize this relationship. Furthermore, not all strains were tested *in vitro*

because they were not viable under the applied lab conditions. Research on the specific growth requirements for reproducible growth of these strains is on-going.

Antimicrobial activity was significantly higher in the strains isolated from dysbiotic patients, suggesting adaptation due to be sourced from more competitive environments. This inhibitory activity ceased following treatment with the protease trypsin, and heating of the supernatants to 85°C for 45 minutes, indicating that the observed antimicrobial properties resulted from a heat-labile protein. This suggests a potential bacteriocin, compounds regarded as being important defence factors for lactobacilli (Borges et al., 2014). These antimicrobial proteins typically have a very narrow spectrum, usually only inhibiting closely related species (Zamfir et al., 1999). However, those produced by *Lactobacillus* spp. are known to have a much broader spectrum of activity, including against Gram-negative bacteria (McGroarty & Reid, 1988). Nonetheless, further studies are required to express and further characterize the putative antimicrobial peptides that are causing this activity.

Strain *L. crispatus* CTV-05 is already being developed as an intravaginal probiotic, but it only appears to be effective if the subject is devoid of indigenous *L. crispatus* (Antonio et al., 2009). This might suggest competition for receptor sites or some other quorum sensing effect that limits foreign strain colonization, but these mechanisms have not been explored to date and its ability to produce or degrade biogenic amines has not been reported.

In summary, the findings from the present study suggests that metabolic readouts along with a strain's genomic capacity should be part of the characterization of candidate probiotics. There is a strong need to improve women's health. The option of using probiotic *L. crispatus* strains to this end, is worthy of further study, but specific, well-documented characteristics must guide the selection.

3.6 Ethics declaration

The research proposal for this study was assessed by the ethics review board of the Academic Medical Center (AMC), University of Amsterdam, The Netherlands. The

review board deemed that no additional ethical approval was required for this study, since the vaginal samples used here were collected as part of routine procedure for cervical examinations at the STI clinic in Amsterdam (document reference number W12_086 # 12.17.0104).

Clients of the STI clinic were notified that their samples could be used for scientific research, after anonymization of client clinical data and samples. The data and samples of clients who refused were discarded. This procedure was approved by the AMC ethics review board (reference number W15_159 # 15.0193).

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Chapter 4

4 Probiotics to reduce biogenic amines that cause urogenital malodour

4.1 Introduction

The biogenic amines (BAs) tyramine, cadaverine, putrescine, and tyramine, have been correlated with malodour in the urogenital tract of women with dysbiosis. Patients with conditions such as bacterial vaginosis (BV) and urinary tract infection (UTI) suffer from malodour, negatively impacting their life-quality. Unfortunately, the gold standard treatment for BV and UTI is a course of antibiotics, but these do not target malodour nor do they provide relief for at least three days. Thus, a remedy that can augment antibiotics or target reduction in malodour more quickly would represent a significant advancement.

Probiotics, defined as ‘live microorganisms that, when administered in adequate amounts, confer a health benefit on the host’ (FAO/WHO Working Group, 2002; Hill et al., 2014), have been used for decades to manage and prevent dysbiosis of the female urogenital tract. *Lacticaseibacillus rhamnosus* GR-1 (LGR-1) and *Limosilactobacillus reuteri* RC-14 have been proven to effectively aid in regulating homeostasis. Indeed, LGR-1 is the most widely studied probiotic for urogenital health (Petrova et al., 2018, 2021), and its genome indicates that it can thrive in the vaginal environment. When used in conjunction with LRC-14 it can decrease recurrence of BV, UTI and effectively inhibit the yeast *Candida albicans*, which is one of the most frequent vaginal pathogens (Beerepoot et al.; Martinez et al., 2009; Reid, 2017; Vujic et al., 2013). Additionally, the combination taken orally with and after antibiotic treatment aids in the replenishment of indigenous species such as *Lactobacillus crispatus*, which are predominant in a healthy urogenital environment (Macklaim et al., 2015).

As shown on Chapter 3, several strains of *L. crispatus* have the potential to degrade BAs. In the present study, five promising candidates were tested against BAs, along with *L. crispatus* ATCC 33820, LGR-1, and LRC-14.

Previous studies suggested that BAs, are not only produced by some pathogens, but they act as virulence factors that increase the urogenital pH, reduce production of lactic acid, and lower the abundance of lactobacilli (Borgogna et al., 2021; Nelson et al., 2015). The relationship between these malodorous compounds and lactobacilli survival was also examined.

The long-term goal was to establish a basis for a novel approach to manage urogenital dysbiosis and malodour, thereby, benefiting millions of women around the world.

4.2 Materials and methods

4.2.1 *Lactobacillus crispatus* clinical strains

The *L. crispatus* strains used in this study were selected based on previous characteristics (Chapter 3) and depending on their potential to degrade BAs. Strains were provided by van der Veer *et al* (2019) with all material approved by each participant with written consent and from the Institutional Review Board (IRB). They originated from vaginal swabs obtained from women attending the Sexually Transmitted Infections clinic in Amsterdam, The Netherlands. The samples came from two groups of women: one with a healthy vaginal microbiota (based upon Nugent score 0-3), or those with dysbiosis (Nugent score 7-10). The swabs were plated on modified Trypticase Soy Agar and incubated anaerobically at 37°C for 24h. Next, single colonies underwent 16S rRNA gene sequencing for identification purposes. The strains were then cryopreserved and stored at -80°C in vaginally defined medium plus peptone (VDMP) (Geshnizgani & Onderdonk, 1992). Additional strains were obtained from four healthy premenopausal women in London, Ontario aged between 25 and 30 years old who consented to partake in the study, approved by the Health Sciences Research Ethics Board at The University of Western Ontario (Collins et al., 2017) (see Appendix E). The subjects self-swabbed their lateral vaginal walls using sterile Dacron swabs. Vaginal pH was confirmed to be approximately 4.5 by using pHem-Alert applicator keys (Gynex Corporation)). Swabs were Gram stained (Becton, Dickinson & Company) and scored using the Nugent system (Nugent et al., 1991; Spiegel et al., 1983).

4.2.2 Biogenic amine production and degradation experiments

A degradation assay using LGR-1, LRC-14, and *L. crispatus* ATCC 33820 was performed, along with a polymicrobial culture containing LGR1 and RC-14. Four individual colonies of each organism were grown in Man-Rogosa-Sharpe (MRS) broth medium (VWR) at 37°C anaerobically for 24 hours. Next, 10 µL of the initial liquid cultures were grown for 24 hours anaerobically at 37°C in 10 mL of VDMP supplemented with 100 ppm of either cadaverine, putrescine, and tyramine or a control of VDMP only. These were subsequently analyzed using LC-MS/MS.

To test their ability to degrade amines at similar concentrations as those in vaginal dysbiosis (Wolrath et al., 2001), three individual colonies of five *L. crispatus* strains (i.e. RL01, RL03, RL05, RL09, and RL12) as well as the type strain (ATCC 33820) were grown in MRS medium at 37°C anaerobically for 24 hours. Then, 10 µL were sub-cultured in 2 mL of either VDMP or amine-VDMP (200 ppm of cadaverine, 200 ppm of putrescine, and 200 ppm of tyramine) and grown anaerobically at 37°C for 24 hours. These samples were analysed using HPLC-UV.

4.2.3 Induction assays

To identify whether *L. crispatus* undergoes adaptation after exposure to BAs, five individual colonies of *L. crispatus* ATCC 33820 were grown in 2 mL of amine-VDMP anaerobically at 37°C for 24 hours, then 10 µL was inoculated into 2 mL of amine-VDMP under the same conditions; these were deemed the ‘induced’ group. Meanwhile, the ‘uninduced’ group was derived from 5 individual colonies of *L. crispatus* ATCC 33820 grown in 2 mL of VDMP anaerobically at 37°C for 24 hours and subsequently sub-cultured in amine-VDMP. Next, HPLC-UV analysis was performed.

To assess whether the metabolites produced by *L. crispatus* had the ability to degrade BAs, the spent media of the original liquid cultures was filtered sterilized (0.22 µm) and incubated under the same conditions as the previous samples in amine-VDMP. These groups were designed ‘induced’ and ‘uninduced’ filtrates.

The effect of pH on the tolerance to amines of ‘induced’ and ‘uninduced’ cultures was measured by inoculating a 1:100 dilution of the liquid cultures in 240 μL of BA-media (amine-VDMP + 50 ppm of TMA). The control consisted on VDMP only ($n = 5$). The pH was adjusted using either NaOH or HCl. Absorbance at 600 nm was measured every 30 min during 24 hours in an automatic plate reader. Finally, pH was measured using pH strips (Eorta). The R package growthcurveR (Sprouffske, 2020) was used to model the bacterial growth kinetics and to calculate relevant growth parameters (i.e. maximum growth capacity, area under the curve, time at inflection point, and doubling time).

Data analysis was performed based on lineal models generated per each growth parameter. Families were defined as either induced or uninduced. Within each family, multiple comparisons and control for type I error were performed using Tukey’s post-hoc test (Tables 4.1, 4.3, 4.5, and 4.7). Next, individual contrasts between both families at every pH were performed (Tables 4.2, 4.4, 4.6, and 4.8).

4.2.4 LC-MS/MS protocol

After incubation, 250 μL of each liquid culture was aliquoted and diluted 1:3 with pure methanol then vortexed and incubated on ice for 30 minutes, followed by centrifugation at $16000 \times g$ for 10 minutes. Supernatants were filtered into vials for analysis (0.22 μm PTFE syringe filters) which was carried out using a Q-Exactive Quadrupole Orbitrap MS, coupled to an Agilent 1290 HPLC system with an Agilent HILIZ-Z (2.1 \times 100 mm, 2.7 μm). The mobile phase A consisted of 20 mM ammonium formate in water, and phase B was 20 mM ammonium formate in 90% acetonitrile. Analytes were eluted with the following gradient: 0 min, 100% B; 0.5 min, 100% B; 5.3 min, 80% B; 9.5 min, 30% B; 13.5 min, 30% B; 14.5 min 100% B; and 16.5 min, 100% B. Conditions for heated electrospray ionization (HESI) were as follows: HESI(+) capillary voltage, capillary voltage, 3.5 kV ; capillary temperature, 250 $^{\circ}\text{C}$; sheath gas, 30.00 units; auxiliary gas, 8.00 units; probe heater temperature, 450 $^{\circ}\text{C}$; S-Lens RF level, 60.00. The instrument was operated with a top 3 data-dependent acquisition protocol with a full MS scan in the mass range of m/z 1.2 and collision energy of 28. Authentic standards were used for calibration curves (cadaverine, putrescine, and tyramine), acquired from Sigma Aldrich (St. Louis,

MO). Compound identification and quantification was performed using the software Xcalibur (Thermo Fisher Scientific, Waltham, MA).

4.2.5 HPLC-UV analysis

Due to pandemic-related restrictions, further access to LC-MS was not possible and the rest of the analyses were carried out in-house using HPLC-UV. Bacterial cultures, and their supernatants, were also prepared by diluting them 1:10 with water, incubating them on ice for 30 min and centrifuging at $16000 \times g$ for 10 minutes. Samples were prepared for derivatization by adding 250 μL of 0.5 KH_2PO_4 buffer (pH = 11) and 10 μL of 1 M NaOH. Next, 500 μL of p-toluenesulfonyl chloride (10 mg/ml, Sigma Aldrich, St. Louis, MO) were added and samples incubated at 56 for 10 min. The reaction was stopped with the addition of 50 μL of 1M HCl and samples were filtered into vials (0.45 μL PTFE syringe filters) (Dziarkowska et al., 2008).

Analysis was carried out using a mobile phase in isocratic mode composed of 60% acetonitrile and 40% HPLC grade water (Thermo Fisher Scientific, Waltham, MA). The flow rate was 1 mg/mL Authentic standards for putrescine, cadaverine, and putrescine were used, and α -aminobutyric acid was an internal standard, all acquired from Sigma Aldrich (St. Louis, MO). Analyte identification and quantification was performed using the Chemstation software (Agilent, Santa Clara, CA).

4.2.6 Analysis of the impact of biogenic amines on the vaginal microbiota

To assess the impact that biogenic amines have on the vaginal microbiota, an adaptation of the *in vitro* polymicrobial vaginal culturing model described previously was used (Collins et al., 2017). Briefly, bacteria collected from vaginal swabs were grown anaerobically for 36 h in different media: VDMP supplemented with either 200 ppm of cadaverine, 200 ppm of putrescine, 200 ppm of tyramine, or 50 ppm of TMA, BA-VDMP, and VDMP.

Microbiota analysis was performed by amplification of the V4 region of 16S ribosomal DNA, which was then sequenced using the Illumina MiSeq to detect shifts in

microbial abundance (Al et al., 2020). Earth Microbiome universal primers, 515F and 806R, were used for PCR amplification. Primers consisted of an Illumina adapter and four random nucleotides, one of 24 unique 12-mer barcodes, and the corresponding annealing primer (Parada et al., 2016). A Biomek® 3000 Laboratory Automation Workstation (Beckman-Coulter, Mississauga, ON) was used for PCR set-up. Amplification was performed in an Eppendorf thermal cycler (Eppendorf, Mississauga, ON) with an initial raise in temperature of 95°, then 25 cycles of one minute each at 95°C, 52°C, and 72°C. Purified amplicons were then paired-end sequenced with 250 cycles on an Illumina MiSeq platform (San Diego, CA) (Parada et al., 2016).

Data were exported as raw fastq files. Quality control was performed following the DADA2 pipeline (Parada et al., 2016). Amplicon sequence variants (SVs) and samples were selected with more than 1,000 filtered reads, SVs present at 1% relative abundance, and SVs larger than 10,000 total reads across all samples. Taxonomy was assigned using the SILVA (c132) training set. (Parada et al., 2016). Downstream analysis was performed with the ALDEx2 (Fernandes et al., 2013, 2014; Gloor et al., 2016), Vegan (Oksanen et al., 2020), rstatix (Kassambara, 2021), and emmeans (Lenth, 2021) R packages.

4.2.7 Statistical analyses

All data analyses were performed using RStudio. One-way analysis of variances (ANOVAs) and all post-hoc tests were done with the R packages rstatix v0.7.0 (Kassambara, 2021). To correct heteroscedasticity marginal means were used and the matrix of co-variance was adjusted. This was performed using the R packages, emmeans v1.6.0 (Lenth, 2021) and sandwich v3.0-1 (Zeileis, 2004). Figure 4.1 was done using the software GraphPad Prism 8. The rest of the figures were made with the R package ggplot2 (Wickham, 2016).

4.3 Results

4.3.1 Lactobacilli growth in biogenic amines

When grown in VDMP supplemented with 100 ppm of cadaverine, putrescine, or tyramine as independent experiments, only *L. crispatus* ATCC 33820 showed statistically

significant reduction of cadaverine and putrescine. Meanwhile, LGR-1 produced a significant amount of tyramine when grown by itself. Results are summarized in Figure 4.1.

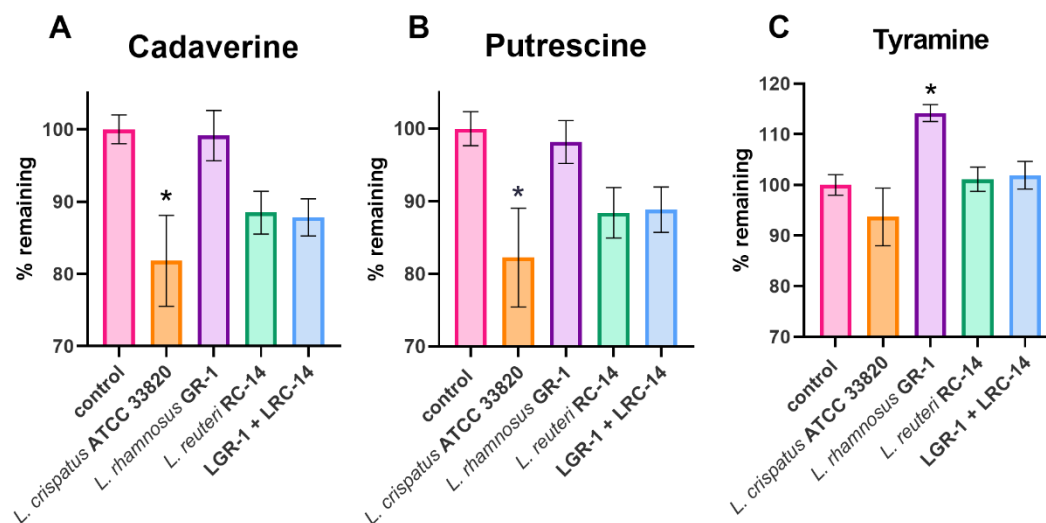


Figure 4.1 Metabolism of biogenic amines by different lactobacilli strains. Data are presented as means of 4 independent experiments \pm 95% confidence intervals (CI). One-way ANOVA with the Dunnet correction for multiple comparisons was used to calculate statistical significance, * $p \leq 0.05$).

Figure 4.2 shows that all the recently isolated *L. crispatus* strains could significantly reduce the concentration of putrescine and cadaverine when supplemented at 200 ppm each, with the exception of strain RL12 which produced cadaverine. Furthermore, all strains completely removed tyramine from the media (data not shown).

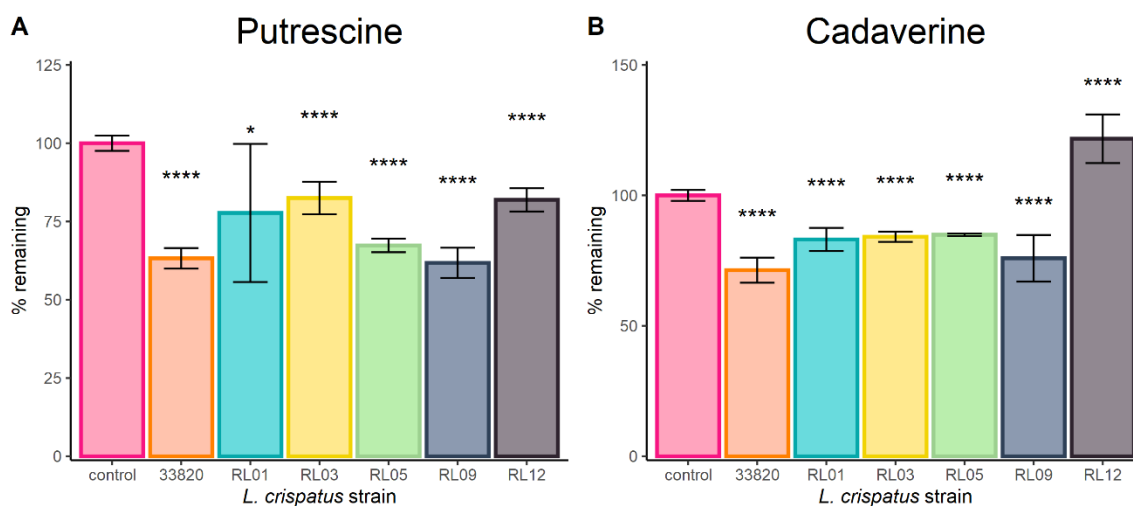


Figure 4.2 Metabolism of biogenic amines by different *L. crispatus* strains in amine-VDMP.

One-way ANOVA with the Dunnet correction for multiple comparisons was used to calculate statistical significance (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Data are presented as means of 3 independent experiments \pm 95% CI.

When degradation assays were carried out using cultures of *L. crispatus* ATCC 33820 that had been originally exposed to putrescine, cadaverine, and tyramine (i.e. ‘induced’ cultures), all preparations from either whole cells or supernatants reduced the amount of all tested amines below 60%. There was no statistically significant difference between treatments for reduction ability of cadaverine. In the case of putrescine, ‘induced’ bacterial cultures showed greater degradation than the rest of the treatments. Both the whole cells and the supernatant of ‘induced’ cultures reduced significantly more tyramine. Results are presented in Figure 4.3.

Due to the physicochemical properties of TMA, none of the analytical methods (i.e. LC-MS/MS and HPLC-UV) were suitable for detecting this compound at relevant concentrations. Therefore, it was excluded.

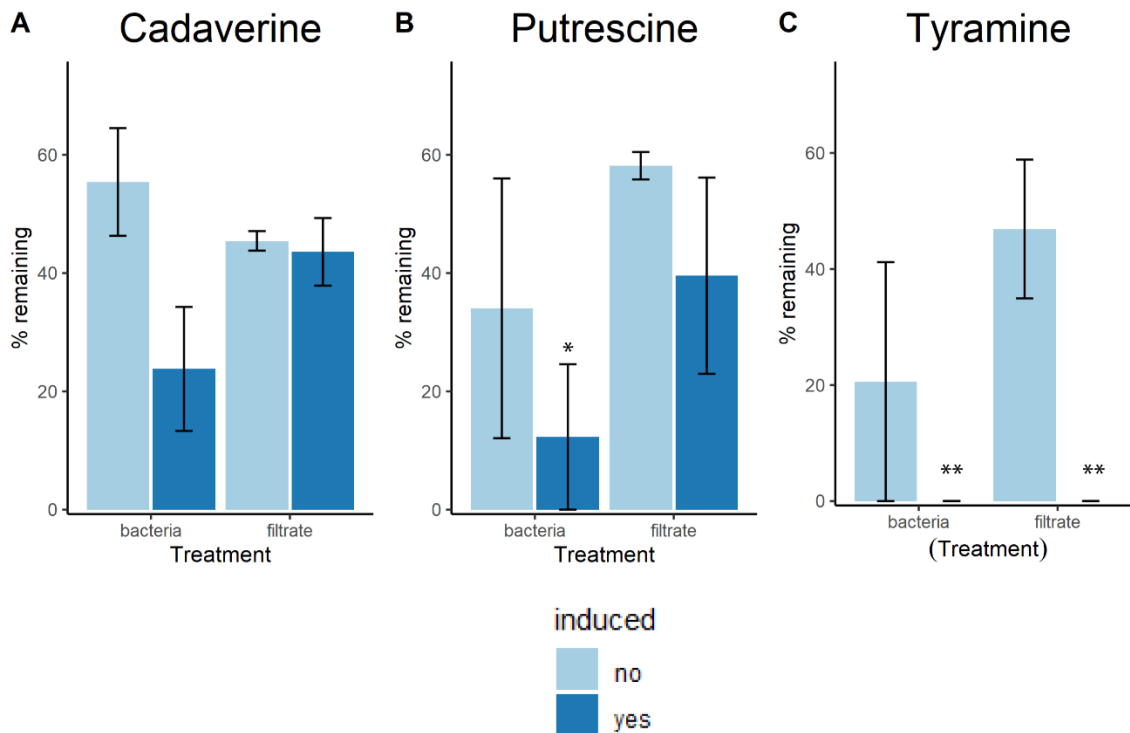


Figure 4.3 Biogenic amines exposure assay.

One-way ANOVA with the Tukey post-hoc test for multiple comparisons was used to calculate statistical significance (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Data are presented as means of 3 independent experiments \pm 95% CI.

4.3.2 Effect of biogenic amines and pH on the growth of *Lactobacillus*.

Originally the pH of non amine-supplemented VDMP (control) was 6.71 and that of BA media was 7.04. An additional set of each media was prepared with adjusted pH that corresponded its counterpart (i.e. control at 7.04 and BA-media at 6.71). At the end of the incubation period all treatments reached a final pH of 4.5.

Results, plotted in Figure 4.4, showed that both ‘induced’ and ‘uninduced’ cultures had the most and fastest growth when cultured in BA-VDMP that had been adjusted to a lower pH. They were followed by ‘induced’ and ‘uninduced’ cultures grown in VDMP at its original pH (6.71). Next, were the bacteria grown in BA-media at its original pH (7.04), in which case ‘induced’ cultures showed better growth than their ‘uninduced’ counterparts. The slowest and lowest growth was observed when bacteria were grown in

VDMP at an adjusted higher pH (7.04). Statistical analyses of different relevant growth parameters between groups are summarized in Tables 4.1 through 4.8.

Analyses on the impact of exposure to biogenic amines on the vaginal microbiota are shown on Figure 4.5. Results revealed that *Lactobacillus* abundance was significantly reduced when cultures were exposed to 200 ppm of cadaverine or to BA-media.

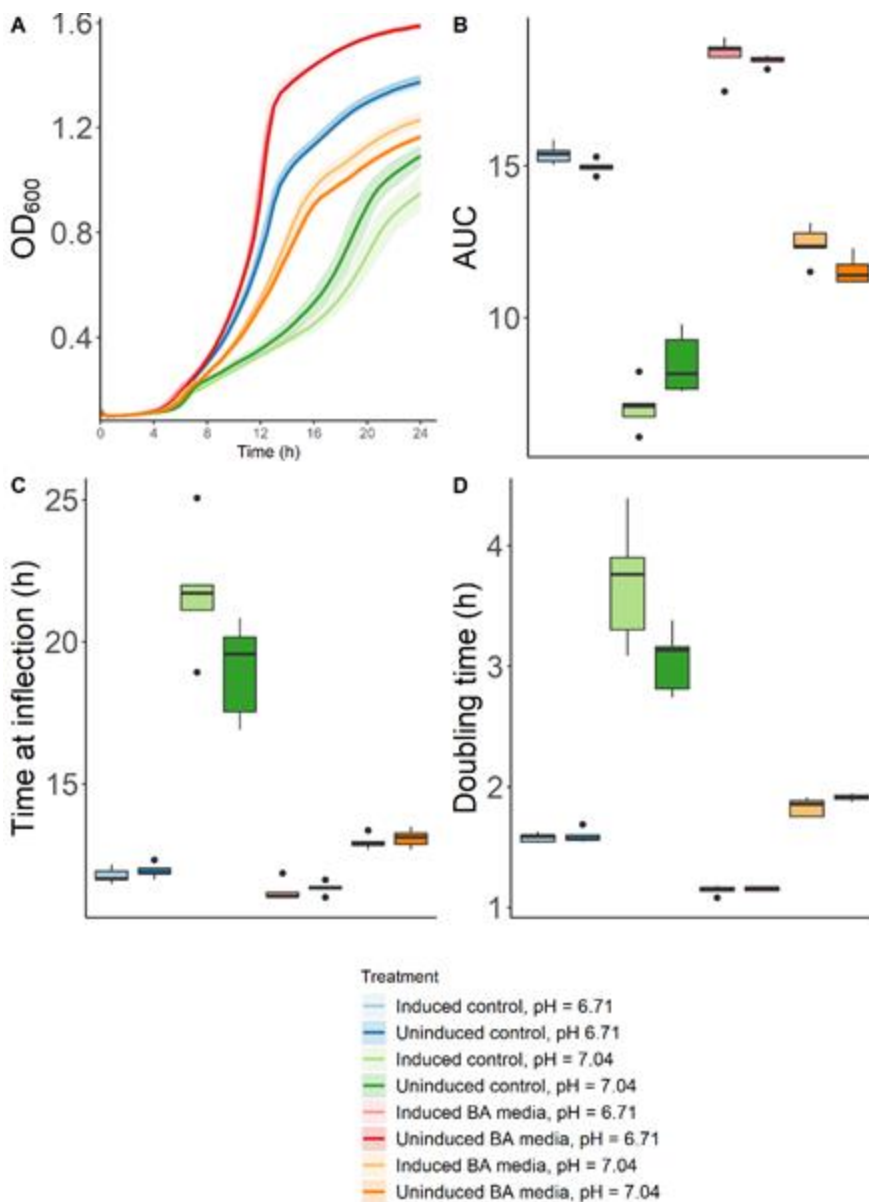


Figure 4.4 Effect of biogenic amines and pH on the growth of *L. crispatus*

Data are presented as the means of 5 independent experiments. A) Bacterial growth curves measured by absorbance at a wavelength of 600 nm. B) Logistic areas under the curve. C) Time at inflection. D) Doubling times.

Table 4.1 Effect of biogenic amine supplemented media on maximum possible population (K)

Multiple comparisons between control group (non-supplemented VDMP media, original pH 6.71) and Biogenic amine (BA) media (original pH). An additional media of each was prepared and its pH adjusted to mimic its supplemented/non-supplemented counterpart. Induced samples were grown from cultures previously exposed to BAs and uninduced from cultures grown in non-supplemented VDMP.

Maximum possible population (K): Multiple comparisons			
Induced vs uninduced samples			
Treatment 1	Treatment 2	P-Value	Significance (α 0.05)
Induced			
Control (pH = 6.71)	Control (pH = 7.04)	0.012	*
Control (pH = 6.71)	BA media (pH = 6.71)	≤ 0.0001	****
Control (pH = 6.71)	BA media (pH = 7.04)	≤ 0.0001	****
Control (pH = 7.04)	BA media (pH = 6.71)	0.995	ns
Control (pH = 7.04)	BA media (pH = 7.04)	≤ 0.0001	****
BA media (pH = 6.71)	BA media (pH = 7.04)	≤ 0.0001	****
Uninduced			
Control (pH = 6.71)	Control (pH = 7.04)	0.067	ns
Control (pH = 6.71)	BA media (pH = 6.71)	≤ 0.0001	****
Control (pH = 6.71)	BA media (pH = 7.04)	≤ 0.0001	****
Control (pH = 7.04)	BA media (pH = 6.71)	0.415	ns
Control (pH = 7.04)	BA media (pH = 7.04)	≤ 0.0001	****
BA media (pH = 6.71)	BA media (pH = 7.04)	≤ 0.0001	****

Table 4.2 Effect of previous amine exposure on maximum possible population (K)

Multiple comparisons between induced samples grown from cultures previously exposed to BAs and uninduced from cultures grown in non-supplemented VDMP. Cultures grown in (non-supplemented VDMP media, original pH 6.71) and biogenic amine (BA) media (original pH). An additional media of each was prepared and its pH adjusted to mimic its supplemented/non-supplemented counterpart.

Maximum possible population (K): Multiple comparisons			
Induced vs uninduced samples			
Treatment 1	Treatment 2	P-Value	Significance (α 0.05)
Control (pH = 6.71)			
Induced	Uninduced	0.217	ns
Control (pH = 7.04)			
Induced	Uninduced	0.387	ns
BA media (pH = 6.71)			
Induced	Uninduced	0.865	ns
BA media (pH = 7.04)			
Induced	Uninduced	0.001	**

Table 4.3 Effect of biogenic amine supplemented media on time at inflection

Multiple comparisons between control group (non-supplemented VDMP media, original pH 6.71) and Biogenic amine (BA) media (original pH). An additional media of each was prepared and its pH adjusted to mimic its supplemented/non-supplemented counterpart. Induced samples were grown from cultures previously exposed to BAs and uninduced from cultures grown in non-supplemented VDMP.

Time at inflection: Multiple comparisons			
Induced vs uninduced samples			
Treatment 1	Treatment 2	P-Value	Significance (α 0.05)
Induced			
Control (pH = 6.71)	Control (pH = 7.04)	≤ 0.0001	****
Control (pH = 6.71)	BA media (pH = 6.71)	0.086	ns
Control (pH = 6.71)	BA media (pH = 7.04)	≤ 0.0001	****
Control (pH = 7.04)	BA media (pH = 6.71)	≤ 0.0001	****
Control (pH = 7.04)	BA media (pH = 7.04)	≤ 0.0001	****
BA media (pH = 6.71)	BA media (pH = 7.04)	≤ 0.0001	****
Uninduced			
Control (pH = 6.71)	Control (pH = 7.04)	≤ 0.0001	****
Control (pH = 6.71)	BA media (pH = 6.71)	0.005	**
Control (pH = 6.71)	BA media (pH = 7.04)	≤ 0.0001	****
Control (pH = 7.04)	BA media (pH = 6.71)	≤ 0.0001	****
Control (pH = 7.04)	BA media (pH = 7.04)	≤ 0.0001	****
BA media (pH = 6.71)	BA media (pH = 7.04)	≤ 0.0001	****

Table 4.4 Effect of previous amine exposure on time at inflection

Multiple comparisons between induced samples grown from cultures previously exposed to BAs and uninduced from cultures grown in non-supplemented VDMP. Cultures grown in (non-supplemented VDMP media, original pH 6.71) and biogenic amine (BA) media (original pH). An additional media of each was prepared and its pH adjusted to mimic its supplemented/non-supplemented counterpart.

Time at inflection: Multiple comparisons			
Induced vs uninduced samples			
Treatment 1	Treatment 2	P-Value	Significance (α 0.05)
Control (pH = 6.71)			
Induced	Uninduced	0.378	ns
Control (pH = 7.04)			
Induced	Uninduced	0.056	ns
BA media (pH = 6.71)			
Induced	Uninduced	0.598	ns
BA media (pH = 7.04)			
Induced	Uninduced	0.49	ns

Table 4.5 Effect of amine supplemented media on doubling time

Multiple comparisons between control group (non-supplemented VDMP media, original pH 6.71) and Biogenic amine (BA) media (original pH). An additional media of each was prepared and its pH adjusted to mimic its supplemented/non-supplemented counterpart. Induced samples were grown from cultures previously exposed to BAs and uninduced from cultures grown in non-supplemented VDMP.

Doubling time: Multiple comparisons			
Induced vs uninduced samples			
Treatment 1	Treatment 2	P-Value	Significance (α 0.05)
Induced			
Control (pH = 6.71)	Control (pH = 7.04)	≤ 0.0001	****
Control (pH = 6.71)	BA media (pH = 6.71)	≤ 0.0001	****
Control (pH = 6.71)	BA media (pH = 7.04)	≤ 0.0001	****
Control (pH = 7.04)	BA media (pH = 6.71)	≤ 0.0001	****
Control (pH = 7.04)	BA media (pH = 7.04)	≤ 0.0001	****
BA media (pH = 6.71)	BA media (pH = 7.04)	≤ 0.0001	****
Uninduced			
Control (pH = 6.71)	Control (pH = 7.04)	≤ 0.0001	****
Control (pH = 6.71)	BA media (pH = 6.71)	≤ 0.0001	****
Control (pH = 6.71)	BA media (pH = 7.04)	≤ 0.0001	****
Control (pH = 7.04)	BA media (pH = 6.71)	≤ 0.0001	****
Control (pH = 7.04)	BA media (pH = 7.04)	≤ 0.0001	****
BA media (pH = 6.71)	BA media (pH = 7.04)	≤ 0.0001	****

Table 4.6 Effect of previous amine exposure on doubling time

Multiple comparisons between induced samples grown from cultures previously exposed to BAs and uninduced from cultures grown in non-supplemented VDMP. Cultures grown in (non-supplemented VDMP media, original pH 6.71) and biogenic amine (BA) media (original pH). An additional media of each was prepared and its pH adjusted to mimic its supplemented/non-supplemented counterpart.

Doubling time: Multiple comparisons			
Induced vs uninduced samples			
Treatment 1	Treatment 2	P-Value	Significance (α 0.05)
Control (pH = 6.71)			
Induced	Uninduced	0.724	ns
Control (pH = 7.04)			
Induced	Uninduced	0.033	*
BA media (pH = 6.71)			
Induced	Uninduced	0.593	ns
BA media (pH = 7.04)			
Induced	Uninduced	0.062	ns

Table 4.7 Effect of amine supplemented media on logistic area under the curve

Multiple comparisons between control group (non-supplemented VDMP media, original pH 6.71) and Biogenic amine (BA) media (original pH). An additional media of each was prepared and its pH adjusted to mimic its supplemented/non-supplemented counterpart. Induced samples were grown from cultures previously exposed to BAs and uninduced from cultures grown in non-supplemented VDMP.

Area under the curve (AUC): Multiple comparisons			
Induced vs uninduced samples			
Treatment 1	Treatment 2	P-Value	Significance (α 0.05)
Induced			
Control (pH = 6.71)	Control (pH = 7.04)	≤ 0.0001	****
Control (pH = 6.71)	BA media (pH = 6.71)	≤ 0.0001	****
Control (pH = 6.71)	BA media (pH = 7.04)	≤ 0.0001	****
Control (pH = 7.04)	BA media (pH = 6.71)	≤ 0.0001	****
Control (pH = 7.04)	BA media (pH = 7.04)	≤ 0.0001	****
BA media (pH = 6.71)	BA media (pH = 7.04)	≤ 0.0001	****
Uninduced			
Control (pH = 6.71)	Control (pH = 7.04)	≤ 0.0001	****
Control (pH = 6.71)	BA media (pH = 6.71)	≤ 0.0001	****
Control (pH = 6.71)	BA media (pH = 7.04)	≤ 0.0001	****
Control (pH = 7.04)	BA media (pH = 6.71)	≤ 0.0001	****
Control (pH = 7.04)	BA media (pH = 7.04)	≤ 0.0001	****
BA media (pH = 6.71)	BA media (pH = 7.04)	≤ 0.0001	****

Table 4.8 Effect of previous amine exposure on logistic area under the curve

Multiple comparisons between induced samples grown from cultures previously exposed to BAs and uninduced from cultures grown in non-supplemented VDMP. Cultures grown in (non-supplemented VDMP media, original pH 6.71) and biogenic amine (BA) media (original pH). An additional media of each was prepared and its pH adjusted to mimic its supplemented/non-supplemented counterpart.

Area under the curve (AUC): Multiple comparisons			
Induced vs uninduced samples			
Treatment 1	Treatment 2	P-Value	Significance (α 0.05)
Control (pH = 6.71)			
Induced	Uninduced	0.045	*
Control (pH = 7.04)			
Induced	Uninduced	0.029	*
BA media (pH = 6.71)			
Induced	Uninduced	0.68	ns
BA media (pH = 7.04)			
Induced	Uninduced	0.032	*

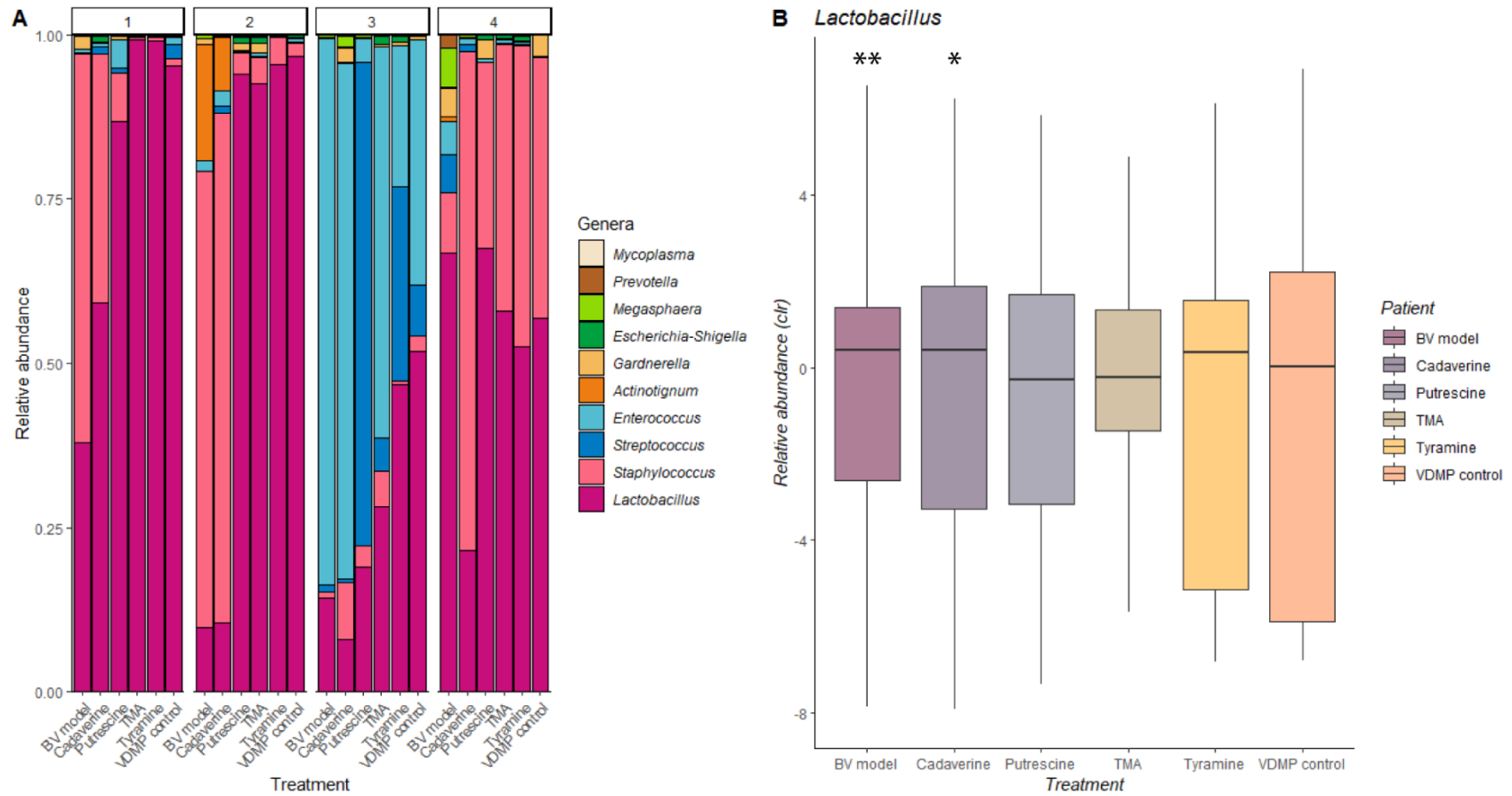


Figure 4.5 Impact of biogenic amines on the vaginal microbiota.

- A) Bar plot of relative abundances. Each colour represents a genus and top numbers indicate each subject from which the sample originated. X-axis denotes which treatment each swab underwent.
- B) Relative abundances of *Lactobacillus* spp. in different media (centered log ratios (CLR) are plotted)

4.4 Discussion

Probiotic strains LRC-14 and LGR-1 showed some degradation of cadaverine and putrescine (both individually and as a co-culture). Interestingly, LGR-1 cultured individually produced a statistically significant amount of tyramine; however, when co-cultured with LRC-14 this effect was not observed. These two strains are administered in combination, and these findings suggests a further benefit of this cooperation (Anukam et al., 2006; Karlsson et al., 2012; Rafael Martinez et al., 2009; Reid et al., 2001, 2003).

Of significant note, *L. crispatus* ATCC 33820 and five vaginal *L. crispatus* strains showed statistically significant reduction of putrescine, cadaverine and tyramine within 24 hours. The exception was strain RL12 which produced tyramine. Cultures of the type strain which had been either exposed ('induced') or not ('uninduced') to biogenic amines prior to the experiment resulted in reduced concentration of biogenic amines from the media. Furthermore, bacteria originating from 'induced' cultures had a higher capacity to degrade amines, better resistance to high pH, and they grew better in the presence of amines. Similarly, filtered supernatants from 'uninduced' and especially 'induced' cultures were able to reduce the concentration of amines.

Interestingly, when the high pH caused by BA was adjusted, *L. crispatus* grew better than in the medium alone. This novel finding suggests that under low pH conditions lactobacilli are capable of using biogenic amines as nutrient sources. The buffering capacity of vaginal fluids has been previously suggested as an indicator of reproductive health (Noguchi et al., 2016). Thus, approaches that aid in the maintenance of an acidic environment, such as the use of prebiotics (Collins et al., 2017), are highly relevant to regulate the concentration of biogenic amines, thereby preventing dysbiosis and malodour.

Previous observations determined that biogenic amine exposure slowed down and reduced the growth capacity of lactobacilli (Borgogna et al., 2021). However, we identified that this inhibitory effect can be reversed under ideal pH conditions. This might explain why some lactobacilli strains can produce biogenic amines whereby some produce small amounts of biogenic amines to support the growth of others, thereby maintaining an acidic environment that prevents the proliferation of pathogenic bacteria.

The microbiota study on vaginal samples showed that at concentrations of biogenic amines similar to those found in the vaginal fluids of patients with BV, *Lactobacillus* are depleted, potentially due to an increase in pH. This, in turn, allows for further proliferation of pathogens towards a state of dysbiosis. Furthermore, only media containing cadaverine had the ability to significantly decrease the proportion of *Lactobacillus* spp., suggesting that this compound plays a key role in the infection process and onset of malodour. The impact of other amines is not clear and should be further explored with larger sample sizes.

Overall, these findings provide a strong rationale for assessing candidate probiotic lactobacilli strains for their capacity to degrade biogenic amines. This study also highlights the relevance of maintaining a low pH in the vaginal environment, since this can turn biogenic amines from inhibitory compounds to energy sources that support the growth of lactobacilli.

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Chapter 5

5 Topical probiotics for women's urogenital health: selection of the best oil-based vehicle

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5.1 Abstract

Vaginal care products are widely used by women to relief discomfort such as pain, itching, or malodour, all of which are commonly caused by conditions resulting from microbiota dysbiosis. Previous studies showed that probiotic strains *Lacticaseibacillus* (formerly *Lactobacillus*) *rhamnosus* GR-1 (LGR-1) and *Limosilactobacillus* (formerly *Lactobacillus*) *reuteri* RC-14 (LRC-14), can aid in restoring homeostasis in the vaginal microbiome when taken orally. A topical product containing these strains could be of value for reducing malodour and improving quality of life. However, the formulation of such a product is a challenge, given that its ingredients must maintain shelf-life viability by excluding moisture. Here, we tested petroleum jelly, mineral oil, coconut oil, and olive oil for how well they maintained the viability of freeze-dried probiotic strains over a six-month timeframe. None of the oils caused excessive loss of bacterial viability, with petroleum jelly and coconut oil showing the most promise. Based on existing knowledge of these oils on the female genitalia, coconut oil and petroleum jelly could be suitable probiotic carriers for clinical testing.

5.2 Introduction

Globally, feminine hygiene product revenue amounts to over 38 billion dollars, with a growth of 3.24% expected annually (*Feminine Hygiene-Worldwide*, n.d.). These products include washes, wipes, creams and sprays intended to clean, soothe, and treat the vaginal area. They are marketed for daily use or to relieve symptoms such as malodour. Although these products are intended to maintain vaginal comfort, many of them may induce adverse effects and disrupt the vaginal

microbiota (Chen et al., 2017; Fashemi et al., 2013; Jenkins et al., 2021), inducing a state of dysbiosis that can predispose women to conditions such as bacterial vaginosis (BV), urinary tract infection (UTI), pregnancy complications (Hillier et al., 1995), and sexually transmitted diseases (Martin et al., 1999), as well as have a negative emotional impact on wellbeing (Bilardi et al., 2013). Therefore, new topical over-the-counter (OTC) therapies could be beneficial if they relieve the cause of aberrant symptoms and signs, and if they help maintain and restore vaginal homeostasis.

Probiotics, defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014), have shown potential to improve female urogenital health. *Lactobacillus* species are dominant in the vaginal microbiome and have properties that contribute to health, such as lactic acid production (Tachedjian et al., 2017) and various mechanisms that compete with pathogens (D’Alessandro et al., 2021). *Lacticaseibacillus* (formerly *Lactobacillus*) *rhamnosus* GR-1 (LGR-1), taken orally, aids to maintain vaginal health and is the most studied probiotic strain for vaginal health (Petrova et al., 2021). Genomic analysis revealed that it is well adapted to the vaginal environment, specifically, due to a unique exopolysaccharide production cluster and its ability to metabolize lactose and maltose, as well as having increased resistance to oxidative stress (Macklaim et al., 2015).

Another strain of interest, *Limosilactobacillus* (formerly *Lactobacillus*) *reuteri* RC-14 (LRC-14), in combination with LGR-1 can reduce infection recurrence (Reid, 2017; Vujic et al., 2013). This combination of strains also has antifungal activity against common uropathogenic yeast *Candida albicans* (Martinez et al., 2009a), and reduces the symptoms of vulvovaginal candidiasis (i.e. discharge, itching, and dysuria), when used in conjunction with an antifungal agent (Martinez et al., 2009b). Furthermore, the symbiotic relationship between strains LGR-1 and LRC-14 can aid in the recovery from dysbiosis, replenishing indigenous species such as *Lactobacillus crispatus* and *Lactobacillus iners*, which are present in high abundance in healthy vagina (Macklaim et al., 2015).

The aim of the present study was to test the viability of a commercial blend of LGR-1 and LRC-14 over a period of 6 months in olive oil, mineral oil, coconut oil, and petroleum jelly, as a means of developing a topical application of strains to counter pathogens and malodour. Current cream and oil-based products are invariably not probiotic by definition, nor do they guarantee that their ingredients can retain the viability of the bacterial contents (Puebla-Barragan & Reid, 2021). The use of preservatives with bactericidal activity is ill advised unless the compounds have proven safety in humans. However, this creates the problem of an increased risk of contaminants being in products. The use of oil-based compounds has the advantage of reduced (or null) water content making them less prone to contamination (Puebla-Barragan & Reid, 2021).

The oils used in the present study can be categorized as plant or petrolatum-based. The former includes olive oil and coconut oil, commonly used in cosmetics due to their low cost and moisturizing properties. Both oils are composed of 95% triglycerides and have been shown to act as an emollient and improve skin barrier function (Sarkar et al., 2017; Vaughn et al., 2018). Petroleum jelly and mineral oil are petrolatum-based and have a long standing history of use in dermatology, dating back to the 1800s (Draelos, 2018). These oils are not absorbed into the skin, have a reduced allergenic profile, can act as an occlusives that reduce moisture loss (Chuberre et al., 2019; Patzelt et al., 2012) and are highly stable (Nash et al., 1996; Rawlings & Lombard, 2012).

5.3 Materials and methods

5.3.1 Oils

Petroleum jelly (Vaseline, Walmart), mineral oil (Life, Shopper's Drug Mart), unrefined coconut oil (Nutiva, Walmart), and extra virgin olive oil (Gallo, Walmart), were purchased from local stores and kept under sterile conditions throughout the duration of the experiment.

5.3.2 Microorganisms

Capsules from a commercial probiotic, stating to contain 5 billion CFUs (colony forming units) of a blend of freeze-dried LRC-14 and LGR-1, were used in this study. Contents consisted of 1 g

of bacteria-containing powder along with the following excipients: glucose anhydrate, microcrystalline cellulose, potato starch, magnesium stearate, gelatin, titanium dioxide, and milk.

5.3.3 Immersion of bacteria in the oil vehicles

Capsules were opened and their entire contents were added to 2 mL tubes with 500 μ L of each oil, and vortexed for 3 minutes. Control consisted of powder only. Petroleum jelly and coconut oil were liquified by incubation in a heating block (40°C) for 3 minutes prior to vortexing. Five replicates were used per oil per time point. Samples in petroleum jelly and coconut oil solidified after the final vortexing step and remained in this state until extraction.

Tubes were stored for 6 months in a dry ice cube in the dark at a temperature of 20°C \pm 2 and at a relative humidity of 50% \pm 5 (temperature and humidity were monitored using a ThermoPro TP50 digital hygrometer). Five tubes were prepared for each time-point for each treatment and discarded after each measurement.

5.3.4 Bacterial extraction

The contents of the oils were extracted by adding 500 μ L of sterile phosphate buffered saline (PBS, pH 7.4), vortexing for 5 minutes, and centrifuged at 5000 x *g* for 15 minutes. PBS was heated to 40°C before being added to the samples with petroleum jelly.

After centrifugation, two layers were formed, PBS with the precipitated bacteria at the bottom, and the oil at the top. Coconut, olive, and mineral oils were removed with a Pasteur pipette, and petroleum jelly with a sterile scoopula. Then, tubes were vortexed for 1 minute to thoroughly resuspend the contents.

5.3.5 Bacterial quantification

Measurements were made at 0, 1, 2, 3, 4, 5, and 6 months. Serial dilutions and CFU enumeration were performed using the drop plate method, which involved inoculating 12 mL of Man-Rogosa-Sharpe (MRS) (De Man et al., 1960) agar plates with rows of 5 μ L drops of PBS containing bacteria using a multichannel pipette. Each row was a miniature serial dilution with each drop down the row having a dilution factor increased 10-fold. The agar plate was incubated

anaerobically for 24 h at 37°C, and the number of colonies was determined by counting the row corresponding to the 10⁸ dilution.

5.3.6 Statistical analysis

The proportion of viable bacteria remaining in comparison to time 0 was calculated for each treatment at each time point. Statistical analysis was carried out in RStudio V1.2.1335, using the ‘emmeans’ package V1.6.0 (Lenth, 2021) factorial two-way analysis of variance (ANOVA) with the Dunnett method post-hoc for multiple comparisons was used to calculate statistical significance. Data was plotted using ‘ggplot2’ (Wickham, 2016).

5.4 Results

5.4.1 Survival of freeze-dried probiotics stored in different oil vehicles

The percentage of bacterial survival is shown in Figure 5.1. Regardless of the treatment, all samples decreased in viability across time points, including the control, which consisted on powder only. After 6 months, all samples had an average reduction in viability of 59.3%. After 3 months, samples stored in coconut oil showed a significantly less viable than those in the control. Petroleum jelly retained the viability of the probiotics significantly better than the control after 5 months of storage.

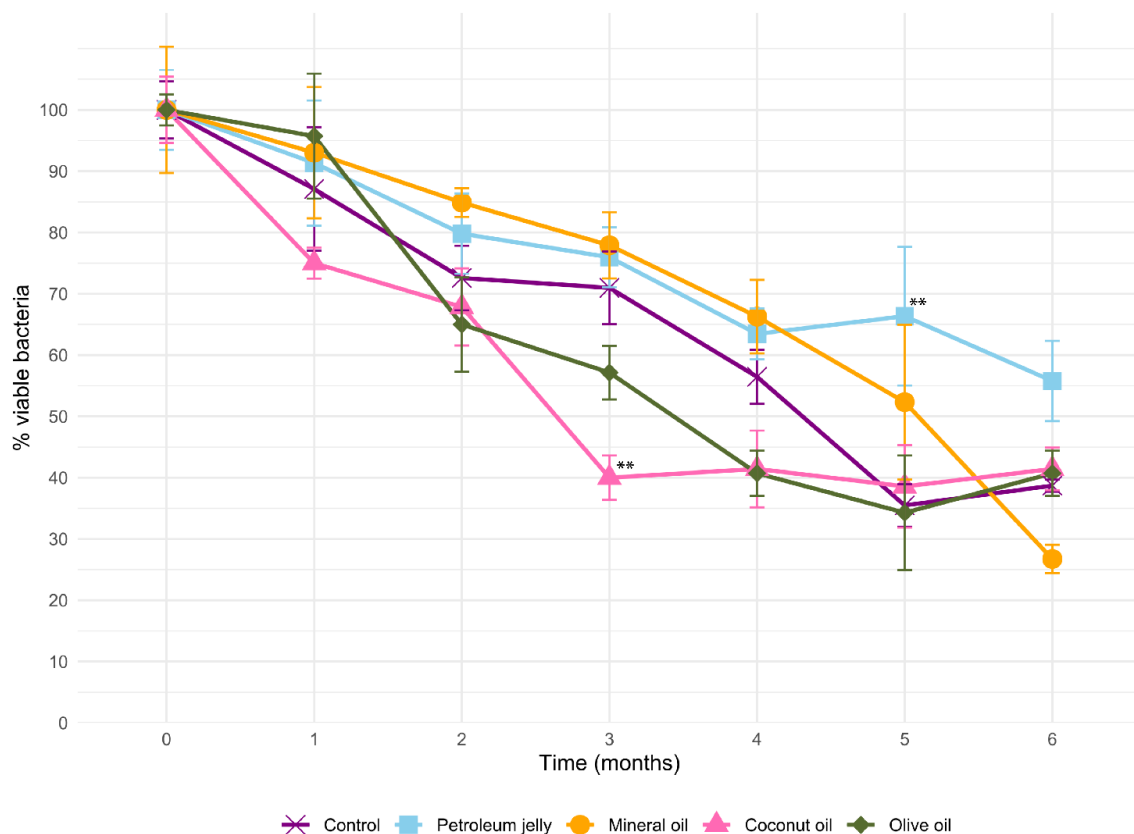


Figure 5.1 Effect of different oils on the viability of freeze-dried probiotics

Proportion of CFUs remaining at time 0 and after months 1-6. One gram of a commercial blend of LGR-1 and LRC-14 was immersed in 500 μL of petroleum jelly (blue line, squared symbols), mineral oil (orange line, circular symbols), coconut oil (pink line, triangular symbols), or olive oil (green line, diamond symbols). The control consisted on freeze-dried bacteria and excipients only. Five independent experiments were carried out at every time point per treatment. The 100% represents 2 billion live organisms. Statistical significance was calculated with a factorial two-way analysis of variance (ANOVA) with the Dunnett method post-hoc for multiple comparisons (** $p \leq 0.001$).

5.5 Discussion

The present study showed that several mineral oils approved for vaginal use can be used to retain urogenital probiotic strain viability over six months. The use of personal care products either externally or intravaginally is common practice around the world (Brown et al., 2013; Crann et al., 2018; Hassan et al., 2007). These include vaginal washes, lubricants, and wipes ostensibly to relieve itching, dryness, malodour, or burning sensations, as well as to improve their sexual lives. Of critical importance in using local applications, in addition to safety, is that strains are selected for appropriate properties and they can survive in the delivery vehicle. Unfortunately, some locally applied products can increase the risk of vaginal dysbiosis and subsequent BV or UTI (Brown et al., 2013, 2016; Chen et al., 2017).

There is generally a loss of bacterial viability within freeze dried preparations. The product label states a guarantee that at least 1 billion of CFU would remain viable at the end of the shelf life of the product (approximately three years after manufacture), which would represent 20% of the initial amount added. The viability of two probiotic strains was not reduced in mineral and olive oil compared to controls, indicating the oils were not antimicrobial. Of note, the powder used for these studies was from capsules which also provide protection from viability loss (Wilcox et al., 2020).

Both coconut oil and petroleum jelly are solid at room temperature, but they behaved differently. The strains incubated in coconut oil had decreased viability by almost 20% more after three months, yet no further loss occurred. The explanation is not known but suggests an equilibrium is reached perhaps after adaptation to the oil's properties. The viable count for strains incubated in petroleum oil was higher at five compared to four months which we suspect was within experimental error despite the statistical significance, since the monthly trend followed a similar pattern between months one to four then five to six.

The nature of the coconut oil and petroleum jelly provided a relatively even dispersion of the bacterial powder. This is a desired attribute in this type of product, to ensure that every application contains the probiotic organisms. Therefore, if approximately 5 mL was dispensed from a tube, at six months, it would still be expected to deliver one billion live organisms.

The knowledge of the impact of these oils in the vaginal microbiome is sparse. This is a factor that must be considered before formulating a product of this nature. External use of mineral oil is common (i.e. perianally or on the vulval area) (Araújo & Oliveira, 2008; Thorstensen & Birenbaum, 2012), and despite it being linked to adverse reactions (Crann et al., 2018) and *Candida* colonization (Brown et al., 2013), it is a mainstay ingredient of medical pomades intended for vaginal use (Tirri, 2011).

In considering the use of petroleum jelly to deliver probiotic bacteria, it should be noted that when applied intravaginally it is associated with a lower prevalence of *Lactobacillus* species and an increase in the abundance of BV-associated morphotypes (Brown et al., 2013; Hassan et al., 2007). Yet, similarly to mineral oil, external use of petroleum jelly is generally considered safe and is common in clinical practice, either perianally (Araújo & Oliveira, 2008), or on the skin of vulval vestibule to treat symptoms associated to dermatological inflammatory conditions of the vulva (Thorstensen & Birenbaum, 2012). The inclusion of lactobacilli strains antagonistic to BV organisms, could prove to counter these negative attributes of the jelly in the vagina. Better still, if the probiotic strains in petroleum jelly were only applied to the outer urogenital skin, this would reduce further the risk of BV organisms propagating.

Olive oil has antimicrobial, antioxidant, and anti-inflammatory activities, mostly due to their high content of phenolic compounds. However, little is known on its impact on the microbiota (Cicerale et al., 2012). Its high content of oleic acid could harm the skin barrier and be an irritant, thereby damaging the native microbiota, which could in turn allow pathological organisms to colonize and cause inflammation (Vaughn et al., 2018). However, olive oil has been successfully used intravaginally in a clinical setting to relief breast cancer patients of dyspareunia (genital pain caused by intercourse) and it can also inhibit *Candida* species (Alwan & Alwan, 2019).

Although coconut oil was the only tested oil to show a significant decrease in viability, it is also the one with most potential for vaginal health and, as mentioned previously, its ability to remain solid at room temperature allows for a better dispersion of freeze-dried bacteria. Coconut oil contains monolaurin, which is an antimicrobial monoglyceride formed from lauric acid, which is a short fatty acid that can disrupt the membranes of microbial organisms; it is particularly efficient at inhibiting common skin pathogens such as *Propionibacterium acnes* and

Staphylococcus aureus. However, this antimicrobial effect could be the reason why there was a drastic decrease in the viability of probiotics after three months so, if a probiotic product were to be formulated based on coconut oil, it would be important to market it as to be used no more than 2 months after purchase (Vaughn, 2018). This could pose challenges for distribution, perhaps requiring refrigeration. Coconut oil has been shown to enrich commensals and decrease the expression of the pathogenesis pathway of fungi found on the scalp (Saxena et al., 2021). In addition, it is highly effective in reducing *Candida albicans* (Ogbolu et al., 2007) and inhibiting the production of exotoxins by vaginal pathogens (Schlievert et al., 2008). When applied vaginally to rhesus macaques, coconut oil did not affect the compositions of the vaginal microbiota (Schlievert et al., 2008).

In summary, the present study suggests that petroleum jelly and coconut oil are good candidates as vehicles for the delivery of topical probiotics to the external female genitalia. Future studies could assess the impact on the vaginal microbiota and metabolic read-out as well as the effects on epithelial cells. A previous study has shown that application of LGR-1 to the vagina can stimulate antimicrobial peptides (Jayaram et al., 2014), making it a good choice for this type of application. An advantage of applying live bacteria rather than compounds such as lactic acid (Bruning et al., 2020) is that the probiotic strains can adapt to the environment and produce other substances important to health maintenance.

It is also clear that packaging will play a major role for this type of product, both for enhancing viability, as well as to guarantee it is designed to be used within the ideal shelf-life time. Additionally, a product that has to be kept in refrigeration would be an excellent approach to extend the utility life of the product while avoiding the use of additional ingredients (e.g. preservatives) that could affect the bacteria in the product as well as the urogenital microbiota of the consumer. Preparations formulated by apothecaries could be made by adding freeze-dried bacteria to the carrier with a defined short-term shelf life, this would warrant the viability of the probiotics.

Of note, all of the tested oils retained the viability of probiotics in a comparable manner as the control, suggesting that they can also be used when formulating topical probiotic products with other stains that target different organs, such as the skin. Still, there is a major area of

opportunity in the investigation of impact of these and other lipids on the microbiota of different organs. It is pressing to bridge these gaps in knowledge to widen the uses and benefits of probiotics.

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Chapter 6

6 General discussion

Interest in probiotic research and applications has increased dramatically throughout the past two decades (Puebla-Barragan & Reid, 2019). However, challenges remain, particularly that although new products claiming to be probiotic are flooding the market, the vast majority do not comply with the requirements to be labelled as such (Puebla-Barragan & Reid, 2021).

A key aspect of probiotics is that they must provide a documented health benefit on the host (FAO/WHO Working Group, 2002; Hill et al., 2014). Therefore, it is crucial to keep investigating different strains with specific benefits in mind, rather than proposing them as a one cure-all (Reid et al., 2019).

On that note, certain probiotic strains have been well documented to aid in the maintenance and restoration of women's urogenital health; the most prominent strains for this purpose are *Lactocaseibacillus rhamnosus* GR-1 (LGR-1) and *Limosilactobacillus reuteri* RC-14 (LRC-14) (Petrova et al., 2018; Reid et al., 2003). Other probiotics, such as *Lactobacillus crispatus* CTV-05, are being tested for intravaginal use (Hemmerling et al., 2009; Stapleton et al., 2011) That strains has shown to reduce recurrence of urinary tract infection (UTI), but is not being pursued further.

The approach taken by our lab is to focus on strain properties and align them with the target for their use. In doing so, it is important to identify ailments amenable to probiotic applications. This thesis focused on urogenital malodour, a problem that affects millions of women around the globe (Bilardi et al., 2013), but one that has been overlooked such that no effective solution exists yet. The idea being explored was whether lactobacilli strains could counter the production of malodourous compounds or degrade them.

6.1 The chemistry of malodour in the female urogenital tract

The female urogenital microbiome in healthy women is populated mostly by *Lactobacillus* species (Miller et al, 2016; Ravel et al., 2011). When homeostasis is lost and a state of dysbiosis arises, the host become susceptible to infection. Bacterial vaginosis (BV) and urinary tract

infection (UTI) are two of the most prevalent conditions that can result from the disruption of the vaginal microbiome (Salvatore et al., 2011; Tandogdu & Wagenlehner, 2016). A factor that these conditions have in common is malodour, which is one of the main reasons patients seek medical advice.

Previous work in our lab by Dr. Amy McMillan used metabolomics and genomics to better characterize the vaginal microbiome. One of her main findings was that the biogenic amines (BAs) cadaverine, putrescine, tyramine, and trimethylamine (TMA), were biomarkers of BV (McMillan et al., 2015). This suggests that, by targeting these compounds, relief from urogenital malodour could be provided.

At the beginning of my doctoral studies, there was a tremendous knowledge gap in terms of which compounds were responsible for malodour during the course of a UTI. Moreover, this sign has been commonly overlooked both in research and clinical settings (Sj et al., 2004; Subramanian et al., 2012). The study outlined in Chapter 1 established metabolomic methodologies and determined that BAs were responsible for urinary malodour (Puebla-Barragan et al., 2020)

6.2 Lactobacilli and biogenic amines: a complex relationship

Preliminary experiments suggested that BAs affected the growth and metabolism of indigenous lactobacilli, thereby reducing the defenses of the urogenital tract and promoting dysbiosis (Borgogna et al., 2021; Nelson et al., 2015). Findings from Chapters 3 and 4 confirmed that there is indeed an interaction between vaginal lactobacilli and BAs since. Some lactobacilli were found able to degrade these compounds while others used them as an energy source. Genomic analyses showed that biocatalytic and biosynthetic pathways were present.

In Chapter 4, these interactions were further investigated, and it was discovered that although BAs indeed reduce the growth of the mainstay vaginal species *Lactobacillus crispatus*, when pH conditions remain low these organisms can use the amines as energy sources that allow for better and faster growth. This implies a change in paradigms were, compounds previously thought to negatively impact vaginal lactobacilli (Borgogna et al., 2021), can actually have the opposite

effect and be beneficial under the right conditions. Future studies should further investigate this relationship in order to develop a strategy to manage the levels of malodour.

Chapter 4 also proved that *L. crispatus* undergoes metabolic adaptations when exposed to BAs, making them less susceptible to these compounds at higher vaginal pH conditions.

Overall, this thesis revealed the potential to develop probiotics capable of degrading BAs, maintaining a pH less than 4.7, and allowing the indigenous strains to use BAs to retain homeostasis. Specific prebiotics (Collins et al., 2017) and buffering products (Mayer et al., 2001) could further assist in lactobacilli stimulation. Although probiotic strains LGR-1 and LRC-14 did not significantly degrade BAs, they can robustly maintain an acidic environment through the production of lactic acid (Reid et al., 2003). Nonetheless, further studies, with human subjects and larger sample sizes are still required to better characterize the mechanisms and relationships between biogenic amines and vaginal lactobacilli.

6.3 Topical probiotics: an efficient solution

The significant burden that urogenital malodour represents for women, as well as the lack of effective and safe treatments, has led to the widespread use of feminine products such as lotions, wipes and vaginal douches, which, are not only ineffective since at best they only mask the odours, but they actually increase the risk of vaginal dysbiosis, making users more prone to infection (Fashemi et al., 2013). The fact that these products exist and are commonly purchased, reflects the market demand for treatments.

The use of probiotics has already shown promise to restore and maintain homeostasis which, albeit indirectly, aids to reduce malodour by preventing the establishment of pathogens that produce them in large quantities. In cases where infection arises, antibiotics do not provide full relief from signs and symptoms for several days, and therefore there is a place for product that can provide faster relief by degrading malodourous compounds. Our findings from Chapter 3 and 4 confirmed that there are several strains, particularly of *L. crispatus*, capable to significantly decrease the amount of BAs. The discovery that cell-free extracts can achieve the same result opens up the possibility for formulations accessible to over-the-counter purchase. In order to

develop a topical application that contains live lactobacilli as well as the cell-free extracts, the delivery system must support the retention of the viable organisms.

If a topical probiotic product were to be developed, there are three key factors to take into account: 1) Bacteria should remain live throughout the shelf-life, 2) The formula must have a low or null water content to prevent contamination, and 3) All components should be safe for human use (Hill et al., 2014; Puebla-Barragan & Reid, 2021; Tkachenko et al., 2017). The results of the proof-of-concept experiments carried out in chapter 5 suggest that an excellent approach would be an oil-based formula, predominantly based on coconut oil or petroleum jelly or as a mixture of different oils. Coconut oil appears to be the most promising choice since there is evidence that it can be favourable for the vaginal microbiota, although based on our observations such product would have a short shelf-life and require specific packaging and storage conditions (Anzaku, 2017; Ogbolu et al., 2007). However, despite products being designed for intravaginal application, there is a major knowledge gap on the impact of their ingredients on the vaginal microbiota (Brown et al., 2013; Fashemi et al., 2013; Łaniewski et al., 2021). More research on this is needed, which takes into account the biochemistry of the different ingredients in personal care formulas, as well as their interaction with probiotic strains. Although our findings in this matter are preliminary, they are the first step towards better understanding of the impact of vehicles in probiotics. Next steps should focus on metabolic and transcriptomic adaptations that bacteria might undergo due to exposure to different ingredients, with a view of getting a clearer picture of how they can impact the health benefits that the product aims to have on the host.

6.4 Where is the field heading?

The market for probiotic products is growing at a very steady rate. Unfortunately current regulations are not sufficient to guarantee that what consumers acquire is indeed a probiotic (FactMR, 2020; Foligné et al., 2013). The term ‘probiotic’ has become a buzz word too often misused to inflate the merits of some products (Puebla-Barragan & Reid, 2019; 2021). While the onus is on regulatory agencies to prevent misleading claims, the scientific community should provide stewardship by demanding that strains only be termed probiotic if backed by appropriate evidence.

In terms of urogenital health, it should be possible to create rapid tests that identify microbiota and metabolic profiles thereby improving the accuracy in diagnoses. In theory this should allow physicians to tailor a better management plan (Cartwright et al., 2018; Hong et al., 2016), but with no alternatives to antibiotics, this is difficult. Hopefully, if probiotic strains and compounds that degrade BAs can be developed as efficacious products, they could either be prescribed or purchased by women to restore and maintain urogenital health. This will be an interesting challenge for treating urinary malodour since these compounds are likely produced by constituents of the bladder and urethral microbiota. Two approaches are conceivable: ingestion of prebiotic compounds to stimulate the lactobacilli within the microbiota; and topical application of the BA-degrading lactobacilli to see if they ascend the urethra and influence the bladder microbiome. Hopefully the work described in this thesis will help towards these goals and lead to tangible improvements in the health of women.

6.5 References

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Appendix D: supplementary material for Chapter 4

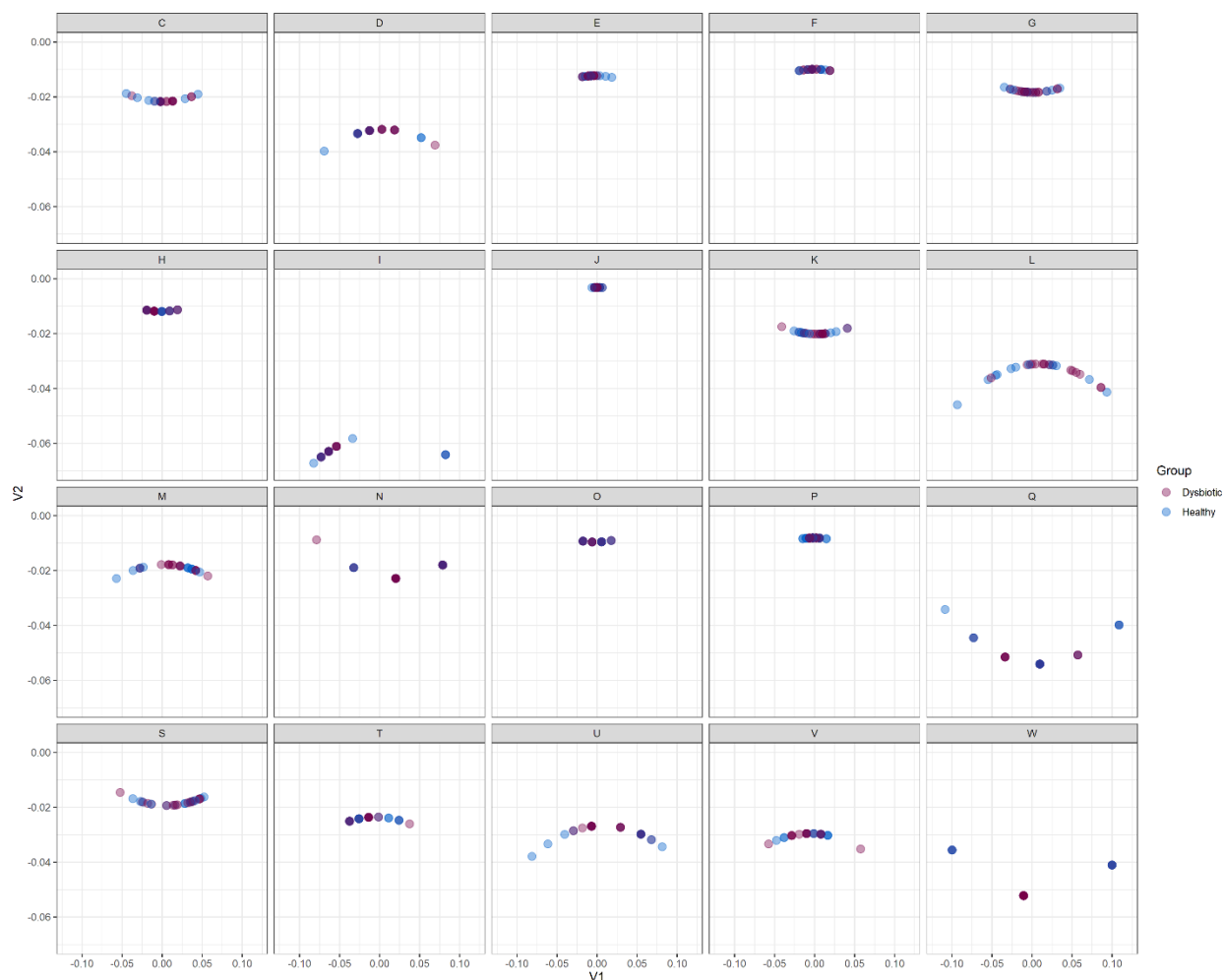


Figure S1. PCoA of predicted functional capacity of strains based on mapping of genes to the EggNOG database. Each letter represents a different functional category. [A] RNA processing and modification, [B] Chromatin structure and dynamics, [C] Energy production and conversion, [D] Cell cycle control, cell division, chromosome partitioning, [E] Amino acid transport and metabolism, [F] Nucleotide transport and metabolism, [G] Carbohydrate transport and metabolism, [H] Coenzyme transport and metabolism, [I] Lipid transport and metabolism, [J] Translation, ribosomal structure and biogenesis, [K] Transcription, [L] Replication, recombination and repair, [M] Cell wall/membrane/envelope biogenesis, [N] Cell motility, [O] Post-translational modification, protein turnover, and chaperones, [P] Inorganic ion transport and metabolism, [Q] Secondary metabolites biosynthesis, transport, and catabolism, [R] General function prediction only, [S] Function unknown, [T] Signal transduction mechanisms, [U] Intracellular trafficking, secretion, and vesicular transport, [V] Defense mechanisms, and [W] Extracellular structures. Groups with <5 genes per strain were removed.

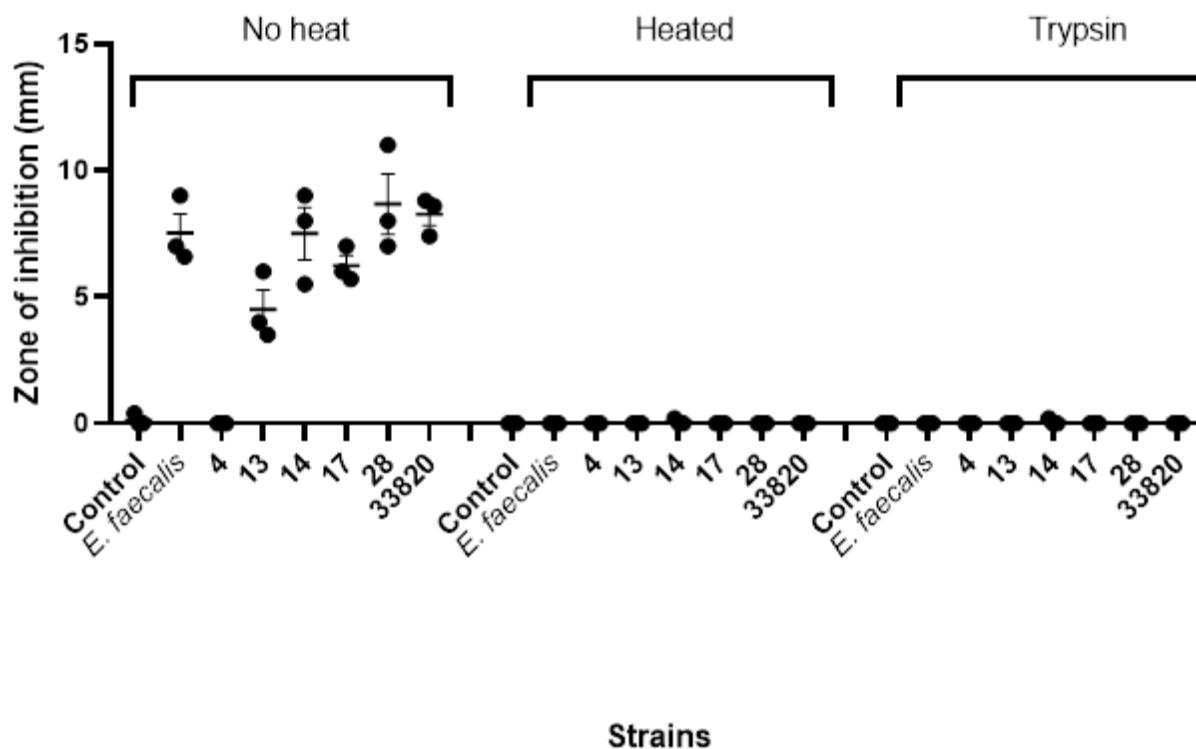


Figure S2. Zone of inhibition measurements (mm) from agar well diffusion assays featuring *L. crispatus* supernatants that were heated, treated with trypsin, and unheated against *E. faecium* indicator strain.

Strains were grown for 24 hours on CBA plates and the individual supernatants were collected using the freeze/thaw method. *L. crispatus* supernatant volumes were then normalized, neutralized using HCl and NaOH, and filter sterilized. Additional CBA plates as controls without bacterial growth, were subjected to the same incubation and collection method. 1/3 each supernatant was heated to 85°C for 45 minutes, and 1/3 of the supernatants were treated with 1mg/mL of trypsin. Included in the controls was a sample that was neutralized, one that was left at its original pH, one that was brought to a pH of 5 using lactic acid and then neutralized using NaOH, and one that was brought to a pH of 5. All controls were then filter sterilized. 250µl of the indicator strain was plated on m17 agar, 1cm holes were bored, and 50 µl of each supernatant was deposited into each well. Following incubation at 37°C for 48 hours the zones of inhibition were measured (N=4).

Table S1. *L. crispatus* strains used in this study. Strains were isolated from human vaginal swabs with *Lactobacillus*-dominated vaginal microbiota (LVM) or dysbiotic vaginal microbiota (DVM). BioSample Database accession numbers (NCBI: PRJNA390079) and bacterial collection strain numbers.

Strain	BioSample Database DDBJ/ENA/GenBank	Collection	Strain	BioSample Database DDBJ/ENA/GenBank	Collection
RL02	SAMN07213039	NCCB 100711	RL21	SAMN07213201	NCCB 100723
RL03	SAMN07213108	not available	RL22	not available	not available
RL05	SAMN07213187	not available	RL23	SAMN07213203	NCCB 100724
RL06	SAMN07213188	not available	RL24	SAMN07213204	NCCB 100725
RL07	SAMN07213189	NCCB 100713	RL25	SAMN07213205	NCCB 100726
RL08	SAMN07213190	not available	RL26	SAMN07213206	not available
RL09	SAMN07213191	NCCB 100714	RL27	SAMN07213207	NCCB 100727
RL10	SAMN07213192	NCCB 100715	RL28	SAMN07213208	NCCB 100728
RL11	SAMN07213193	not available	RL29	SAMN07213209	not available
RL13	SAMN07213194	NCCB 100716	RL30	SAMN07213210	NCCB 100730
RL14	SAMN07213195	NCCB 100717	RL31	SAMN07213211	not available
RL15	SAMN07213196	NCCB 100718	RL32	SAMN07213212	not available
RL16	SAMN07213197	NCCB 100719	RL33	SAMN07213213	NCCB 100731
RL17	SAMN07213198	NCCB 100720			
RL19	SAMN07213199	NCCB 100721	RL01	not available	not available
RL20	SAMN07213200	NCCB 100722	RL04	Not available	Not available
			RL12	not available	not available
			RL22	not available	not available

Appendix E: ethical approval for swab collection



**Western
Research**

Research Ethics

**Western University Health Science Research Ethics Board
HSREB Amendment Approval Notice**

Principal Investigator: Dr. Gregor Reid

Department & Institution: Schulich School of Medicine and Dentistry\Microbiology & Immunology, Western University

Review Type: Expedited

HSREB File Number: 106089

Study Title: Acquisition and analysis of vaginal swabs from healthy woman and those with bacterial vaginosis

Sponsor: Kimberly Clark Corp

HSREB Amendment Approval Date: June 15, 2016

HSREB Expiry Date: January 13, 2017

Documents Approved and/or Received for Information:

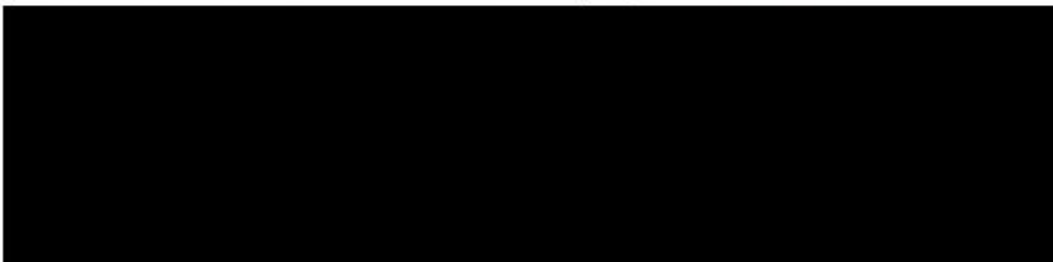
Document Name	Comments	Version Date
Revised Letter of Information & Consent	Revised ICF clean	2016/05/19
Revised Western University Protocol		2016/06/14

The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the amendment to the above named study, as of the HSREB Initial Approval Date noted above.

HSREB approval for this study remains valid until the HSREB Expiry Date noted above, conditional to timely submission and acceptance of HSREB Continuing Ethics Review.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice Practices (ICH E6 R1), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.



Curriculum Vitae

Name: Scarlett Puebla Barragan

Post-secondary Education and Degrees: Tec de Monterrey
Monterrey, Nuevo Leon, Mexico
2009-2013 B.Sc.

The University of Western Ontario
London, Ontario, Canada
2015-2017 M.Sc.

The University of Western Ontario
London, Ontario, Canada
2017-2021 Ph.D.

Honours and Awards: Scholarship from the Mexican Council of Science and Technology
2015-2017, 2017-2021

F.W. Luney Travel Award
2018, 2019

Related Work Experience Teaching Assistant
The University of Western Ontario
2015-2017, 2018-2021

Publications:

Puebla-Barragan, S., & Reid, G. (2019). Forty-five-year evolution of probiotic therapy. *Microbial cell*, *6*, 184–196.

Puebla-Barragan, S., Renaud, J., Sumarah, M., & Reid, G. (2020). Malodorous biogenic amines in *Escherichia coli*-caused urinary tract infections in women—a metabolomics approach. *Scientific reports*, *10*, 9703.

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Giallourou, N., Urbaniak, C., **Puebla-Barragan, S.**, Vorkas, P., Swann, J., & Reid, G. (2021) Characterizing the lipidomic signature of breast cancer and its interaction with the tissue microbiota. [Manuscript submitted for publication].