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# Metabolomic and Genomic Analysis of Lactobacillus crispatus Vaginal Isolates

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

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# Abstract

*Lactobacillus crispatus* is the dominant species in the vagina of many women. To add to our knowledge of its properties, the metabolic readout of twenty strains was analyzed using liquid chromatography-mass spectrometry (LC-MS). This led to a separation of the strains into two groups, Groups A and B. Notably, five Group B strains produced tyramine, known to raise vaginal pH, and make the environment more favourable to vaginal pathogens. The origin of Group B strains did not correlate with their host having bacterial vaginosis (BV), a condition associated with elevated pH. Five bacteriocins were detected in the genomes. Therefore, differences between *L. crispatus* vaginal strains based upon their genomes and metabolism. In absence of a correlation with the health status of the donor, it remains to be fully determined if this species is an active participant in creating a healthy state, or a bystander easily displaced by organisms causing dysbiosis.



**Key Words:** *Lactobacillus crispatus,* bacterial vaginosis, vaginal dysbiosis, metabolomics, bacteriocins, pathogen inhibition





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## Summary for Lay Audience

Within the vagina there is a collection of microorganisms termed the vaginal microbiome. Of these organisms the most common type in healthy women of reproductive age is *Lactobacillus crispatus*. To better understand this species, twenty strains were isolated from either healthy women, or women with vaginal dysbiosis, which is an imbalance of the vaginal bacteria that can have severe reproductive outcomes. The findings separated the twenty strains into two groups A and B, but this was not based on whether the women from whom they were collected were healthy or not. Rather, group B strains had characteristics that could negatively influence the vaginal environment, including the production of compounds that elevate pH to one more favourable to disease-causing bacteria. Five compounds were identified in the DNA that have the ability to inhibit or kill infection-causing bacteria. In summary, this research identified that not all *L. crispatus* strains are the same. Therefore, although these types of organisms have been suggested to be good probiotics, which are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host," care must be taken in selecting which strain has the necessary properties to improve the vaginal environment.



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# Co-Authorship

**Chapter 2:** Watson, E., van der Veer, C., Collins, S., Renaud, J., Sumarah, M., Kort, R., Reid, G.

Van der Veer, C.- Provided guidance for experimental design

Collins, S.- Assisted with metabolomics analysis

Renaud, J.- LC-MS analysis, provided guidance for experimental design

Sumarah, M - Provided guidance for experimental design

Kort, R. - Supplied *Lactobacillus crispatus* isolates

Reid, G. - Principal investigator, provided guidance for experimental design and critical comments for manuscript

**Chapter 3:** Watson, E., van der Veer, C., Gloor, Tandon, P., G., Kort, R., Reid, G.

Van der Veer, C. - Provided guidance for experimental design and performed genome annotation

Gloor, G. - Provided guidance for experimental design

Tandon, P. - Assisted with sequence alignment of bacteriocin genes

Kort, R. - Provided *Lactobacillus crispatus* isolates

Reid, G. - Principal investigator, provided guidance for experimental design and critical comments for manuscript

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## Dedication

To my dad, you're the best:

Byron Watson



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# List of abbreviations

| | |
|---|---|
| BV | Bacterial vaginosis |
| UTI | Urinary tract infection |
| CBA | Columbia blood agar |
| MRS | De Man Rogosa and Sharpe |
| BHI | Brain heart infusion |
| °C | Degrees Celsius |
| L | Litre |
| μL | Microlitre |
| mL | Millilitre |
| DNA | Deoxyribonucleic acid |
| Bp | Base pairs |
| Mbp | Million base pairs |
| pH | Potential hydrogen |
| LC-MS | Liquid chromatography mass spectrometry |
| GC-MS | Gas chromatography mass spectrometry |
| M/z | Mass to charge ratio |
| mm | Millimetre |
| cm | Centimetre |
| CFU | Colony forming units |
| LAB | Lactic acid bacteria |
| FDA | Federal drug administration |
| N | Number |



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# Chapter 1: General introduction

## 1.1 *Lactobacillus crispatus*

*Lactobacillus crispatus* is a gram positive, rod-shaped species classified as a lactic acid bacterium (LAB), producing lactic acid as a major metabolic product.[1,2] Found in the human urogenital tract and the chicken gastrointestinal tract, it is considered to be an extremely beneficial species.[3,4] As the dominant organism among healthy women, *Lactobacillus crispatus* is thought to be important to maintain vaginal health. [1,5–8] It has been shown to have the ability to adhere to vaginal epithelial cells and block the adherence of uropathogenic bacteria.[9–11] Due to these attributes and clinical observations, the species has been postulated to be an excellent candidate for a probiotic to restore a healthy vaginal microbiota.[12–14] The definition of a probiotic is "live microorganisms that when administered in adequate amounts confer a health benefit on the host".[15] The prevalence of *L. crispatus* in the vagina is likely due to its superior ability to survive in this environmental niche, albeit certain strains have superior adherence ability and higher rates of vaginal colonization.[16–18] The ability of this species to mitigate and control disease is an ongoing area of research.

### 1.1.1 The vaginal environment

Based upon studies from around the world, a healthy vaginal microbiota is commonly composed of *Lactobacillus jensenii, Lactobacillus gasseri,* and *L. crispatus,* or *Lactobacillus iners*, or a fifth strain that can be *Lactobacillus vaginalis, Lactobacillus reuteri,* or *Lactobacillus rhamnosus.* [19–22] The incidence of these species fluctuates based on hormonal changes throughout a woman's life, and can be altered by several processes including menstruation, pregnancy, and sexual activity.[23–26] Their dominance is associated with a decreased risk of



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sexually transmitted infections, such as gonorrhea and human immunodeficiency virus (HIV), and infection by *Candida albicans* and *E.coli*, as well as the organisms associated with BV. [1,27–30] When lactobacilli are displaced by pathogenic bacteria, vaginal dysbiosis occurs.[8,31,32]

The term BV is a catchall of several conditions that can be symptomatic or asymptomatic[33]. A dysbiotic state is universal under these condition, and it has been associated with significant adverse outcomes including pre-term birth, low-weight infants, and increased susceptibility to sexually transmitted infections.[13,34–36] This is one of the most common vaginal conditions, stemming from a decrease in lactobacilli and an increase in anaerobic pathogenic bacteria such as *Gardnerella vaginalis, Atopobium vaginae* and *Prevotella bivia*.[37,38] Characterized by vaginal discomfort and malodorous discharge, BV is diagnosed using two systems. The first is the Amsel criteria which requires the presence of at least three of the following four criteria: a fishy odor following mixture of vaginal secretions with a 10% potassium hydroxide solution, the presence of clue cells, vaginal secretions with a pH of over 4.5, and a white vaginal discharge.[39] Also used, the Nugent scoring system[40] based upon Gram stained slides of vaginal smears, provides a scale ranging from 1 to 10 with 1-3 being normal dominated by lactobacilli morphotypes. A weighted sum of the present bacteria provides a standardized score of vaginal health.[40-42]

Current treatment of BV is an oral or topical dose of metronidazole or clindamycin. However, these are extremely unreliable, with cure rates ranging from 50 to 80% and a relapse rate greater than 50% within six months.[43,44]

The absence or depletion of lactobacilli associated with vaginal dysbiosis and BV can be harmful, particularly in pregnancy,[45,46] leading to preterm birth, including those caused by



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rupture of the fetal membrane, and low-weight infants.[13,30,47] A healthy pregnancy has a more stable *Lactobacillus*-dominated composition, especially with *L. crispatus.*

1.1.2 Role in the vaginal microbiome

An important contribution to vaginal health by lactobacilli is the production of lactic acid, which lowers pH making the environment less favorable to pathogens.[2,48,49] Lactic acid levels are highest when the vaginal environment is dominated by *L. crispatus*, further supporting its role in health maintenance.[21] Women who are colonized by this species appear to have a fivefold decreased risk for developing BV as well as a decrease in urinary tract infection recurrence.[50] However, other mechanisms for the maintenance of health have been postulated, such as the production of antimicrobial proteins, which may include bacteriocins, and hydrogen peroxide.[5,21,24]

Of the most abundant vaginal lactobacilli, *L. crispatus* has the largest genome with the highest number of proteins, ranging from 1.83 to 2.7 Mb and encoding up to 2,688 proteins.[51] Strains can also have unique DNA polymerase, bacteriocin, toxin-antitoxin systems, and genes encoding mobile genetic elements.[5]

The primary source of carbon and energy within the vagina is thought to be glycogen produced by host epithelial cells.[48,52] This can be used by bacteria to produce lactic acid. [21,45] Until recently the core genome of *L. crispatus* was not found to contain the enzymes necessary for the degradation of glycogen, which meant the species relied on the host for access to the sugar. However, *L. crispatus* vaginal strains have now been discovered that degrade glycogen.[53]

Bacteriocins have been applied as food preservatives, treatments for bacterial infection and prevention of disease, and as cancer therapy.[54] These antimicrobial polypeptides are



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produced by lactobacilli tend to have a narrow spectrum of activity. There are three classes of bacteriocins produced by LAB: class I are small in size (<5kDa) and encompass the lantibiotics where post-translationally modified amino acid resides form a covalent bridge between residues, resulting in a ring formation; class II are small heat-stable peptides (<10kDa) composed of an N-terminal leader peptide, which acts as a recognition site and is not antimicrobial on its own, and a C-terminal propeptide; class III are heat-labile antimicrobial proteins, also referred to as bacteriolysins due to their lytic activity (>30kDa).[55,56] Among class III, four bacteriocins from LAB have been characterized.[57]

Conducting studies involving the production of bacteriocins has recently become popularized due to the increase in antibiotic resistant pathogens.[28,54,58–60] Those produced by LAB are especially promising due to their heat and pH tolerance. Of these the best characterized, and one of two approved by the FDA, is nisin from class III, a compound used quite successfully as a food preservative as well as for treatment of ulcers.[61]

The mechanism of antimicrobial activity is class specific, however all bacteriocins have the same initial reaction to cell membranes. Their positive charge creates an electrostatic interaction with the negatively charged bacterial cell membrane, facilitating the arrival of the molecule.[55] This is why bacteriocins are less effective against Gram negative species without the presence of a surfactant. Lantibiotics disrupt the cell membrane through pore formation causing termination of cellular processes. Pediocin-like bacteriocins (class IIa) induce membrane permeabilization leading to cell death.[55] Two-peptide bacteriocins (class IIb) have a similar mode of action to lantibiotics but cause a smaller pore leading to the leakage of small molecules.[62] Circular bacteriocins (class IIc) are also pore forming causing an efflux of intracellular solutes.[63] Bacteriolysins (class IIIa) proteolytic enzymes which cause dissolution of



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the cell wall, resulting in lysis. The other branch of class III bacteriocins (class IIIb) disrupt plasma membrane potential.[57]

### 1.1.3 Antimicrobial activity

Due to its production of hydrogen peroxide ($H_2O_2$), *L. crispatus* CTV-05, termed Lactin-V by Osel Inc., California, was chosen for clinical trials to evaluate the effect it has on vaginal health. The pilot study demonstrated persistence of CTV-05 following vaginal insertion in women who were not already colonized by a $H_2O_2$-producing *L. crispatus*. [18] This implied that indigenous strains did not allow persistence of the exogenous CTV-05. A randomized double-blind study using CTV-05 showed that persistence of the strain resulted in minimal adverse outcomes and reduced the risk of recurrent urinary tract infection (UTI) by around 50%. [64] Ironically, the company has since decided not to pursue UTI claims for business reasons, which is unfortunate as CTV-05 applied vaginally could have offered another option in management recurrence.

Overall, *L. crispatus* isolates have exhibited broad-spectrum inhibition against both Gram positive and negative bacteria. Among those inhibited include *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Gardnerella vaginalis, Escherichia coli,* and *Neisseria gonorrhoeae.* [9,13,24,65,66] Although lactic acid was determined to play a role in the inhibition of pathogens, several studies have suggested that there are other molecules, such as bacteriocins, that can contribute to its antimicrobial activities. This broad inhibitory potential suggests promise for the use of *L. crispatus* isolates as probiotics for the prevention and treatment of infection, and as preservatives.



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1.2 Metabolomics

Metabolomics refers to the study of metabolites present within, or produced by, an organism, cell, or tissue. [67] Through a careful experimental design, the compounds present can be examined using a targeted or untargeted approach. The latter allows for a comprehensive analysis of all metabolites present in a sample, both known and unknown. This approach tends to be favoured as it allows for the identification of novel biomarkers. However, the difficulty lies with how time-consuming it is to analyze the extensive raw data generated and to identify and characterize the unknown molecules.[68]

Targeted metabolomics measures metabolites that have already been chemically and biochemically annotated. Using standards of known compounds, biochemical pathways can be analyzed. This approach allows for the optimization of sample preparation and the analysis protocol, so that compounds of interest can be clearly defined. [68]

Analytical approaches include gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS), among others. The more common of the two, GC-MS, begins with a complex sample preparation and the vaporization of the sample using the gas phase, before being carried through a column by a gas, and ionized by the mass spectrometer. In the final step the ions are sorted based on their mass-to-charge values (m/z). However, this technique is only ideal for volatile and low-weight metabolites.

In LC-MS analysis the prepared sample is run through a column, the type of which depends on the analysis being conducted. The column retains or releases compounds based on the compound's characteristics. The eluted compounds are then introduced to a mass spectrometer which ionizes the sample and separates the ions based on their m/z.[69,70] A detector



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then analyzes the ions when they emerge, and it generates a chromatogram, which can consist of hundreds of peaks.[71] This is then used for compound identification and quantification.

#### 1.2.1 Vaginal metabolites

In a study by Borgogna *et al.* [72] in 2017 it was determined that women with low colonization of lactobacilli have a higher concentration of biogenic amines (BAs) cadaverine, putrescine, and agmatine when compared to women with a microbiota dominated by lactobacilli. It was also determined that an increase in BAs and decrease in amino acids, such as arginine, lysine, and ornithine is found when lactobacilli are outgrown by other bacteria.

In addition, BAs have been shown to increase transmission and virulence of sexually transmitted infections. [73] This collectively shows that the presence of BAs is associated with a dysbiotic vaginal microbiota.

Furthermore, several metabolites have been identified which are associated with a dysbiotic microbiota, such as 2-hydroxyisovalerate and γ-hydroxybuterate (GHB), trimethylamine, and acetate. [74–76]

### 1.3 The psychosocial impact of vaginal dysbiosis

Bacterial vaginosis has a significant bearing on women's quality of life. It negatively impacts their body-image, self-esteem, personal lives, and mental health.[77] This is the most common vaginal condition among reproductive aged women, affecting up to 70% of women worldwide at some point in their life.[78] Despite this high prevalence very little progress has been made towards developing a treatment alternative to antibiotics, which, as mentioned above, are not an ideal option given their side effects, suboptimal efficacy, and high recurrence rate. One of



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the few studies conducted in this area, by Anukam *et al.,* [79] suggests potential for probiotic therapy as an alternative treatment to metronidazole gel.

The majority of women with recurrent BV report that the symptoms associated with the condition, especially malodor, cause them a significant amount of distress, both due to the embarrassment and shame associated with their symptoms, and the lack of control they feel during frequent recurrence.[77]

It is extremely surprising that given the adverse health outcomes associated with this condition more research has not been conducted on this topic, as recurrent BV can not only have a huge impact on the quality of life but is also responsible for a severe economic drain on the healthcare system.[77]

## 1.4 Thesis objectives and hypothesis

We hypothesize that *Lactobacillus crispatus* vaginal isolates differ in their intraspecific metabolite profile and ability to inhibit growth of pathogens.

The objectives of this project were:

1. To determine if the genomes and metabolites of *L. crispatus* isolates from the vagina of healthy women differ from those with dysbiosis;

2. To test 20 strains for the ability to inhibit *E. coli, E. faecalis,* and bacteria associated with bacterial vaginosis.



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## 1.5 Key findings

The *L. crispatus* isolates were found to have varying metabolomic profiles with some strains capable of producing molecules that could confer health benefits. These variations did not correspond to the health status of the individual from whom they were isolated, namely women with BV or those who were deemed healthy. This means that simply selecting a strain from a healthy woman may not result in a suitable probiotic.

The strains separated into two groups based upon metabolic profile, with one set having much more beneficial characteristics. Irrespective of their host origin, several bacteriocins were identified within the genomes of the strains. The isolates showed inhibition of standard indicator strain *E. faecium* to varying degrees, as well as against *G. vaginalis* ATCC 14018, and *C. albicans* TIMM 1768, as well as a decrease in growth of *E. coli* UTI 89 in co-culture.

Altogether, this project provides a better understanding of the beneficial qualities of *L. crispatus* as well as some of the variation seen between isolates. This highlights the need to consider what attributes might best suit a strain to be used as a probiotic for vaginal health, alone or in combination with antimicrobial therapy. The variation between strains could explain why although colonized by *L. crispatus* some women still develop BV or other diseases.



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## Chapter 2: Metabolomic profiling of *Lactobacillus crispatus* vaginal isolates

### 2.1. Introduction

In identifying and developing a probiotic several characteristics are considered in addition to its ability to be propagated commercially and its safe history of use. These can include the potential for pathogenicity, the production of antimicrobial compounds such as lactic acid, bacteriocins, biosurfactants and hydrogen peroxide ($H_2O_2$), its ability to coaggregate, to disrupt pathogenic biofilms, and how it interacts with the host including patients at high risk.[1–11] However, what has not been considered until recently is the range of metabolites they produce.

Metabolomics is the study of the metabolites present in an environment. An efficient method for performing these studies is to use liquid chromatography-mass spectrometry (LC-MS).[12,13] It has also been referred to as metabonomics by Nicholson and others who have tended to use nuclear magnetic resonance (NMR) methods.[14] These techniques allow for the identification and quantification, or semi-quantification, of compounds present in a given tissue or fluid sample.[12-15]

In general, *Lactobacillus crispatus* is associated with vaginal health, making it a good organism to study.[16-19] The absence of which is associated with vaginal dysbiosis and conditions such as bacterial vaginosis (BV) and increased risk of sexually transmitted infections.[20-23] *Lactobacillus crispatus* is one of the highest lactic acid producers among the vaginal lactobacilli, expressing broad spectrum inhibition of bacterial pathogens, making it a good candidate for probiotic application.[7,24-26]

*Lactobacillis crispatus* CTV-05, branded as Lactin-V, was chosen by Sharon Hillier for its $H_2O_2$ production.[27] Thus far, it has been determined that the application of Lactin-V using a vaginal applicator is a safe and efficient method for the introduction of *L. crispatus* into the



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vaginal environment.[27,28] Persistence of CTV-05 reduced the risk of recurrent urinary tract infections (UTI) in 50% of ninety-eight women tested in a randomized, placebo-controlled study.[29]

In the present study, the aim was to use an untargeted liquid chromatography mass spectrometry-based approach to compare the metabolomic profiles of 19 vaginal isolates of *L. crispatus*, 8 of which were isolated from women with vaginal dysbiosis and 11 isolated from healthy women.

## 2.2. Materials and Methods

### 2.2.1. Collection and growth of strains

Nineteen *Lactobacillus crispatus* strains were obtained from Charlotte van der Veer in Amsterdam, the Netherlands. The strains were isolated from nurse-collected vaginal swabs, 8 from women with vaginal dysbiosis, and 11 from women with a lactobacilli-dominated vaginal microbiota (Table 2.1).[30] The *L. crispatus* type strain ATCC 33820 was obtained from ATCC. All *L. crispatus* strains were grown at 37°C in vaginally defined media with 5% proteose peptone (VDMP), a media which mimics vaginal secretions, prepared as described by Anukam and Reid.[31]



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Table 2.1 Strains used in this study

| Strain | Status of patient | Other pathogens present |
|---|---|---|
| **01** | Healthy | |
| **02** | Dysbiotic | |
| **03** | Healthy | |
| **04** | Healthy | |
| **05** | Healthy | |
| **09** | Healthy | |
| **10** | Healthy | |
| **12** | Healthy | |
| **13** | Dysbiotic | |
| **14** | Dysbiotic | *Chlamydia trachomatis* |
| **16** | Healthy | |
| **17** | Dysbiotic | |
| **22** | Healthy | |
| **23** | Dysbiotic | |
| **25** | Dysbiotic | |
| **27** | Healthy | |
| **28** | Dysbiotic | |
| **29** | Healthy | |
| **33** | Dysbiotic | *Trichomonas vaginalis* |
| **33820** | Type strain | |

2.2.2. LCMS protocol

The *L. crispatus* strains were grown for 24 hours in vaginally defined medium peptone (VDMP) at 37°C. Following the addition of 50% methanol in a 1:1 ratio, supernatants were collected after centrifugation for 10 minutes at 10,000 x g, filtered using 0.45μm syringe filters and deposited into HPLC vials. The samples were then analyzed using an Agilent 1290 Infinity HPLC coupled to a Q-Exactive Orbitrap mass spectrometer with a heated electrospray ionization source, in positive and negative ionization mode. Two LCMS runs were conducted in both positive and negative ionization mode, the first was a preliminary run of N=1 for the purpose of method verification and identification of significant metabolites, and the second was a run of N=3 replicates to verify the results from the preliminary run. Strain 4 is absent from runs 1 and 2 and strain 33 is absent from run 2 due to growth issues.



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2.2.3. Metabolite identification

The raw files were converted into .MZML format using ProteoWizard [32] and chromatogram alignment and deconvolution was completed using the XCMS package in R.[13] Features were detected at a 1ppm tolerance and the prefilter was set to 3 to 5,000, noise to 1000, and a signal-to-noise threshold of 5. Principal component analysis was completed using FactoMineR in R and the data was exported and analyzed. Metabolites determined to be statistically significant between the groups, as well as those significantly different from what was found in the media, serotonin for example (Figure S4), using a Mann-Whitney test were identified based on accurate mass, retention time, and MS/MS spectra using METLIN.

2.2.4. Statistical analysis

Statistical analysis during metabolite identification was conducted using Excel. The remainder of the statistical analysis was completed using GraphPad Prism 8.0.1. As mentioned above, Mann-Whitney tests were used to identify metabolites which differed between the groups. Kruskal-Wallis tests were utilized to determine if organic acid production differed between strains.

## 2.3 Results

2.3.1. Acid production

As organic acids are thought to play a role in vaginal health, the production of succinic acid, glutamic acid, lactic acid, and tyrosine, technically an amino acid, by the strains was measured. Initially, production was compared between strains isolated from dysbiotic and healthy individuals, however, no differences were found based on that grouping (Figure 1). So, production was simply compared between strains (Figures 2-5). Again, there was little variation observed.



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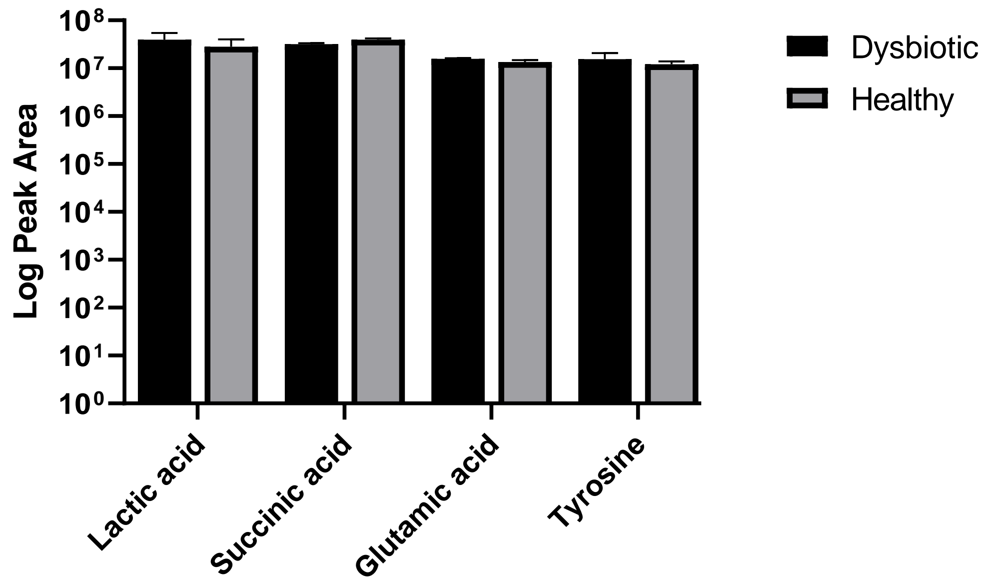


**Figure 1. Production of acidic compounds grouped by health status of patient.** Production of acidic compounds grouped by health status of individual the strain was isolated from (P>0.05).



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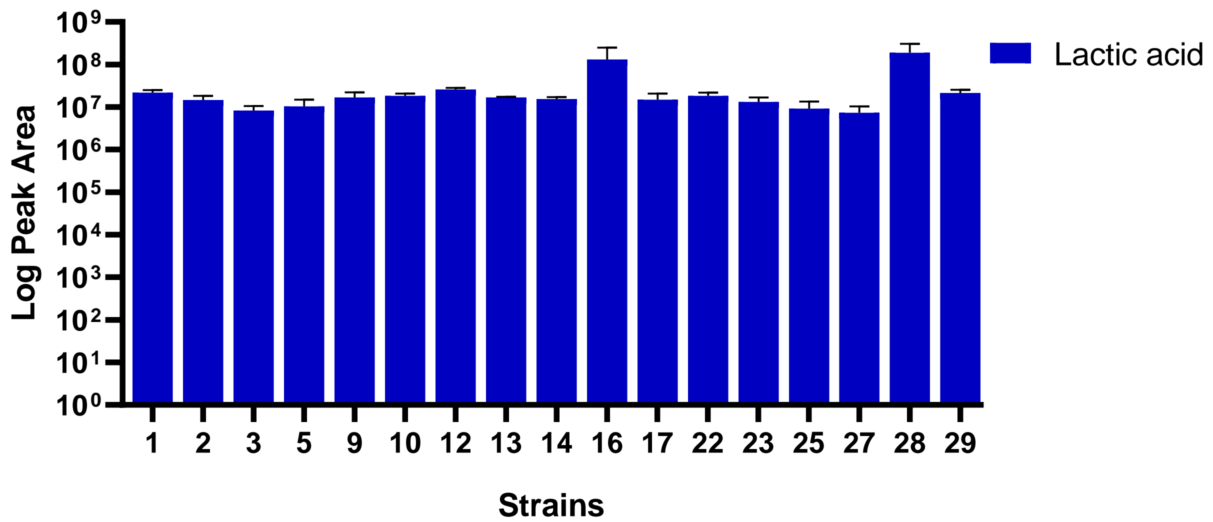


**Figure 2**. **Lactic acid production by strain.** Lactic acid production of *L. crispatus* isolates grown in VDMP for 24 hours (N=3±SEM) (P>0.05).

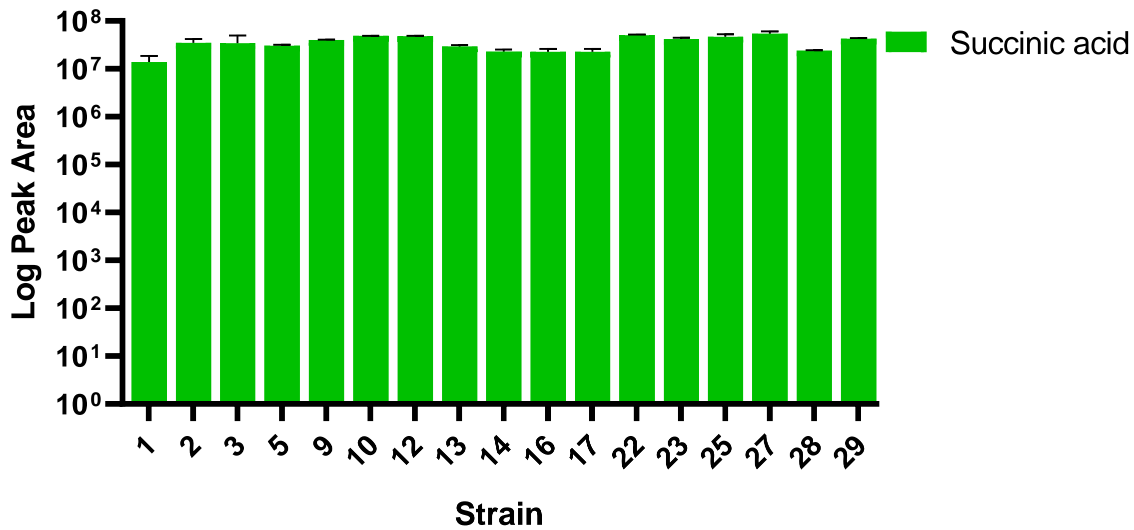


**Figure 3. Succinic acid production by strain.** Succinic acid production of *L. crispatus* isolates following 24 hour growth in VDMP (N=3±SEM) (P>0.05).



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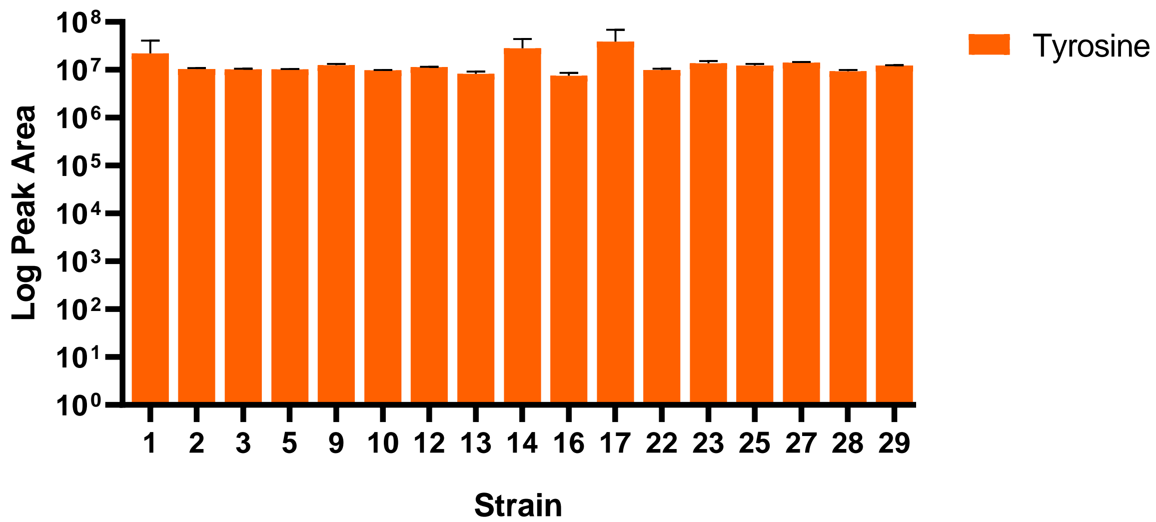


**Figure 4. Tyrosine production by strain.** Tyrosine production of *L. crispatus* isolates following 24 hour growth in VDMP (N=3±SEM) (P>0.05).

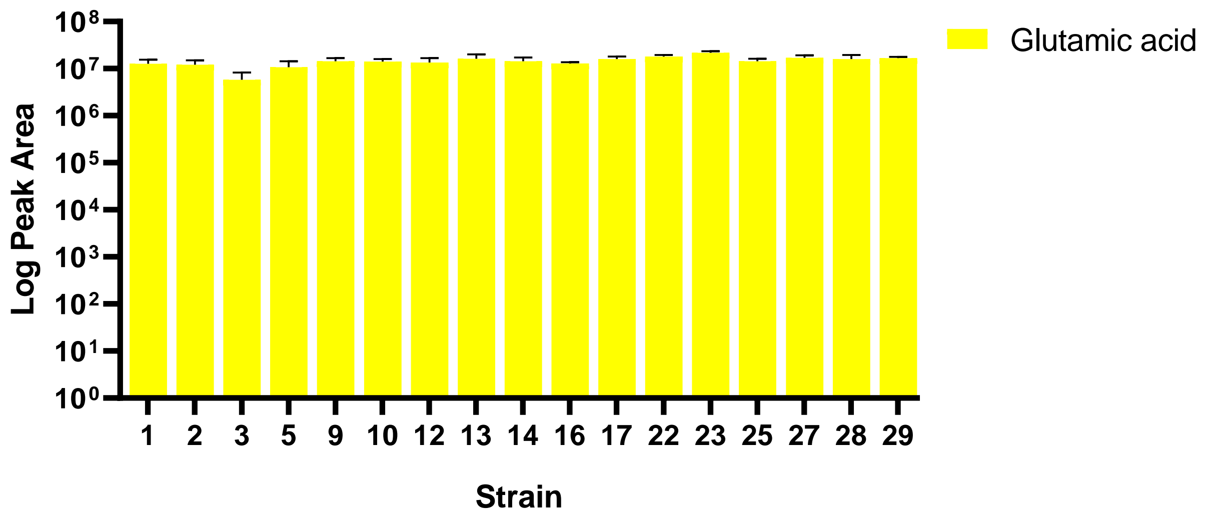


**Figure 5. Glutamic acid production by strain.** Glutamic acid production by *L. crispatus* isolates following 24 hour growth in VDMP (N=3±SEM) (P>0.05).



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### 2.3.2 Clustering of metabolic profiles

Other potential differences between the strains were examined. To accomplish this, a PCA plot of metabolites was created to look for patterns within the metabolic profiles (Figure 6). It was discovered that the strains clustered into two groups, however, these did not correlate with the health status of the individual from whom they were isolated (Table 2.1). The groups will henceforth be referred to as Group A and Group B (Table 2.2). Metabolites that were determined to be statistically significant between the groups were identified. Strain 5 was omitted from further metabolomic analysis as it appears to be completely distinct from Groups A and B.

Table 2.2 Grouping of strains based upon metabolic profiles

| GROUP A | GROUP B |
| --- | --- |
| 1 | 13 |
| 2 | 14 |
| 3 | 16 |
| 9 | 17 |
| 10 | 28 |
| 12 | |
| 22 | |
| 23 | |
| 25 | |
| 27 | |
| 29 | |
| 33 | |



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**Figure 6. PCA of metabolites in positive ionization mode for *L. crispatus* strains.** Within this plot two clusters can be seen, these have been termed Group A and Group B.



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2.3.3. Metabolomic differences between Groups A and B

Metabolite production and degradation was compared between the groups. Seen in figure 7, the tyrosine-tyramine pathway appears not to function within Group A strains but it does within Group B strains. This was also seen in the second run. Additionally, Group B strains were unable to consume guanosine, producing less guanine than Group A strains (Figure 8). Evident in figure 9, the Group A *L. crispatus* strains reduced hypoxanthine and inosine, whereas the Group B strains did not. A significantly higher reduction in inosine was also seen by Group A in the second LCMS run, when compared to Group B in the same run, the method was unable to detect hypoxanthine.

The arginine deiminase (ADI) pathway (Figure 10) is comprised of enzymes that degrade arginine into citrulline and ammonia; ornithine transcarbamoylase which cleaves citrulline into carbamoyl phosphate and ornithine; carbamate kinase which produces ATP, ammonia and carbon dioxide through the dephosphorylation of carbamoyl phosphate. Evident in figures 11-13 the ADI pathway appears to be running in overdrive within the Group B strains. The degradation of L-arginine and production of citrulline and ornithine by Group B strains was also noted in the second LCMS run and was again determined to be significantly different.



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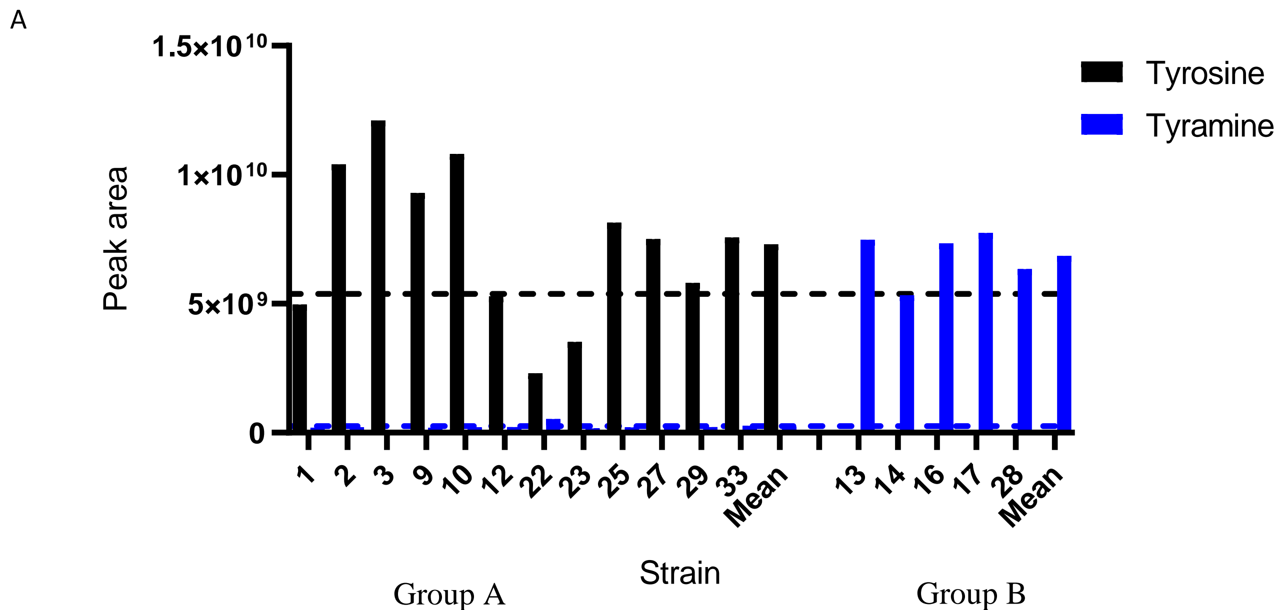


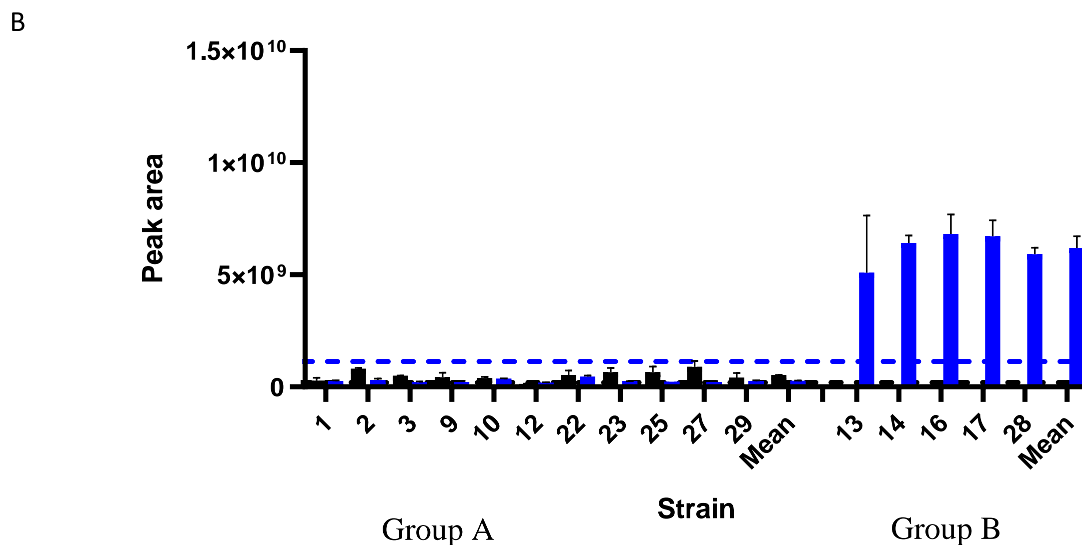


**Figure 7. Tyrosine and tyramine abundance within *L. crispatus* isolates separated into Groups A and B. evident on PCA plot.** Tyrosine and tyramine abundance within spent supernatant following 24 hour growth of *L. crispatus* isolates in VDMP conducted in two runs. Preliminary run (A) expressed as N=1. Run 2 (B) values are expressed as a mean of N=3±SEM (Tyrosine $P=2.17x10^{-07}$, Tyramine $P=5.53x10^{-22}$). Dashed lines represent presence in the media.



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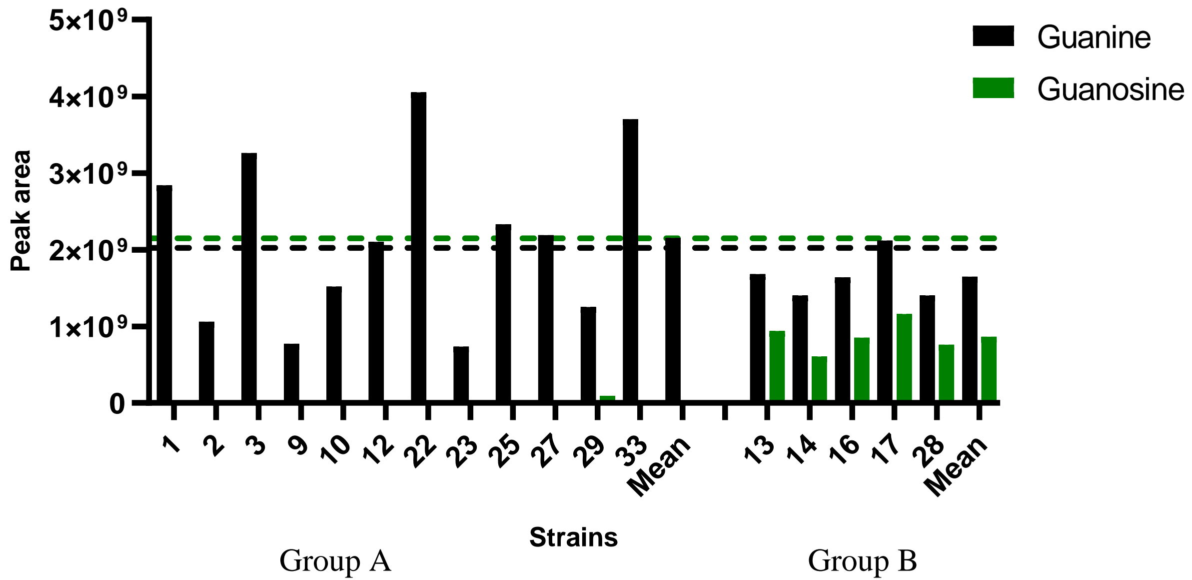
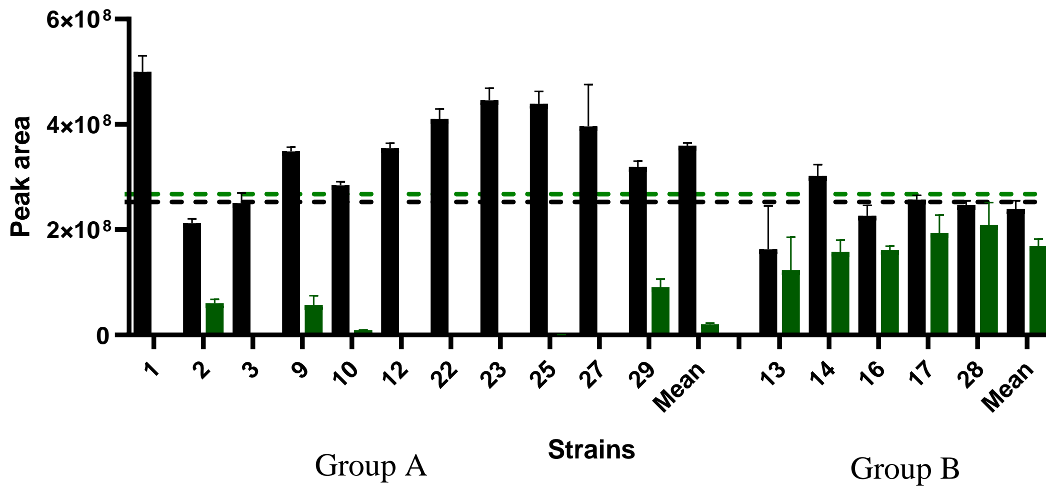
A




Group A Group B

B




Group A Group B

**Figure 8. Guanosine degradation by *L. crispatus* isolates separated into Groups A and B evident in PCA plot.** Guanine and Guanosine abundance within spent supernatant following 24 hour growth of *L. crispatus* isolates in VDMP over two runs. Preliminary run (A) values are expressed as N=1. Run 2 (B) values are expressed as a mean of N=3±SEM ($P=8.0x10^{-05}$ for guanine, $P=6.6x10^{-14}$ for guanosine). Dashed lines represent presence in the media.



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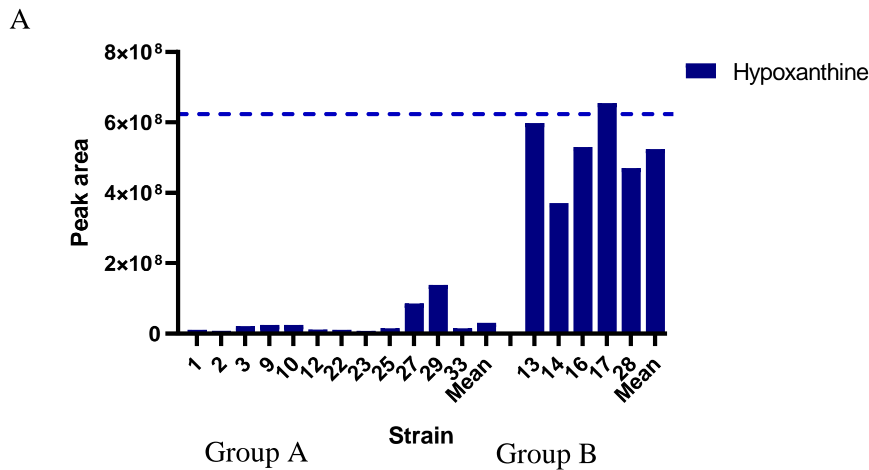
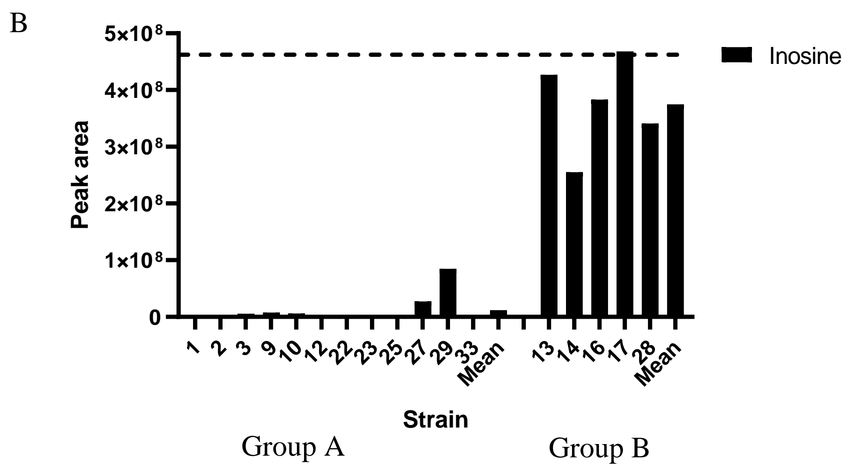
A




B

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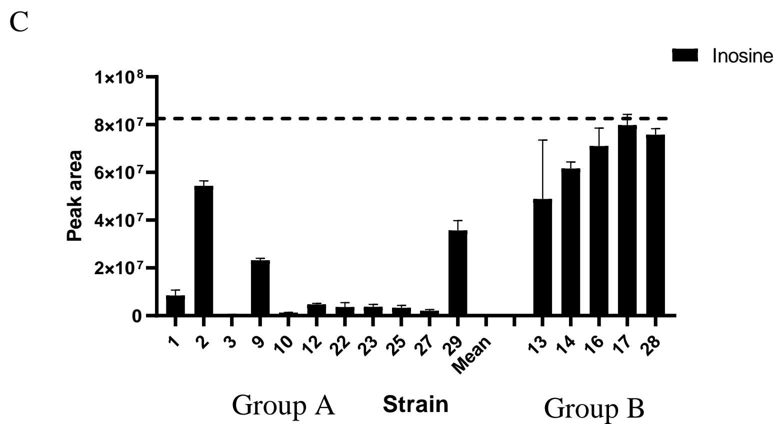


**Figure 9. Purine degradation separated by Groups A and B of *L. crispatus* strains as shown in the PCA plot**. (A) Hypoxanthine and (B) Inosine abundance within spent supernatant following 24 hour growth of isolates in VDMP from preliminary run expressed as N=1. (C) Inosine abundance within spent supernatant following 24 growth of *L. crispatus* isolates in VDMP. All values are expressed as a mean of N=3±SEM. Inosine presence in the media was $3x10^7$ ($P=1.82x10^{-12}$). Dashed lines represent presence in media.



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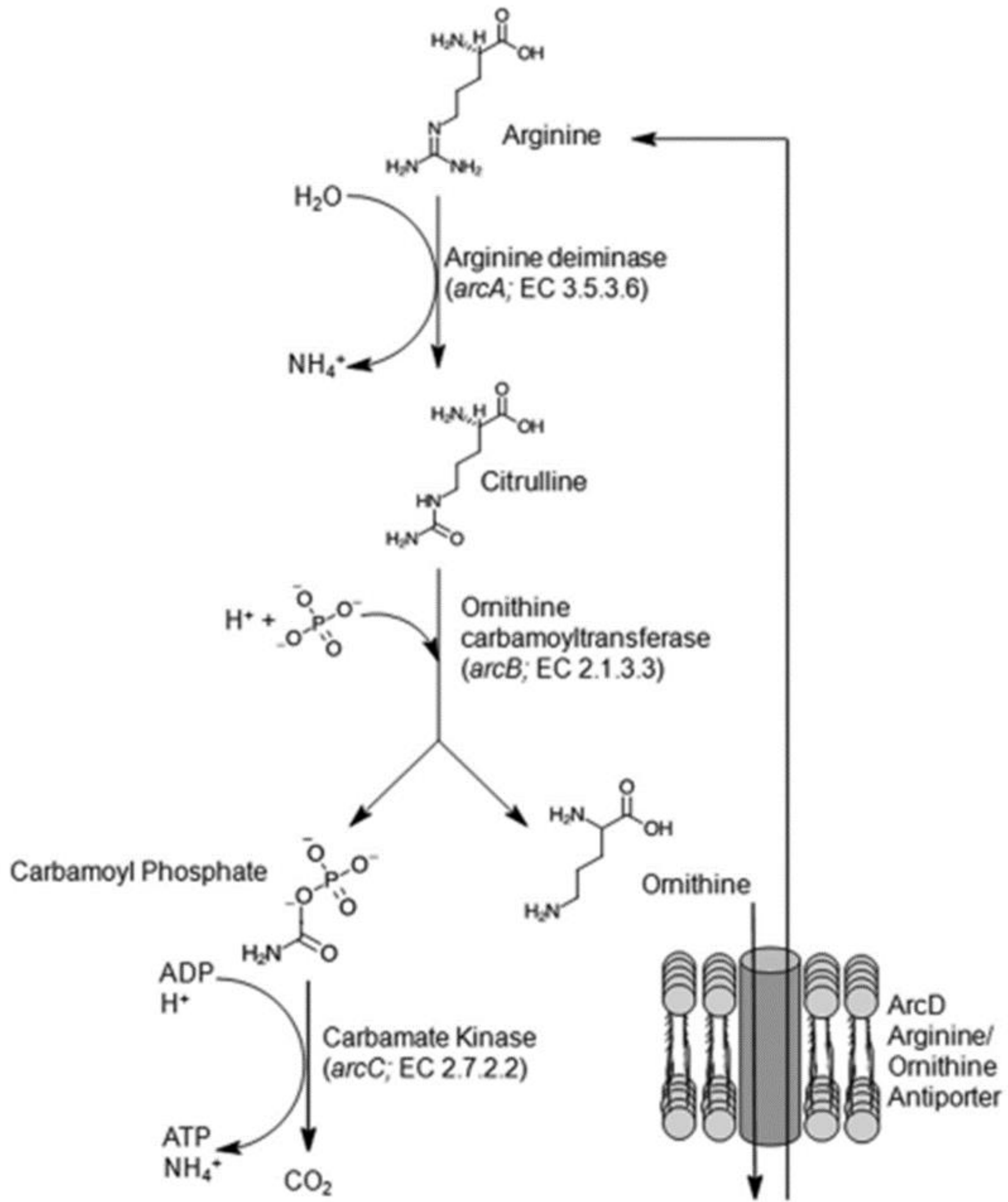


**Figure 10.** The arginine deiminase pathway. [33,34]



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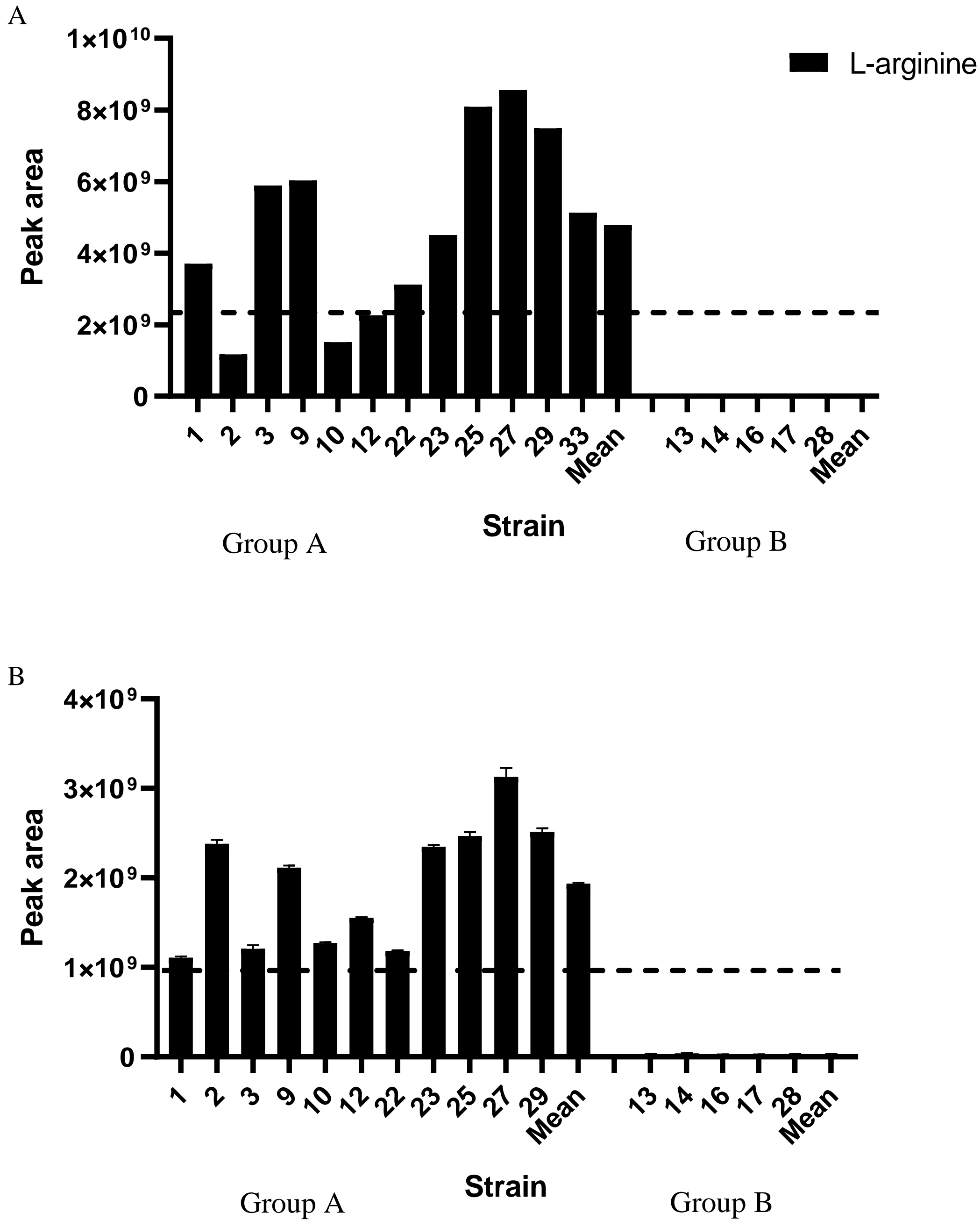


**Figure 11. L-arginine degradation by *L. crispatus* strains from Groups A and B identified within PCA plot.** L-arginine abundance within spent supernatant following 24 hour growth of isolates in VDMP over two runs. Preliminary run (A) values are expressed as N=1. Run 2 (B) values are expressed as a mean of N=3±SEM ($P=2.56x10^{-14}$). Dashed lines represent presence in media.



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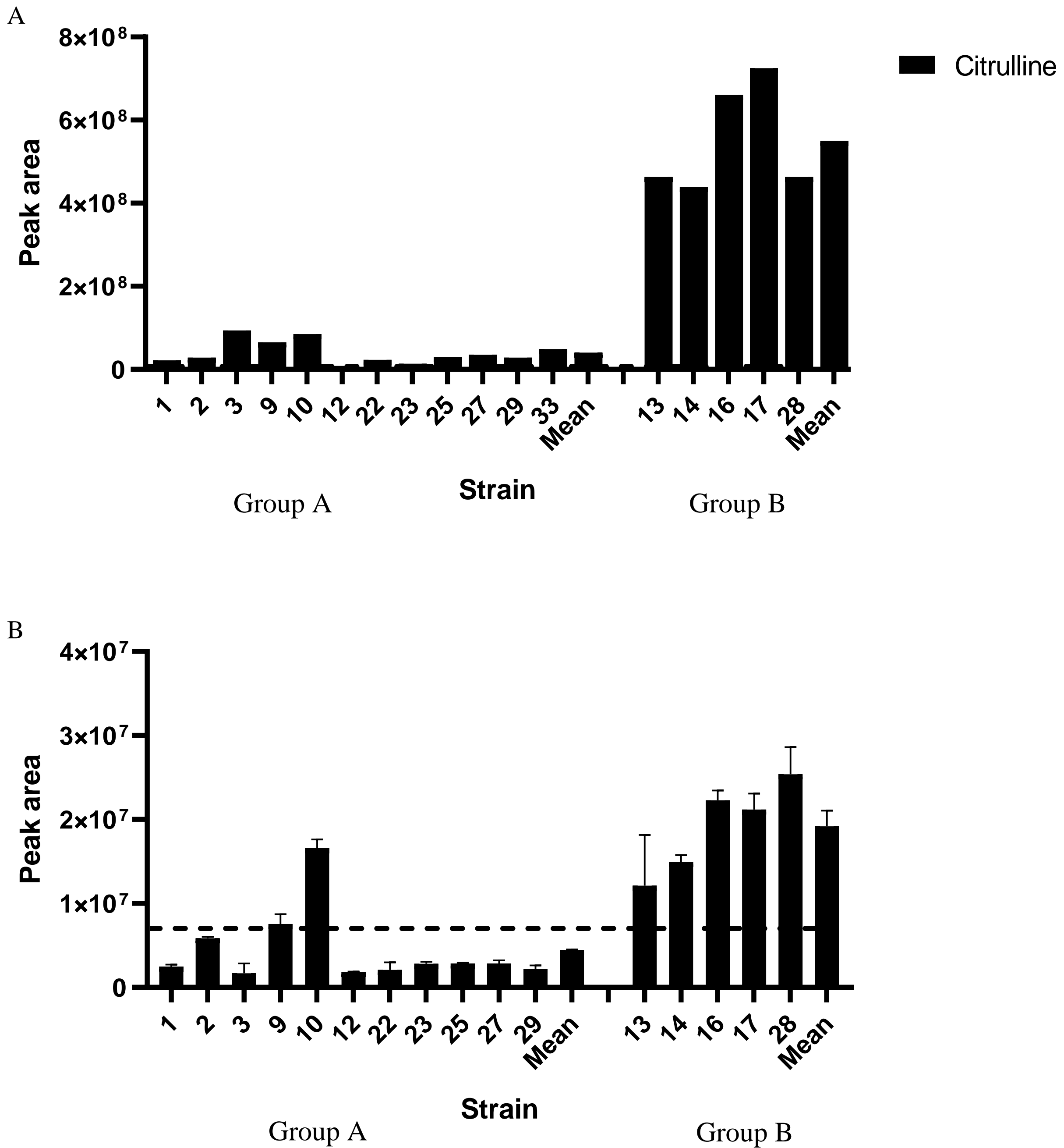


**Figure 12. Citrulline production by *L. crispatus* isolates from Groups A and B evident on PCA plot.** Citrulline abundance within spent supernatant following 24 hours growth of isolates in VDMP over two runs. Preliminary run (A) values expressed as N=1. Run 2 (B) values are expressed as a mean of N=3±SEM ($P=1.27x10^{-11}$). Dashed lines represent presence in media.



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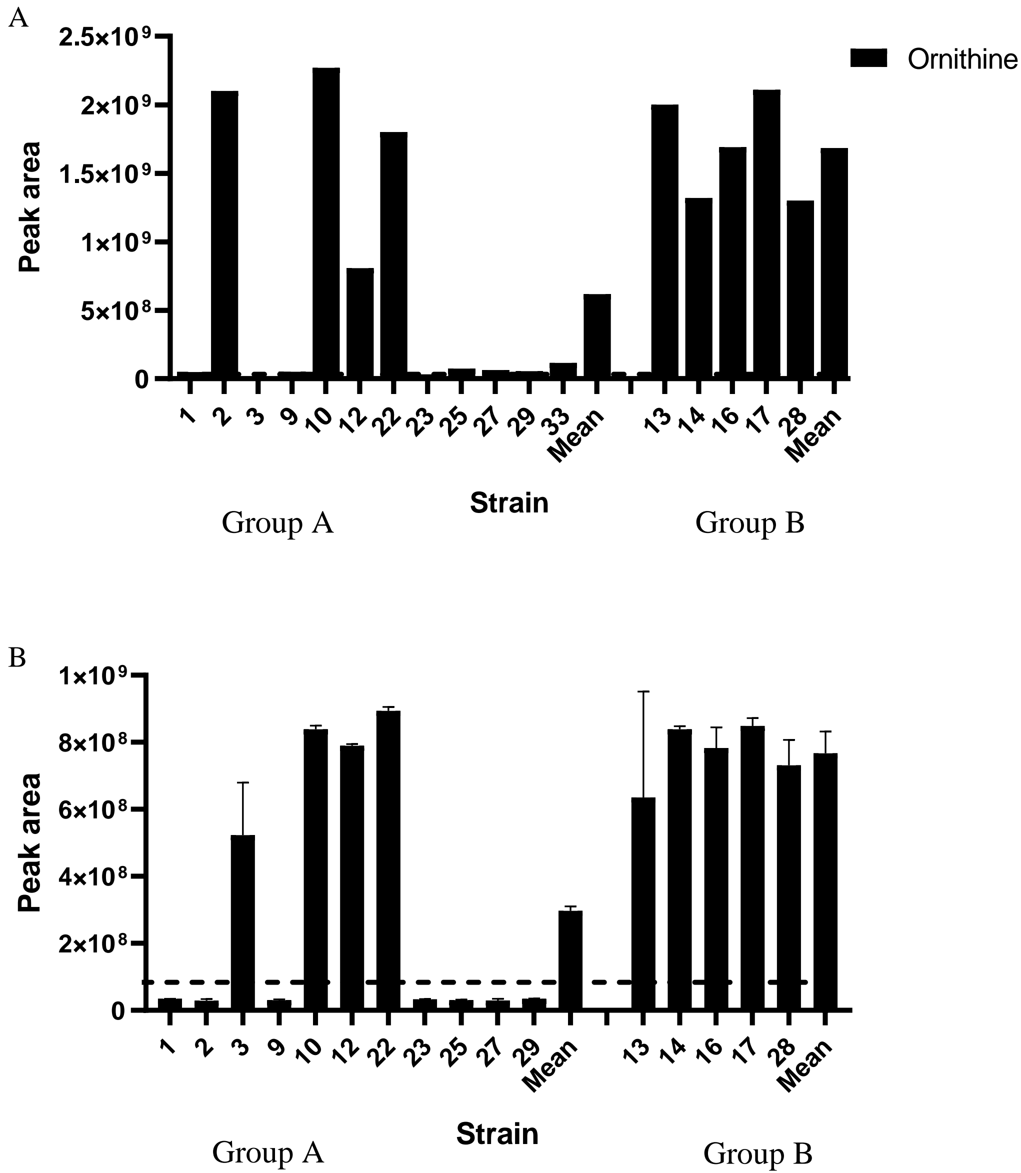


**Figure 13. Ornithine production by *L. crispatus* strains from Groups A and B evident in PCA plot.** Ornithine abundance within spent supernatant following 24 hour growth of isolates in VDMP over two runs. Preliminary run (A) values are expressed as N=1. Run 2 values (B) are expressed as a mean of N=3±SEM ($P=4.76x10^{-5}$). Dashed lines represent presence in media.

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## 2.4 Discussion

As acidic compounds are thought to play a vital role in vaginal health, the production of organic acids succinic acid, glutamic acid, lactic acid, and tyrosine, an amino acid, by the strains was measured. Initially, production was compared between strains isolated from dysbiotic and healthy individuals, however, no differences were found (Figure 1). This is interesting as Dr. van der Veer had hypothesized that strains from women with dysbiosis may be deficient in critical 'protective' properties and therefore have a different genomic and metabolic profile. The fact that this was not the case raises the question of how effective *L. crispatus* are in preventing dysbiosis and BV.

Following this finding, organic acid production was compared on a strain to strain basis (Figures 2-5), and again, no differences were noted. Then a PCA plot of metabolites was created (Figure 6) to look for other patterns. Within the plot, two groupings were found and designated the strains under Group A and Group B. Two LCMS runs were conducted, the second with the purpose of verifying the initial, preliminary, run. The tyrosine-tyramine pathway appears not to function within Group A strains but does within Group B (Figure 7).This is interesting because tyramine is formed by the decarboxylation of tyrosine, and in a study of another lactic acid bacterium strain, *Enterococcus faecium* E17, the tyrosine decarboxylation pathway was found to be important in low acid and nutrient-depleted conditions.[35] This is viewed as a marker for its chance of survival. Within enterococci the functioning of this pathway also contributes to the binding and immunomodulation of enterocytes. Strain *Enterococcus durans* IPLA655 uses this ability as a survival and colonization mechanism that enhances adhesion to the intestinal epithelium.[36] The ability 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.[37] Alternatively, the production of biogenic amines (Bas), such as tyramine,



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within the vagina is linked to an increase in pH which promotes colonization by BV-associated bacteria. This suggests that strains 13, 14, 16, 17, and 28, all in Group B, produce biogenic amines, raising vaginal pH, and making the environment more favourable to pathogens. Other Bas, trimethylamine, putrescine, and cadaverine did not differ significantly between the strains, though some production and degradation was evident (Figures S1-3).

Nucleotides are required for a variety of biological processes. During cellular proliferation, increased nucleotide synthesis is necessary for DNA replication and RNA production to support protein synthesis during the cell cycle. This process is energy intensive and required for cell viability.[38] Evident in figure 8, Group B strains are unable to consume guanosine, producing less guanine than Group A strains. A depletion of DNA and RNA precursors results in an almost complete cessation of growth in most species.

An excessive intake of purine-rich foods results in elevated serum levels of uric acid, a decrease in inosine and related purines, including hypoxanthine, results in a decrease of serum uric acid levels. Meats, including fish and shrimp, contain high levels of these purines. Following intestinal absorption, the end-product is uric acid. Currently, to lower the production of uric acid, which can result in hyperuricemia and gout, individuals are recommended to reduce consumption of purine-rich food. An alternative theory being investigated is the oral intake of *Lactobacillus* species able to utilize purines such as inosine and hypoxanthine.[39] Evident in figure 9, the Group A *L. crispatus* strains reduce inosine and hypoxanthine, whereas the Group B do not. Although, as only inosine was identified in the second LCMS run, it is unclear if the difference in hypoxanthine abundance observed between Groups A and B in figure 9 is significant. The implications for the vaginal environment are not yet clear, but perhaps Group A strains propagate in the intestine of meat-eating women thereby providing a health benefit. When



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the strains ascend from the rectum to the vagina, this property remains but has less functional aptitude at that site.

The arginine deiminase (ADI) pathway (Figure 10) is comprised of enzymes that degrade arginine into citrulline and ammonia; ornithine transcarbamoylase which cleaves citrulline into carbamoyl phosphate and ornithine; and carbamate kinase which produces ATP, ammonia, and carbon dioxide through the dephysphorylation of carbamoyl phosphate. This pathway may be responsible for providing ATP for microbial growth under different environmental conditions, and thereby protecting bacteria against damage caused by environmental stressors including acid and starvation.[33] Evident in figures 11-13 the ADI pathway appears to be running in overdrive within the Group B strains, suggesting they are less tolerable to acid conditions and may be affected by other environmental stressors such as starvation. This suggests that Group B strains 13, 14, 17, and 28 are under stress when grown in VDMP, the medium that simulates vaginal secretions. The same pattern was observed in the second LCMS run. The end products of this pathway are putrescine and ammonia, both of which result in an increase in pH. However, no difference in putrescine production was evident (Figure S1), and ammonia was not detectable with the LCMS method used.

Although two distinct groups were evident and the distinguishing compounds between the groups were determined to be statistically significant, variation was noted between individual strains within each group. This further indicates that no two strains are alike within this species.

Overall strains belonging to Group A seem to have a much more beneficial metabolomic profile than Group B strains whose characteristics such as the production of biogenic amines are not ideal for vaginal probiotics. The release of such compounds would result in an increase in vaginal pH and allow for the colonization of urogenital pathogens that favour a less acidic



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environment. This could then facilitate a transition from a *Lactobacillus spp.* dominant vaginal microbiome to a dysbiotic environment.[40] Furthermore, BA presence as been linked to an increase in virulence of pathogens, including *Neisseria gonorrhoeae,* as well as an increase in pathogen resistance to lactic acid.[40,41]

## 2.5. Conclusion

The results highlight the importance of studying metabolic profiles of lactobacilli strains.[42] They show that generalizations are not advisable, for example *that all L. crispatus are beneficial to vaginal health*. This is countered by the fact that strains were isolated from women with BV and those who were healthy, and a group of six of eighteen had properties not conducive to conveying health to that niche. Therefore, in selecting a *L. crispatus* for probiotic application, it would be counter-intuitive to have one that produced biogenic amines or molecules that might stimulate dysbiosis.



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# Chapter 3: Genomes and antibacterial activity of *Lactobacillus crispatus* vaginal isolates

## 3.1. Introduction

Bacteriocin production is thought to be one of the most important defense mechanisms for bacteria. These are proteins which are secreted for the purpose of killing closely related species.[1-3] Lactic acid bacteria (LAB), which as the name suggests are those that produce lactic acid, typically also produce bacteriocins which have greater antagonistic activity than those produced by bacteria belonging to other groups.[1,4]

Bacteriocins are divided into three main classes. Class I bacteriocins are small heat-stable proteins termed lantibiotics. Class II bacteriocins are small proteins belonging to IIa, with a strong anti-listerial effect, or group IIb, which have two polypeptide chains. Class III bacteriocins are large heat-labile proteins. These compounds are currently used in the food industry as preservatives, however, they are becoming increasingly interesting to the medical field as an alternative to antibiotic treatment of infection.[4,5]

The aim of the present study was to investigate bacteriocin production in vaginal *Lactobacillus crispatus* strains as a possible contributor to retention of a health-associated microbiota dominated by these lactobacilli. [6-8] In addition, a brief overview of the genomes of 29 *L. crispatus* isolates is provided, 19 of which were used in this thesis.



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## 3.2. Materials and Methods

### 3.2.1. Strain collection

The strains (Table 2.1) were collected as outlined by van der Veer *et al.*[9]. In brief, swabs were obtained from the Sexually Transmitted Infections clinic in Amsterdam, the Netherlands. Samples were isolated from women with a healthy vaginal microbiota (Nugent score 0-3) or a dysbiotic microbiota (Nugent score 7-10). The swabs were plated on modified Trypticase Soy Agar and incubated anaerobically. Single colonies underwent 16S sequencing for identification purposes. The strains were then kept in vaginally defined media.[10]

### 3.2.2. Genome assembly and annotation

Following sequencing using the Illumina Miseq to generate FASTQ workflow, the isolates were assembled and deposited at DDBJ/ENA/GenBank. The genomes were assembled, reordered, and annotated by Charlotte van der Veer.[9]



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Table 3.1. Characteristics of the *L. crispatus* genomes

| Strain | Genome size(bp) | Contigs | Proteins with functional assignments | GC content | tRNA | Transporter | Drug target | Antibiotic resistance genes | Virulence factor |
|---|---|---|---|---|---|---|---|---|---|
| **02** | 2252111 | 299 | 1808 | 36.99 | 60 | 13 | 2 | 41 | |
| **03** | 2566581 | 354 | 1939 | 36.96 | 66 | 9 | 2 | 70 | |
| **05** | 2589338 | 446 | 1980 | 36.56 | 64 | 10 | 2 | 62 | |
| **06** | 2199194 | 379 | 1861 | 37.00 | 59 | 10 | 2 | 63 | |
| **07** | 2192815 | 396 | 1821 | 37.01 | 59 | 9 | 2 | 49 | |
| **08** | 2315703 | 387 | 1869 | 37.15 | 68 | 10 | 2 | 60 | |
| **09** | 2289537 | 308 | 1859 | 36.95 | 65 | 14 | 2 | 53 | |
| **10** | 2325626 | 309 | 1855 | 37.11 | 60 | 12 | 2 | 55 | |
| **11** | 2193818 | 289 | 1821 | 36.98 | 68 | 10 | 2 | 63 | |
| **13** | 2277921 | 505 | 1939 | 37.22 | 68 | 10 | 2 | 71 | 1 |
| **14** | 2637488 | 442 | 2002 | 36.86 | 70 | 9 | 2 | 74 | |
| **15** | 2335510 | 406 | 1852 | 37.25 | 67 | 10 | 3 | 63 | |
| **16** | 2626072 | 416 | 1925 | 36.66 | 69 | 10 | 2 | 67 | |
| **17** | 2484879 | 664 | 1931 | 38.41 | 72 | 10 | 2 | 66 | 1 |
| **19** | 2327246 | 577 | 1913 | 37.72 | 67 | 10 | 2 | 64 | |
| **20** | 2451667 | 378 | 1920 | 36.59 | 69 | 10 | 2 | 66 | 1 |
| **21** | 2582757 | 517 | 1972 | 37.25 | 66 | 9 | 2 | 65 | |
| **22*** | 3807741 | 1115 | 3246 | 36.10 | 114 | 15 | 5 | 81 | |
| **23** | 2334218 | 316 | 1829 | 36.89 | 67 | 10 | 2 | 62 | |
| **24** | 2395592 | 325 | 1862 | 36.77 | 65 | 10 | 2 | 64 | |
| **25** | 2362879 | 340 | 1830 | 36.97 | 67 | 9 | 3 | 63 | |
| **26** | 2286115 | 469 | 1899 | 37.47 | 67 | 9 | 2 | 62 | |
| **27** | 2612254 | 528 | 2063 | 37.32 | 67 | 9 | 2 | 80 | 1 |
| **28** | 2253331 | 464 | 1911 | 37.33 | 69 | 10 | 2 | 71 | |
| **29** | 2262306 | 434 | 1895 | 37.20 | 68 | 10 | 2 | 49 | |
| **30** | 2338000 | 442 | 1918 | 36.99 | 68 | 9 | 2 | 42 | |
| **31** | 2421383 | 528 | 2018 | 37.31 | 68 | 12 | 2 | 70 | |
| **32** | 2386968 | 447 | 1954 | 37.33 | 68 | 9 | 2 | 69 | |
| **33** | 2419049 | 477 | 1956 | 36.96 | 67 | 9 | 2 | 56 | |
| **CTV 05** | 2364583 | 25 | 1403 | 36.04 | 45 | 6 | 2 | 17 | 1 |

*Poor genome quality: too many contigs



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3.2.3. Bacteriocin identification

The genomes were searched for putative bacteriocins using BAGEL4, which identifies areas of interest within a genome, taking genomic context into account.[11] PATRIC 3.5.30, a web resource used to assist in data analysis, was used to identify specialty genes within the genomes and to search for genes of interest.[13]

3.2.4. Genome alignment

A conserved domain search was conducted using BLAST CD-search to determine similarity between bacteriocin sequences within the strains and verified sequences of the bacteriocins. One-hundred per cent identity to a verified sequence was considered a positive identity for that bacteriocin.[12]

3.2.5. Agar well diffusion assays

A series of agar well diffusion assays, a standard method used to detect bacteriocins and other antimicrobial compounds, were performed. [14,15] The strains were grown for 48 hours on Columbia blood agar (CBA) plates anaerobically in a BD GasPak™ EZ container systems at 37°C. The plates were then frozen at -80°C for 1 hour and thawed at room temperature for 1 hour. Following thawing the resulting supernatant was collected from the lid of the plate, the volumes were normalized, the samples were neutralized using NaOH and HCl, and then all samples were filter sterilized. The indicator strains were plated on M17 agar. Next, wells 1cm in diameter were bored into the agar using the base of a 1000µl pipette tip and 50µl of each individual supernatant sample was deposited into the wells. Following an incubation period of 48



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hours the plates were imaged with a scale and the zones of inhibition were measured using ImageJ software.[16]

Included in all assays were neutralized *Enterococcus faecalis* ATCC 19433, *Lactobacillus helveticus,* and *Pediococcus pentosaceus* supernatants and a CBA supernatant brought to pH 5 using lactic acid as positive controls 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 *Enterococcus faecium* ATCC 19434, *Escherichia coli* UTI 89, *E. faecalis* ATCC 19433, *Gardnerella vaginalis* ATCC 14018, *Prevotella bivia* ATCC 29303, and *Candida 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* as 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 treatment with trypsin, a protease, using 1 mg/mL final concentration and an agar well diffusion assay was conducted.
Several conditions were also altered to determine if these influenced inhibitory potential. The assays were repeated using *L. crispatus* isolates grown in vaginally defined medium peptone (VDMP) with varying amounts of NaCl. Media prepared with 1, 0.5, and 0 grams of NaCl in 500mL of media. The strains were also grown in VDMP with lactulose in place of dextrose. Lastly, to determine if the media used for strain growth impacted inhibitory potential a selection of strains were grown on MRS without tween, CBA, M17, and BHI.



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3.2.6. Co-culture experiments

Pictured in figure 23, the co-culture apparatus can be used to compare the growth of an organism grown challenged with another organism to the organisms grown independently. The two chambers of the apparatus are separated by a filter which allows for mixing of the media as wells as bacterial products but not the bacteria. By comparing the growth of the bacteria in the chambers to the growth of bacteria grown outside of the apparatus you can determine how the bacteria react to growth with other bacteria. A strain of *L. crispatus* was grown in one side, with a urogenital pathogen in the other. The strains used were *L. crispatus* isolates 1, 2, 25, ATCC 33820 and urogenital pathogens *P. bivia* ATCC 29303, *G. vaginalis* ATCC 14018, *E. coli* UTI 89, and *C. albicans* TIMM 1768. The growth in each chamber, as well as the growth in the cultures outside of the chamber, was measured after 24hours by CFU counts. The reason for selecting strains 1, 2, and 25 was because they were in Group A with more potential for probiotic application, as per Chapter 2.

3.2.7. Statistical analysis

Statistical analysis was completed using GraphPad Prism 8.0.1. To determine if significant differences existed between groups Wilcoxon signed-rank tests were conducted. Significance was determined if $P<0.05$.

## 3.3. Results

3.3.1. Adhesion and host colonization

A fibronectin/fibrogen binding protein was identified within the genomes of all the strains including CTV-05. This protein has been implicated in increased adhesion to human fibronectin. [17]



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3.3.2. Specialty genes

Out of the *L. crispatus* strains scanned, including *L. crispatus* CTV-05, strains 13, 17 (Group B), 20, 27 (Group A), and CTV-05 were found to have 'virulence factors.' GlnA, was found in strain 13, Chyl found in strain 17, CvfA was identified within strain 20, and CTP synthase was found within the CTV-05 genome.

3.3.3. Bacteriocin presence

Bacteriocins Enterolysin A, Helveticin J, and Penocin A, as well as two putative bacteriocins, which will be referred to as putative bacteriocin 1 and putative bacteriocin 2, were identified within the genomes of the *L. crispatus* isolates. A single bacteriocin, Penocin A, was identified within the CTV-05 genome. Table 3, summarises the bacteriocins identified and the strains in which they were found. Using sequence alignment Enterolysin A, Helveticin J, putative bacteriocin 1, which received a specific hit with the Helveticin J family and exhibited 57.49% similarity with Helveticin J, and putative bacteriocin 2, which received a specific hit for the bacteriocin class II c family, were confirmed. The alignment for Penocin A determined that there were sequence gaps which suggest that this bacteriocin is not produced by the strains and is likely a fragment.



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Table 3.2. Putative bacteriocins identified within the *L. crispatus* genomes

| Bacteriocin: | Enterolysin A | Helveticin J | Penocin A | Putative bacteriocin 1 | Putative bacteriocin 2 |
|---|---|---|---|---|---|
| **2** | + | + | - | + | + |
| **3** | + | + | + | + | + |
| **5** | + | + | + | + | + |
| **6** | + | + | + | + | + |
| **7** | + | + | + | + | + |
| **8** | + | + | + | + | + |
| **10** | + | + | - | + | + |
| **11** | + | + | + | + | + |
| **13** | + | + | + | + | + |
| **14** | + | + | + | + | + |
| **15** | + | + | + | + | + |
| **16** | + | + | + | + | + |
| **17** | + | + | + | + | + |
| **19** | + | + | + | + | + |
| **20** | + | + | + | + | + |
| **21** | + | + | + | + | + |
| **23** | + | + | + | + | + |
| **24** | + | + | + | + | + |
| **25** | + | + | + | + | + |
| **26** | + | + | + | + | + |
| **27** | + | + | + | + | + |
| **28** | + | + | + | + | + |
| **29** | + | + | + | + | + |
| **30** | + | + | + | + | + |
| **31** | + | + | + | + | + |
| **32** | + | + | + | + | + |
| **33** | + | + | + | + | + |
| **CTV-05** | - | - | + | - | - |



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3.3.4. Antibacterial activity

Agar well diffusion assays were conducted using 20 of the *L. crispatus* strains, including the type strain ATCC 33820 as this is the most studied *L. crispatus* strain, against *E. faecium*, *E. faecalis, E. coli, G. vaginalis, P. bivia,* and *C. albicans* as indicator strains. The lactobacilli inhibited the indicators in varying amounts, except for *E. faecalis,* and strains 1, 2, 25 had the largest impact on the widest spectrum of indicators (Figures 14-18).

Following heating of the supernatants and treatment with trypsin, no inhibition of *E. faecium* was seen (Figure 19). When grown in VDMP supplemented with prebiotic lactulose *L. crispatus* strain ATCC 33820 inhibition of *E. faecium* significantly increased in agar well diffusion assay (Figure 20). When grown in VDMP with varying amounts of NaCl (1g, 0.5g, and 0g) inhibition of *E. faecium* by *L. crispatus* ATCC 33820 increased (Figure 21). When grown on MRS without tween, BHI, CBA, and m17 prior to solid supernatant collection and agar well diffusion assay inhibition of *E. faecium* by *L. crispatus* ATCC 33820 and *L. crispatus* 17 did not change (Figure 22).

Strains 1, 2, and 25, in addition to the type strain, were chosen for co-culture experiments due to the high inhibition observed in the agar well diffusion experiments. The strains, as well as *L. crispatus* ATCC 33820, were tested for their ability to affect the growth of *G. vaginalis* ATCC 14018*, P. bivia* ATCC 29303*, E. coli* UTI 89*,* and *C. albicans* TIMM 1768. All strains were able to decrease the growth of the pathogens in co-culture. Strain 25 significantly decreased growth of *G. vaginalis* ATCC 14019 and *P. bivia* ATCC 29303 and strain 2 significantly decreased growth of *P. bivia* ATCC 29303 and *E. coli* UTI 89 (Figures 24-27).



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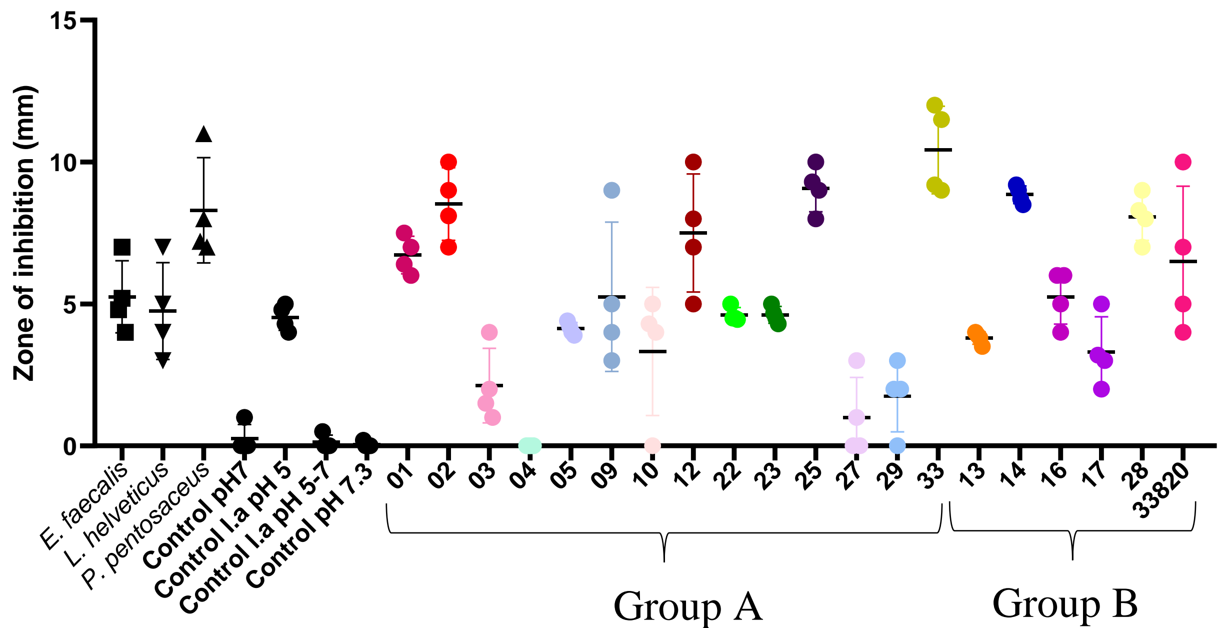


**Figure 14. Zone of inhibition measurements (mm) from agar well diffusion assays featuring *L. crispatus* supernatants plated against *E. faecium* ATCC 19434 indicator strain separated into Groups A and B**. Strains were grown for 24 hours on CBA plates and the individual supernatants were collected 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. 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 neutralized. 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 aerobic incubation at 37°C for 48 hours the zones of inhibition were measured (N=4).



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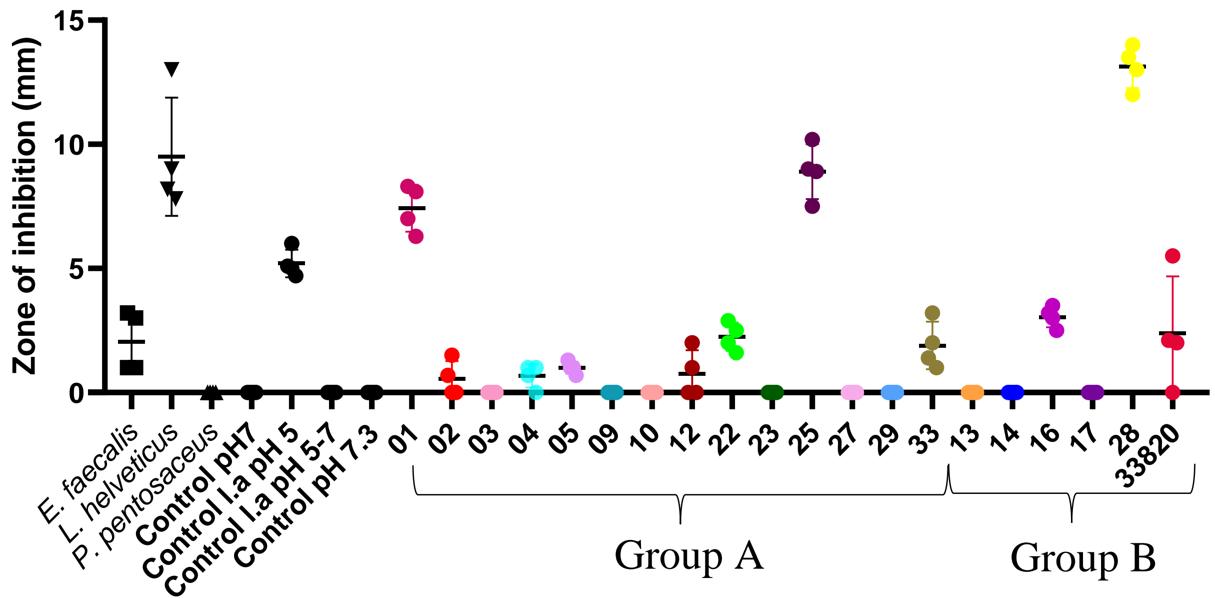


**Figure 15. Zone of inhibition measurements (mm) from agar well diffusion assays featuring *L. crispatus* supernatants plated against *G. vaginalis* ATCC 14018 indicator strain separated into Groups A and B.** Methodology was carried out as described in figure 24, except for final incubation which was carried out anaerobically because of the nature of the indicator strain.



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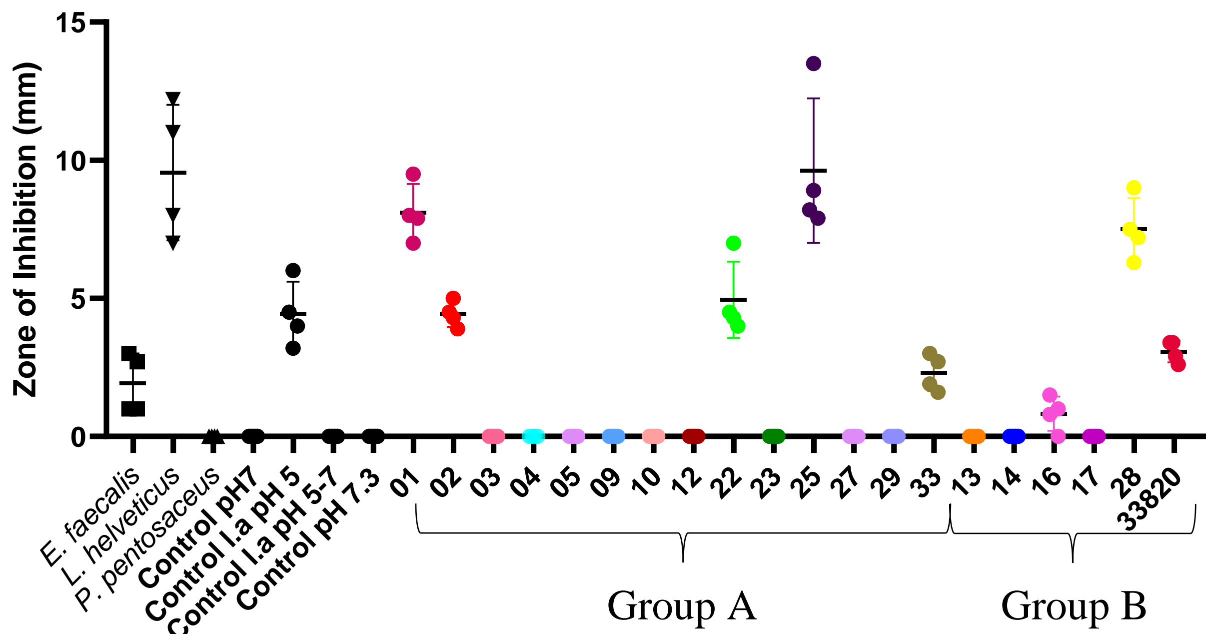


**Figure 16. Zone of inhibition measurements (mm) from agar well diffusion assays featuring *L. crispatus* supernatants plated against *P. bivia* ATCC 29303 indicator strain separated into Groups A and B.** Methodology was carried out as described in figure 24, except the assay was conducted using CBA in place of m17 and final incubation which was carried out anaerobically because of the nature of the indicator strain.



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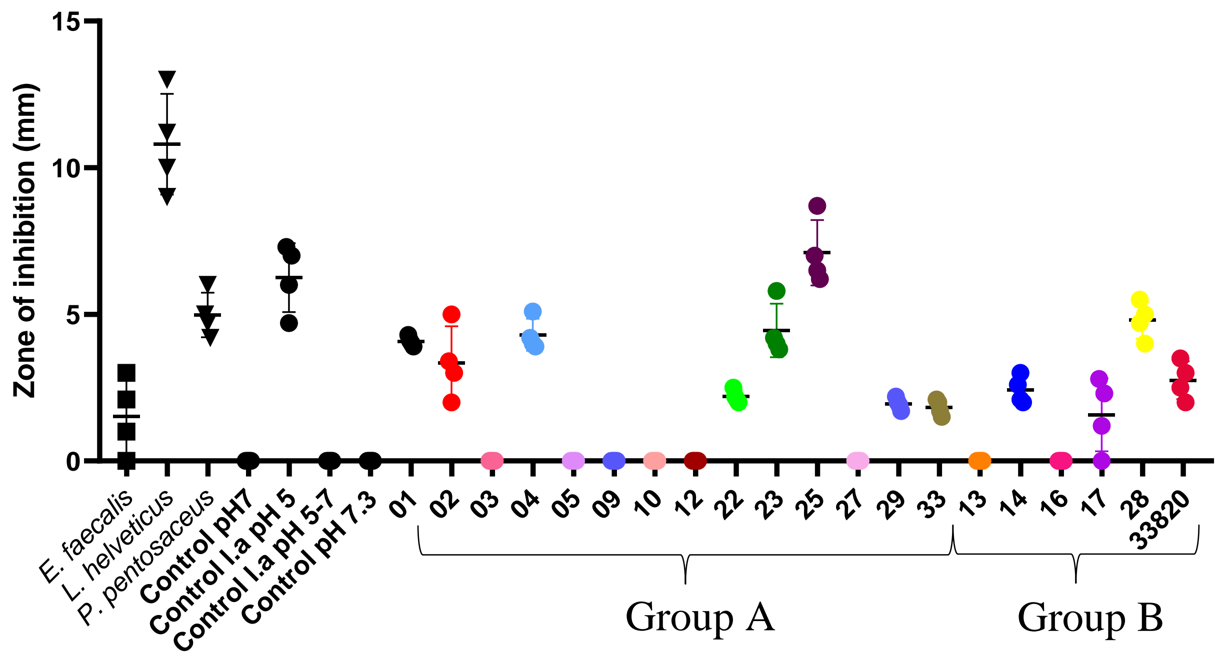


**Figure 17. Zone of inhibition measurements (mm) from agar well diffusion assays featuring *L. crispatus* supernatants plated against *E. coli* UTI 89 indicator strain separated into Groups A and B.** Methodology was carried out as described in figure 24, except the assay was conducted using CBA in place of m17.



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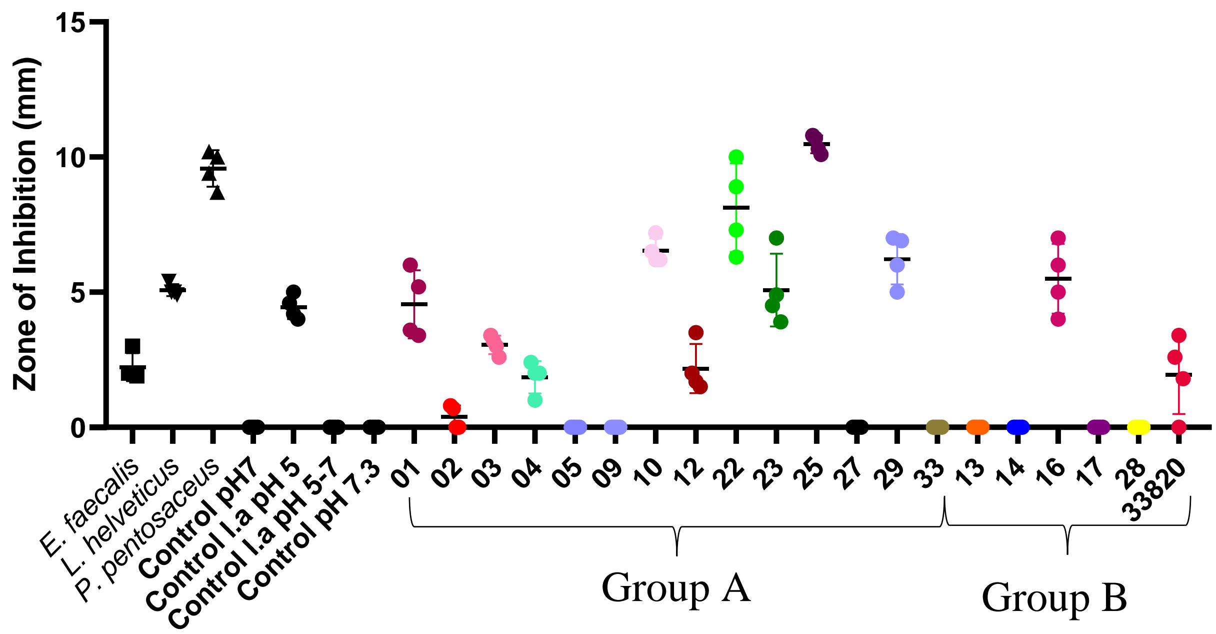


**Figure 18. Zone of inhibition measurements (mm) from agar well diffusion assays featuring *L. crispatus* supernatants plated against *C. albicans* TIMM 1768 indicator strain separated into Groups A and B.** Methodology was carried out as described in figure 24, except for final incubation which was carried out anaerobically because of the nature of the indicator strain.



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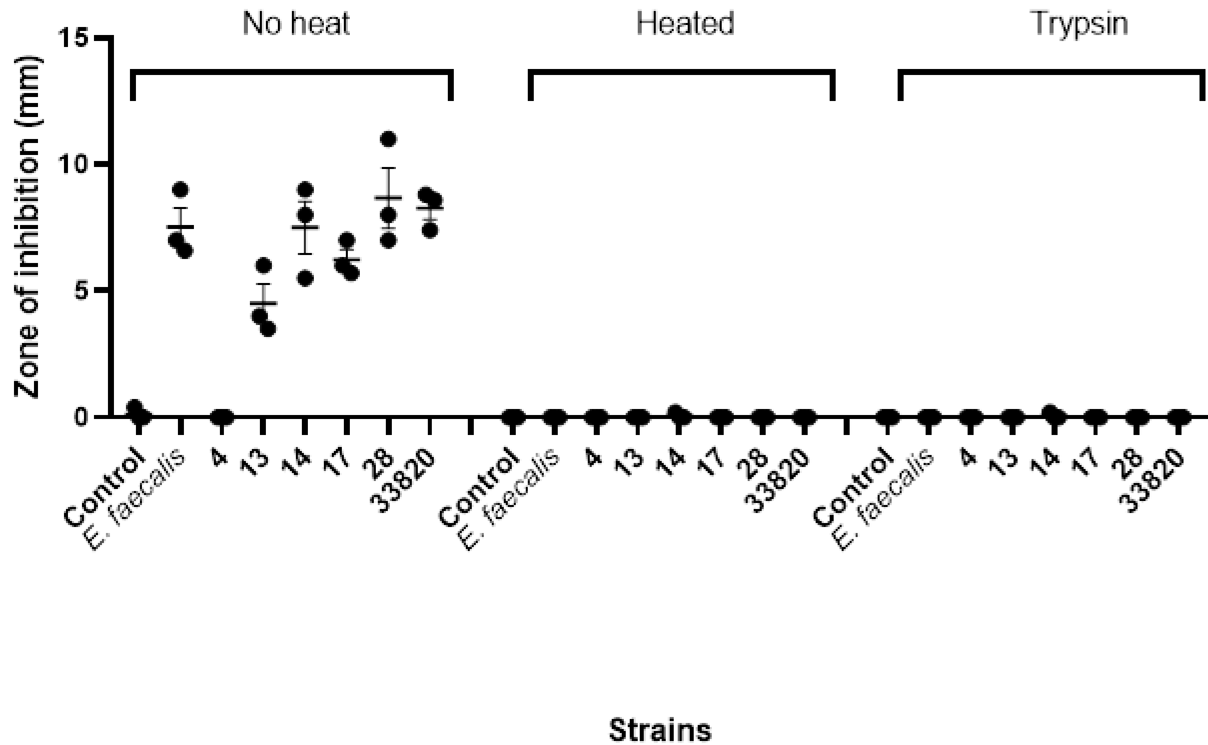


**Figure 19. Zone of inhibition measurements (mm) from agar well diffusion assays featuring *L. crispatus* supernatants that were heated, treated with trypsin, and unheated against *E. faecium* indicator strain.** Strains were grown for 24 hours on CBA plates and the individual supernatants were collected 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 neutralized. 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).



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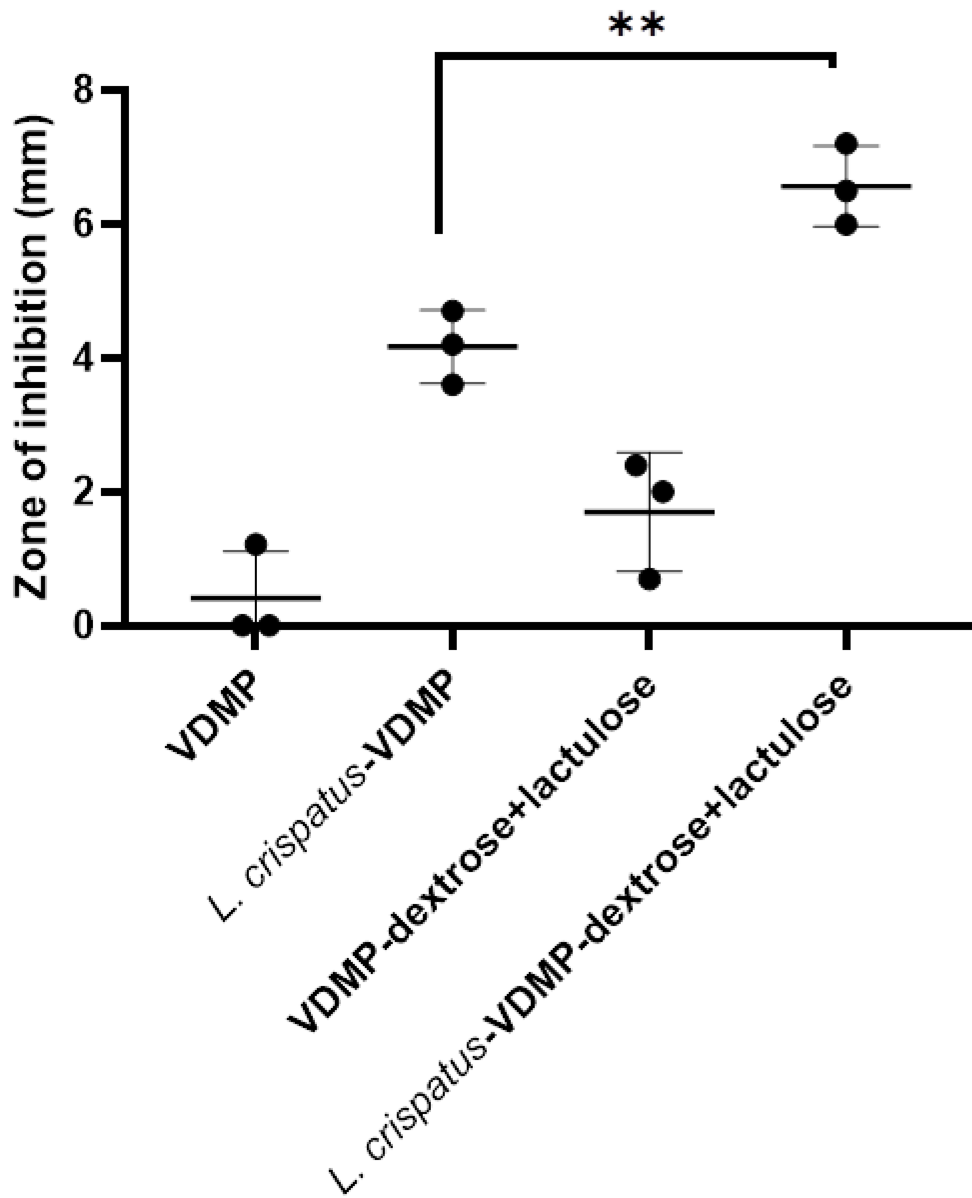


**Figure 20. Lactulose supplementation of *L. crispatus* 33820.** Zone of inhibition measurements (mm) from agar well diffusion assays conducted using supernatants collected from *L. crispatus* ATCC 33820 grown in VDMP and VDMP where dextrose was replaced with lactulose for 24 hours (P=0.0070). The supernatants were collected using centrifugation for 10 minutes, neutralized using NaOH and HCl, and filter sterilized. VDMP and VDMP with lactulose in place of dextrose was used as controls and prepared using the same method as the experimental samples. 250µl *E. faecium* 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=3, P=0.007).



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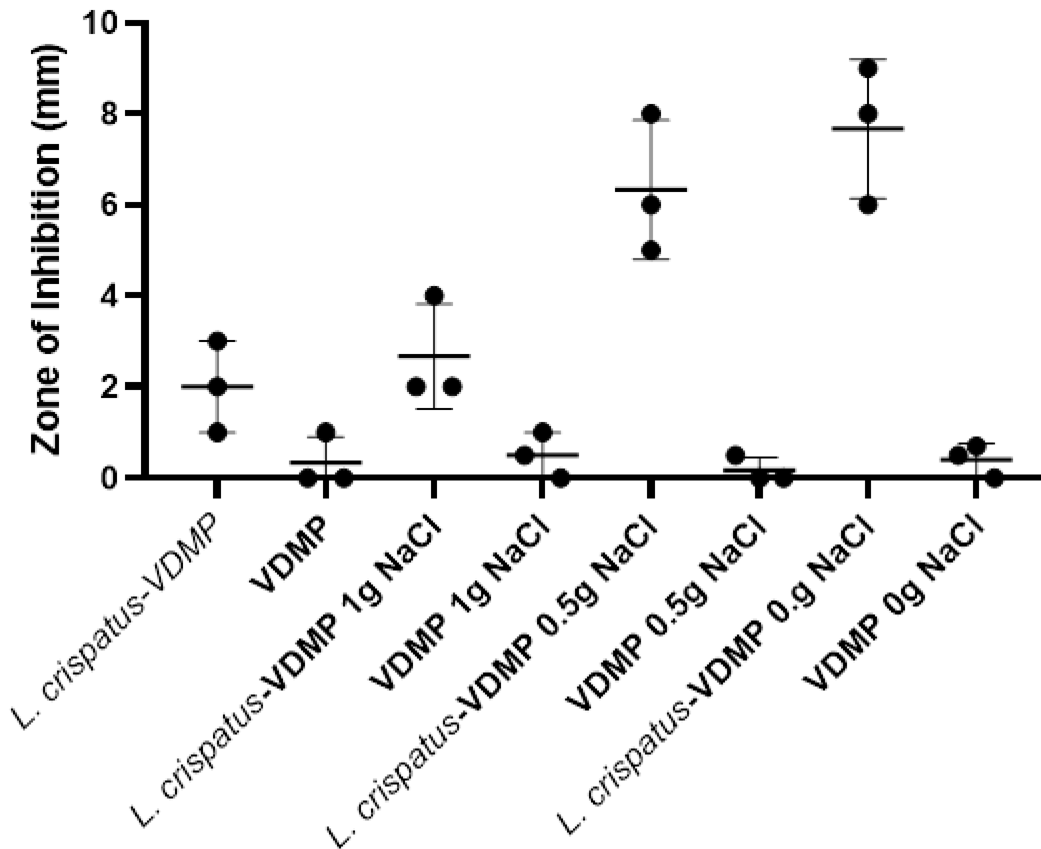


**Figure 21. Inhibition of *E. faecium* by *L. crispatus* isolates following growth in VDMP prepared with different amounts of NaCl**. Zone of inhibition measurements (mm) from agar well diffusion assays conducted using supernatants collected from *L. crispatus* ATCC 33820 grown in VDMP, VDMP with 1g, 0.5g, and VDMP prepared with 0g NaCl. The supernatants were collected using centrifugation for 10 minutes, neutralized using NaOH and HCl, and filter sterilized. VDMP with each salt concentration was used as controls and prepared in the same method as the experimental samples. 250µl *E. faecium* 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=3±SEM, P>0.05).



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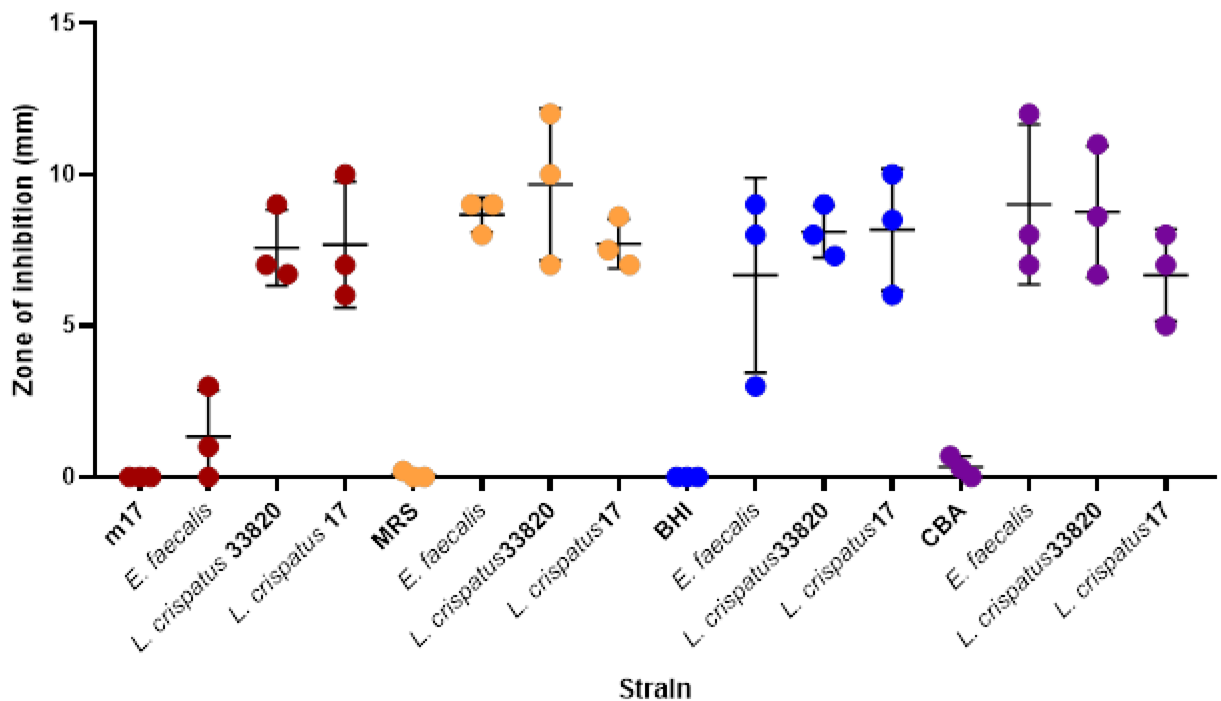


**Figure 22. Inhibition of *E. faecium* by *L. crispatus* isolates ATCC 33820 and 27 following growth on m17, MRS, BHI, and CBA**. Strains were grown for 24 hours on m17, MRS without tween, BHI, and CBA plates. The individual supernatants were collected using the freeze/thaw method. *L. crispatus* supernatant volumes were then normalized, neutralized using HCl and NaOH, and filter sterilized. Additional plates of each type were used as controls without bacterial growth and subjected to the same incubation and collection method. 250µl of the indicator strain was plated on CBA agar, 1cm holes were bored, and 50 µl of each supernatant was deposited into each well. Following aerobic incubation at 37°C for 48 hours the zones of inhibition were measured (N=3).



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**Figure 23. Co-culture apparatus.** The apparatus was assembled, sterilized, and filled with VDMP (50mL)**.** Pictured are two chambers clamped together with a 0.45µm filter paper placed between.



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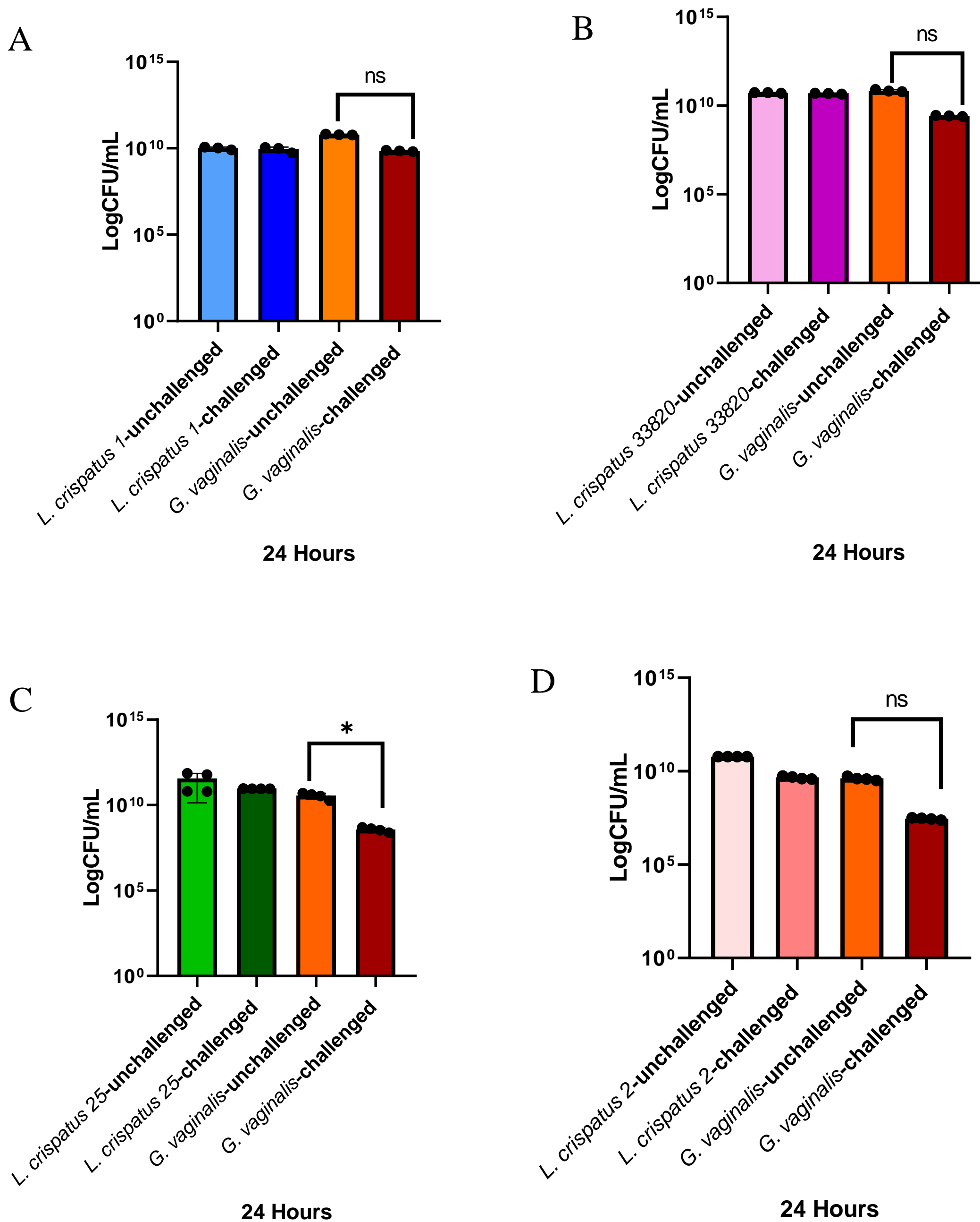


**Figure 24.** Co-culture experiment where *G. vaginalis* was challenged with (A) *L. crispatus* 1 (P>0.05), (B) *L. crispatus* ATCC 33820 (P>0.05), (C) *L. crispatus* 25 (P=0.0417), and (D) *L. crispatus* 2 (P>0.05). *L. crispatus* strains were grown on one side with *G. vaginalis* on the other for 24 hours. The growth of each was compared to the same strain grown individually by calculating CFU/mL.



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***P. bivia***

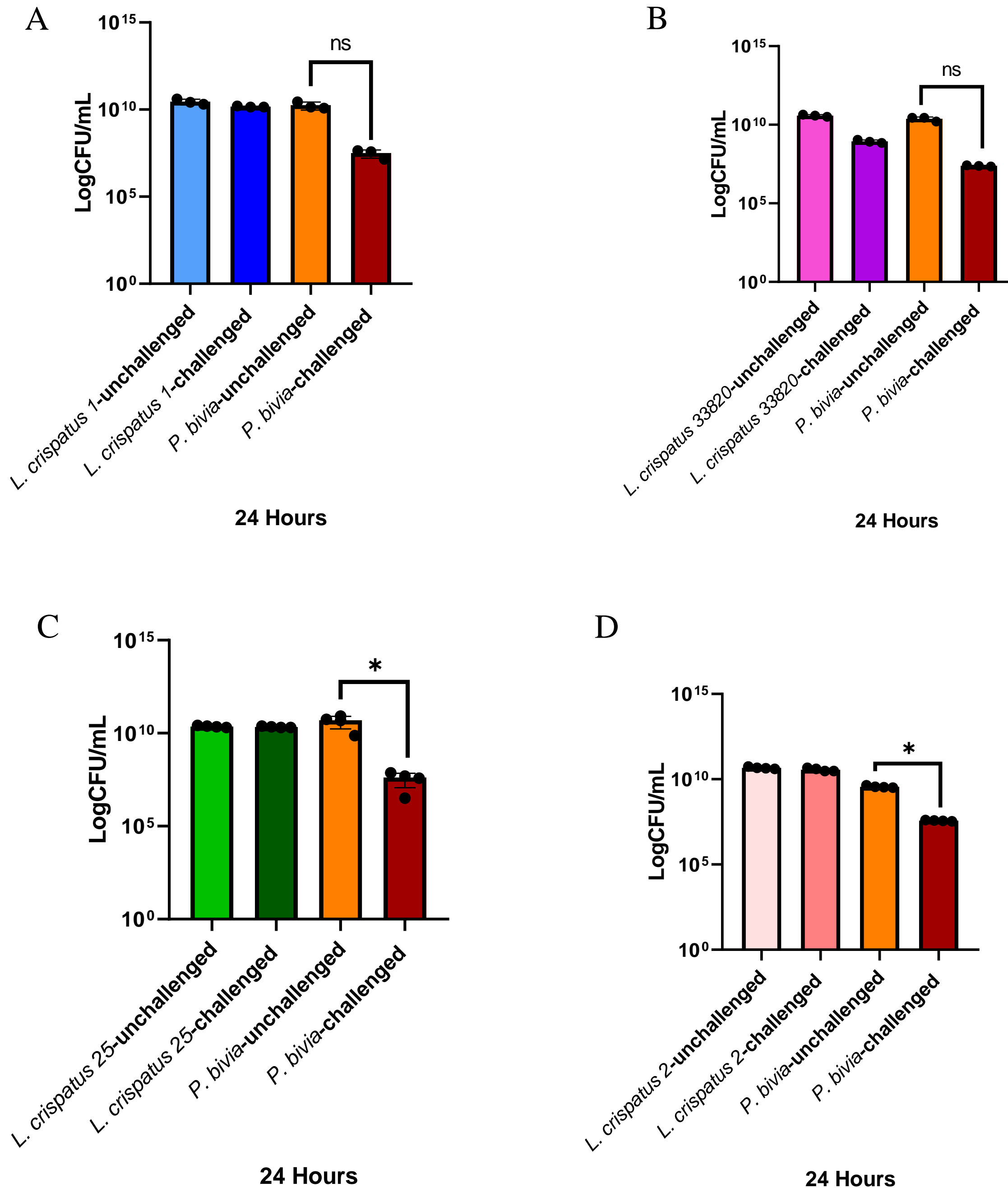


**Figure 25.** Co-culture experiment where *P. bivia* ATCC 29303 was challenged with (A) *L. crispatus* 1 (P>0.05), (B) *L. crispatus* ATCC 33820 (P>0.05), (C) *L. crispatus* 25 (P=0.0286), and (D) *L. crispatus* 2 (P=0.0008). *L. crispatus* strains were grown on one side with *P. bivia* on the other for 24 hours. The growth of each was compared to the same strain grown individually by calculating CFU/mL.



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## *E. coli*

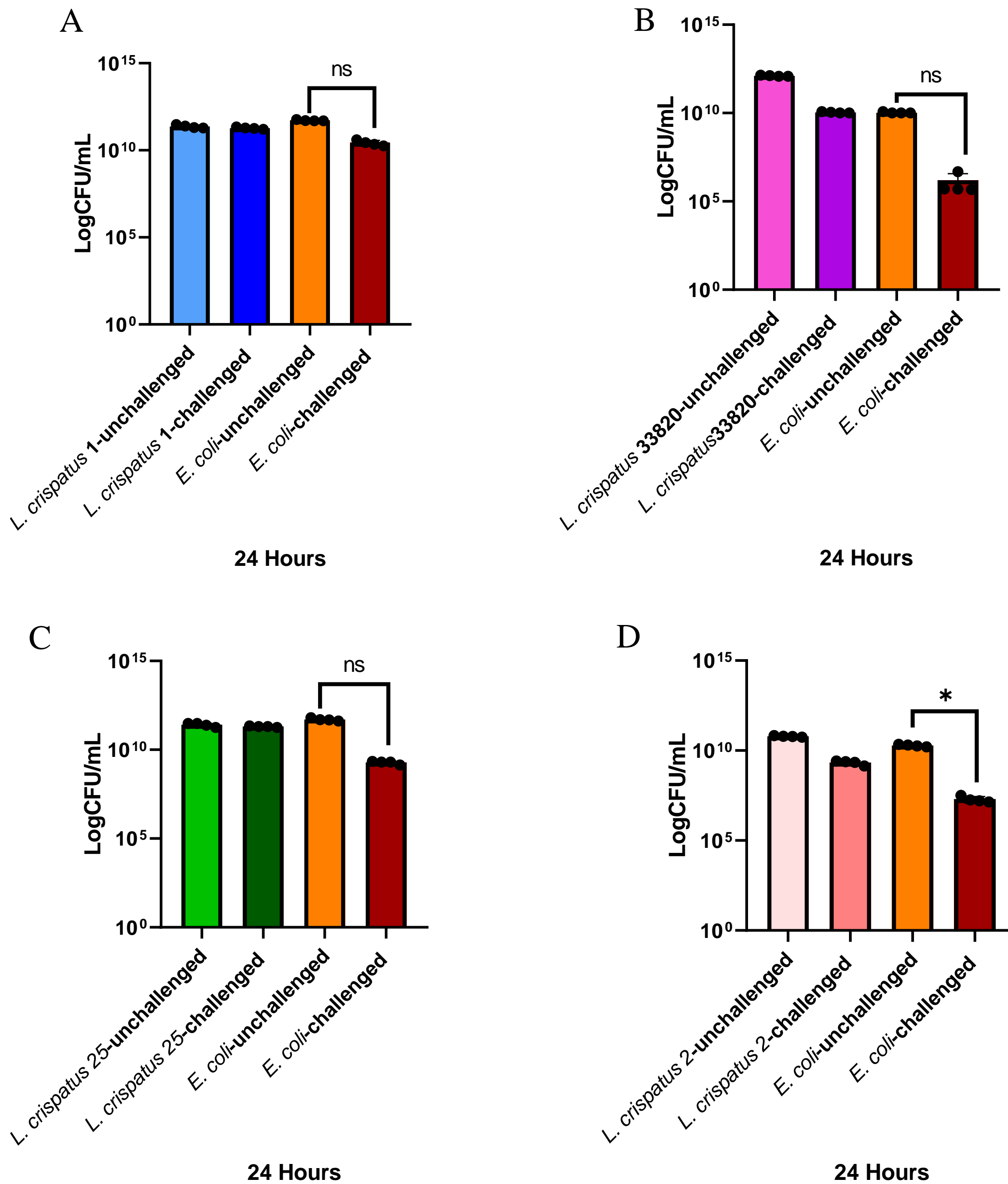


**Figure 26.** Co-culture experiment where *E. coli* UTI 89 was challenged with (A) *L. crispatus* 1 (N=4)(P>0.05), (B) *L. crispatus* ATCC 33820 (N=4)(P>0.05), (C) *L. crispatus* 25 (N=4) (P>0.05), and (D) *L. crispatus* 2 (N=4)(P=0.0286). *L. crispatus* strains were grown on one side with *E. coli* on the other for 24 hours. The growth of each was compared to the same strain grown individually by calculating CFU/mL.



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***C. albicans***

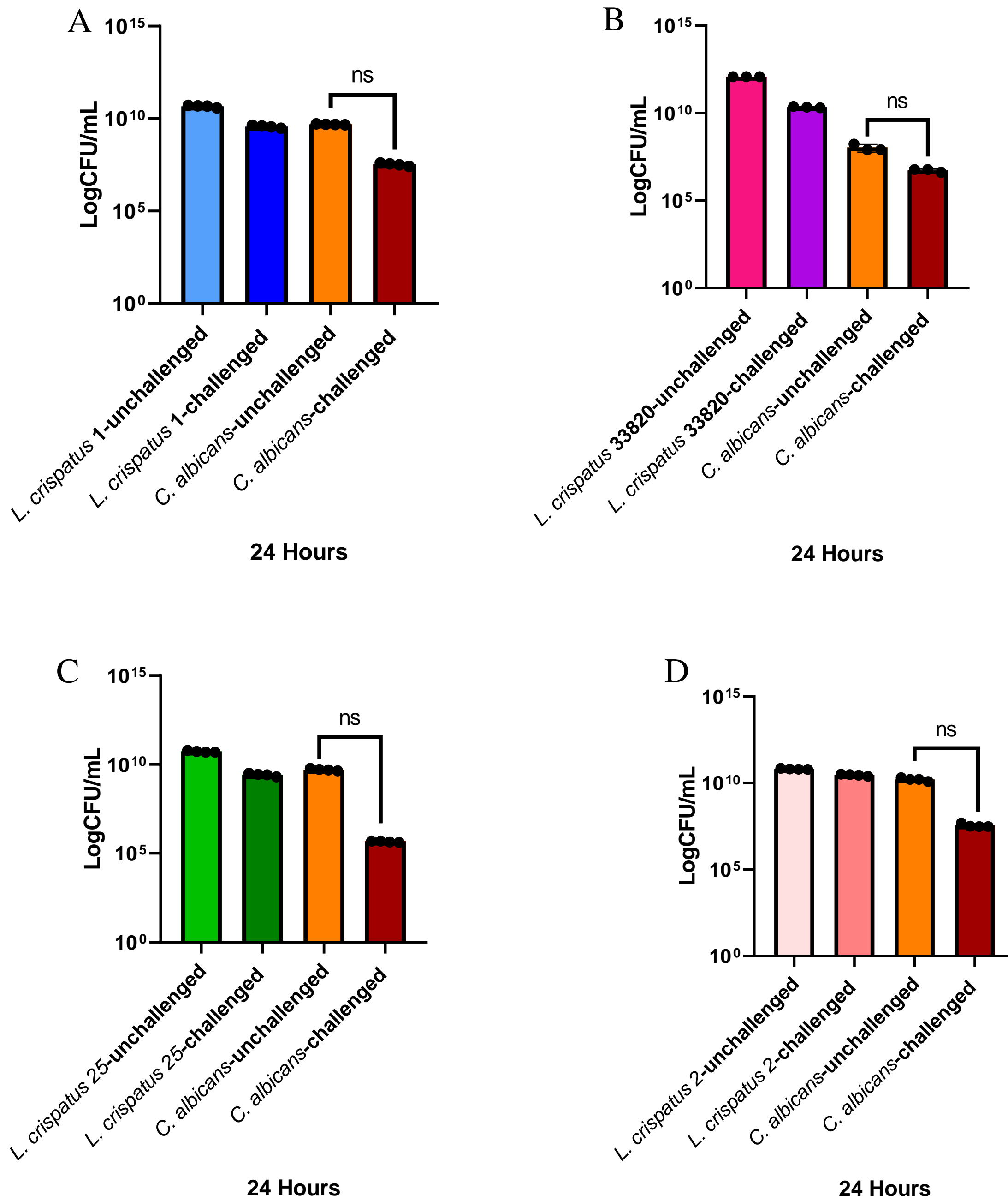


**Figure 27.** Co-culture experiment where *C. albicans* TIMM 1768 was challenged with (A) *L. crispatus* 1 (N=4)(P>0.05), (B) *L. crispatus* ATCC 33820 (N=3)(P>0.05), (C) *L. crispatus* 25 (N=4)(P>0.05), and (D) *L. crispatus* 2 (N=4) (P>0.05). *L. crispatus* strains were grown on one side with *C. albicans* on the other for 24 hours. The growth of each was compared to the same strain grown individually by calculating CFU/mL.



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## 3.4 Discussion

In this study, the presence of five putative bacteriocins were identified within the genomes of *L. crispatus* isolates. Several of the genomes contained all four bacteriocins, suggesting that these strains may be more effective than CTV-05 at the inhibition of pathogens, given that the latter only had Penocin A. Of the bacteriocins identified four had 100% sequence identity with those identified on BLAST, and Penocin A was determined to be an incomplete sequence.

Bacteriocins are regarded as being important defence factors of lactobacilli.[19] These antimicrobial proteins typically have a very narrow spectrum of activity, usually only inhibiting closely related species.[20] However, those produced by *Lactobacillis spp.* have been shown to have a much broader spectrum of activity, some of which have been shown to be effective against gram negative bacteria.[21] Production of bacteriocins can be influenced by a number of factors such as temperature, pH, and nutrient presence.[5]

The *L. crispatus* isolates produced bacteriocins with varying zones of inhibition against the indicator strains with isolates 01, 02, 12, 14, 25, 28, and 33 being particularly efficient against *E. faecium*, a standard indicator used to evaluate bacteriocin production. However, the strains examined were less effective at inhibiting growth of *G. vaginalis*. Only strains 1, 25, and 28 showed relevant inhibition, the minimum of which is a zone of inhibition measuring over 5mm. This is likely due to the mode of action of class III bacteriocins, which are cell wall-degrading and more effective against Gram positive bacteria.[22] However, it may be due to the fact that there are conditions that affect bacteriocin production and activity, such as neutralizing the supernatants to account for the effect of lactic acid, which may result in decreased activity.



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While the media used for solid supernatant collection had no apparent effect on inhibition, the effect was notably increased in liquid collection following the addition of lactulose prior to growth and a decrease in NaCl suggesting a stress response, similar conditions are seen during menstruation. This is interesting as lactulose has been proposed as a prebiotic for application to the vagina.[23] Inhibitory activity ceased following treatment with trypsin, a protease, which suggests the active compound is a protein, and following heating of the supernatants at 85°C for 45 minutes, indicative of a heat-labile protein.

Co-culture showed a decrease in growth of *P. bivia* ATCC 29303 when inoculated with *L. crispatus* strains 2 and 25, a decrease in growth of *G. vaginalis* ATCC 14018 when inoculated with *L. crispatus* strain 25, and a decrease in growth of *E. coli* UTI 89 when inoculated with *L crispatus* strain 2. This suggested *L. crispatus* has the potential to be used as a probiotic to treat vaginal dysbiosis caused by these uropathogens. Furthermore, in a study completed by van der Veer et al. (2019) it was determined that *L. crispatus* supernatants, from the same strains in my collection, were able to inhibit *Neisseria gonorrhoeae* growth by 27.9±15.8%, further depicting the potential benefits of a vaginal *L. crispatus* probiotic as women with BV are at higher risk of acquiring sexually transmitted infections.

An overview 29 *L. crispatus* strains is shown in Tabl3 3.1, 19 of which were used in this thesis. Additional notable characteristics identified within the genomes included the presence of 'virulence factors' within strains 13, 17, 20, 27, and CTV-05 and a fibronectin/fibrogen binding protein within all of the strains, CTV-05 included. GlnA, found in strain 13, expression has been shown to be important for colonization and survival of *S. pneumoniae* during an infection.[24] Chyl genes, the presence of which was found in strain 17, are involved in bacterial survival in species that grow in plant wound environments.[25] CvfA, identified within strain 20, was found to



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influence the expression of virulence genes.[26] The role of CTP synthase, found within the CTV-05 genome, may act as sensor for carbon sources, or may play a role in morphological alterations in parasites.[27] These genes, as well as the fibronectin/fibrogen-binding genes, may confer a competitive advantage to strains. Of the vaginal lactobacilli only *L. iners* and *L. crispatus* were found to contain the fibronectin-binding protein, suggesting it may play a role in the increased adhesion to human fibronectin seen among these species but not other lactobacilli.[28]

## 3.5. Conclusion

Five bacteriocins were detected within the genomes of fresh humans *L. crispatus* strains tested in this study, one of which is likely non-functional, with commercial isolate *L. crispatus* CTV-05 only having one partial bacteriocin sequence. Strains 2 and 25 inhibited the growth of key vaginal pathogens. However, it is not clear the extent to which bacteriocins are important in vivo, since lactic acid production is high and likely the main deterrent for pathogens. It remains unclear why strain CTV-05 was chosen for clinical testing, given its main attribute appears to be production of $H_2O_2$, a compound that is not a key driver of health.



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# Chapter 4: General discussion and future perspectives

## 4.1 General Discussion

The main goals of this project were to evaluate the strain to strain differences of *Lactobacillus crispatus* vaginal isolates, in particular their metabolomic and genomic profiles, and to examine the probiotic potential of these strains through interfering with the growth of pathogens.

The findings provide new insight into how one might go about selecting a probiotic strain for a vaginal application. The long-held belief that inhibition of pathogens is a key property should no longer be the only consideration. This thesis describes the importance of metabolic readouts along with a strain's genomic capacity as being part of any selective process. These need to take into account the intent of the probiotic. For example, if it is to treat vaginal dysbiosis, then it needs to disrupt pathogen biofilms perhaps by production of biosurfactants as well as acids, or $H_2O_2$ or possibly bacteriocins. Its ability to modulate host defences, such as immune mediators and mucus could also be factors in the selection. The type of lactic acid that is produced was not assessed by the LC-MS run. The D-isomer is primarily produced by *L. gasseri, L. jensenii,* and *L. crispatus*, whereas the L-isomer is produced by *L. iners* and the vaginal epithelium.[1] The higher production of L-lactate compared to D-lactate has been linked to altered cervical integrity, which may allow entry of human papillomavirus to the basal layer of the vagina.[2] Furthermore, higher concentrations of D-lactate increase vaginal mucus viscosity, which has been shown to increase viral particle trapping.[3]

The strains examined here seemed to cluster within two distinct groups based on their metabolomic profile. The most striking observation was biogenic amine production by group B



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strains. These compounds can raise vaginal pH and enhance the colonization of pathogens. In addition to being associated with infection, BA production has also been associated with an increased risk of acquiring STIs, especially *N. gonorrhoeae*, which is also more resistant to host mediated defenses in the presence of BAs.[4] that the production of BA compounds may allow strains to overcome a pH inhibition and facilitate the outgrowth of BV-associated bacteria.[5]

The bacteriocins Enterolysin A, Penocin A, and Helveticin J, as well as two putative bacteriocins were identified within the genomes, a summary of those found in each strain is available in chapter 3. However, only one bacteriocin, Penocin A, was identified within the *L. crispatus* CTV-05 genome, and similar to the other isolates analyzed, this bacteriocin is not likely functional due to the incomplete gene sequence. It is certainly possible that the more strains analyzed the more bacteriocins would be found. The data here do not allow conclusions as to which bacteriocin would give a strain more of an advantage in performing restorative functions in the vagina.

To verify production of an antimicrobial compound agar well diffusion assays were conducted. Included in the assays as controls were supernatants from bacteria-free plates that were treated with lactic acid and then neutralized. Since these controls did not cause inhibition of the indicator strains it can be assumed that the resulting inhibition is not due to lactic acid production. The heat and trypsin treatment of the supernatants and resulting loss of inhibition suggests that the inhibition found during the assays is the result of a heat-labile antimicrobial protein, likely one of helveticin J or enterolysin A, perhaps in conjunction with the other bacteriocins evident in the genomes or a biosurfactant.

Microbiome studies of the vagina, first reported by Reid's group in London Ontario, demonstrated the prevalence of *L. iners* and the array of pathogens that can inhabit this niche. [6-8]



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It soon became obvious that metronidazole, the primary treatment of BV for forty or more years, was never selected to kill these organisms and is indeed a sub-optimal therapy. Furthermore, the increase in antibiotic resistance, low cure-rate with antibiotic treatment and high recurrence rate of BV, as well as disruption of the microbiota with metronidazole and clindamycin, demonstrate that alternative therapies have long been needed. Even when successful, antibiotic treatment does not result in restoration of the vaginal microbiota, so a way to promote regrowth of endogenous species associated with health is necessary. An oral dose of *Lactobacillis rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 is intended to reduce re-seeding of the vagina by pathogens and also disrupt their colonization. When administered with antimicrobial agents, the net result is improved cure and restoration of indigenous lactobacilli.[9] An improved cure rate of BV when these strains are administered alone into the vagina illustrates the potential of probiotics.[10]

In the case of *L. crispatus*, CTV-05 which is being developed as an intravaginal drug, it only appears to be effective if the subject is devoid of indigenous *L. crispatus.*[11] 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. For now, the use of antimicrobial agents plus oral probiotic lactobacilli appears to be the best management practice.[9, 12,13]

The case of highly prevalent vulvovaginal candidiasis is different in that the yeast infects irrespective of lactobacilli abundance. [14] This is interesting and experiments showing that *L. rhamnosus* GR-1 and *L. reuteri* RC-14 can affect the yeast's susceptibility to anti-fungals and penetrate its biofilms suggests that indigenous lactobacilli cannot do this.[15] All the more reason for using probiotic strains as part of the therapy, as shown in two human studies. [16,,17]

Another approach could be the use of prebiotics, which stimulate the growth of beneficial



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microorganisms. Defined as “a substrate that is selectively utilized by host microorganisms conferring a health benefit,” one substance, lactulose, showed promise as a vaginal prebiotic as it stimulates the growth of *L. crispatus, L. gasseri, L. vaginalis,* and *L. jensenii*, but not vaginal pathogens. [18,19]

In an era where the reproductive rights of women have been widely recognized and emphasis has been placed on women’s health the use of probiotics offers, in some cases, preferable treatment to use of antimicrobial agents. This includes prevention of recurrence of BV and urinary tract infection as well as possibly increasing the rate of successful IVF outcomes.[20-23] At the very least, the impact on female quality of life makes probiotics worthy of further study.

## 4.2 Summary and future directions

From this series of experiments, it has become clear that not all strains of the same species have probiotic potential. This further emphasizes that generalizations should not be made about strains, such as *lactobacilli do this*, or *L. acidophilus does that*. There is no substitute for assessing each strain’s properties.

Although the metabolomic analyses showed a separation of the *L. crispatus* strains into two groups, these did not correspond to the health status of the women from whom they were collected. Group B stains appeared to have a more unfavourable metabolic profile than Group A strains, appearing to be stressed in VDMP. Thus, selection of candidate probiotics cannot be based on the assumption that just because a woman is healthy, her strain is ideal for propagation.

Five bacteriocins were detected within the *L. crispatus* strains’ genomes, with commercial isolate *L. crispatus* CTV-05 only having one. This adds to our knowledge of



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properties possessed by vaginal lactobacilli. But, the extent to which these bacteriocins play a role in protecting the host remains to be determined. Studies are needed that acquire vaginal samples and determine whether these compounds are actually being produced and if there is any evidence of impact on the microbiota.

In addition, evaluating the metabolomic profiles of the *L. crispatus* isolates while grown with a vaginal cell line could provide more insight on the *L. crispatus*/host interactions. Perhaps there are compounds produced in response to recognition of the epithelial cells.

It is hoped that these studies will contribute to the framework of which properties may be useful for lactobacilli applied to the vagina. In future, candidate probiotic strains should also be evaluated for the ability to inhibit *A. vaginae*, a pathogen that could not be included in this study due to its extreme anaerobic growth requirements. A *L. crispatus* strain exhibiting the ability to inhibit pathogen growth, with an ideal metabolic profile, and the ability to degrade glycogen, could be a promising probiotic candidate worthy of clinical testing. The creation of a synbiotic, "A synbiotic is a mixture, comprising live microorganisms and substrate(s) selectively utilized by microbes, which confers a health benefit on the host" with *L. crispatus* and lactulose would also be worth assessing.



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## Supplemental Figures

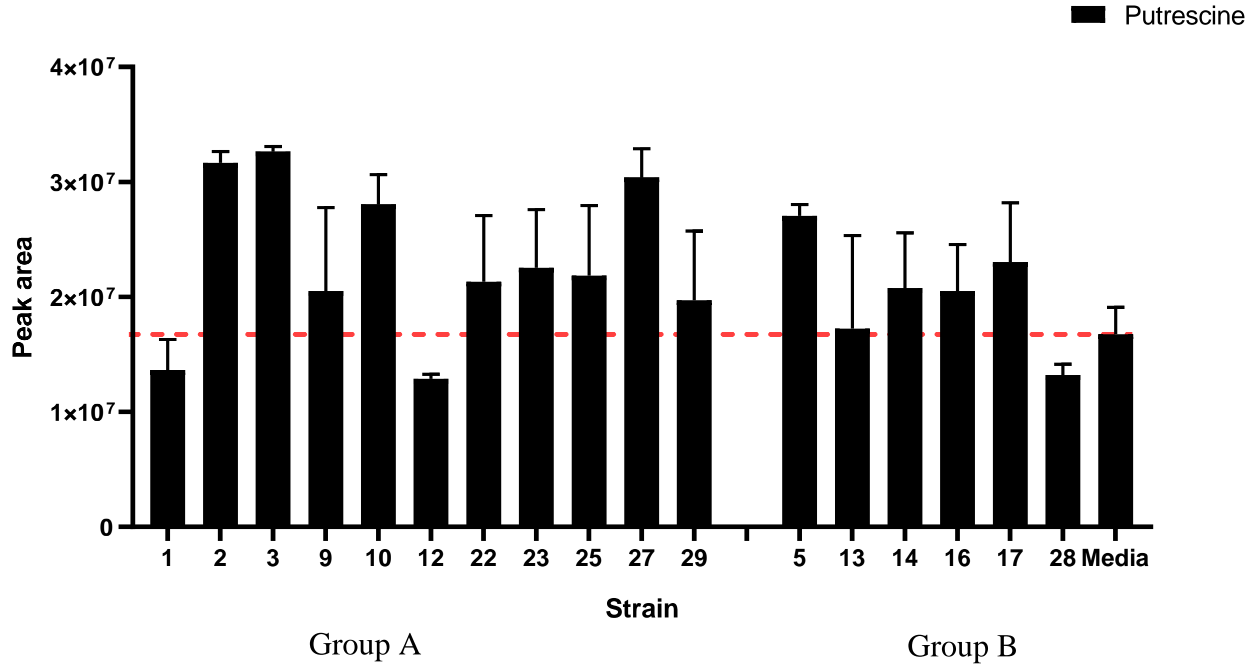


**Figure S1. Putrescine presence in spent *L. crispatus* supernatant.** The presence of putrescine in *L. crispatus* supernatants following 24 hour growth in VDMP separated into Groups A and B (N=3±SEM, P>0.05). Dashed line represents presence in media.



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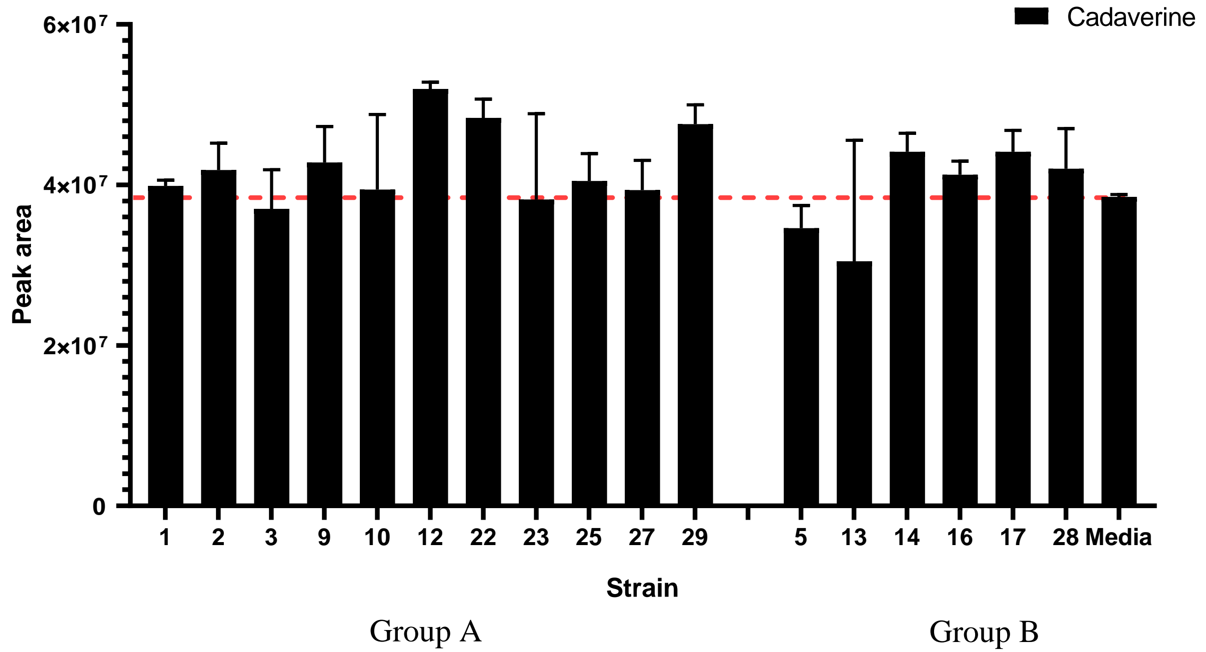


**Figure S2. Cadaverine presence in spent *L. crispatus* supernatant.** The presence of cadaverine in *L. crispatus* supernatants following 24 hour growth in VDMP separated into Groups A and B (N=3±SEM, P>0.05). Dashed line represents presence in media.



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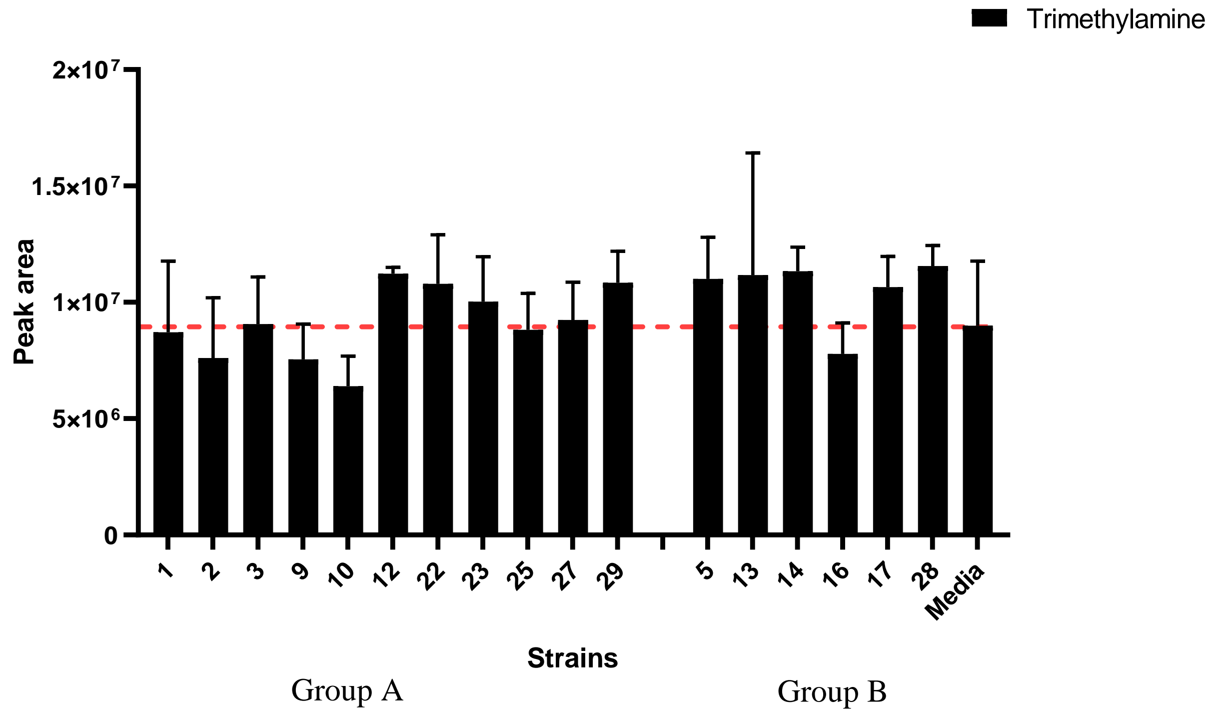


**Figure S3. Trimethylamine presence in spent *L. crispatus* supernatant.** The presence of trimethylamine in *L. crispatus* supernatants following 24 hour growth in VDMP separated into Groups A and B (N=3±SEM, P>0.05). Dashed line represents presence in media.



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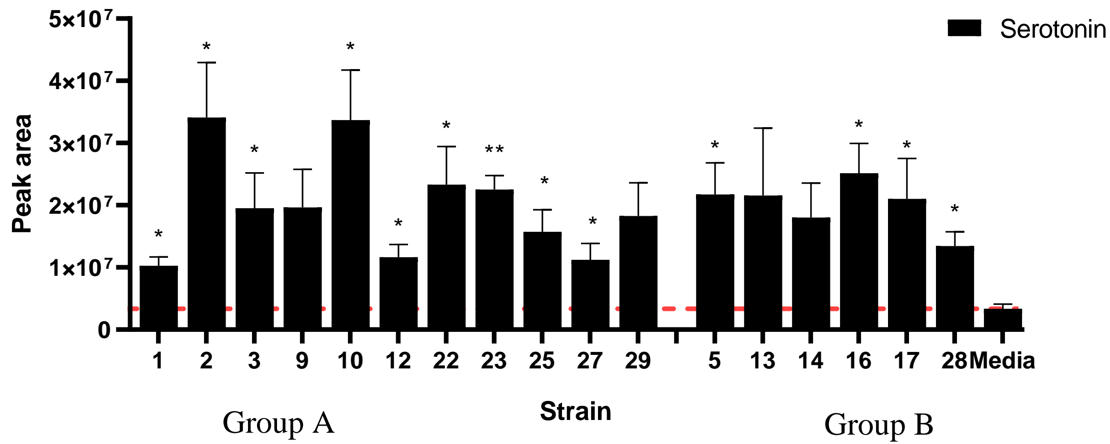


**Figure S4. Serotonin presence in spent *L. crispatus* supernatant.** The presence of serotonin in *L. crispatus* supernatants following 24 hour growth in VDMP separated into Groups A and B (N=3±SEM). Presence in spent supernatant compared to presence in media. ($P<0.05$(*), $P<0.01$(**)). Dashed line represents presence in media.



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SPECIAL ISSUE

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# Metabolomics as a clinical testing method for the diagnosis of vaginal dysbiosis

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Microbes play an important role in vaginal health, with lactobacilli a particularly abundant species. When dysbiosis occurs, the tools to determine whether it is a condition such as bacterial vaginosis, and whether it warrants antibiotic treatment, are currently suboptimal. We propose that standardization and implementation of an affordable metabolomics-based diagnostic technique could reduce instances of false positives, stress associated with misdiagnosis, and potentially save time and money. Basing diagnosis on the detection of pH elevated above 4.5 and specific polyamines could provide a better method to assist a physician determine whether treatment is warranted.

KEYWORDS
bacterial vaginosis, metabolomics, vaginal metabolome, vaginal microbiota

## 1 | INTRODUCTION

The reproductive health of women is determined to a large extent by the microbes that inhabit the region. The presence of lactobacilli, especially a select number of species, has not only been associated with cervicovaginal health, but also with sperm motility.[1-3] When dysbiosis, a disruption in the microbiota, occurs through sexual contact, douching, antimicrobial disruption, or unknown causes, a condition referred to as bacterial vaginosis (BV) can occur. This is exceedingly common among premenopausal and pregnant women and has been associated with an increased risk of acquiring sexually transmitted infections, and premature delivery of low-weight infants.[4-6]

Recently, questions have been raised as to whether BV is actually a group of conditions, rather than one.[7,8] One of the main reasons for women to seek medical assistance is the odor that is associated with dysbiosis, which contributes to emotional and social detriments for women.[9] The odor is due to specific polyamines.[10,11]

We hereby make a case that a metabolomic approach could be used for a more accurate diagnosis of BV. Metabolomics couples mass spectrometry with chromatography, and with postfiltering of artifacts and careful statistical analysis, it enables the accurate identification of small-molecule metabolites in a sample.[12-14] Applying these techniques, along with microbiome analysis, proteomics, and transcriptomics, allows for the elucidation of microbial profiles and biomarker metabolites, which then enable physicians to distinguish between a diseased and a nondiseased state.[15-19] Indeed, in one study, the vaginal metabolome mostly correlated with bacterial diversity.[15] This gets to the core of functionality, rather than only demonstrating which organisms are present.

## 2 | LIMITATIONS OF CURRENT DIAGNOSTIC METHODS

Diagnostically, BV is confirmed at most sites using the Amsel criteria or the Nugent scoring system.[20,21] The former requires the presence of three of four of the following criteria: homogenous vaginal discharge, a vaginal pH higher than 4.5, positive whiff test or release of amine odor after addition of 10 per cent KOH, and the presence of clue cells associated in a microscopic evaluation. The Nugent scoring system provides a 0-10 scale for the evaluation of vaginal microbiota; a weighted sum of the presumptive lactobacilli based upon morphology, and presumptive identification of *G. vaginalis* and *Mobiluncus* spp.[9,19,21,22] However, these methods are often inaccurate, in part due to the diverse morphology and Gram staining of bacteria found in the vagina, which makes it difficult even for expert observers to differentiate bacterial species. For example, a Gram-positive *Atopobium* associated with BV may be counted as Gram-positive *Lactobacillus*, while *L. iners* may appear Gram variable and be counted as *Gardnerella*


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or another Gram-negative species. In addition, microscopic diagnosis of BV relies largely on the assumption that the vagina of unhealthy women contains low numbers of lactobacilli. This is built on the premise that healthy women are colonized predominantly by *Lactobacillus* species, which is not always the case.[22] A Gram stain assessment would identify the community state type IV, described by Ravel et al[22] as being dysbiotic and therefore BV, yet it was found in healthy women with no other indication of BV. This makes microscopic analysis of vaginal samples even more unreliable to diagnose a condition like BV that may require antibiotic treatment. Variations also arise within individuals, due to menstruation, age, and sexual activity,[23-27] and often, a secondary sample is needed to verify BV.

## 3 | ADVANTAGE OF METABOLOMICS

The increasing availability of new analytical tools, providing a highly specific detection of various biomarkers, contributes to the development of genomics, proteomics, metabolomics, and transcriptomics.[12,16,28-34] Through production of a molecular catalog and comparing metabolic data from healthy and non-healthy individuals, a statistically significant difference in specific biomarkers between groups can lead to the detection of certain conditions.[12,31,32]

Metabolomics, a study of the metabolome of an environment, is an area that is especially applicable to studies involving the vagina. It allows for quantification of the metabolites in an environment that has fluctuating conditions and is easily accessible for sample collection. Arguably, more relevant than other -omic techniques, due to closely reflecting cellular activity, it allows for the identification of biomarkers associated with BV. Using GC-MS analysis for non-polar compounds, or LC-MS for polar compounds, the relative abundance of metabolites in a sample can be determined. Following metabolite identification and clustering, differences between the relative concentrations of metabolites can be observed.[12,32]

Metabolites are produced through the bacterial metabolism of nutrients. This production is central to the maintenance of the host's health, and alterations in their composition and abundance reflect a changing microbiota. Molecular composition within the vaginal environment may give an indication of health due to a metabolic variation that can be seen in a diseased state,[12,23] In the case of BV, dysbiosis results from a displacement of *Lactobacillus crispatus*, *L. iners*, *L. jensenii*, *L. gasseri*, or other *Lactobacillus* spp. by anaerobic bacteria, including *Gardnerella vaginalis*, *Atopobium sp.*, *Prevotella* spp., and *Clostridium* spp.[33] This is detected by 16S rRNA, but dysbiosis alone does not provide a reason, in most cases, to prescribe treatment. Thus, the ability to detect concomitant malodorous discharge is much more informative, and to understand which compounds are present, such as γ-hydroxybutyrate (GHB) detected by LC-MS,[12] helps better guide intervention.

Several studies have been conducted indicating promise for use of metabolomics as a diagnostic method for clinical BV (Table 1). Yeoman et al[32] were able to distinguish between patients with and without BV using their metabolomic profiles following a metabolomic characterization of BV. After GC-MS analysis to examine the relative abundances of metabolites in each of 36 samples, 176 metabolites were identified. Their primary findings were that after comparing women with high Nugent scores to those with low to moderate scores, a decrease in lactic acid was found. In addition, when comparing BV-positive samples, as defined by the Amsel criteria, to BV-negative samples, a reduction in 1,2 propanediol, a lactic acid derivative, was evident in the BV-positive samples.

Vitali et al[34] correlated vaginal colonization with metabolic profiles to determine possible biomarkers of BV. They identified 17 unreported small molecules associated with BV, including various amines, amino acids, and monosaccharides. Specifically, during a BV state, there was an increase in tyramine, trimethylamines, and cadaverine.

Srinivasan et al[10] analyzed samples from women with and without BV and were able to detect 279 metabolites, of which 173

**TABLE 1** Key metabolites determined to increase in a BV state when compared to a healthy state

| Study reference | Sample | Detection method | Metabolites detected | Clinical relevance |
|---|---|---|---|---|
| 32 | Vaginal lavage | GC-MS | Putrescine, cadaverine, 2-methyl-2- hydroxybutanoic acid, diethylene glycol | Relevant to symptomatology |
| 35 | Vaginal secretion | GC | Trimethylamine | Fishy odor is easy to detect when in high amounts, but compound can be present when patient is deemed "healthy" by Gram stain scoring of vaginal cells |
| 34 | Vaginal lavage | $^{1}$H-NMR | Acetate, succinate | Succinate determined to be irrelevant by McMillan et al[12] |
| 10 | Cervicovaginal lavage | LC-MS | Putrescine, cadaverine | Relevant to symptomatology |
| 12 | Vaginal swab | LC-MS | 2-hydroxyisovalerate, γ-hydroxybutyrate | 2-hydroxyisovalerate: tyrosine ratio 0.621 found to be diagnostically relevant |

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differed between patients and controls. Furthermore, a group of bacteria associated with BV were significantly correlated with cadaverine, succinate, putrescine, tyramine, and deoxycarnitine. Following treatment of BV with metronidazole, eight women returned for a follow-up visit 4 weeks later, four of whom no longer had BV. These women had high concentrations of lactobacilli, and those with high abundances of *L. crispatus* had elevated concentrations of metabolites negatively associated with BV.

Prior to metabolomic analysis, McMillan et al[12] defined BV in vaginal samples using the Nugent method. Then, using LC-MS, 49 metabolites were then detected and determined to be significantly different between women with BV and those who were healthy. Next, conditional logistic regression of the metabolites was conducted to identify whether the metabolites were associated with a higher bacterial diversity and whether the method could be diagnostic for BV. The best metabolites for accurate indication of BV were high 2-hydroxyisovalerate, γ-hydroxybutyrate, and low lactate and tyrosine, with a 0.621 ratio of GHB to tyrosine and a 0.882 ratio of 2-hydroxyisovalerate to tyrosine being the optimal cut points. The biomarkers were validated in a blinded cohort of 45 women, with the 2-hydroxyisovalerate:tyrosine ratio being 89% selective and 94% specific for BV. They found 91% of BV cases were predicted.

The use of trimethylamine as an identifier of BV has been proposed previously, as its reduction is one cause of the fishy odor associated with BV. However, although present in women with Nugent scores over 7, low levels of trimethylamine are not always indicative of BV and have been found in women with negative Nugent scores, likely a result of their diets.[35]

Taken together, these data suggest that a 2-hydroxyisovalerate: tyrosine ratio, an increased concentration of putrescine and cadaverine, as well as a lower concentration of lactate, could be indicative of BV, and could be used to diagnose BV in conjunction with pH.

## 4 | THE FUTURE OF METABOLOMICS IN A CLINICAL SETTING

There is no doubt great potential for metabolomic methods to be incorporated into practical diagnostic systems within the healthcare system. Metabolomics allows for rapid and accurate determination of disease and health markers at a molecular level. With continuing advances to chromatography and mass spectrometry equipment, and as the rather expensive price of these machines continues to drop, they could become more widely used in clinical settings, replacing outdated and inaccurate tests for BV diagnostics. Although variability is expected between metabolomic profiles, the use of key metabolite biomarkers will allow for a quick and more specific diagnosis of the condition.

In addition to expertise needed to run the equipment, service it when minor adjustments are needed, and appropriately prepare samples, there are important databases needed of reference compounds produced by microbes and the host. It may be some time before automation can be employed to run samples and identify key compounds. Targeted metabolomics measure defined groups of chemically characterized and biochemically annotated compounds and could provide a rapid and effective method for use in healthcare settings.[36]

Biases need to be considered in the sample origins, including those involving subjects of different ages, ethnicity, and dietary or lifestyle variations. Where possible, control samples are pivotal, as are collection of multiple samples from the same subject at different timepoints. As with any clinical sampling, until sufficient data have been generated to define what constitutes health vs dysbiosis vs a disease that merits treatment, appropriate sample sizes must be used to ensure significance of any results obtained.

The tremendous enthusiasm for microbiome analysis is now being somewhat curtailed by the realization of limitations in 16S rRNA methods and analytical tools leading to potentially wrong conclusions.[37,38] In addition, it is clear that functional data are vital to know what is happening at the site where microbes are metabolically active, or where their molecules or those indirectly resulting from their presence, are influencing the host. While metagenomic methods are growing in popularity for the genomic information they provide on the microbes in a given sample,[39,40] this expensive and time-consuming method does not confirm functionality. Transcriptomic analysis of vaginal samples combined with metabolomics would provide the most information, but the former is extremely time-consuming and requires RNA recovery, which is not easy from swab samples.[19]

## 5 | IN SUMMARY

The female reproductive tract is vital for human survival. The process of pregnancy is influenced greatly by microbes, but in recent times, it has been the beneficial bacteria that have garnered much interest in restoration and maintenance of health. Molecular -omics methods that reveal what microbes are present, in what abundance, and how they affect the host, represent the most significant progress made in understanding women's health in forty or more years. During that time, hundreds of millions of women worldwide have been diagnosed and treated for bacterial vaginosis, when in reality the diagnosis has been far from optimal, and the condition itself poorly defined.[7]

Targeted and untargeted metabolomic analysis has the potential to provide significant improvements in diagnosing health and disease in the reproductive tract, and for being a tool to monitor treatment outcomes. With standardization and further development of less expensive metabolomic protocols, as well as data analyses, the potential for application to the clinical setting and personalized medicine is high.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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# CURRICULUM VITAE

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## List of Publications and Presentations

a. Articles published or submitted

**Watson, E.** and Reid, G. (2018) Metabolomics as a clinical testing method for the diagnosis of vaginal dysbiosis. *American Journal of Reproductive Immunology* **80**(2), e12979

**Watson, E**.,van Der Veer, C., Collins, S., Renaud, J., Sumarah, M., Kort, R., and Reid, G. Metabolomic profiling of *Lactobacillus crispatus* vaginal isolates(2019) manuscript in preparation

**Watson, E.**, van Der Veer, C., Kort, R., and Reid, G. Genomes and antibacterial activity of *Lactobacillus crispatus* vaginal isolates (2019) manuscript in preparation

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b. Non-refereed contributions (Poster presentations)

**Watson, E**. and Reid, G. (2019) Potential beneficial attributes of *Lactobacillus crispatus*. Presented at the International Scientific Association for Probiotics and Prebitoics, Antwerp, Belgium, June 2019.

**Watson, E.** and Reid, G. (2019) Potential beneficial attributes of vaginal *Lactobacillus crispatus*. Presented at London Hospitals Research Day, London, Canada, April 2019

**Watson, E.** and Reid, G. (2018) Bacteriocin production by vaginal *Lactobacillus crispatus* isolates. Presented at Infection and Immunity Research Forum, Stratford, Canada, October 2018

**Watson, E.** and Reid, G. (2018) Production of Enterolysin A by *Lactobacillus crispatus* vaginal isolates. Presented at LHRD, London, Canada, May 2018

**Awards and Scholarships**

a. Scholarships

Western Graduate Research Scholarship (2018)
University of Western Ontario

FW Luney Entrance Scholarship (2017)
University of Western Ontario

b. Awards

OUA Scholar Athlete Award (2018)
University of Western Ontario

FW Luney Travel Award (2019)
University of Western Ontario

ISAPP SFA Travel Award (2019)
ISAPP Students and Fellows Association

Microbiome and Probiotic Student Research Award (2018)
St. Joseph's Health Care Foundation, Anonymous donor

NSERC Undergraduate Student Research Award (2016)
Nipissing University

Carl Sanders Scholar award (2016)
Nipissing University

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