Investigating the Role of *Lactobacillus* Isolates from South African Young Women in the control of Bacterial Vaginosis (BV) - associated *Gardnerella vaginalis* and Candida.

By

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Supervisor: Dr Pamela Gumbi.

This thesis has been prepared according to **Format 1** as outlined in the information for guidance of examiners of higher degrees, which states:

Format 1: A single coherent book, with a single Introduction, Materials and Methods, Results, Discussion, Conclusion, and References.

PREFACE

The data presented in this thesis was collected at the School of Life Sciences at the University of KwaZulu-Natal Pietermaritzburg campus, South Africa. The experimental work was carried out while registered at the University of KwaZulu-Natal in the Pietermaritzburg campus, under the supervision of Dr. Pamela Gumbi.

This thesis submitted for the degree of Master of Science in the School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg campus, is the author's original work and has not been submitted in any form at any other university.

15 July 2024 

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As the candidate's supervisor, I certify that the above information is correct, and I have approved this thesis for submission.

15 July 2024 

Dr Pamela Gumbi

DECLARATION – PLAGIARISM

I, Busisiwe Nomnganga, declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs, or other information unless specifically acknowledged as being sourced from other persons.
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ABSTRACT

Background: *Lactobacillus* (*L.*) strains offer a promising approach for preventing and treating viral, bacterial, and fungal infections, serving as alternatives or complements to traditional antibacterial and antifungal therapies. Current treatments for bacterial vaginosis (BV) often entail side effects such as gastrointestinal disturbances, antibiotic resistance, and high recurrence rates, while antifungal treatments may lead to skin irritation and gut health issues, particularly in pregnant women. These challenges underscore the urgent need for alternative strategies to manage vaginal infections. This study investigated the probiotic characteristics of *Lactobacillus* species isolated from healthy African women and assessed their effects on pathogenic microbes, specifically *Gardnerella* (*G.*) vaginalis and *Candida* (*C.*) albicans, alongside their production of antimicrobial substances, including lactic acid and H_2O_2.

Methods: Thirteen strains of vaginal *Lactobacillus* species (*L. crispatus*, *L. gasseri*, *L. jensenii*, *L. mucosae*, and *L. vaginalis*) were pre-isolated from HIV-negative young women from South Africa. The cell morphology of the bacterial strains was confirmed with the Gram staining technique. The ability of *Lactobacillus* strains to acidify culture media was measured by monitoring the pH changes over time during incubation using a calibrated pH meter. The production of antimicrobial compounds, specifically D- and L-lactate and hydrogen peroxide (H_2O_2), were quantified using colorimetric assay kits. The inhibitory activity of the isolated *Lactobacillus* strains against *G. vaginalis* and *C. albicans* was assessed using standardised *in vitro* assays, which typically involved co-culturing the *Lactobacillus* culture supernatants (LCS) with the pathogens and measuring growth inhibition by optical density (OD600nm) over time. The study compared differences in inhibitory effects, lactic acid, H_2O_2 production, and pH reduction among the *Lactobacillus* species and strains. The Mann-Whitney non-parametric t-test was used to analyse the data for two groups, while the Kruskal-Wallis one-way ANOVA unpaired test was used with the unadjusted Dunn's multiple comparisons tests.

Results: The growth kinetics of the *Lactobacillus* isolates were comparable, with their optimum growth reached at 48 to 72 hours. The pH of MRS cultures decreased over time from 6 to 3.5 by all the vaginal isolates. The isolates produced relatively low but measurable H_2O_2, D- and L-lactic acid concentrations ranging from 253 to 440 µMol/L, 1.05 to 2.9 ng/µL, and 0.09 to 0.13 mM, respectively. *Lactobacillus crispatus* (70.7pa and 94.79pa) showed better production of metabolites, followed by *L. gasseri* and *L. jensenii* (95.1pa). *Lactobacillus gasseri* and *L. crispatus* demonstrated vigorous antibacterial activity against *G. vaginalis*, decreasing its growth to about 50%. *Candida albicans* grew to approximately 70% in the

presence of *L. crispatus* (70.7pa and 94.79pa) and *L. vaginalis* 88.5b, showing better inhibitory activity than the other tested *Lactobacillus* strains.

Conclusion: This study highlights the significant potential of African-derived vaginal *Lactobacillus* species as probiotics for preventing and treating vaginal infections, with *L. gasseri* and *L. crispatus* showing the most potent properties. These strains effectively inhibited pathogenic microbes and produced higher amounts of lactic acid and H_2O_2, essential for maintaining a healthy vaginal environment. The results indicate that probiotic efficacy is both species- and strain-dependent, underscoring the need for alternative treatments given the limitations of current therapies.

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LIST OF ABBREVIATIONS

~	Approximately
°C	Degrees Celsius
x g	Times gravity
µg	Microgram
µL	Microlitre
µM	Micromolar
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
B.	*Bacillus*
BHI	Brain heart infusion
BSL	Biosafety level
BV	Bacterial Vaginosis
C.	*Candida*
CA	California
CD	Clusters of differentiation
CFU	Colony forming units.
CO_2	Carbon dioxide
DH_2O	Distilled water
DMSO	Dimethyl sulfoxide
E.	*Escherichia*
G.	*Gardnerella*
GM	Gastrointestinal microbiome
HCl	Hydrochloric acid
h	Hour
H_2O_2	Hydrogen peroxide

HIV	Human immunodeficiency virus
HPV	Human papillomavirus
KOH	Potassium hydroxide
L.	*Lactobacillus*
LAB	Lactic acid bacteria
LCS	*Lactobacillus* culture supernatants
LPCB	Lactophenol cotton blue
Ltd	Limited company
M	Molar
MO	Missouri
min	Minute/s.
ml	Millilitre
mM	Millimolar
MRS	De Man, Rogosa and Sharpe
NaOH	Sodium hydroxide
NaCl	Sodium Chloride
nm	Nanometre
OD	Optical density
P.	*Prevotella*
PBS	Phosphate buffered saline.
PCR	Polymerase chain reaction
pH	Potential of Hydrogen
Rpm	Revolutions per minute
RT	Room temperature
S	Seconds
S.	*Staphylococcus*
SA	South Africa

SCFAs	Short-chain fatty acids
Sd	Standard deviation
SDA	Sabouraud Dextrose Agar
Spp.	Species
STIs	Sexually Transmitted infections
UK	United Kingdom
USA	United States of America
UTI	Urinary tract infection
VC	Vaginal candidiasis
v/v	Volume per volume
VK	Vaginal keratinocyte
VM	Vaginal microbiome
WHO	World Health Organization
w/v	Weight per volume
YEPD	Yeast extract peptone dextrose

Chapter 1

Literature Review

1.1 Introduction

The mechanisms by which the vaginal microbiome (VM) aids women in evading urogenital illnesses and maintaining health are the subject of ongoing investigation (Stapleton, 2016). There is an urgent need for improved management of bacterial and fungal infections among African women (Chersich and Rees, 2008). In healthy women of reproductive age, the VM is primarily composed of commensal bacteria, such as *Lactobacillus* (*L.*) species (spp.), which inhibit pathogen growth by producing antimicrobial compounds (Anderson *et al.*, 2014). A strong association exists between a highly diverse VM community and genital inflammation, which negatively impacts reproductive health and increases the risk of sexually transmitted infections (STIs) and Human Immunodeficiency Virus (HIV) threefold (Masson *et al.*, 2015; McClelland *et al.*, 2018). Notably, young South African women with a more diverse microenvironment face a fourfold greater likelihood of acquiring HIV (Gosmann *et al.*, 2017). This study identified that anaerobes, such as *Prevotella* (*P.*) *bivia*, were linked to HIV infection and inflammation, while *L. crispatus* was associated with reduced vaginal inflammation. Their research examined the relationship between pathogenic anaerobes and CD4 helper T cell counts in an animal model, revealing that *L. crispatus* decreased CD4 cell numbers, whereas pathogenic anaerobes increased them, suggesting that these anaerobes enhance the risk of HIV transmission by stimulating relevant HIV cells on the mucosal surfaces of the VM.

Pathogenic anaerobic bacteria have also been linked to VM alterations in the sub-Saharan African region (Bayigga *et al.*, 2019). These authors connected anaerobic species to vaginal inflammation and the transmission of HIV. A less diverse VM, dominated by healthy *Lactobacillus* bacteria, is found to clear infections such as human papillomavirus (HPV) more effectively (Brotman *et al.*, 2014). Furthermore, in bacterial communities with a low abundance of *Lactobacillus* spp., the prevalence of *Gardnerella* (*G.*) or *Ureaplasma* spp., which are associated with increased pregnancy complications (DiGiulio *et al.*, 2015). These findings indicate that *Lactobacillus* species are essential for eliminating harmful microorganisms, preventing infection, and regulating inflammation in the genital tract. The depletion of *Lactobacillus* spp. can lead to an altered VM, a condition known as vaginal dysbiosis. Vaginal dysbiosis occurs when *Lactobacillus* numbers are diminished and are gradually or entirely replaced by anaerobic bacteria, such as *G. vaginalis*, *P. bivia*, and *Chlamydia trachomatis* and

fungi such as *C. albicans* (Lagenaur *et al.*, 2021). An increase in a diverse array of unhealthy anaerobes correlates with a rise in vaginal pH, a condition referred to as bacterial vaginosis (BV) (McLaughlin *et al.*, 2018).

The primary aim of this research is to characterise *L. crispatus*, *L. gasseri*, *L. jensenii*, *L. mucosae*, and *L. vaginalis* strains isolated from young HIV-negative South African women and to investigate their probiotic potential for the prevention of bacterial and fungal vaginal infections.

1.2 Definition of a Healthy Reproductive Tract Microbiome and its impact on women's health.

The VM is a compartment within the human microbiome, consisting of microorganisms that colonise the vagina (McKinnon *et al.*, 2019). These microorganisms are vital for women's overall health (Anderson *et al.*, 2014). There exists a well-balanced mutualistic relationship between the female genital tract and the bacterial species residing there (Peric *et al.*, 2019). In this relationship, the human vagina supports microbial communities by supplying essential nutrients necessary for bacterial growth (Van De Wijgert *et al.*, 2000). This support is crucial, as bacteria are continually shed from the body through vaginal secretions, necessitating their growth to replenish lost populations (Hillier *et al.*, 1992). Skin-peeled cells contribute some essential nutrients, while hormonal secretions provide others (Klebanoff *et al.*, 1991; Sobel, 1999). Conversely, indigenous bacterial populations play a protective role, preventing potentially infectious microorganisms from colonising the host. These include pathogens such as *Escherichia* (*E.*) coli, yeast infections such as vaginal candidiasis (VC), sexually transmitted infections (STIs) like *Chlamydia trachomatis*, and urinary tract infections (UTIs) (Sobel, 1999).

1.2.1 Abundance of *Lactobacilli* bacteria

A healthy VM is predominantly composed of *Lactobacilli* bacteria (Figure 1.1A), including species such as *L. crispatus*, *L. gasseri*, *L. mucosae*, *L. reuteri*, *L. iners*, *L. jensenii*, and *L. vaginalis*. In contrast, an unhealthy VM features a diverse array of anaerobic bacteria alongside the healthy *Lactobacilli* spp. (Figure 1.1B) (Nunn and Forney, 2016). *Lactobacillus* species are Gram-positive anaerobic bacteria (Cousin *et al.*, 2013) that adapt well to varying environmental conditions and are found to not produce the catalase enzyme (catalase-negative), indicating that they do not use oxygen as a terminal electron acceptor during respiration (Antonio *et al.*, 1999). Additionally, they are non-spore-forming and tend to thrive

better in low-oxygen environments than in those with atmospheric oxygen levels (Amanatidou *et al.*, 2001). Their Gram stain morphology is characterised by small, fat, long, narrow, and chain-like rods. *Lactobacilli* can be cultivated in media such as Man, Rogosa, and Sharpe (MRS), resulting in colonies that appear white and milky (Goldstein *et al.*, 2015).

1.2.2 An acidic Vaginal pH

Lactobacilli species lower vaginal pH through the production of lactic acid (Daniel Johannes Rönnqvist *et al.*, 2006; Vaneechoutte, 2017; Zhou *et al.*, 2007). Research has shown that lactic acid significantly adjusts vaginal pH to a balanced acidic environment, although this effect diminishes at lower lactate levels (Tachedjian *et al.*, 2017). Lactic acid acts as a potent virucidal and bactericidal agent with broad-spectrum activity (O'Hanlon *et al.*, 2013). It increases apoptosis in vaginal keratinocyte (VK) cells, facilitating the degradation of viruses and other infections, such as HIV. Moreover, lactic acid reduces inflammatory responses and inhibits histone deacetylases (HDAC), enhancing DNA repair and gene transcription processes (Witkin and Linhares, 2017). Consequently, therapeutic efforts are underway to enhance vaginal health using oral and vaginal probiotic products derived from *Lactobacillus* spp. For example, the antibacterial effect of *L. paracasei* cell-free culture supernatant against *G. vaginalis* has been documented (Moon *et al.*, 2022), and it was found that *L. paracasei* culture supernatants at neutral pH exhibited antibacterial activity against *G. vaginalis*. These findings suggest that antibacterial molecules beyond lactic acid may contribute to the antibacterial effects of *Lactobacillus* cell-free culture supernatants at neutral pH

1.2.3 Inhibition of pathogen invasion and production of antimicrobials

Lactobacilli bacteria are renowned for their ability to inhibit pathogen growth, effectively maintaining an optimal environment within the vagina (Wessels *et al.*, 2017). They produce antimicrobial substances that restrict pathogen proliferation in the female reproductive ecosystem (Wiesenfeld *et al.*, 2003). These *Lactobacilli* bind to uterine epithelial cells as competitors, thereby preventing colonisation by harmful bacteria, viruses, or fungi (Figure 1.1A). Additionally, they generate antimicrobial compounds such as bacteriocins, hydrogen peroxide (H_2O_2), short-chain fatty acids (SCFAs), and lactic acid (Figure 1.1A; Table 1.1). These substances collectively reduce the risk of infection and mitigate inflammation that may arise from shifts in the vaginal bacterial balance or existing infections (Witkin and Linhares, 2017). For instance, in a study focused on bacteriocin purification from *Bacillus* (*B.*) subtilis, *G. vaginalis* was inhibited by a bacteriocin produced by *L. acidophilus* (Lee and Chang, 2018).

Furthermore, intravaginal treatment with *L. crispatus* in patients with BV led to the restoration of a healthy VM (Ravel *et al.*, 2011).

The H_2O_2 produced by *Lactobacilli* inhibits the growth of viruses and fungi, as well as harmful bacteria like *Neisseria* (*N.*) *gonorrhoeae* (Klebanoff *et al.*, 1991). Certain strains of *L. reuteri* have demonstrated antibacterial action against methicillin-resistant *Staphylococcus* (*S.*) aureus, attributed to their H_2O_2 production (Voravuthikunchai *et al.*, 2006). Notably, some *Lactobacillus* species, such as *L. iners*, do not produce H_2O_2, a characteristic previously used to differentiate favourable from unfavourable vaginal *Lactobacillus* isolates (Antonio *et al.*, 1999). H_2O_2 production has been shown to inhibit the growth of *G. vaginalis in vitro* (Klebanoff *et al.*, 1991; Pascual *et al.*, 2006) and to eliminate endogenous harmful bacteria, thereby maintaining a healthy VM (Aroutcheva *et al.*, 2001). An investigation in Argentina examined *Lactobacillus* species obtained from the vaginal secretions of reproductive-age women, revealing that out of 100 tested strains, 62-including *L. acidophilus*, *L. casei*, *L. brevis*, *L. fermentum*, *L. gasseri*, and *L. jensenii*-produced H_2O_2, while 38 did not (Pascual *et al.*, 2006).

Bacteriocin production by vaginal *Lactobacillus* species has also been documented (Sobel, 1999). Research has explored the potential of using vaginal *Lactobacillus*-derived bacteriocins to control bacterial overgrowth in women. Specifically, *L. paragasseri* was found to produce a substantial amount of bacteriocins effective against *L. iners*, a species found in the VM alongside bacteria associated with BV. While *L. iners* is not thought to protect against BV, further studies are investigating its potential as a treatment for BV and related conditions (Nilsen *et al.*, 2020). Bacteriocins are proteinaceous antibacterial substances that exert a bactericidal effect against anaerobic pathogens and other *Lactobacillus* species associated with BV, such as *L. iners* (Cintas *et al.*, 2001). As peptides, these substances contain fewer than 60 amino acids and suppress bacterial growth under specific conditions (Zacharof and Lovitt, 2012). Additionally, *Lactobacilli* defend host cells from infections by competing for nutrients and inhibiting pathogen adherence to vaginal epithelial cells (Reid and Burton, 2002; Vesterlund *et al.*, 2006).

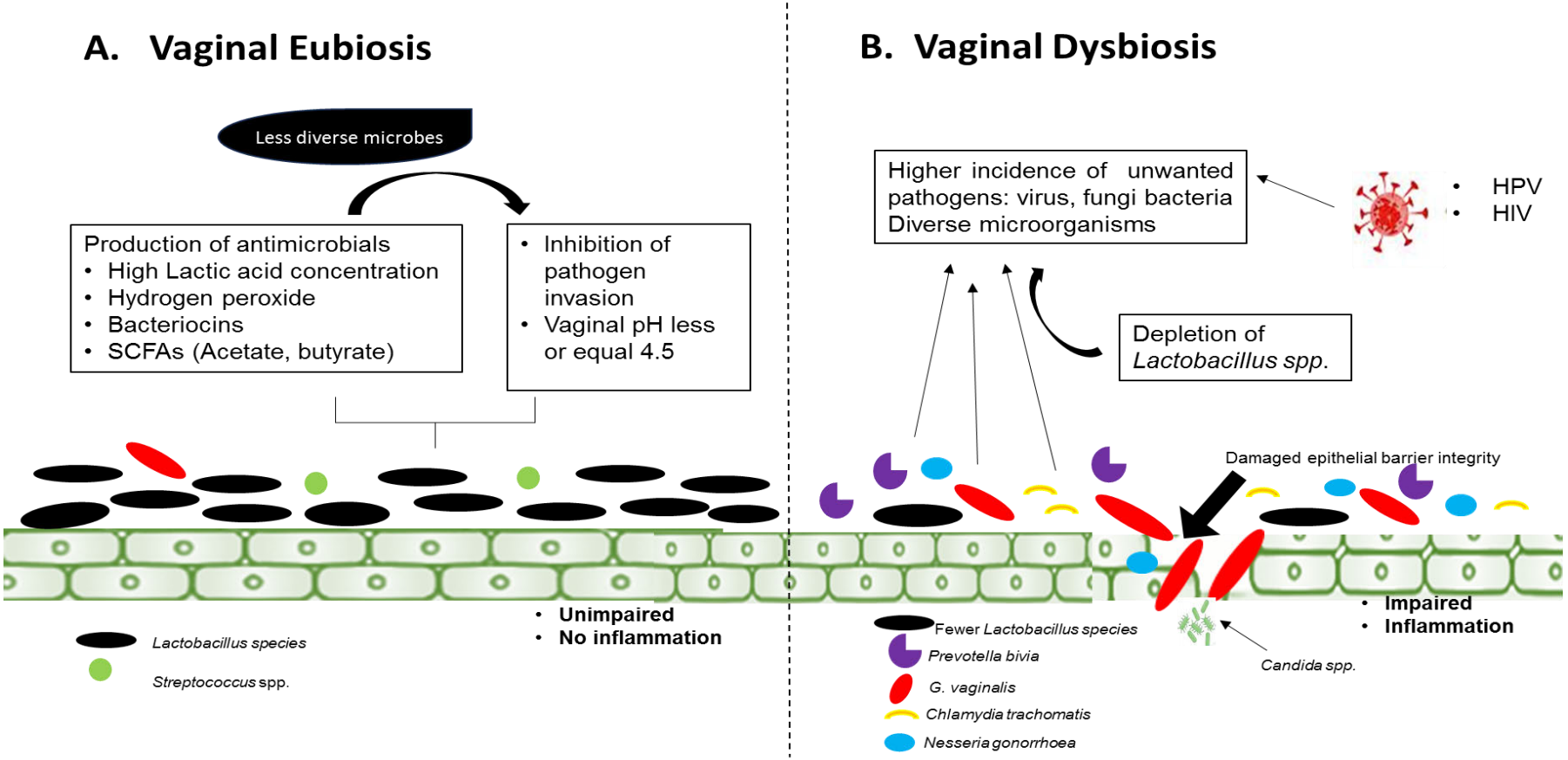

Figure 1.1: Differences between Eubiosis and Dysbiosis vaginal state. The predominant bacteria in the vaginal microbiome during a normal vaginal environment (A) are *Lactobacillus*. They produce lactic acid, which acidifies the vaginal microenvironment and effectively inactivate undesired vaginal infections. Hydrogen peroxide also contributes to the generation of a non-inflammatory environment. The vaginal environment has an altered vaginal microbiome (B), also known as vaginal dysbiosis, which is favourable to the growth of pathogens. The various anaerobic bacteria produce factors that contribute to virulence that break down mucosae, impair the effectiveness of the epithelial barrier, causing competition between microorganisms for nutrition and adhesion to the vaginal epithelium, and thus result in genital inflammation.

Gram-positive bacteriocins are classified into four groups (Class I, II, III, and IV) based on amino acid sequences, molecular mass, post-translational modifications, and genetic characteristics. Class I bacteriocins are linear or globular proteins comprising fewer than 28 amino acids, including lanthionine, and are heat-stable, maintaining their activity under elevated temperatures (Mokoena, 2017). Class II bacteriocins are unmodified, containing 30–60 amino acids; they are positively charged and heat-stable due to the absence of non-lanthionine (Cotter *et al.*, 2013; Ditu *et al.*, 2014). Class III bacteriocins are larger, water-soluble, and inactivated within 30 minutes at temperatures above 100°C, with *Lactobacilli* typically producing Class III bacteriocins like Lactacin B, J, and V helveticins. Class IV bacteriocins are more complex, incorporating lipid and carbohydrate components into their structure, contributing to their antibacterial effects (Ghodhbane *et al.*, 2015).

Probiotic strains that produce bacteriocins have been explored in previous studies as potential treatments for microbe-associated illnesses and cancer (Nishie *et al.*, 2012; Yang *et al.*, 2014). Bacteriocins can prevent infections caused by pathogenic Gram-positive bacteria such as *Staphylococcus*, *Streptococcus*, and *Micrococcus* (Nigam *et al.,* 2014). Additionally, many Gram-negative bacteria, including *E. coli*, *Salmonella*, *Shigella*, *Listeria monocytogenes*, and *Vibrio*, have been used as test organisms to evaluate the inhibitory effects of newly identified antimicrobial peptides. Although bacteriocins have been extracted from various strains, including *L. plantarum*, *L. bulgaricus*, *L. acidophilus*, and *L. lactis*, studies showing significant antibacterial effects against multiple pathogens remain limited. Nonetheless, researchers are investigating emerging strains from diverse food sources to address the prevalence of food-borne diseases (Yang *et al.*, 2014).

Short-chain fatty acids (SCFAs), including acetic, propionic, and butyric acids, can be produced by lactic acid bacteria (LAB). These SCFAs impact the intestinal mucosae and can be absorbed into the body, influencing the circulatory, respiratory, and urinary systems (Pessione *et al.*, 2015). While SCFAs have primarily been associated with gut dysbiosis, their role in relation to vaginal *Lactobacillus* species remains under investigation. Studies have suggested that when lactic acid is present at normal levels within an acidic VM environment dominated by *Lactobacillus*, it induces protective activity in VK2 cells, thus inhibiting inflammation. However, treatment of VK2 cells with a VM characterised by BV-associated lactic acid and SCFAs, particularly at lower lactic acid levels and higher SCFA concentrations at neutral pH, showed no beneficial activity. Instead, prolonged exposure disrupted the immune response in VK2 cells due to the lactic acid and SCFAs associated with BV (Delgado-

Diaz *et al.*, 2020). Interestingly, a study on vaginal candidiasis (VC) noted that the antifungal properties of SCFAs, such as propionate, butyrate, and acetate, depend on their concentration in the VM. Thus, it can be argued that the acidic pH levels of the VM dictate the antibacterial and antifungal actions of SCFAs, with higher pH levels resulting in reduced SCFA efficacy (Baldewijns *et al.*, 2021). Moreover, SCFAs linked to BV demonstrated diminished activity against bacteria and may contribute to inflammation-related alterations in the VM (Aldunate *et al.*, 2015).

Table 1.1: Common metabolites produced by *Lactobacillus* species and their mechanisms of action on vaginal microbial infections.

Metabolites	Mechanism of action & examples	References
Bacteriocins	• Break down the cell wall of the pathogen and creates pores in their cytoplasmic membrane. • Examples: Class 1 Plantaricin	(Pérez-Ramos *et al.*, 2021)
Organic acids	• Breakdown glycogen to create an acidic vaginal environment. • Prevent the fermentation of glucose which in turn prevents glycolysis and diminish energy in the form of ATP thus limiting the growth of Candida and other infections. • Examples: Lactic acid, sorbic, acetic, benzoic	(Krebs *et al.*, 1983; Valenti *et al.*, 2018)
SCFAs	• Alter the internal ultrastructure of the pathogen's cell membrane by interfering with the size and form of its cell wall. As a result, the pathogen's cell membranes become less cohesive and retains more fluid, which increases permeability and spontaneous release of chemicals. • Reduce the synthesis of cytokines that promote inflammation and promotes T lymphocytes in the vagina by inhibiting HDAC. • Examples: 3-Hydroxy Fatty Acids, Acetate, Butyrate	(Sjögren *et al.*, 2003) (Amabebe and Anumba, 2020)
Biosurfactants	• Disrupt cell membrane structure, thus increasing permeability. • Use anti-adhesive properties to cause pathogenic cells that are already adhering to the epithelium to separate.	(Gomaa, 2013)
H_2O_2	• Functions as an oxidative antimicrobial agent, producing reactive oxygen molecules that cause oxidative damage to proteins, DNA, and lipids found in cells membranes of the invaders. • Anti- inflammatory properties • Signalling activities.	(Miko and Barakonyi, 2023)

1.3 Factors affecting a Healthy Vaginal Microbiome and their contribution to an Altered Vaginal Microbiome (dysbiosis).

A healthy VM can be disrupted by various factors, leading to an altered VM, known as dysbiosis (Floch *et al.*, 2016). Hormonal changes, sexual activities, vaginal practices, use of antibiotics, social and genetic factors, and the dominance of competing species within the VM are some of the many factors leading to vaginal dysbiosis (Lagenaur *et al.*, 2021). The following sections provide explanations of the most common factors.

1.3.1 Hormonal changes

Specific periods of life, such as puberty, menopause, pregnancy, menstruation, and the use of contraceptives, can lead to dramatic hormonal changes (Hickey *et al.*, 2015). The VM remains relatively pure until puberty, when *Lactobacilli* begin to populate due to hormonal shifts (Nishida *et al.*, 2021). Premenopausal and postmenopausal women have different microbial species in their vaginal mucosae. These stages are related to the decline in oestrogen levels that occurs with menopause, leading to low *Lactobacilli* numbers in postmenopausal women. In premenopausal women, increased oestrogen levels promote *Lactobacilli* adhesion to the vaginal epithelium, resulting in improved microbiome composition stability (Chan *et al.*, 1984; Mirmonsef *et al.*, 2015). The vaginal lining becomes thinner and less elastic as oestrogen and progesterone levels decrease following menopause (Taylor-Robinson *et al.*, 2002). Changes in vaginal pH, cellular glucose metabolism, and VM occur due to these hormonal changes, influencing vulvovaginal symptoms, including vaginal infections (Kim *et al.*, 2015).

During pregnancy, high oestrogen levels tend to make pathogens less common in the VM (Amabebe and Anumba, 2018; Hur *et al.*, 2021). These hormone levels promote the retention of glycogen in vaginal epithelial cells, supporting *Lactobacilli* species colonisation and preserving VM balance (Nunn and Forney, 2016). However, during menstrual periods, concentrations of *G. vaginalis* and *L. iners* increase while those of *L. crispatus* and *L. jensenii* drop (Dabee *et al.*, 2021). This can be explained by the presence of iron, a critical growth component for *G. vaginalis*. Menstrual blood flow may also prevent *Lactobacillus* species from adhering, giving BV-associated microbiota an advantage (Lopes dos Santos Santiago *et al.*, 2012). The use of oestrogen-containing contraceptive vaginal rings has been shown to reduce BV-associated *G. vaginalis* and *Atopobium vaginae* while promoting *Lactobacilli* growth (Crucitti *et al.*, 2018). Women who use condoms are more likely to have a normal Nugent

score (0-3) than those who use oral contraceptives, which is relatively in line with a vaginal microbiome dominated by *Lactobacillus* (Ma *et al.*, 2013).

1.3.2 Sexual behaviours

Sexual behaviours, including unprotected or protected intercourse, the use of lubricants, and male circumcision, are linked to changes in the vaginal microbiota and an increased risk of BV (Cherpes *et al.*, 2008). Women who have multiple partners or initiate sexual activity at a young age are more likely to develop BV than those with fewer partners or later sexual initiation. Sexual activities between a male and female can affect the healthy VM because semen, with a basic pH, introduced to the acidic vaginal environment increases the overall vaginal pH. A vaginal pH above 4.5 creates an ideal environment for harmful bacteria or fungi to grow and cause infections (Witkin, 2015). There is also a strong connection between vaginal dysbiosis and female sexual partners, as women who have sex with other women are at risk of altering the VM (Koumans *et al.*, 2007; Vodstrcil *et al.*, 2017). Sharing sex gadgets has been linked to a 90% BV acquisition rate among female partners (Marrazzo *et al.*, 2002).

1.3.3 Vaginal practices

The use of hygiene products, such as soap, to keep the genital tract clean can affect the overall composition of microbes in the vagina, and studies show a link to increased BV risk (Brotman *et al.*, 2008). Women commonly use these hygiene products for their daily vaginal cleaning routine; however, these may alter the VM (Hilber, Hull*, et al.*, 2010). Vaginal douching, the process of rinsing or cleansing the vaginal canal with water or other fluids, is also associated with BV (Brotman *et al.*, 2008). Vaginal washing methods differ significantly by region. For instance, women in America tend to wash the inside of their vagina once or twice a month with commercially available products, while African women usually wash more often with water, detergents, or home remedies using a towel or fingers daily (Hilber, Francis*, et al.*, 2010).

1.3.4 The use of antibiotics

The Antibacterial medications used to destroy bacteria or slow microorganism growth can disrupt the delicate balance of yeast and bacteria in the vaginal canal. Antibiotics disrupt this balance, allowing Candida fungi to grow, resulting in symptoms such as itching, burning, and pain (Schwebke *et al.*, 1999). This disruption in VM diversity results in vaginal dysbiosis and a disease-prone microbiota as it impacts the permeability of the cervical epithelial barrier (Ferrer *et al.*, 2017). However, some antibiotics are used to treat BV and STIs, applied as

creams or administered orally (Balkus *et al.*, 2016). Such medications include penicillin, amoxicillin, metronidazole, and clindamycin (Vicariotto *et al.*, 2014). Studies show that antibiotics can eradicate beneficial microbes, possibly leading to microbiome dysbiosis (Keeney *et al.*, 2014). Additionally, BV treatment provides a temporary cure, with approximately 50% of women relapsing within six months (Vicariotto *et al.*, 2014). Antibiotics are common causes of erosion of normal beneficial bacteria, resulting in vaginal inflammation (Schwebke *et al.*, 1999; Workowski and Berman, 2010).

1.3.5 Genetic factors

Genetics can significantly affect VM composition, as people from different regions and ethnicities experience different health issues (Nam *et al.*, 2011).Genetic differences between hosts of different racial backgrounds can influence the species makeup of vaginal communities. A study evaluating genetic components associated with the unique VM makeup of women from Kenya confirmed that immune mechanisms and vaginal cell communication contribute to the type of microorganisms that occupy their vaginal flora (Mehta *et al.*, 2020). Differences in the innate and adaptive immune systems, the content and quantity of vaginal secretions, and molecules on epithelial cell surfaces, among other factors, can all play a role (Zhou *et al.*, 2010). Microbiome diversity among different ethnicities is also associated with varying susceptibilities to pre-term birth, diabetes mellitus, obesity, or cancer. For example, people from the Netherlands consume more milk and use fewer antibiotics compared to other Europeans (Kwint *et al.*, 2012). To understand VM-based diseases, variations in populations' bacterial profiles must be considered (Zhernakova *et al.*, 2016) . Many studies have shown that the gastrointestinal microbiome (GM) composition differs by region or ethnicity. Firmicutes, Actinobacteria, and Bacteroidetes are found in greater abundance in the GM of the American population, Japanese, Korean, and Chinese communities than in developing areas (Nam *et al.*, 2011).

1.3.6 Dominance of non- *Lactobacillus* species

A healthy genital microbiome comprises primarily *Lactobacilli* bacteria. However, other non-*Lactobacillus* species, like fungi, play a minor role in the human microbiome, and no one is entirely free of them. The overgrowth of bacteria in the VM caused by immune system impairment contributes to the entry of opportunistic pathogens into the VM (Chee *et al.*, 2020). BV has been associated with fertility complications, inflammation of the pelvic and uterine lining (Ravel *et al.*, 2021). Metagenomics studies show that 0.1% of fungal species are found in the human abdomen (Arumugam *et al.*, 2011). Fungi may help maintain the microbial

community structure as they produce distinct metabolites and enzymes. A small group of fungal species, such as *Candida spp.*, *Malassezia spp.*, *Cryptococcus neoformans*, and *Pneumocystis jirovecii*, are asymptomatic invaders but can become pathogenic when the host is impaired or altered (Huffnagle and Noverr, 2013). *Candida* species are the most common cause of nosocomial systemic hospital-acquired infections in the US (da Silva Dantas *et al.*, 2016). In humans, *C. albicans* is the most significant cause of vulvovaginal candidiasis (VC) and is found in the mouth, intestinal, and vaginal microbiota (Sobel, 2016). Other human-associated non-albicans *Candida* species include *C. krusei*, *C. glabrata*, *C. tropicalis*, and *C. lusitaniae* (Silva *et al.*, 2012). The dominance of other competing species, such as *Chlamydia trachomatis*, *G. vaginalis*, and *P. bivia*, also contributes to vaginal dysbiosis (Figure 1.1). The BV- associated bacteria can break down the fluids secreted by women, weakening the tissues around the cervix and releasing toxic compounds that contain organic nitrogen, such as amines. This increases the likelihood of recurring HPV infections (Briselden *et al.*, 1992; Ng *et al.*, 2021). A VM with highly pathogenic BV-related anaerobes is prone to diseases like chlamydia, HIV, and candida because bacterial overgrowth in the vagina creates an unfavourable environment for *Lactobacilli* species. Consequently, they cannot perform their protective role if they must coexist with other microbial predators, encouraging the emergence of infection-causing species such as *Candida spp.* in the VM (Fatma *et al.*, 2017). Social factors like stress (Witkin and Linhares, 2017), smoking (Brotman *et al.*, 2013), and diet (Miller *et al.*, 2016) also play a role in the dominance of non-*Lactobacillus* species. Disturbance in a healthy VM by these factors can lead to an altered VM characterised by inflammation and a high risk of infections such as trichomoniasis, vaginitis, candidiasis, chlamydia, and BV (Filardo *et al.*, 2019).

An imbalance in the vaginal flora causes vaginal inflammation, which results in discharge, pain, and itching in the vaginal region. This condition is known as vaginitis (Leclair and Stenson, 2022). Changes in hormone levels during the postmenopausal stage have been linked to several skin illnesses, including vaginitis. According to Medscape Drugs and Diseases in collaboration with Hetal B. Gor (2024), most women with vaginitis (~72%) may not be diagnosed because they are asymptomatic. Among women who experience symptoms, BV accounts for approximately 40% of cases, VC for ≤24% (Yasin *et al.*, 2021), and trichomoniasis for <20%. The treatment plan for vaginitis is determined by the patient's age and the probability of infection. It is essential to distinguish between vaginitis and other possible sources of symptoms, as obtaining a correct diagnosis can be challenging. Cervical swabs and extensive pelvic assessments may be required to confirm the pathogen type. Two

factors of concern in this study are BV and Candida, influenced by the epidemiology of vaginitis.

1.4 Bacterial vaginosis

Bacterial vaginosis (BV) occurs when the normal balance of microorganisms in the vagina is disrupted. During BV, there is a shift from a *Lactobacillus*-dominated VM to one with high concentrations of aerobic and anaerobic pathogens (Greenbaum *et al.*, 2019). Historically, BV was referred to as Gardnerella vaginitis because it was believed that *G. vaginalis* was the primary cause. The more recent term, "bacterial vaginosis," acknowledges that a variety of different bacteria, both anaerobic and aerobic, naturally present in the vagina can proliferate excessively and cause the condition (Greenbaum *et al.*, 2019).

Women diagnosed with BV are more susceptible to contracting other STIs, and pregnant women with BV are at increased risk of preterm birth (Russo *et al.*, 2019). The Centre for Disease Control and Prevention (CDC) reported that BV is most common in early adolescence and in adult females in their mid-forties. BV is not classified as an STI. Instead, it appears to result from an imbalance in the vaginal bacteria population, often due to bacteria-bacteria transmission during sexual activity (Coughlin and Secor, 2010). Supporting this, individuals who have never engaged in sexual activity rarely contract BV (Verstraelen *et al.*, 2010). Activities like regular douching, acquiring a new sexual partner, or engaging in unprotected sexual activity can increase the risk of BV. A study indicated that *G. vaginalis* can be transmitted between sexual partners through close mucosal contact or shared sexual objects (Javed *et al.*, 2019). Moreover, unmarried women with multiple sexual partners, those who initiated sexual activity at a young age, and female sex workers are more prone to the infection. About 25-30% of women affected by BV are of reproductive age (Ellington and Saccomano, 2020; Kamga *et al.*, 2019; Koumans *et al.*, 2007; Peebles *et al.*, 2019), with many experiencing recurrent infections (Ellington and Saccomano, 2020). Globally, BV is generally rare in Asia and Europe but most prevalent in several African regions due to genetic and social factors (Javed *et al.*, 2019). Factors such as contraceptive use, smoking, and previous antibiotic use are also associated with BV. In the US, approximately 30% of women in their late forties and teenagers exhibit symptoms of BV, though prevalence varies among ethnic groups, with higher rates observed in African women (51%) compared to non-white American women from Mexico (27%) (Eastment and McClelland, 2018; Javed *et al.*, 2019). According to the World Health Organization (WHO, 2023), the global incidence of BV among women of reproductive

age is estimated to be between 23% and 29%, though this varies between countries and groups. A comprehensive study in 2019 reported significant prevalence rates: South Asia (29%), North America (27%), sub-Saharan Africa (25%), South America and Africa (24%), and the lowest prevalence in Central Asia and Europe (23%) (Figure 1.2) (Peebles *et al.*, 2019).

Gardnerella vaginalis is assumed to be the primary cause of most BV infections, yet the exact cause is still not well understood. *G. vaginalis* forms a biofilm that allows other opportunistic pathogens, such as *C. albicans*, *Prevotella (P.) bivia*, and *Chlamydia trachomatis*, to proliferate in the vagina (Verstraelen and Swidsinski, 2019). Symptoms of BV may not always be present, but in some cases, patients experience increased vaginal pH levels, abnormal vaginal secretions such as watery discharge, painful urination, pelvic pain, and genital inflammation. Other symptoms can include a foul odour and vaginal itchiness. However, many affected women may not exhibit any symptoms. The relationship between BV and a higher risk of developing STIs is linked to the substitution of protective lactic acid-producing bacteria with anaerobic bacteria, allowing additional pathogenic vaginal bacteria to proliferate (Greenbaum *et al.*, 2019). BV also triggers the release of endotoxins that promote inflammatory cytokines and prostaglandin synthesis, and the presence of enzymes such as dioxygenases that inhibit the ability of host leukocytes to prevent infection (Greenbaum *et al.*, 2019).

Identifying BV involves examining vaginal fluid or cervical specimens microscopically. Epithelial cells from the cervix attached to rod-shaped bacteria indicate BV (Secor and Coughlin, 2013). Alternatively, the purity and Gram status of the bacterial strain in the vaginal fluid can be confirmed through Gram staining. Despite having over 80% sensitivity and specificity, this approach is rarely employed in clinical research (Coleman and Gaydos, 2018). A thorough pelvic examination is necessary to analyse the vaginal discharge and avoid confusion with other infections like gonorrhoea, pelvic inflammation, candidiasis, chlamydia, and herpes (Coughlin and Secor, 2010). Usually, a clinical diagnosis of BV is followed by a confirmation test. A drop of sodium chloride (NaCl) solution is added to a slide containing the specimen, and the specimen is examined microscopically for the presence of BV indicator cells. To test for odour, a small amount of potassium hydroxide (KOH) is added to the vaginal discharge on a slide. The test is considered positive if a distinctive fishy odour is detected. Since BV is associated with dysbiosis, the pH of the VM might be disturbed. Therefore, it is essential to measure the pH of the discharge using a pH strip. The results can be compared to colour controls to determine the acidity or basicity of the discharge. The swab may show an

abnormally high vaginal pH (greater than 4.5) and a positive odour test (Verstraelen and Swidsinski, 2019), The presence of a fishy vaginal discharge odour, unusual vaginal discharge colour, higher or lower than usual vaginal pH (greater than 4.5), and indicator cells are typically sufficient to confirm the diagnosis of BV (Coleman and Gaydos, 2018; Verstraelen and Swidsinski, 2019).

About 76% of BV cases require medical intervention (Munoz-Barreno *et al.*, 2021). When medical or clinical help is needed, prescription antibacterial creams or gels, such as metronidazole and clindamycin, may be used as treatment. These medications work well whether ingested or administered vaginally and can be used safely by pregnant women (Javed *et al.*, 2019). However, according to research by Verstraelen and Swidsinksi (2019), pregnant women with BV symptoms should receive clindamycin treatment before 22 weeks of pregnancy to reduce the likelihood of preterm birth. Recurrence of BV is problematic, with rates typically high within three to twelve months, often necessitating further therapy (Bradshaw *et al.*, 2006; Ya *et al.*, 2010). About a quarter of cases may require additional treatment if symptoms persist after the first round of antibiotics (Hay, 2000). A second course of antibiotics is often prescribed if persistent symptoms are present (Faught and Reyes, 2019).

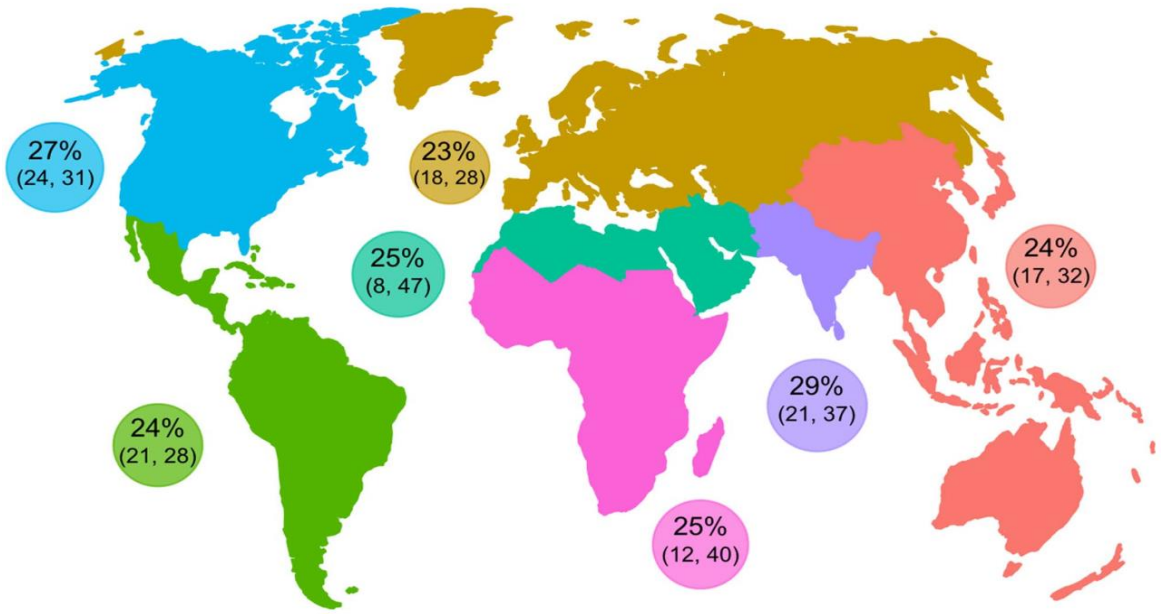

Figure 1.2: The Worldwide Epidemiology of Bacterial vaginosis amongst reproductive- age women. The distribution of BV in each region was shown as: North America (Blue); South Asia (Purple); East Asia (Light pink), America (strong green), Africa (Strong pink), and Europe (Brown). Adapted from: (Peebles *et al.*, 2019)

1.5 Consequences of a Bacterial vaginosis-altered vaginal microbiome

Probiotic use as a treatment or preventative measure for BV is supported by some, but recent studies suggest that there is insufficient evidence to confirm its effectiveness (Park *et al.*, 2023; Verstraelen and Swidsinski, 2019). If a patient with BV is not treated, the risks of contracting STIs, particularly HIV, and experiencing complicated pregnancies increase significantly (Leitich and Kiss, 2007; Redelinghuys *et al.*, 2020). Research indicates that women living with HIV and have been diagnosed with BV are more likely to transmit HIV to their sexual partners compared to BV-negative women (Jain *et al.*, 2018). Additionally, BV has been linked to HIV shedding (Fastring *et al.*, 2014). BV also increases the risk of infection with herpes simplex viruses and HPV. Recent research suggests that BV can trigger the recurrence of HPV, indicating that treating BV in asymptomatic patients may be necessary to prevent HPV recurrence (Bayigga *et al.*, 2019; Eastment and McClelland, 2018).

Long-term reproductive outcomes associated with BV include infertility. Studies have shown that infertile women have a significantly higher occurrence of BV compared to fertile women (Salah *et al.*, 2013; Van Oostrum *et al.*, 2013). Women with BV undergoing in vitro fertilisation experience higher levels of premature pregnancy loss and lower rates of embryo implantation (ORTIZ *et al.*, 2022). Research has also demonstrated that BV increases the risk of endometrial inflammation, amniotic rupture, and chorioamnionitis both during and after pregnancy (Han *et al.*, 2019). The most common consequences of BV are explained below.

1.5.1 Increase in vaginal pH.

A vaginal pH of more than 4.5 creates an ideal environment for harmful bacteria to grow and cause infections (Hemalatha *et al.*, 2013). The pH of a woman's vaginal fluid can alter over time due to various factors, such as drug consumption and unhealthy eating habits. *Lactobacilli* reside in the VM and secrete lactic acid and H_2O_2, maintaining an acidic vaginal pH (Boris and Barbés, 2000). Several diseases and infections can disrupt the pH balance in the vaginal area, primarily by increasing pH values (Witkin, 2015; Witkin and Linhares, 2017). BV primarily changes the pH of the vagina by decreasing lactic acid production. As more pathogenic fungi and anaerobes are introduced to the vaginal flora, they compete for nutrients and deplete beneficial bacteria within the VM, harming the reproduction process and leading to early labour and birth complications (Garg *et al.*, 2010; Garg *et al.*, 2009).

1.5.2 Genital inflammation

Genital inflammation is an irritation of the genitals driven by abnormalities in the normal balance of the VM, which can occur due to various factors, including BV (Onderdonk *et al.*, 2016). Sexually transmitted infections and BV promote inflammation in the genital area, increasing the risk of HIV (Onderdonk *et al.*, 2016). Compared to other bacterial communities, a cervicovaginal microbiota with many *Lactobacilli* species, such as *L. crispatus*, is linked to a lack of genital inflammation. Compared to women whose microbiome is dominated by *L. iners*, those whose microbiome is dominated by *L. crispatus* had a lower risk of contracting HIV (Nunn *et al.*, 2015). Human Immunodeficiency Virus acquisition was also linked to higher levels of pathogenic bacteria and a wide distribution of strict and facultative anaerobic microorganisms (Gosmann *et al.*, 2017). Another reason for lower HIV susceptibility in women with *Lactobacilli*-dominant microbiota is that *Lactobacilli* and lactic acid inhibit inflammatory reactions (Hearps *et al.*, 2017). Inflammatory cytokines and growth factors like interleukin (IL-1, IL-2, IL-6, IL-8), interferon (IFN), and tumour necrosis factor (TNF) affect innate immunity and may compromise mucosal barrier integrity (Bamias *et al.*, 2014). Inflammation can also lead to the recruitment of HIV target cells, such as CD4+CCR5+ T cells (Platz-Christensen *et al.*, 1993). The greater risk of HIV infection in African women is mainly due to a vaginal inflammatory profile (Passmore *et al.*, 2016). In the global South and other underdeveloped countries, STIs and BV are inadequately controlled, remaining significant causes of vaginal irritation and HIV infection risk among women (Francis *et al.*, 2018).

Regardless of the causative agent, inflammation of the female genital tract generates an environment that encourages HIV replication and infection growth. Women with high inflammatory cytokine levels in their genital tracts, such as macrophage inflammatory protein 1 (MIP-1) and interferon-inducible protein 10 (IP-10), are more likely to acquire HIV (Masson *et al.*, 2015). A study showing a correlation between increasing genital inflammatory cytokine concentrations and a greater risk of HIV infection *in vivo* implies that genital inflammatory responses are critical in HIV infection. Hence, the likelihood of young women contracting HIV is influenced by BV, STIs, and other biological stressors (Laga *et al.*, 1993). The overexpression of inflammatory cytokines and the migration of immune cells to the vaginal mucosae are common reactions to BV and STIs (Levine *et al.*, 1998). Although inflammation is crucial for infection removal, it can also damage the infected lining, allowing BV-associated bacteria to access the underlying tissue (Svanborg *et al.*, 1999).

1.6 Candida Infection

Candida albicans are members of the body's natural microflora, commonly found in the intestines, oral cavity, and vaginal area (Sobel, 2007a). The name "albicans" is derived from the Latin word for "white" because the yeast appears white when cultivated on a plate and causes white spots in certain illnesses, such as thrush. Signs and symptoms of a *Candida* infection include acute itchiness, inflamed vulva, dyspareunia, white vaginal discharge, frequent urination, burning sensation when urinating, abdominal or pelvic pain, fatigue, and recurring infections of the genital or urinary systems. Although *Candida albicans* usually do not cause problems, overgrowths and infections can occur when the VM is altered opportunistically (Nobile and Johnson, 2015).

Candida albicans possess a significant level of adaptability, allowing them to thrive in a wide range of conditions, including variations in nutrient availability, temperature, pH, osmolarity, and oxygen availability (da Silva Dantas *et al.*, 2016; Paramythiotou *et al.*, 2014). A form of vaginal fungal infection known as vulvovaginal candidiasis (VVC) is typically caused by an overabundance of *C. albicans*, although it can also be caused by other yeasts that inhabit the vagina. *Candida* species likely emerged in the gastrointestinal microbiome (GM) and invaded the genital area as parasites, initially presenting no symptoms (Lourenço *et al.*, 2019).

Candidiasis is commonly characterized by discomfort, urination problems, vaginal irritation, and unusual secretions from the vagina (Jeanmonod *et al.*, 2017). Current research is investigating whether vaginal microbial populations play a role in reducing the incidence of vaginal candidiasis and inflammation (Zhou *et al.*, 2009). When the vaginal environment is in a commensal state, the vaginal epithelium can tolerate the presence of *C. albicans* to some degree (Figure 1.3A). This indicates that the VM remains healthy when there are numerous *Lactobacillus* species and fewer *Candida* or BV-associated species (Figure 1.3A).

In the VVC state of the VM, reductions in the number of *Lactobacilli* and increased hormone levels, which are linked to increased fungal proliferation and burden, are common (Figure 1.3B). Candidalysin, a fungal peptide toxin produced by *C. albicans*, helps initiate host infection by stimulating the Nod-like receptor (NLR) P3 inflammatory pathway (Naglik *et al.*, 2019). This causes VK2 cells to release chemicals such as chemokines and cytokines,

ultimately resulting in an inflammatory VM (Gaziano *et al.*, 2023). Consequently, the outer layer of the vaginal cavity is damaged, which may cause epithelial cells to produce cytokines, leading to the recruitment of neutrophils and the establishment of a microbial population characterised by inflammation (Figure 1.3B) (König *et al.*, 2020).

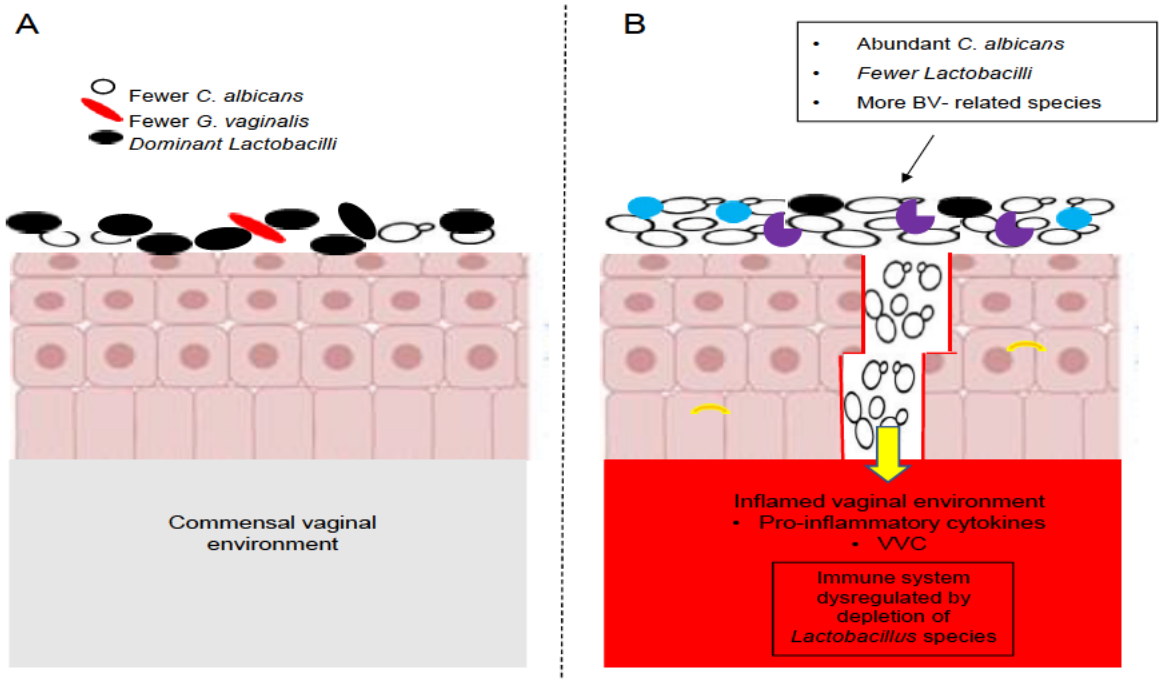

Figure 1.3: The relationship between the Commensal and Vulva Vaginal Candidiasis state of the vaginal environment in the presence and absence of vaginal inflammation. The expansion of undesirable bacteria invites *C. albicans* into the vaginal environment, which is the cause of VVC (B). When there are not many *C. albicans* (A), the vaginal environment remains balanced. Fewer *Lactobacilli* species are linked to a high candida invasion (B), and pro-inflammatory cytokines are produced because of genital inflammation induced by damaged epithelial cells. Modified from (Gaziano *et al.*, 2023)

Candida albicans can enter the vagina through a more delicate cervical lining, often due to the depletion of *Lactobacillus* species and subsequent yeast overgrowth, allowing it to penetrate the epithelial membrane (Drago *et al.*, 2000). The growing hyphal tip of the fungus, which is the actively extending end of its thread-like structure, begins penetrating the cervical lining due to its active permeability, strong adherence, and the enzymatic activity of external hydrolases on the fungal cell (Wächtler *et al.*, 2012).

A hospital study found that the most prevalent yeast species in vaginal samples from non-pregnant women aged 18 to 49 with symptoms of VVC was *C. albicans*, accounting for 51% of the tested fungal isolates. Other common species included *C. parapsilosis* (26%) and *C.*

glabrata (11%), with the remaining fungal isolates each comprising less than 5% (Figure 1.4) (Anh *et al.*, 2021). The study utilised gene sequencing, polymerase chain reaction (PCR), and antifungal susceptibility assays to identify distinct fungal samples and assess their resistance to various antifungal medications (Anh *et al.*, 2021).

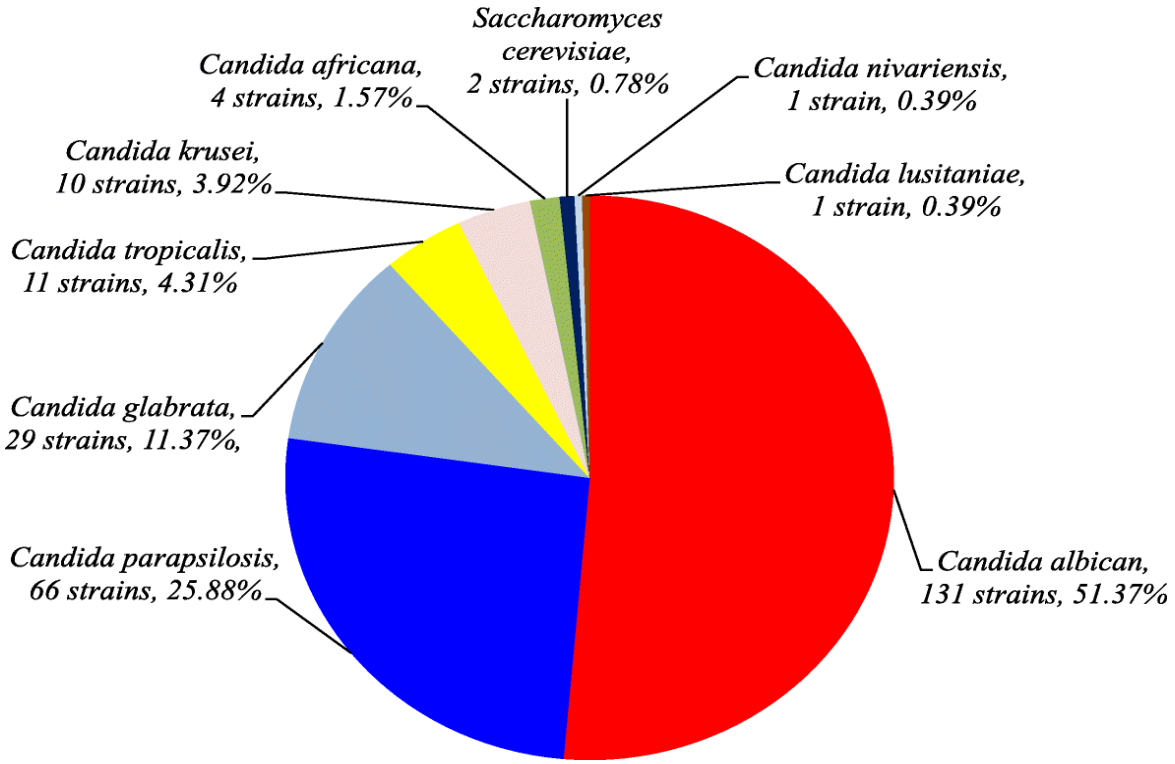

Figure 1.4: The incidence of fungal species isolated from women who experienced symptoms of vulva vaginal candidiasis at the Vietnam Military Hospital. The study investigated the prevalence of vaginal yeast strains from VVC- symptomatic females, and the percentages of distribution are shown as; *C. albicans* (red), *C. parapsilosis* (strong blue), *C. glabrata* (light blue), *C. tropicalis* (Yellow), *C. krusei* (pink), *C. africana* (green), *Saccharomyces cerevisiae* (navvy), *C. nivariensis* (white), and *C. lusitaniae* (brown). Adapted from BMC infectious diseases (Anh *et al.*, 2021).

1.6.1 Current Treatments for Candida Infection

Treatments for *Candida* infection is only recommended for symptomatic patients. Fluconazole (oral) is an antifungal medication widely used in various situations. For moderately severe Candida vaginal infections, a brief course of antifungal ointment, tablets, or powder, available over the counter or by prescription, is typically prescribed (Sobel, 2007a). One tablet of oral antifungal medication, such as Amphotericin B or fluconazole, and topical applications of miconazole and clotrimazole may also be recommended. For more complex infections, a longer course of medication in the form of cream, tablets, or ointment may be provided (Eckert, 2006).

Diet plays a crucial role in managing Candida infections. Consuming refined carbohydrates (such as pasta, rice, and bread) high in antifungal components can help reduce the conditions favourable to Candida overgrowth, as these foods are low in vitamins and minerals and are readily transformed (Ibrahim M and Abdel Baset, 1989). Sugar-filled foods, on the other hand, provide sustenance for Candida (Nomanbhoy *et al.*, 2002). Addressing the underlying causes of Candidiasis and preventing recurrent infections is the most effective strategy.

The balance of healthy and harmful bacteria in the gut is significantly influenced by diet. High-lactose dairy products can promote the growth of Candida and other harmful microbes, especially in individuals with weakened immune systems (Sobel, 2007a). Conversely, certain foods have been shown to support the growth of beneficial bacteria while inhibiting Candida growth (Fernandes *et al.*, 2022). Foods like garlic, coconut oil, aloe vera, curcumin, and probiotics like *Lactobacilli* can reduce the growth of Candida and protect against infections (Sobel, 2007a).

Home remedies can also ease symptoms caused by Candida and adverse reactions from its treatment (Jahdi *et al.*, 2021). These remedies include soaking in an oatmeal bath and using moisturisers to relieve itching and rash, getting enough sleep, eating foods like yogurt and organic honey that contain natural antifungal agents (Jahdi *et al.*, 2021), drinking plenty of water to help remove toxins from the body, and applying a cool compress to reduce muscle pain and heat, which could lead to fever (O'connor and Sobel, 1986).

Biofilm production is significant for pathogenicity and treatment, as biofilms exhibit stronger antifungal resistance than planktonic cells. *In vitro* efficacy against fungal biofilms has been observed with amphotericin B and echinocandins (Tumbarello *et al.*, 2012).

1.6.2 Shortfalls of current Candida treatments

Current treatments for *Candida* infections have several shortcomings. Women may develop allergies to Candida medications, with adverse effects including abdominal pain, diarrhoea, and nausea. Candida die-offs, also known as Jarisch-Herxheimer reactions, occur when the Candida yeast is eliminated too quickly from the body (Eckert, 2006). Antibiotics and antifungal medications remove pathogens and fungi by metabolising yeast and generating toxins, which can induce unpleasant reactions.

Side effects of antifungal medications include muscle aches, rapid heart rate, skin rash, energy loss, low blood pressure, fever, and other flu-like symptoms (McKeny *et al.*, 2019). In some individuals, antifungal medications can trigger anaphylaxis, a severe allergic reaction (Schwebke *et al.*, 1999). Symptoms of anaphylaxis include a swollen face, throat, or tongue; difficulty breathing; wheezing; severe skin rash (blistering or peeling); vomiting; weak pulse; shock; and loss of consciousness (Linhares *et al.*, 2010). These potential adverse effects highlight the need for alternative treatments and preventative measures that minimise the risk of allergic reactions and other negative side effects associated with current Candida medications.

1.6.3 Impact and use of vaginal *Lactobacilli* species as probiotics for Bacterial Vaginosis and Candida treatment.

Probiotics are viable bacteria administered in sufficient proportions to improve the health of recipients. They offer various health benefits, such as lowering serum cholesterol, improving lactose intolerance, enhancing nutritional utilisation, reducing antibiotic use, and providing antidiabetic therapy (Matsubara *et al.*, 2016). Probiotics have been extensively studied in the fields of nutrition and health, with numerous studies highlighting their positive effects on the circulatory and digestive systems (Saad *et al.*, 2013). Commonly used probiotic microorganisms include *Lactobacillus*, *Bacillus*, *Saccharomyces cerevisiae*, *Escherichia coli*, and *Bifidobacterium* (Hong *et al*., 2005).

As mentioned earlier, *Lactobacillus* species produce several antimicrobial peptides and chemicals that defend against bacterial infections (Owen and Katz, 1999). Some *Lactobacilli* spp. have collagen binding proteins with a size of 29 kD on their surfaces, enabling them to bind collagen (Rojas *et al.*, 2002). This binding allows *Lactobacilli* to attach to vaginal tissue cells, blocking pathogen binding to host tissues in large numbers (Donnarumma *et al.*, 2014). According to most investigations, *Lactobacillus* bacteria remain the dominant bacterial species during vaginal candidiasis (VC).

There has been a significant increase in both *in vivo* and *in vitro* studies regarding the advantages of probiotics for treating infections caused by bacteria, fungi, or viruses. These benefits include mitigating the adverse effects of antibiotics, prolonging the remission of chronic inflammatory diseases of the digestive tract, lowering the chances of cancer (Górska *et al.*, 2019; Śliżewska *et al.*, 2020), reducing sensitivities, and positively influencing intestinal

bacteria (Saad *et al.*, 2013). Recent studies have shown that various *Lactobacillus* strains can activate adaptive and innate immune responses by attaching to cell surface receptors on antibodies and other organs, including intestinal tissues. *Lactobacilli* also influence gene expression related to immune system stimulation (Abedin-Do *et al.*, 2015).

More research is needed to determine how *Lactobacilli* isolates can be employed as probiotics to treat *C. albicans*, particularly using African-derived bacterial strains. This is crucial because the vaginal microbiome is a significant determinant of vaginal health, and geographic differences in the composition of the vaginal microbiota have been described.

1.7 Study Rationale

Vaginal infections, including bacterial BV and vulvovaginal candidiasis, represent a significant public health challenge globally, particularly in resource-limited settings such as sub-Saharan Africa. The current therapeutic approaches, primarily involving antibacterial and antifungal agents, are fraught with limitations such as adverse side effects, the emergence of drug-resistant strains, and high rates of recurrence (Schwebke and Desmond, 2007; Sobel, 2007b). For instance, the treatment of BV often results in gastrointestinal disturbances and antibiotic resistance, while antifungal treatments for vulvovaginal candidiasis can cause skin irritation and disrupt gut health, posing additional risks for pregnant women (Reid *et al.*, 2001).

The human vaginal microbiota is dominated by *Lactobacillus* species, which play a critical role in maintaining vaginal health by producing antimicrobial compounds, including lactic acid and hydrogen peroxide (H_2O_2), that inhibit the growth of pathogenic microorganisms such as *G. vaginalis* and *C. albicans* (O'Hanlon *et al.*, 2013; Ravel *et al.*, 2011). Emerging evidence suggests that probiotic interventions using *Lactobacillus* strains offer a promising alternative or adjunct to conventional therapies by restoring and maintaining a healthy vaginal microbiota (Petrova *et al.*, 2015). However, the probiotic potential of *Lactobacillus* strains, particularly those derived from African women, remains underexplored.

This study aims to fill this gap by investigating the probiotic characteristics of *Lactobacillus* species isolated from healthy African women and evaluating their effects on pathogenic microbes. By focusing on strains such as *L. crispatus*, *L. gasseri*, *L. jensenii*, *L. mucosae*, and *L. vaginalis*, this research seeks to identify *Lactobacillus* strains with superior probiotic

properties, including robust production of lactic acid and H_2O_2 and potent inhibitory activity against *G. vaginalis* and *C. albicans*.

The findings of this study could have significant implications for the development of new probiotic-based treatments for vaginal infections. By demonstrating that certain *Lactobacillus* strains can effectively inhibit pathogenic microbes and produce higher amounts of antimicrobial substances, this research underscores the potential for species- and strain-specific probiotic therapies. These insights could pave the way for safer, more effective, and sustainable alternatives to current treatment modalities, addressing the urgent need for improved management of vaginal infections in settings where traditional therapies are often inadequate.

1.8 Study Aim and Objectives

This study examined the probiotic properties of thirteen strains of vaginal *Lactobacillus* species *(L. crispatus*, *L gasseri*, *L. jensenii*, *L. mucosae*, and *L. vaginalis*) isolated from healthy African women and evaluated their impact on pathogenic microbes, specifically *Gardnerella vaginalis* and *Candida albicans*. Additionally, the study analysed the production of antimicrobial substances, including lactic acid and H_2O_2, by these *Lactobacillus* strains.

To achieve this aim, the objectives of this study were:

1. To characterise vaginal isolates by evaluating their growth kinetics at various pH levels and determining how they alter the culture pH over time.
2. To measure the production of lactic acid (D-/L-) and H_2O_2 by the isolates.
3. To perform microscopic analysis of bacterial and *C. albicans* fungal cells to determine their cell morphology.
4. To investigate the antimicrobial and antifungal activity of the *Lactobacillus* culture supernatant from the isolates against BV-associated *G. vaginalis* and *C. albicans* using growth inhibition assays.

Chapter 2

Materials and Methods

2.1 Commonly used reagents & equipment

The following reagents and equipment's were used: 10X phosphate buffered saline (PBS) pH 7.4 (Thermo Fischer Scientific, Waltham, MA, USA); 96-well cell culture plate (U-type) (Thermo Fisher Scientific™, Waltham, MA, (United states (US)); Bifonazole (Sigma-Aldrich Co., St. Louis, Missouri (MO), United states of America (USA)); Brain heart infusion (BHI) agar, and BHI broth (Oxoid ltd, Basingstoke, Hants, United kingdom (UK)); Clindamycin hydrochloride (Glentham Life Sciences Ltd, Corsham, UK); Clotrimazole (Glentham Life Sciences Ltd, Corsham, UK); Glycerol (Sigma-Aldrich Co., St. Louis, MO, USA); Dimethyl sulfoxide (DMSO) (Sigma-Aldrich Co., St. Louis, MO, USA); Metronidazole (Sigma-Aldrich (Pty) LTD, Modderfontein, Johannesburg, SA); MRS agar and MRS broth (Sigma-Aldrich, Darmstadt, Germany); Sabouraud Dextrose Agar (SDA) (Oxoid ltd, Basingstoke, Hants, UK); Trypan Blue solution (Sigma-Aldrich Co., St. Louis, MO, USA); Versa-MAX microplate reader (Molecular Devices, California (CA), USA); yeast extract peptone dextrose (YEPD) Agar (Sigma-Aldrich Co., St. Louis, MO, USA), and YEPD Broth (Scharlab, Gato Perez, Spain).

2.2 Bacterial and Fungal Strains and their growth conditions

2.2.1 Vaginal bacterial isolates

Thirteen strains of five *Lactobacillus* species: *L. crispatus* (70.7apa, 73.55a, 94.79pa), *L. gasseri* (94.98pb), *L. jensenii* (73.2pa, 73.27pa, 95.1pa), *L. mucosae* (80.23a, 85.1pa, 99.10pa), and *L. vaginalis* (88.5b, 91.8a, 100.13pa) were previously isolated at the University of Cape Town as part of the completed WISH cohort study, South Africa (Cape Town, HREC Ethics Ref: 267/2013) from STI-free and HIV-negative South African adolescent girls and young women (AGYW). The bacterial strains were tested for *Chlamydia trachomatis*, *N. gonorrhoeae*, *T. vaginalis*, and *M. genitalium* STIs. The isolated strains of the bacterial species were identified by Sanger sequencing of the 16S ribosomal ribonucleic acid (rRNA) gene before being used for this study (Happel *et al.*, 2020).

The thirteen isolated vaginal bacterial strains were streaked onto MRS agar plates (Merck, Kenilworth, New Jersey (NJ), US), supplemented with 0.05 g/L L-cysteine (Glentham,

Corsham, UK) and 1% (v/v) Tween 80 (Glentham, Corsham, UK), and incubated for 48 hours at 37°C under anaerobic conditions achieved by using an Oxoid anaerobic jar (Thermo Fisher Scientific™, Waltham, Massachusetts (MA), US) and anaerogen 2.5 L sachet (Thermo Fisher Scientific™, Waltham, MA, US). The MRS medium was selected for the isolation and growing of *Lactobacillus* species because it is an improved selective medium that promotes good growth of *Lactobacilli*, in addition, supports growth on strains which demonstrate poor growth. L-cysteine is advantageous because it controls the amount of oxygen present during incubation, favouring the *Lactobacilli*'s anaerobic state. A single colony was taken from the agar plates and cultured in MRS broth. The cultures were maintained for 48 hours at 37°C in the anaerobic jar. After centrifuging at 589 x g, the pellet was resuspended in fresh MRS broth, preserved with 20% v/v final glycerol (Sigma-Aldrich, Darmstadt, Germany), and stored at -80°C for future experiments.

2.2.2 Fungal and bacterial ATCC strains

Gardnerella vaginalis ATCC 49145 strain and *Candida albicans* (ATCC 10231 and 60193, catalogue numbers 0643P, 0443K, and 0425P, respectively) KWIK-STIK, containing inoculating swabs and a lyophilised pellet of the organism, were obtained from Microbiologics (St. Cloud, Minnesota (MN), USA). Clinical isolation of *G. vaginalis* was from vaginal secretions. The *G. vaginalis* swab was streaked onto BHI agar and incubated for 48-72 hours at 37˚C under anaerobic conditions in the presence of 5% carbon dioxide (CO_2). The fungal strains were streaked onto YEPD agar to facilitate colony isolation. Plates were incubated under aerobic conditions at 30˚C for 24-48 hours. After incubation of culture plates, colonies were transferred to BHI broth for *G. vaginalis* and YEPD broth for *C. albicans*. The cultures were grown in the medium for 24 hours under the same physiological conditions.

2.3 Gram staining

A Gram staining kit for microscopy (Catalogue number: 77730) (Sigma-Aldrich Co., St. Louis, MO, USA) was used to confirm the morphology of the isolated *Lactobacillus* and *G. vaginalis* strains. Overnight cultures of *Lactobacilli* and *G. vaginalis* were grown in MRS and BHI, respectively, under anaerobic conditions for 48 hours at 37ºC. *Gardnerella vaginalis* was grown in the presence of 5% CO_2. A smear was prepared by placing a drop of distilled water onto a clean microscope slide and aseptically collecting a single colony of the bacterial culture from the surface of the MRS agar plates. The mixture was spread over the entire slide using

a sterile loop. The suspension was allowed to air dry completely and then heat-fixed by passing it through a flame four times before staining the slide.

The smear was stained with crystal violet solution for one minute and then rinsed with distilled water (DH_2O). Subsequently, an iodine solution (Sigma-Aldrich, Darmstadt, Germany) was added for one minute and gently washed off with DH_2O. Ethanol was added for 30 seconds to decolorize, followed by a rinse with DH_2O. Finally, safranin (Sigma-Aldrich, Buchs, Switzerland) was added for two minutes and then rinsed with DH_2O. Slides were blot-dried and visualized with phase contrast light microscopy (100x magnification, scale bar 10–20 µm) in the presence of immersion oil. Images were captured with an Axiocam 105 colour camera and saved using Zen 2.5 lite software.

2.4 Microscopic examination of *Candida albicans* cells morphology.

Overnight cultures of both *C. albicans* strains from the orbital shaking incubator (United Scientific (Pty) LTD, Congella, Durban, SA) were grown on SDA agar for 24-48 hours under aerobic conditions. A single colony was collected from each plate and inoculated on a clean slide on the four sides of a small square/block of fresh SDA agar. The inoculated SDA block was covered with a coverslip and incubated under aerobic conditions at 30˚C for 48 hours. After incubation, lactophenol cotton blue (LPCB) stain was used to stain the cultured coverslip on the slide. Microscopic examination of the fungal strains was done using fluorescence and phase contrast light microscopy under 100x magnification with immersion oil.

2.5 *Lactobacilli* Growth Kinetics

Cultures of *Lactobacilli* were grown in MRS and incubated under anaerobic conditions at 37ºC for 48 hours. The next day, the cultures were diluted to an optical density at 600 nm wavelength (OD600nm) of 0.1 ± 0.01, equivalent to 1 x 10^5 colony forming units (CFU)/ml, before being added to each well of a 96-well cell culture plate. Although growth was observed for 96 hours, readings were only obtained at 3, 24, 48, 72, and 96 hours using a Versa-MAX microplate reader. MRS was the control. Experiments were performed in triplicates.

2.6 Assessing the effect of *Lactobacilli* on pH

2.6.1 Growth of *Lactobacilli* at various pH levels

Lactobacilli bacterial overnight cultures were adjusted to OD600nm 0.1 ± 0.01 in MRS broth. Using a 1M hydrochloric acid (HCl) solution (Sigma-Aldrich Co., St. Louis, MO, USA), the cultures' pH levels were lowered to 3.5, 4, 4.5, 5, 5.5, and 6. MRS was the control and cultured under the same pH conditions as the bacterial cultures. All cultures were added to their respective wells of a 96-well cell culture plate and incubated at 37˚C for 48 hours in an anaerobic environment. Growth kinetics were measured with a Versa-MAX microplate reader at A600nm. Readings were taken at 0, 3, 24, and 48 hours. Experiments were performed in triplicates.

2.6.2 Evaluation of the effect of *Lactobacilli* on pH level of culture medium

Bacterial strains were cultured in MRS, serially diluted to OD600nm 0.1 ± 0.01, and incubated at 37°C for 48 hours under anaerobic conditions. pH was measured with a VioLab pH meter (Lasec, Cape Town, SA) at 0, 3, 24, and 48 hours. Experiments were performed in triplicates.

2.7 Lactic Acid Production by *Lactobacilli*

2.7.1 D- lactic Acid Production

Adjusted *Lactobacilli* cultures were prepared according to section 2.3.4. D-lactate standards and reaction mixes were prepared following the D-lactate colorimetric assay kit (Catalogue Number MAK058) (Sigma-Aldrich Co., St. Louis, MO, USA) according to the manufacturer's instructions. The control was MRS only. D-lactate standards, samples, and control were added to the respective wells of the 96-well plates to make 100 µL/well. The plates were incubated in a dark environment at room temperature (RT) for 30 minutes. After 30 minutes of colour development, A450nm was measured using a Versa-MAX microplate reader. D-lactate concentrations in all the bacterial cell cultures were measured using a standard curve (Figure 2.1). Experiments were repeated three times, each performed in triplicates.

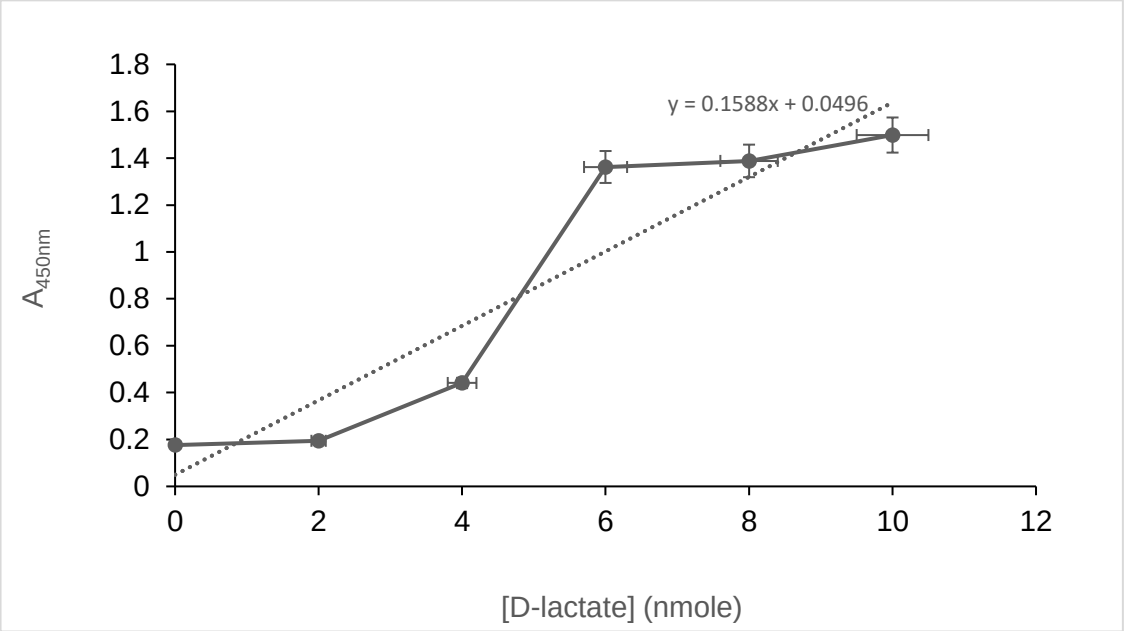

Figure 2.1: D-lactate Colorimetric Assay Standard curve. Overnight cultures of bacteria, D-lactic acid standards were prepared and added to the 96-well plate wells to make up 100 µL/well. Plates were incubated at RT for 20 minutes in a dark environment. A_{450nm} was measured. The D-lactate standard curve was used to determine the D-lactate concentrations in the bacterial samples. Error bars represent mean of triplicates and standard error.

2.7.2 L- lactic Acid Production

Overnight cultures of isolates, L-lactate standards, and reaction mixes were prepared following the L-lactate Assay kit (Catalogue Number MAK329) (Sigma-Aldrich Co., St. Louis, MO, USA) procedure, according to the manufacturer's instructions. The MRS control did not contain L-lactate. L-lactate standards, bacterial cultures, and the control were added to their respective wells of the 96-well plate. The initial A565nm was measured, and the plate was incubated for 20 minutes at room temperature. Final A565nm was measured using a Versa-MAX microplate reader. The L-lactate assay standard curve (Figure 2.2) was used to determine the concentrations of L-lactate in the cultures. Experiments were repeated three times, each performed in triplicates.

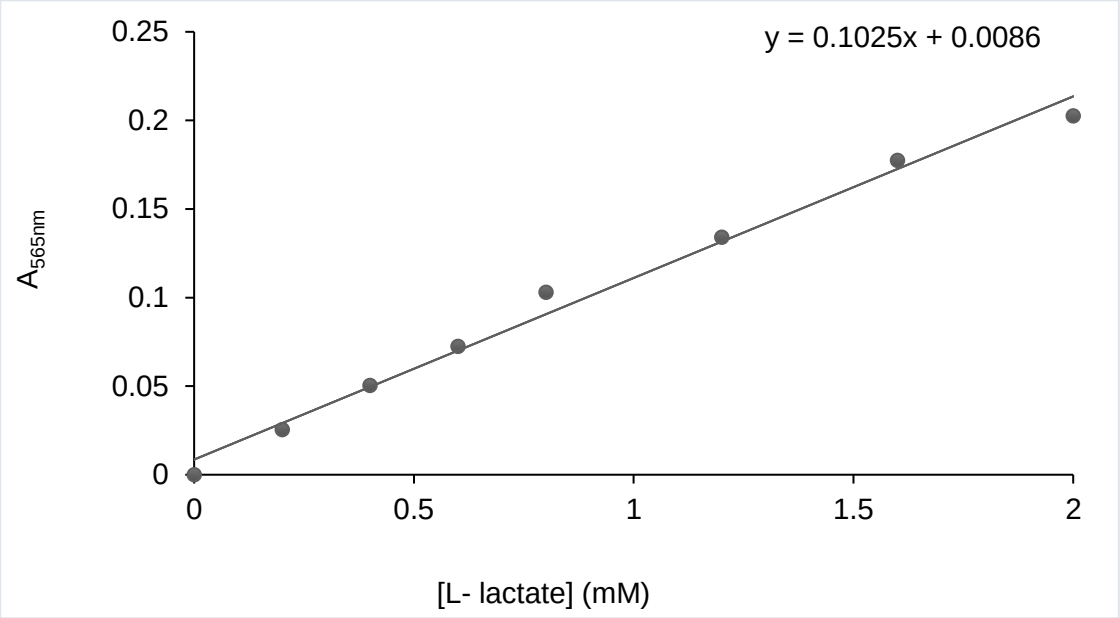

Figure 2.2: L-lactate assay relationship with Absorbance. Overnight cultures of bacteria, L-lactic acid standards were prepared and added to the 96-well plate wells to make up 100 µL/well. Plates were incubated at RT for 20 minutes and A565nm was measured. The L- lactate standard curve was used to determine the L- lactate concentrations in the bacterial samples. Experiment performed in triplicates

2.8 Hydrogen Peroxide Production

Following the aqueous-compatible procedure of the Pierce quantitative peroxide assay kit (Aqueous compatible, Catalogue number: 23280) (Thermo Fisher Scientific, Fairland, SA), H_2O_2 concentration generated by *Lactobacillus* isolates was detected in the presence of xylenol orange based on ferrous to ferric ion oxidation. The H_2O_2 stock solution was prepared with 30% H_2O_2 (Sigma-Aldrich Co., St. Louis, MO, USA). The peroxide standards (concentrations of hydrogen peroxide ranging from 1000 to 0 µM) and working reagent (WR) were prepared following the manufacturer's instructions. 200 µL of WR was added to all the wells, and 20 µL of peroxide standards and cells were added to the wells of a 96-well plate. The plates were incubated at room temperature for 20 minutes. A595nm was measured using a Versa-MAX microplate reader. The concentration of H_2O_2 produced by each *Lactobacillus* strain was calculated using the H_2O_2 assay standard curve (Figure 2.3). Experiments were performed in duplicates.

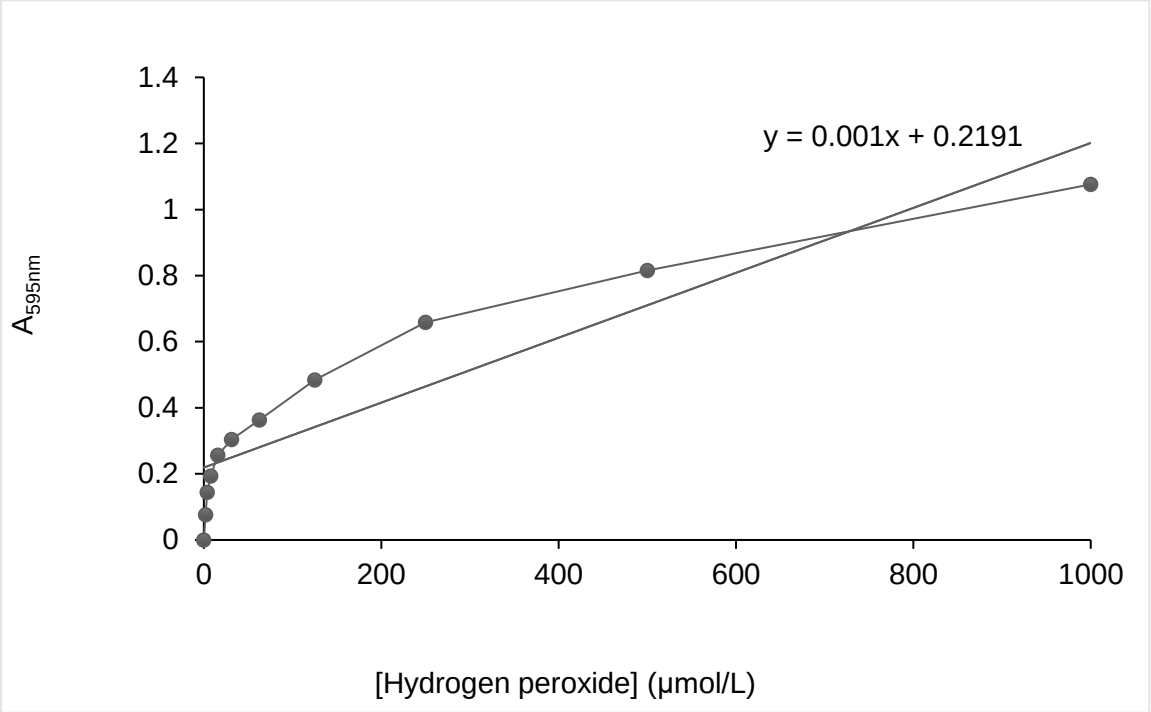

Figure 2.3: Hydrogen peroxide assay standard curve. Different concentrations of H_2O_2 (1000 to 0 µm were prepared using the 30% H_2O_2 stock solution. Overnight cultures of bacteria were serially diluted before being added to the 96-well plate. A colour reagent (Working reagent = 200µL) was added to all the. Samples were agitated and incubated at RT for 20 minutes. A595nm was measured using a 96-well plate reader. The standard curve was generated and used to calculate the concentrations of hydrogen peroxide in the bacterial samples according to the instructions of the kit.

2.9 Preparation of *Lactobacillus* Culture Supernatants for Growth Inhibition assays

Lactobacilli were grown under anaerobic conditions in MRS, incubated for 48 hours at 37°C, and then adjusted to OD600nm 0.1 ± 0.01. After 48 hours of incubation, cultures were centrifuged at 4000 xg for 10 minutes at RT to remove the cells. The pellet was discarded, and the LCS was transferred to an empty centrifuge tube. The cell-free suspensions were filtered using a sterile Acrodisc 0.22 µm pore size, 25 mm diameter syringe filter (Cytvia, Marlborough, USA). The filtered LCS were stored at -20°C and used in the growth inhibition experiments.

2.10 Determination of Bacterial Numbers by Dry Weight Method

Gardnerella vaginalis was grown in BHI for 48 hours at 5% CO_2 under anaerobic conditions. To determine the dry weight, the overnight sample was serially diluted in five pre-weighed 2

ml Eppendorf tubes (Labocare, London, UK) using the KERM ADB weighing scale (KERN & SOHN GmbH, Balingen, Germany). The OD600nm of the diluted samples was determined with a Nanophotometer spectrophotometer (Implen, Munich, Germany). The supernatant was discarded after centrifuging the tubes at 12,000 xg for 5 minutes. The pellet was dried in a 100°C drying oven. The cells were observed at 15-minute intervals until they were dehydrated and weighed. Bacterial counts were determined using the generated standard curve of dry weight (g/L) vs. OD600nm (Appendix A, Figure A1 (A)) to calculate the g/L value using the equation of the straight line obtained. Experiments were performed in duplicates.

2.11 *Gardnerella vaginalis* Growth Kinetics

Gardnerella vaginalis was grown on BHI agar and incubated for 72 hours at 37°C with 5% CO_2 under anaerobic conditions. Subsequently, individual colonies were inoculated in BHI culture and left to culture for 48 hours. The overnight culture was adjusted to OD600nm 1.2 ± 0.1, corresponding to a cell concentration of 1.27 g/L, before being added to each well of a 96-well plate. The growth of *G. vaginalis* was observed for 96 hours, with measurements taken at 3, 24, 48, 72, and 96 hours. The control was BHI. The experiment was performed in triplicates for each of the three independent experiments.

2.12 Antimicrobial Activity of *Lactobacillus* culture Supernatants against *Gardnerella vaginalis*

Overnight cultures of *G. vaginalis* in BHI were adjusted to OD600nm 1.2 ± 0.01, equivalent to 1.27 g/L of cells. The modified cell cultures were incubated for 48 hours under anaerobic conditions at 37°C with 5% CO_2. *Gardnerella vaginalis* cells (100 µL) and 100 µL of each standardised LCS filtrate were added in triplicates to their respective wells of a 96-well plate. Plain MRS (100 µL) and *G. vaginalis* cells (100 µL) only were added to the control wells. The plates were incubated under *G. vaginalis* suitable conditions. The growth of *G. vaginalis* in the presence and absence of LCS was measured at OD600nm at 3, 24, 48, 72, and 96 hours using a Versa-MAX microplate reader. The inhibitory effects of LCS from *Lactobacilli* strains on *G. vaginalis* growth were determined by calculating the percentage (%) of growth inhibition in the presence and absence of cell-free culture supernatants. The experiment was performed in triplicates and repeated in the same way in four independent experiments.

2.13 *Candida albicans* Growth Kinetics

Individual colonies of *C. albicans* (both strains grown independently) were cultured in YPED media in a shaking incubator for 24 hours at 30˚ C and 100 rpm. One hundred thousand cells were added to each well after overnight cultures were counted using an Invitrogen countess 3 FL automated cell counter (Thermo Fischer Scientific, Waltham, MA, USA) and adjusted to 1 x 10^6 CFU/ml by diluting them to the fresh YPED growth medium. The growth kinetics of *C. albicans* were monitored and recorded for 96 h; however, readings were taken at 3, 24, 48, 72, and 96 hours using a Versa-MAX microplate reader. YPED was used as the control. Each experiment was performed in triplicates and repeated two times.

2.14 *Candida albicans* Growth Inhibition Assay

Candida albicans cells were grown in a shaking incubator in YPED under aerobic conditions (24 h, 30˚C, and 100 rpm). After 24 hours, cultures were centrifuged for 7 minutes (4000 xg, RT). The supernatant was discarded, and the pellet was resuspended in 3 ml of diluted 1X PBS buffer thrice to wash the cells. The cells were then added to fresh 10 ml YPED media. Ten µL from this cell suspension was used for cell counting. Cells were stained with equal volumes of trypan blue and counted using an automated cell counter. The cells were diluted to make 1x10^6 cells per ml, and a final concentration of 100,000 cells per well was added to each well. From the prepared LCS filtrates, 100 µL was added to 100 µL of *C. albicans* cell suspension in the wells of a 96-well plate. For the control, 100 µL MRS was added instead of filtered LCS, with the same amount of *C. albicans* cells. Plates were incubated for 24 hours under aerobic conditions at 30˚C and 100 xg. Following LCS treatment, the growth of *C. albicans* was measured (OD600nm) at 3, 24, 48, 72, and 96 hours. The percentage growth (%) inhibition of *C. albicans* by LCS was calculated using the OD600nm. The experiment was performed in triplicates and repeated in four independent experiments.

2.15 Statistical Data Analysis

Data analysis and the generation of assay graphs were performed using GraphPad Prism9 (GraphPad Software version 9.0.0, San Diego, CA, USA), while standard curves were generated using Microsoft Excel (2016). The Normal (Gaussian) distribution and Shapiro-Wilk tests were used to confirm the data's normality and lognormality to determine whether the data

were uniformly distributed. For triplicates or more groups, the Kruskal-Wallis one-way ANOVA unpaired test was employed with the unadjusted Dunn's multiple comparisons test. The Mann-Whitney non-parametric t-test was used to analyse the data for two groups. For multiple comparisons, the individual p-values were compared to those of the control and among the strains, given in the following order: *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$.

Chapter 3

Results

3.1 Gram Staining

The Gram staining technique was employed for the microscopic evaluation of *Lactobacillus* species and *Gardnerella vaginalis* to differentiate bacterial cells as Gram-positive or Gram-negative. All thirteen vaginal *Lactobacillus* strains (Figures 3.1A - 3.1M) stained purple, confirming their classification as Gram-positive bacteria. The intensity of the purple colour varied among the cells, reflecting the degree to which they retained the crystal violet dye. For instance, *L. mucosae* (80.23a) appeared faint purple (Figure 3.1H), whereas strains like *L. vaginalis* (100.13pa) exhibited a darker purple hue (Figure 3.1M). The rod-like morphology of all the *Lactobacillus* strains corroborates their identification as members of the *Lactobacillus* genus. Overall, the cell morphologies of the *Lactobacilli* strains were uniform.

The *G. vaginalis* (ATCC 49145) cells displayed a range of colours from purple to faint pink (Figure 3.1N), indicating that this strain is Gram-variable (ranging from Gram-positive to Gram-negative). The bacterium exhibited pleomorphism, appearing as chains of rod-like structures, and the cells were observed in motion under the microscope. The colour and morphological characteristics of the *G. vaginalis* cells support their classification within the *Gardnerella* genus.

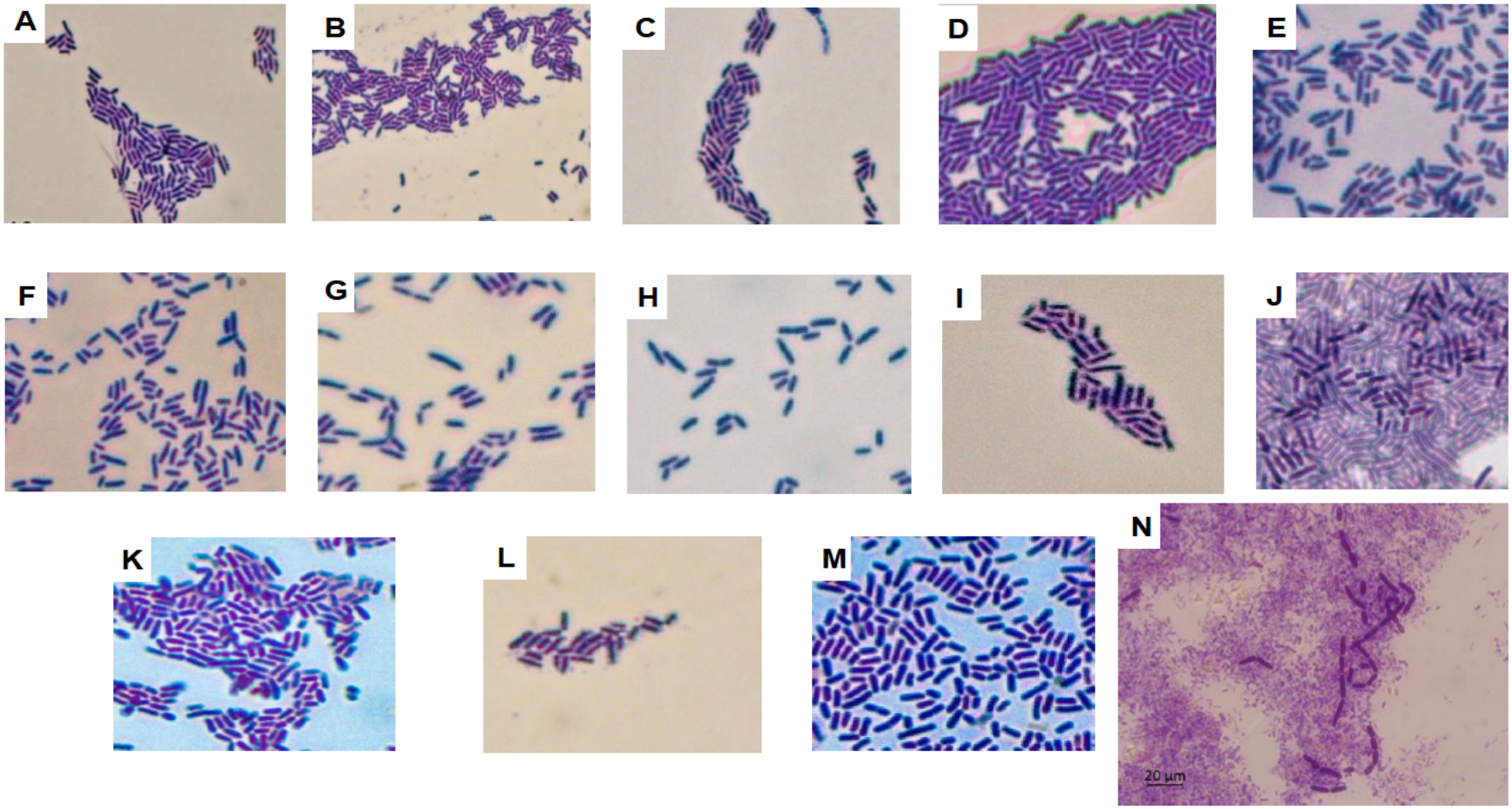

Figure 3.1: Gram stain microscopic analysis of Bacterial strains. Single colonies of *L.crispatus* 70.7pa (A), 73.55a (B), 94.79pa (C), *L. gasseri* 94.98pb (D), *L. jensenii* 73.2pa (E), 73.27pa (F), 95.1pa (G), *L. mucosae* 80.23a (H), 85.1pa (I), 99.10pa (J), *L. vaginalis* 88.5b (K), 91.8a (L), 100.13pa (M), *G. vaginalis* (N) were heat-fixed and smeared in a slide before being stained. The stained specimens were viewed using phase contrast light microscopy (1000X magnification); images were captured with Axiocam 105 colour and the Zen 2.5 lite software. Scale bar (10µm).

3.2 Microscopic Examination of *Candida albicans* Cells Morphology

The slide culture method was used to examine the morphology of the *C. albicans* fungal strains. This approach is appropriate for the microscopic analysis of yeast cultures and confirmed the identity and cell morphologies of the fungal strains. The yeast cells of *C. albicans* ATCC 10231 were observed to be round to oval and budding (Figure 3.2A). The *C. albicans* ATCC 60193 cells were aggregated, budding, and shaped from oval to spherical (Figure 3.2B). No hyphae were observed in either strain. Both fungal strains retained the blue colour from the LPCB.

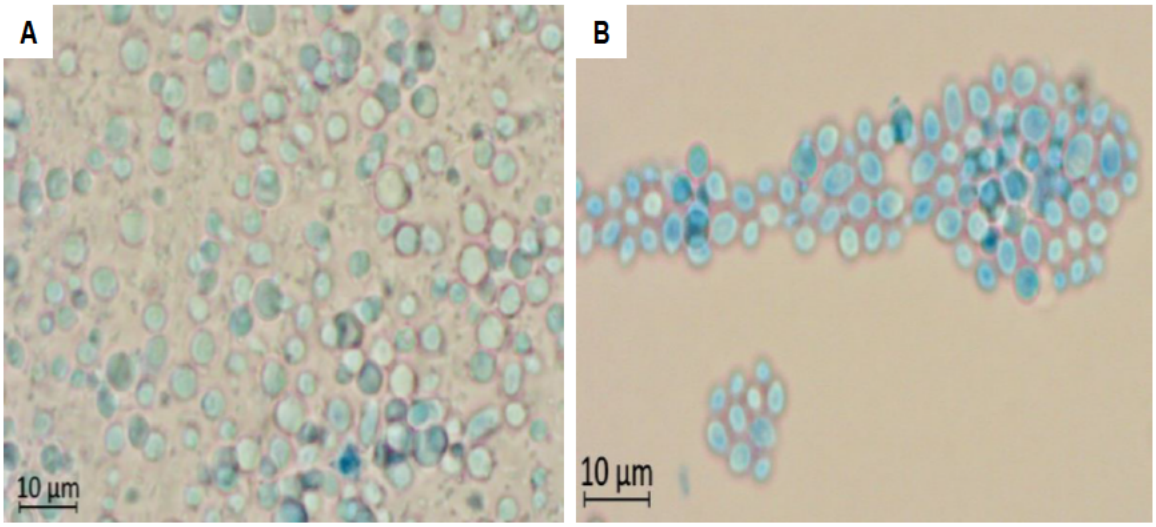

Figure 3.2: Microscopic analysis of *Candida albicans* ATCC 10231 and 60193 strains from Sabouraud Dextrose Agar plates. A single colony from SDA plates was collected and inoculated on four sides of a block of fresh SDA which was incubated under aerobic conditions at 30°C for 48 hours. The coverslip containing the fungus was stained with Lactophenol cotton blue and viewed using fluorescence microscopy. Phase contrast microscopy (40x magnification) was used to view strain ATCC 10231 (A), image was captured using Axiocam 105 colour camera and Zen 2.5 lite software. ATCC 60193 strain (B) was viewed using Olympus AX70 fluorescent microscope at 40X magnification, image was captured using Nikon DS-R1I camera and the NIS-Elements BR software.

3.3 *Lactobacilli* Growth Kinetics

To achieve optimal results for the upcoming investigations, the characterisation of the various groups of bacterial organisms relied on understanding their growth kinetics over time, as there could be a relationship between the growth rate of the cells and their performance. After confirming the Gram-status and morphologies of the isolated bacterial strains, their cultures were further grown under suitable anaerobic conditions to investigate their growth kinetics in

MRS over time. The growth dynamics of the different strains were monitored and measured at 3, 24-, 48-, 72-, and 96-hours post-incubation.

For *L. crispatus*, the exponential phase occurred between 3 and 24 hours of incubation (Figure 3.3). After that, the 94.79pa *L. crispatus* strain experienced a slight decline at 48 hours and remained in the stationary phase between 48 and 72 hours, entering the death phase after 72 hours. *Lactobacillus crispatus* 70.7pa continued to grow exponentially until 48 hours, reaching the death phase afterwards. The 73.55a *L. crispatus* strain had the longest survival, reaching the stationary phase after 24 hours and continuing until 96 hours of incubation.

The *L. gasseri* strain grew exponentially between 3 and 24 hours, then entered the stationary phase, which continued until 96 hours of incubation. The *L. jensenii* strains proliferated exponentially between 3 and 24 hours, with 73.2pa and 95.1pa strains continuing to grow until 48 hours. They entered the stationary phase after 48 hours, continuing until 96 hours post-incubation, whereas the 73.27pa strain reached the death phase after 48 hours.

The *L. mucosae* strains (85.1pa and 99.10pa) grew exponentially until 24 hours, continued slowly until 48 hours, and began to decline after 48 hours, moving towards the death phase. Notably, the 80.23pa *L. mucosae* strain did not grow and was similar to the negative control. The *L. vaginalis* strains grew exponentially between 3 and 24 hours, reaching the stationary phase between 48 and 72 hours. Between 72 and 96 hours, they entered the death phase, which was more pronounced in the 91.8a strain.

Overall, the ability of all *Lactobacillus* strains to grow anaerobically over time demonstrated comparable trends. However, *L. mucosae* (80.23a) had the lowest growth rate (OD600nm of less than 0.5) at all time points, while all other strains had a visibly higher growth rate compared to the negative control (MRS only) (Figure 3.3).

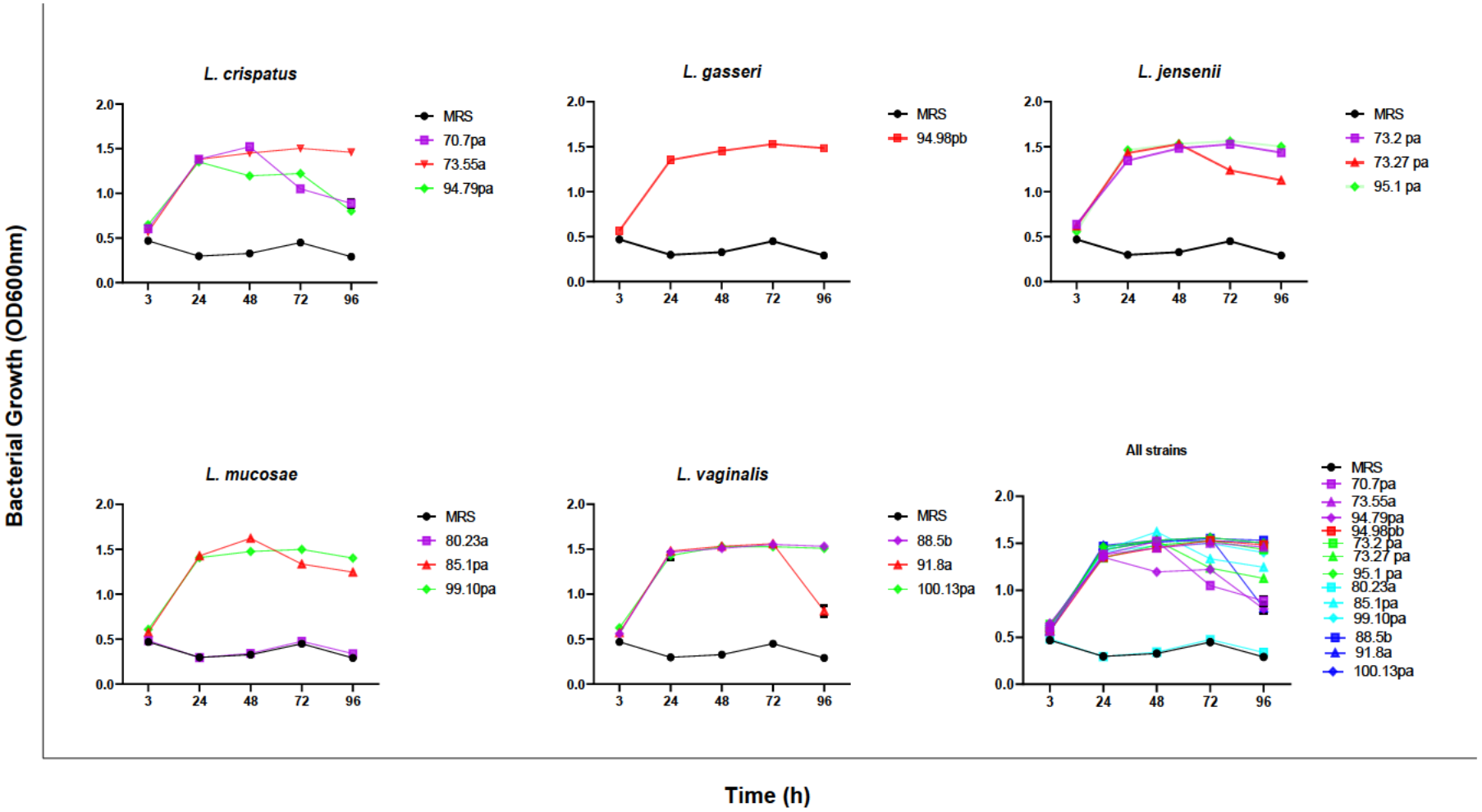

Figure 3.3: Growth kinetics of *Lactobacillus* species over time. Growth of *L.crispatus* (70.7pa, 73.55a, 94.79pa), *L. gasseri* 94.98pb, *L. jensenii* (73.2pa, 73.27pa, 95.1pa), *L. mucosae* (80.23a, 85.1pa, 99.10pa), and *L. vaginalis* (88.5b, 91.8a, 100.13pa) was measured over 96 hours under anaerobic incubation at 37°C. Readings were recorded at OD_{600nm} after 3, 24, 48, 72 and 96 hours using a Versa-MAX microplate reader. Growth summary of all the strains is shown at bottom right. *L. crispatus* (Purple), *L. gasseri* (Red), *L. jensenii* (green), *L. mucosae* (light blue), and *L. vaginalis* (dark blue), MRS control (Black). Error bars represent standard error and standard deviation (Sd) (n=3).

3.4 Growth of *Lactobacilli* at various pH levels

To maintain the health of the vaginal microbiome, *Lactobacilli* species must survive at lower, acidic pH levels. The ability of these vaginal strains to grow well under various pH conditions, specifically from pH 3.5 to 6, was investigated over 48 hours. This incubation period was chosen based on the findings in Section 3.3, which showed that most bacterial strains reached optimum growth between 48 and 72 hours (Figure 3.3). At pH 3.5 and pH 4, most strains did not grow well, with only a few showing some growth (*L. crispatus* 70.7a, *L. jensenii* 73.27pa, *L. mucosae* 99.10pa, and *L. vaginalis* 88.5b) (Figure 3.4). At pH 4.5, bacterial growth started to increase for most strains, particularly *L. crispatus* 73.55a, *L. mucosae* 85.1pa, *L. jensenii* 73.27pa, and *L. vaginalis* 88.5b. At pH 5, the growth rate was reasonable and slightly higher than at pH 4.5. At pH 5.5 and pH 6, all strains grew well after 48 hours of incubation, indicating that pH 5.5 to pH 6 is the most ideal range for *Lactobacilli* (Figure 3.4)

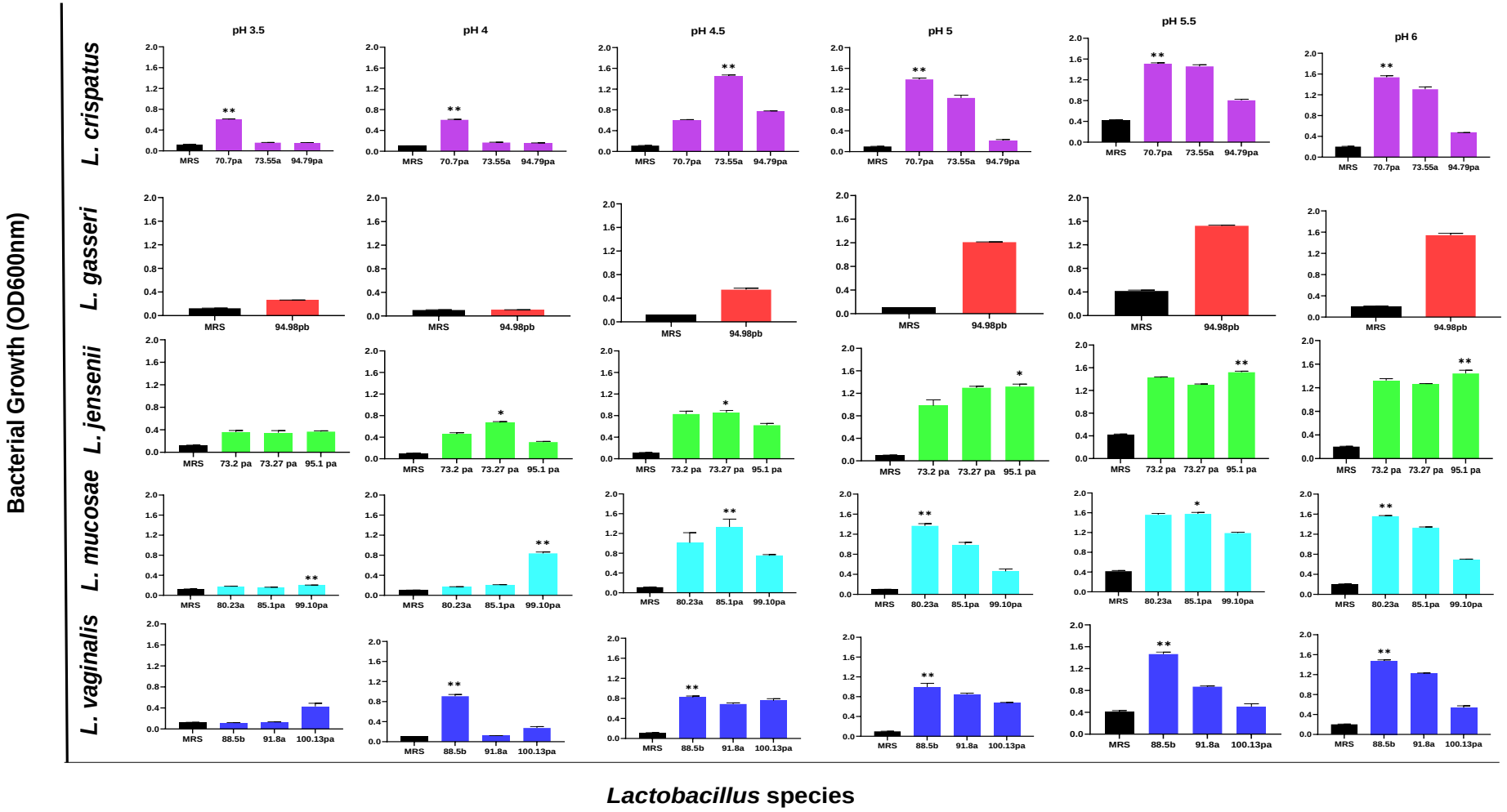

Figure 3.4: *Lactobacilli* Growth under varying pH conditions. The growth of *L. crispatus* (purple), *L. gasseri* (red), *L. jensenii* (green), *L. mucosae* (light blue), and *L. vaginalis* (dark blue) at various adjusted pH levels in comparison to the pH unadjusted MRS control (black) was measured by recording microplate readings (n=3) at OD_{600nm} after 48 hours of anaerobic incubation at 37˚C. Various pH levels of bacterial cultures were achieved by adjusting MRS pH with either Sodium hydroxide (NaOH) or HCL. The pH was measured with a calibrated pH meter. The error bars represent mean of the microplate reading (n=3) at OD600nm with Standard deviation. The Kruskal Wallis test was used to conduct non-parametric multiple comparisons. Unadjusted *p<0.05, **p<0.01 values were reported.

3.5 Evaluating the effect of *Lactobacilli* on pH level of culture medium.

The potential of *Lactobacillus* isolates to lower the culture pH was investigated. The ability of each strain to reduce the pH of the MRS cultures was comparable after 48 hours of anaerobic incubation. Overall, the vaginal *Lactobacillus* strains varied in their ability to lower pH. However, all strains significantly reduced the culture pH after 48 hours of incubation compared to the MRS control (Figure 3.5). This reduction was particularly notable for *L. crispatus* (70.7pa), *L. jensenii* (73.2pa), *L. mucosae* (99.10pa), and *L. vaginalis* (100.13pa) with p-values of 0.0067, 0.0067, 0.0067, and 0.0066, respectively (Figure 3.5). According to the kinetics data, a noticeable decrease in pH began after 24 hours of incubation (Appendix B, Figure B1).

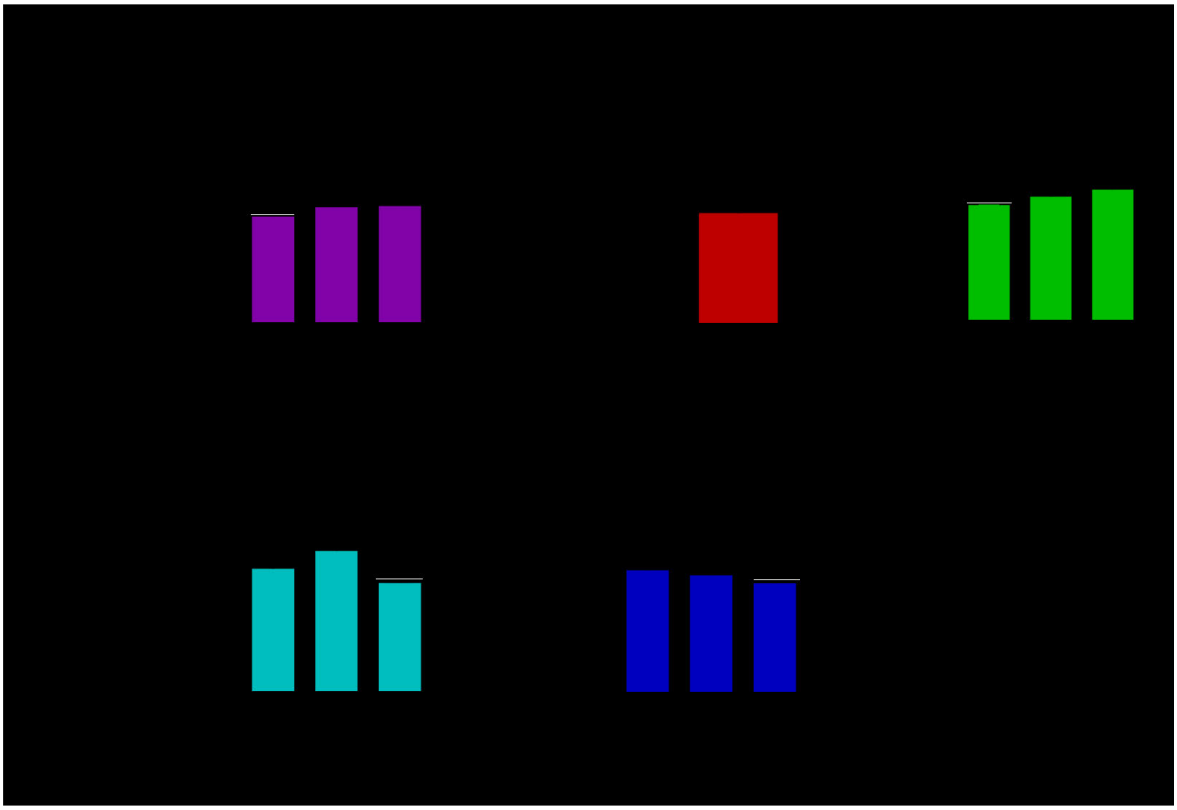

Figure 3.5: Ability of *Lactobacillus* species to lower Culture pH. The pH of *L. crispatus* (70.7 pa, 73.55 a, 94.79 pa), *L. gasseri* (94.98 pb), *L. mucosae* (80.23 a, 85.1 pa, 99.10 pa), *L. jensenii* (73.2 pa, 73.27 pa, 95.1 pa), and *L. vaginalis* (88.58 b, 91.8 a, 100.13 pa) MRS cultures were measured at 48 hours after anaerobic incubation at 37˚C. The control was MRS medium only. The error bars represent mean of the pH meter readings (n=3) with standard deviation. Statistical comparisons were done using multiple comparison adjustment from Kruskal-Wallis one-way ANOVA unpaired test to compare more than 3 groups and Mann Whitney test was used to compare *L. gasseri* with the MRS control. P- value of ≤0.05 indicated by ** was considered significant.

3.6 Lactic Acid Production by *Lactobacilli*

Since *Lactobacillus* strains varied in their ability to lower pH (Section 3.5), lactic acid production, known to protect against pathogens, is likely responsible. Therefore, in this study, the ability of *Lactobacillus* strains to produce lactic acid under anaerobic conditions was assessed. All *Lactobacillus* strains produced higher D-lactate levels than the MRS control, except *L. gasseri* (94.98pb) and *L. mucosae* (99.10). D-lactate levels produced by the strains varied from 0.7 to 2.9 ng/µL. Significant D-lactate production was observed in *L. crispatus* 94.79pa ($p = 0.0429$) and *L. vaginalis* 91.8a ($p = 0.0143$) compared to the MRS control (Figure 3.6A). The optimum time for D-lactate production was 48 hours, corresponding to the strains' optimum growth time. All three strains of *L. crispatus* (94.79pa, 70.7pa, and 73.55a) and *L. vaginalis* (91.8a) produced the most D-lactate after 48 hours compared to the MRS control.

The L-lactate levels produced by the *Lactobacillus* strains were generally low, ranging from 0.09 to 0.13 mM (Figure 3.6B). As with D-lactate, *L. gasseri* (94.98pb) did not produce measurable L-lactate compared to the MRS control. Among the *L. jensenii* species, only the 73.27pa strain produced measurable L-lactate ($p = 0.0429$) compared to the MRS control (Figure 3.6B). At 48 hours, *L. vaginalis* strains produced the most L-lactate overall (88.5b; $p = 0.0237$, 100.13pa; $p = 0.05901$), followed by *L. mucosae* (85.1pa; $p = 0.0742$). While *L. crispatus* was better at producing L-lactate than *L. gasseri*, the L-lactate production was insignificant compared to the MRS.

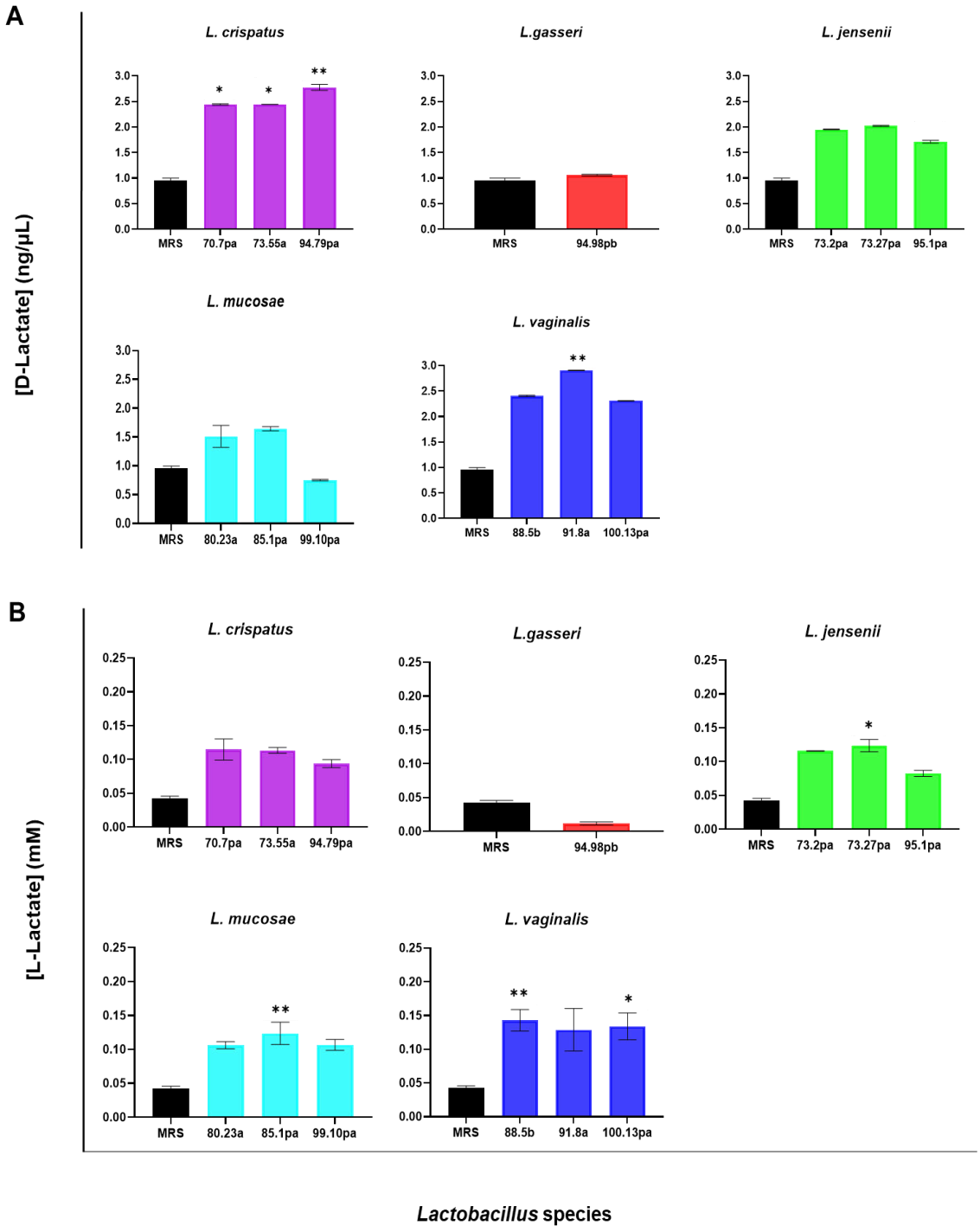

Figure 3.6: Production of lactic acid by *Lactobacillus* species. Overnight cultures of *Lactobacillus* isolates were incubated under anaerobic conditions for 48 hours. D- lactate (A) was measured at 450nm and L- lactate (B) at 565nm. L- lactate (mM) D- lactate (ng/µL) concentrations were measured with reference to the assay standard curves. The error bars represent mean of the microplate readings (n=3) at OD_{450nm} for D- lactate and OD_{565nm} for L- lactate with standard deviation. After adjusting for numerous comparisons with the Kruskal- Wallis Dunn's test, the level of significance was shown as * indicating p≤0.05.

3.7 Hydrogen Peroxide Production

The hydrogen peroxide produced by *Lactobacilli* species may help prevent pathogen growth and enhance their ability to produce lactic acid. The concentration of H_2O_2 produced by each vaginal strain was determined, varying from 250 to 450 µMol/L (Figure 3.7). The H_2O_2 concentration was significantly higher in *L. crispatus* 94.79pa (p = 0.0067), *L. jensenii* 73.27pa (p = 0.0139), *L. mucosae* 80.23a (p = 0.0067), and *L. vaginalis* 88.58b (p = 0.0097) compared to the MRS control (Figure 3.7).

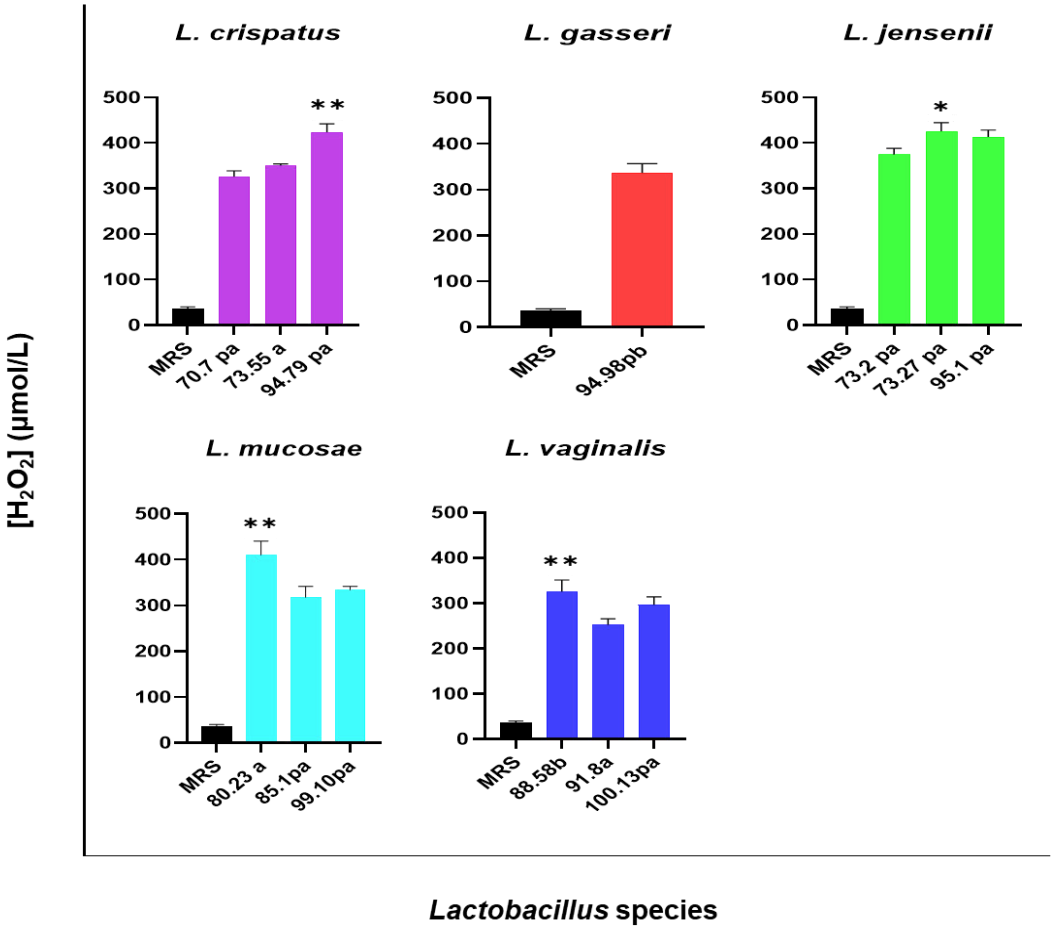

Figure 3.7: Production of Hydrogen peroxide by vaginal *Lactobacillus* isolates. Overnight cultures of *L. crispatus* (purple), *L. gasseri* (red), *L. jensenii* (green), *L. mucosae* (light blue), and *L. vaginalis* (dark blue) were prepared and incubated for 48 hours under anaerobic conditions. The H_2O_2 (µmol/L) produced by the strains was measured at 595 nm. Concentrations of H_2O_2 produced by the isolates were calculated with reference to the H_2O_2 assay standard curve. The error bars represent mean of the microplate readings (n=2) at OD_{595nm} with standard deviation. After adjusting for numerous comparisons with the Kruskal- Wallis Dunn's test, P values were reported, * representing p ≤0.05 and ** representing p ≤0.01.

3.8 Summary data comparing characteristics of *Lactobacillus* species.

After adjusting for multiple comparisons using the non-parametric Kruskal-Wallis's test (Figure 3.8; Table 3.1), the significance of each isolate's ability to reduce pH and produce D-Lactate, L-lactate, and H_2O_2 was compared and reported. Overall, different *Lactobacillus* species were able to reduce pH, particularly *L. crispatus*, *L. gasseri*, *L. jensenii*, and *L. mucosae*. *Lactobacillus crispatus* 70.7pa strongly reduced pH, followed by the mildly pH-reducing *L. gasseri* 94.98pb, *L. jensenii* 95.1pa, and *L. mucosae* 85.1pa. *Lactobacillus jensenii* 73.27 and *L. mucosae* 99.10 also reduced pH, albeit somewhat. Regarding D-lactate production, fewer species were involved, mainly *L. crispatus* and *L. vaginalis*. *Lactobacillus crispatus* 94.79pa produced the highest level of D-lactate, followed by *L. crispatus* 70.7pa, 73.55a, and *L. vaginalis* 88.5b. Although lower than these, the production of D-lactate by *L. gasseri* 94.98pb and *L. mucosae* 85.1 pa and 99.10pa was still significant. Fewer species produced L-lactate, mainly *L. gasseri* 94.98pb, *L. jensenii* 95.1pa, and *L. crispatus* 94.79pa (Figure 3.8). Many *Lactobacillus* species produced H_2O_2, with *L. crispatus* 94.79pa, *L. jensenii* 73.27pa, and *L. mucosae* 80.23a producing the highest amounts. These were followed by *L. crispatus* 70.7pa, *L. jensenii* 73.2pa, and *L. vaginalis* 88.5b. Lastly, *L. crispatus* 73.55a produced significant amounts of H_2O_2, with *L. vaginalis* (91.8a and 100.13pa) and *L. gasseri* producing the least H_2O_2.

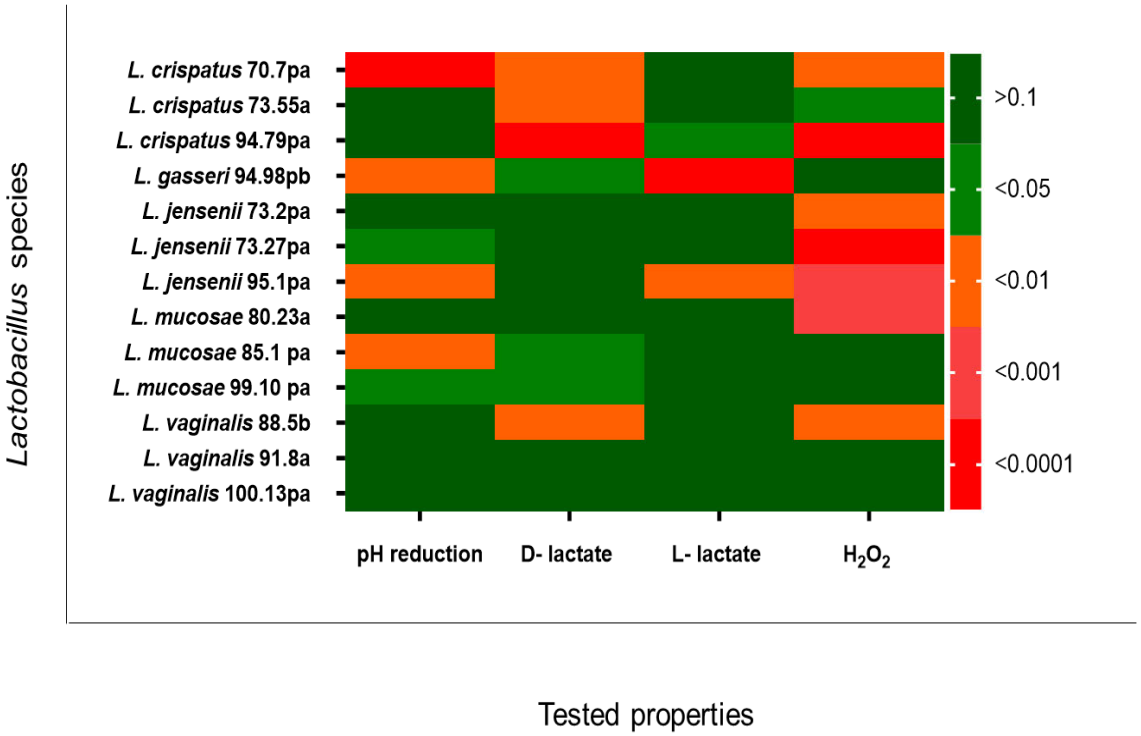

Figure 3.8: Summary of *Lactobacillus* isolates properties. The ability to reduce pH, produce D-lactate, L- lactate and H_2O_2 was tested in vaginal *Lactobacillus* species (*L. crispatus, L gasseri, L jensenii, L. mucosae* and *L. vaginalis*). The Kruskal Wallis test was used to conduct non-parametric multiple comparisons. After adjusting for numerous comparisons, P- values were reported. The level of significance was shown as p> 0.01(Dark green), p< 0.05 (Light green), p< 0.01 (Orange), p< 0.001(Pink), and p< 0.0001 (Red). The heat map was generated in Graph Pad Prism 8.4.3.

3.9 *Gardnerella vaginalis* Growth Kinetics

The growth kinetics of the *G. vaginalis* strain were observed over 96 hours to determine its optimum growth time and suitable growth conditions. After the initial 3-hour anaerobic incubation period, *G. vaginalis* continued to grow and entered an exponential growth phase, which lasted for 24 hours (Figure 3.9). After 24 hours, the growth rate began to decelerate gradually. This decrease in cell numbers continued at 48 and 72 hours until the cells reached the death phase at 96 hours, where a portion of the cells lost their viability. The OD600 nm for BHI alone (used as a negative control) was relatively low compared to that of *G. vaginalis*.

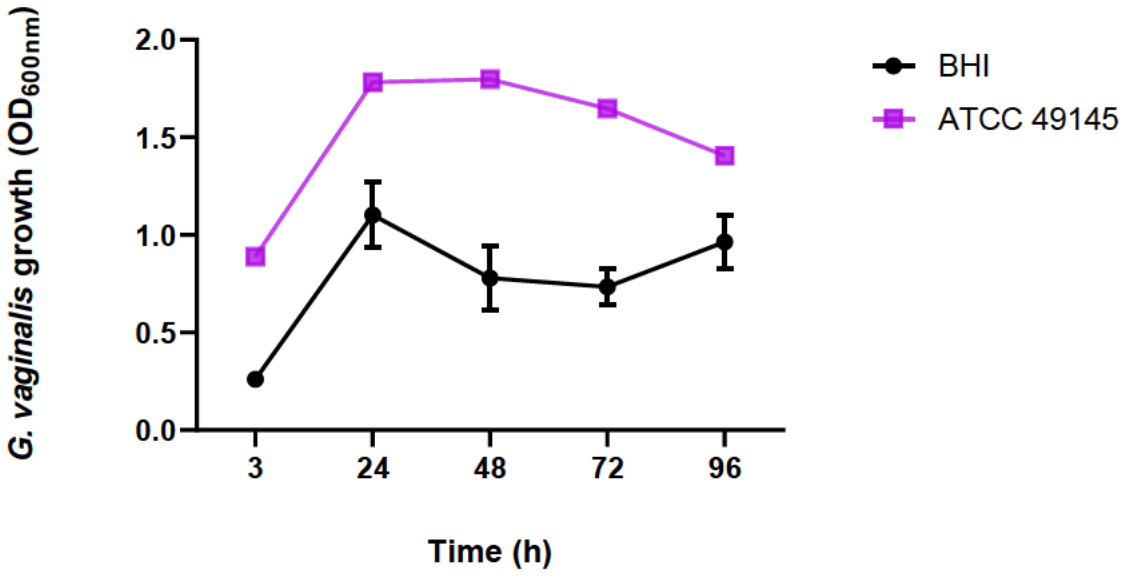

Figure 3.9: Growth kinetics of *Gardnerella vaginalis* over time. *Gardnerella vaginalis* was grown in BHI under anaerobic conditions for 96 hours in a 5% CO_2 incubator. Growth kinetics was measured at 3, 24, 48, 72 and 96 hours post incubation at OD_{600nm}. The control was BHI. Error bars represent the mean and standard error. Experiments was done in triplicates (n=3).

3.10 Antimicrobial Activity of *Lactobacillus* Culture Supernatants against *Gardnerella vaginalis*

Bacterial infections are currently treated with antimicrobial drugs such as metronidazole and clindamycin. However, their ability to cure prevalent BV infections is threatened by the emergence of antibiotic-resistant microorganisms and the recurrence of BV (Javed *et al.*, 2019). The potential of *Lactobacillus* strains to produce lactic acid, H_2O_2, and decrease vaginal pH is one of the many mechanisms behind their antibacterial activity against BV bacteria. The antibacterial activity of the isolates was determined using the *G. vaginalis* growth inhibition assay as previously described (Happel *et al.*, 2020; Jang *et al.*, 2019) with minor modifications. *Gardnerella vaginalis* cells were stimulated with LCS from the vaginal *Lactobacillus* isolates to investigate their ability to grow in the presence of the LCS. *Gardnerella vaginalis* growth was inhibited by the LCS of *L. vaginalis* (91.8a and 100.13a), *L. crispatus* (70.7pa and 73.55a), and *L. gasseri* (Figure 3.10). Compared to the other *L. crispatus* strains, 94.79pa did not significantly suppress the development of *G. vaginalis*; however, it inhibited growth by up to 88% compared to the MRS control (100%) and LCS from other *Lactobacillus* species.

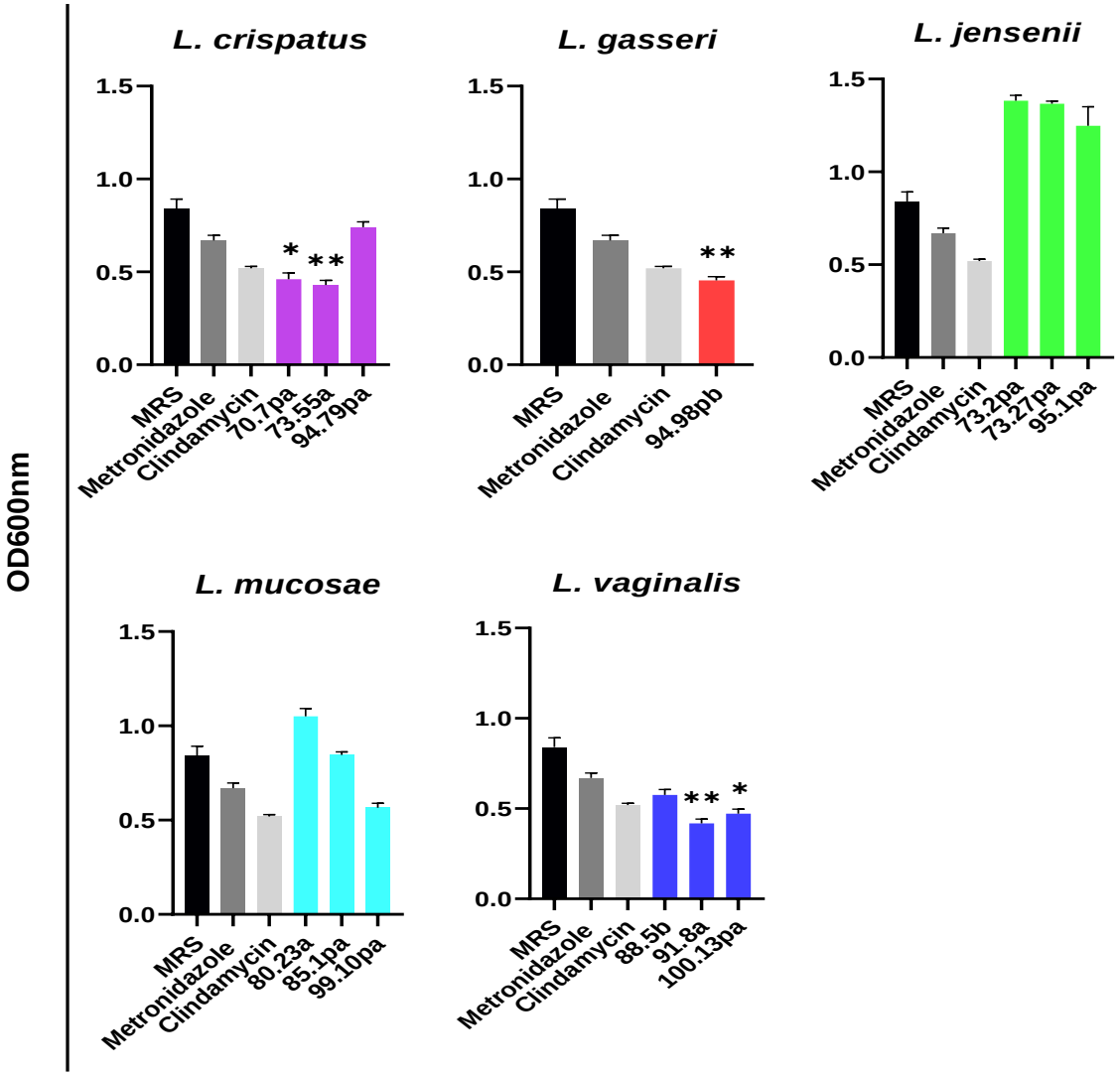

Figure 3.10: Antimicrobial activity of cell-free *Lactobacillus* Culture Supernatants against *Gardnerella vaginalis*. The growth (OD600nm) of *G. vaginalis* was measured after 48 hours of stimulation with LCS from the 13 vaginal *Lactobacillus i*solates under anaerobic conditions at 37˚C. MRS is the negative control. Antifungal agents (Clotrimazole and Bifonazole) were used as positive controls. Data was plotted as average triplicate values. After adjusting for numerous comparisons with the Kruskal- Wallis non-parametric test, P<0.05 was reported and the significance of the results is compared to the MRS. Bars represent mean with standard deviation.

Compared to MRS alone, *G. vaginalis* growth decreased to 60% in the presence of clindamycin and to 65% in the presence of metronidazole. *Lactobacillus gasseri*, *L. crispatus* (73.55a), and *L. vaginalis* (91.8a) inhibited the growth of *G. vaginalis* to about 52%, followed by 56% inhibition by *L. vaginalis* (100.13pa) and *L. mucosae* (99.10pa) (Figure 3.11). On the other hand, all *L. jensenii* strains and *L. mucosae* (except for 99.10pa) did not inhibit the growth of *G. vaginalis*, with growth levels above 100%. Although the inhibition by *L. crispatus* 94.79pa was not statistically significant compared to MRS alone, the LCS from *L. crispatus* 94.79pa, *L. gasseri*, and *L. vaginalis* 91.8a showed the best inhibitory action against *G. vaginalis*.

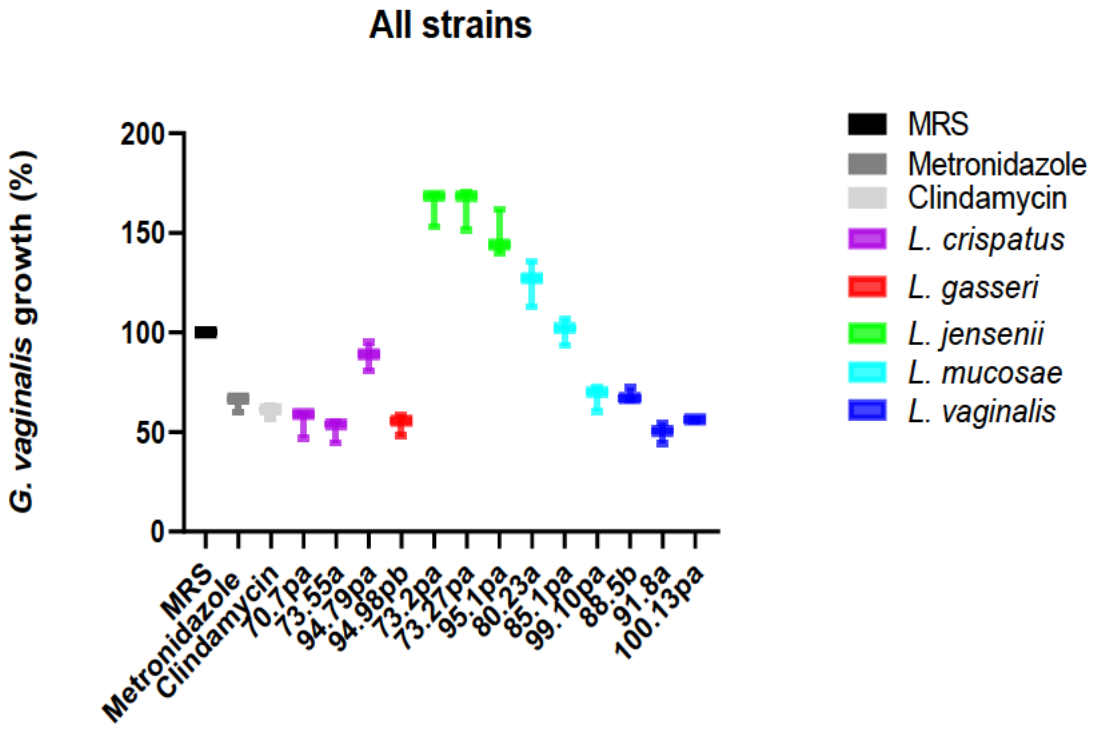

Figure 3.11: Growth inhibition of *Gardnerella vaginalis* by *Lactobacillus* Culture Supernatants. The OD_{600nm} values were used to calculate the growth inhibition rate (%) calculated as [(OD_{MRS}-OD_{LCS})/OD_{MRS} x 100)]. Data is plotted as average triplicate values. Bars represent minimum to maximum.

3.11 *Candida albicans* Growth Kinetics

The growth of the fungal strains was observed over time before starting the antifungal activity tests of *Lactobacillus* strains. When grown aerobically, both fungal strains exhibited similar growth kinetics. However, strain ATCC 60193 generally grew more favourably than strain ATCC 10231 (Figure 3.12). YPED alone was used as a negative control and showed minimal detectable growth, as predicted. Both strains developed exponentially up to 24 hours; after that, the growth of strain ATCC 10231 was reduced. At 48 hours, ATCC 60193 reached its peak growth, after which it began to decline, continuing this trend for 96 hours, similar to ATCC 10231.

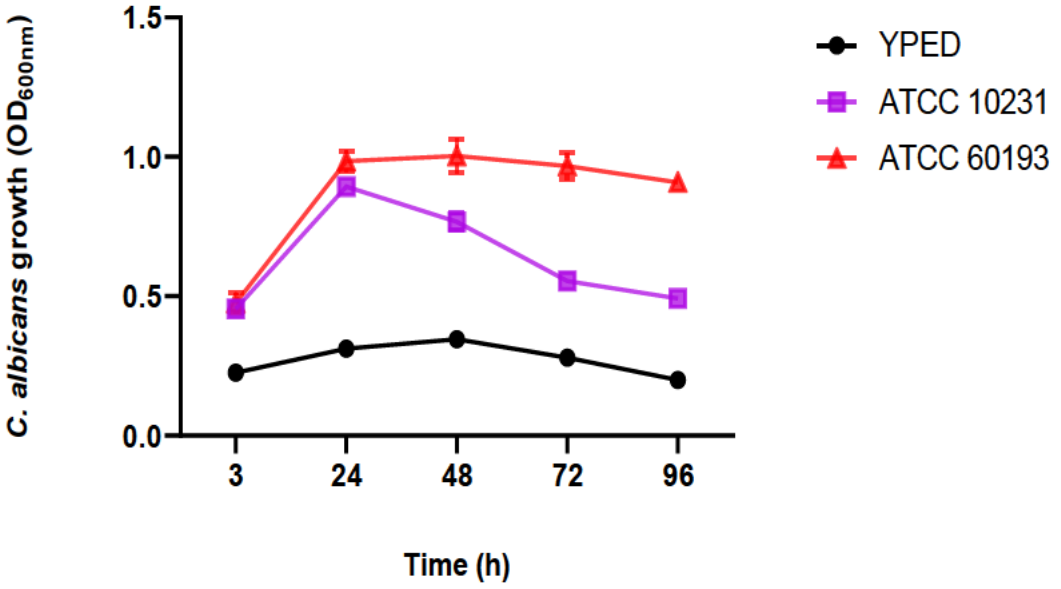

Figure 3.12: Growth kinetics of *Candida albicans* over time. *Candida albicans* ATCC 10231(red) and ATCC 60193 (purple) were grown in YEPD medium under aerobic conditions for 96 hours at 30˚C. Growth was measured at 3, 24, 48, 72 and 96 hours. YPED media (Black) was grown under the same conditions and used as a negative control. Error bars represent mean and standard error (n=3).

3.12 *Candida albicans* Growth Inhibition Assay

Antifungal medications may not be as effective as before on *Candida* infections, making treatment challenging. The current treatment for *Candida* overgrowth involves administering an antifungal medication, such as fluconazole, clotrimazole, or bifonazole (Yano *et al.*, 2010). The potential of LCS harvested from the *Lactobacillus* isolates to inhibit the growth of *C. albicans* was investigated using the previously described *Candida albicans* growth inhibition assay (Jang *et al.*, 2019). The growth of fungal cells stimulated with cell-free LCS was measured after incubating under suitable conditions for *C. albicans* growth. The LCS considerably lowered the growth of *C. albicans* (ATCC 10231) compared to the ATCC 60193 strain (Figure 3.13). Inhibition appeared to be more *C. albicans* strain-specific, as almost all LCS inhibited ATCC 10231, while only a few inhibited ATCC 60193. The ATCC 10231 strain reached its optimal growth at 24 hours (Figure 3.12; Purple), slightly behind ATCC 60193, and the cells rapidly slowed down after 24 hours. In contrast, ATCC 60193 grew more quickly than the 10231 strain and reached its optimum growth at 48 hours (Figure 3.12; Pink).

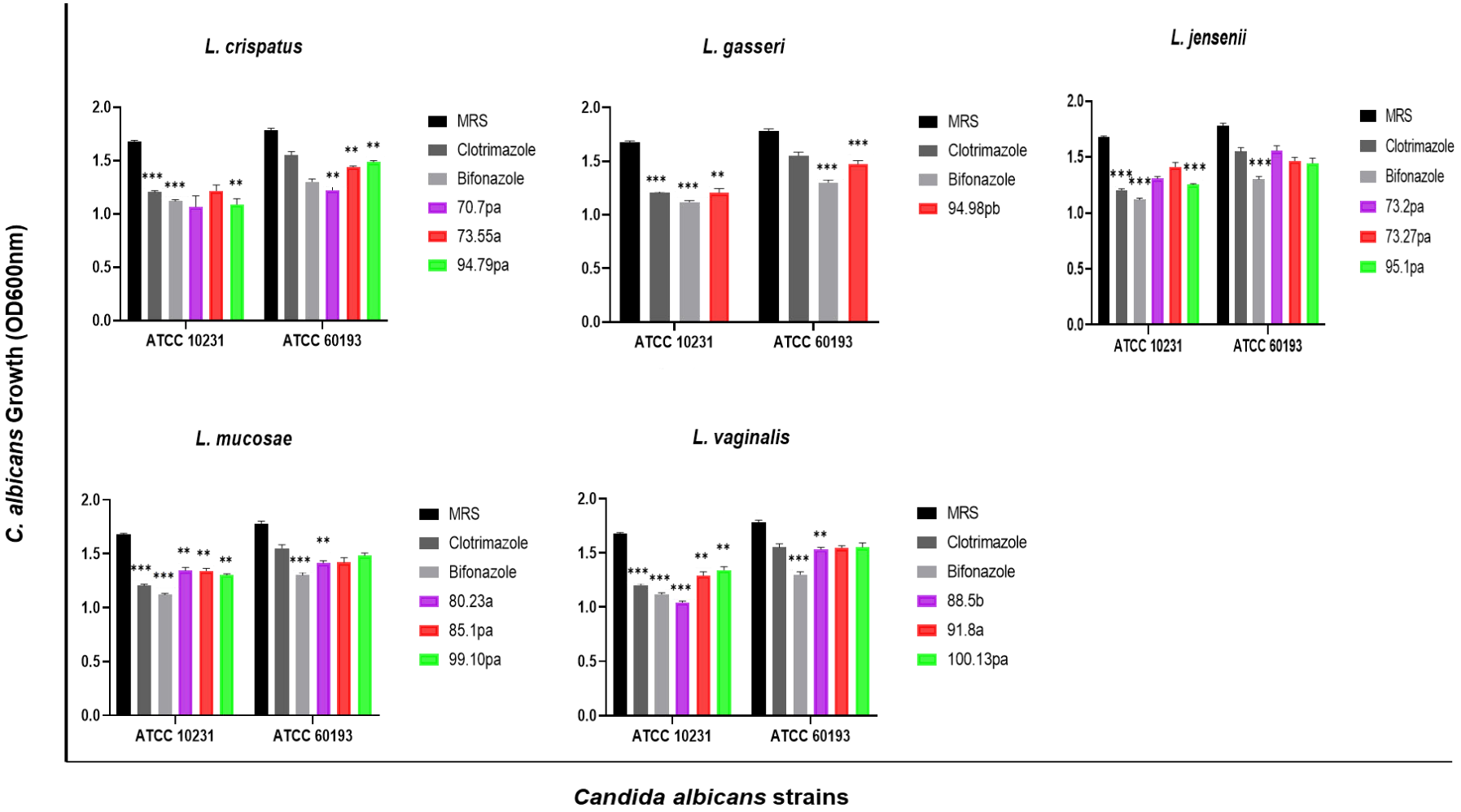

Figure 3.13: Antifungal activity of cell-free *Lactobacillus* Culture Supernatants against *Candida albicans*. The growth of *C. albicans* was measured 24 hours post stimulation with LCS from all 13 vaginal *Lactobacillus* strains. The stimulations were incubated under aerobic conditions at 30˚C. Clotrimazole, Bifonazole, and MRS were the controls. Each bar shows the mean and standard deviation for each strain. After adjusting for numerous comparisons with the Kruskal- Wallis non-parametric test, P<0.05 was reported and the significance of the results was compared to the MRS.

The LCS-free MRS was used as a negative control, while antifungal drugs, including clotrimazole and bifonazole, served as positive controls. The growth of the ATCC 10231 strain was inhibited by antifungal drugs to some degree, with growth reduced to 65-75%, surpassing the inhibition seen with other LCS strains (Figure 3.14). The ability of vaginal strain LCS to inhibit the growth of *C. albicans* varied across strains and did not follow a consistent pattern within the corresponding species. Based on activity level and unadjusted p-values, the antifungal activities of the 13 LCSs were assessed and compared (Figure 3.15). Overall, all LCS exhibited varied inhibitory effects on *C. albicans*. The *Lactobacillus* culture supernatant of *L. crispatus* (70.7pa; 35% inhibition), *L. gasseri*, and *L. vaginalis* (88.5b; 35% inhibition) slightly affected the growth of ATCC 10231. LCS from *L. gasseri* strongly inhibited the growth of ATCC 60193. In the case of ATCC 60193, *L. mucosae* (8.23a), *L. vaginalis* (100.13pa), and all three *L. crispatus* LCS moderately inhibited the growth of *C. albicans*. No noticeable antifungal activity was observed when the ATCC 60193 strain was tested against *L. jensenii*, *L. mucosae* (85.1pa and 99.10pa), and *L. vaginalis* (100.13pa, 91.8a). Unlike the ATCC 10231 strain, the ATCC 60193 strain appeared resistant to most LCS.

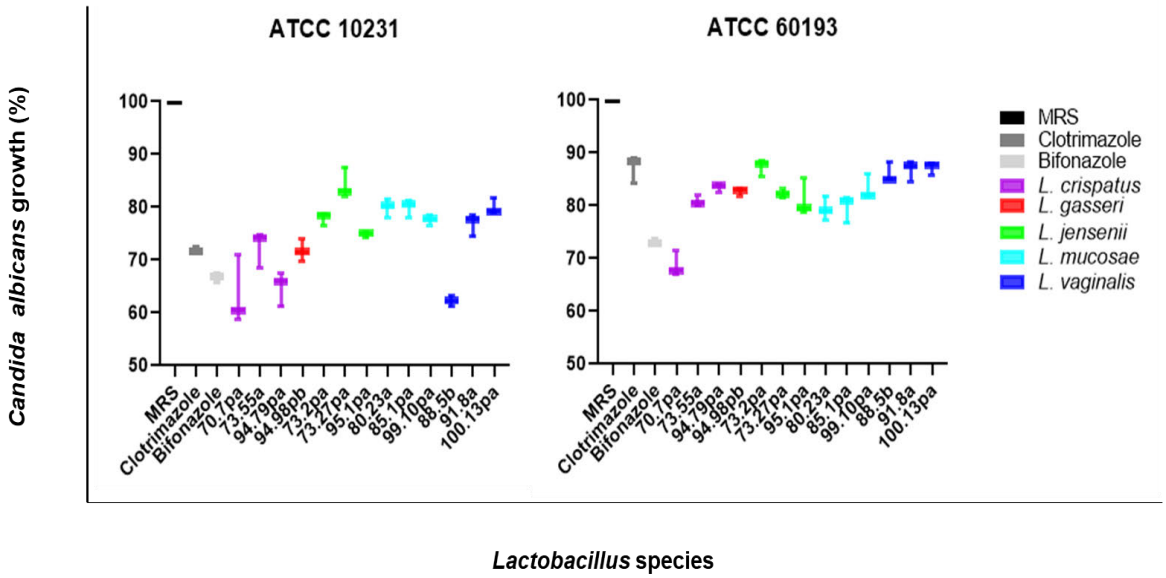

Figure 3.14: Growth inhibition of *Candida albicans* by *Lactobacillus* Culture Supernatants. The growth inhibition (%) of *C. albicans* was calculated as [(OD_{MRS}-OD_{LCS})/OD_{MRS} x 100)]. Data is plotted as average triplicate values. Bars represent minimum to maximum.

3.13 Summary data comparing the ability of *Lactobacillus* species to inhibit bacterial and fungal infections.

In summary, *G. vaginalis* was significantly inhibited by a few *Lactobacillus* strains, mainly *L. crispatus* (73.55a; p<0.01), *L. gasseri* (94.98pb; p<0.01), and *L. vaginalis* (91.8a; p<0.01),

followed by *L. crispatus* (70.7pa; p<0.05) and *L. vaginalis* (100.13pa; p<0.05). *Candida albicans* ATCC 10231 was significantly inhibited by most *Lactobacillus* strains, especially *L. jensenii* (95.1pa; p<0.001), followed by *L. crispatus* (94.79pa; p<0.01), *L. gasseri* (94.98pb; p<0.01), *L. mucosae* (80.23a; p<0.01), *L. mucosae* (85.1pa; p<0.01), *L. mucosae* (99.10pa; p<0.01), *L. vaginalis* (88.5b; p<0.01), and *L. vaginalis* (91.8a; p<0.01) (Figure 3.15). *Candida albicans* ATCC 60193 was significantly inhibited by a fair number of *Lactobacillus* strains, mainly *L. gasseri* (94.98pb; p<0.001), followed by *L. crispatus* (70.7pa; p<0.01), *L. crispatus* (73.55a; p<0.01), *L. crispatus* (94.79pa; p<0.01), *L. mucosae* (80.23a; p<0.01), and *L. vaginalis* (88.5b; p<0.01) (Table 3.1; Figure 3.15). *Lactobacillus gasseri* demonstrated strong antifungal activity against the tested *C. albicans* infections and the BV-associated *G. vaginalis*. *Lactobacillus crispatus* (94.79pa) performed well in inhibiting *Candida albicans* infection, followed by *L. mucosae* (80.23a). The ability of these species to inhibit infections was superior to that of the MRS (without LCS) and the currently used bacterial and antifungal agents (Table 3.1).

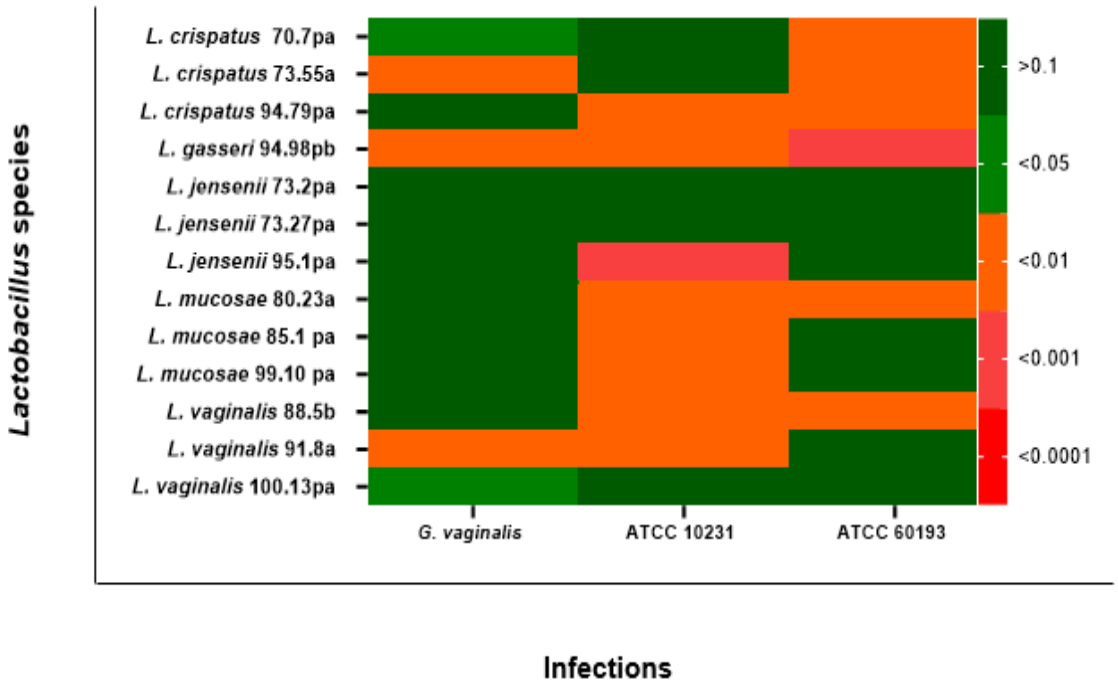

Figure 3.15: Summary of the Growth inhibition activity of *Lactobacillus* Culture Supernatants against *Gardnerella vaginalis* and *Candida albicans*. The ability to reduce pH, produce D-Lactate, L-Lactate, and H_2O_2 was tested in vaginal *Lactobacillus* species (*L. crispatus, L gasseri, L jensenii, L. mucosae, and L. vaginalis*). The Kruskal Wallis test was used to conduct non-parametric multiple comparisons. After adjusting for numerous comparisons, p- values were reported. The level of significance was shown as p> 0.01(Dark green), p< 0.05 (Light green), p< 0.01 (Orange), p< 0.001(Pink), and p< 0.0001 (Red). The heat map was generated in Graph Pad Prism 8.4.3

3.14 Summary data for the metabolites produced by *Lactobacillus* species and their inhibition ability against vaginal microbial infections.

Table 3.1 summarises the production of antimicrobial substances and inhibition of microbes by vaginal *Lactobacillus* species. *Lactobacillus gasseri* ranked the highest in displaying probiotic properties, followed by all strains of *L. crispatus*. The probiotic effectiveness of *Lactobacillus jensenii* and *L. vaginalis* was strain-dependent, with *L. jensenii* (95.1pa) and *L. vaginalis* (88.5b) performing well, following the *L. crispatus* strains. All *L. mucosae* strains were next in effectiveness. The remaining *L. jensenii* (73.2pa, 73.27pa) and *L. vaginalis* (91.8a and 100.13pa) strains displayed the least probiotic properties.

Table 3.1: Summary of the production of antimicrobial substances by vaginal *Lactobacillus* species and the inhibition of *Gardnerella vaginalis* and *Candida albicans* infections by the species.

		Significance						
***Lactobacillus* species**	**Strains**	**pH reduction**	**D-Lactate production**	**L-Lactate production**	**H_2O_2 Production**	***G. vaginalis* growth inhibition**	***C. albicans* ATCC 10231 growth inhibition**	***C. albicans* ATCC 60193 growth inhibition**
L. crispatus	70.7 pa	Yes	Yes	No	Yes	Yes	No	Yes
	73.55 a	No	Yes	No	Yes	Yes	No	Yes
	94.79 pa	No	Yes	Yes	Yes	No	Yes	Yes
L. gasseri	94.98 pb	Yes	Yes	Yes	No	Yes	Yes	Yes
L. jensenii	73.2 pa	No	No	No	Yes	No	No	No
	73.27 pa	Yes	No	No	Yes	No	No	No
	95.1 pa	Yes	No	Yes	Yes	No	Yes	No
L. mucosae	80.23 a	No	No	No	Yes	No	Yes	Yes
	85.1 pa	Yes	Yes	No	No	No	Yes	No
	99.10 pa	Yes	Yes	No	No	No	Yes	No
L. vaginalis	88.5 b	No	Yes	No	Yes	No	Yes	Yes
	91.8 a	No	No	No	No	Yes	Yes	No
	100.13 pa	No	No	No	No	Yes	No	No

The Kruskal Wallis test was used to conduct non-parametric multiple comparisons. After adjusting for numerous comparisons between each strain, p- values were reported and indicated by Yes ($p<0.05$; highlighted in red) or No ($P>0.05$).

Chapter 4

General Discussion and Conclusion

4.1 General Discussion

Lactobacillus strains possess beneficial properties that can prevent viral, bacterial, and fungal infections, and they can also complement existing antibacterial and antifungal therapies (Happel *et al.*, 2020; Jang *et al.*, 2019). Current treatments for bacterial vaginosis (BV) with antibacterial medications often cause side effects such as gastrointestinal issues, antibiotic resistance, and infection recurrence. Similarly, antifungal ointments applied to the skin can lead to increased itching or irritation, and oral antifungals can negatively impact gut health, particularly in pregnant women (Bookstaver *et al.*, 2015). Additionally, oral antifungal treatments pose significant concerns regarding drug-drug interactions, potentially causing severe side effects like migraines, light-headedness, diarrhoea, and nausea (Xie *et al.*, 2017). These challenges underscore the urgent need for alternative treatments for vaginal infections.

Few studies have focused on antimicrobial regulators present in the female reproductive tract. This study investigated the probiotic features of various African-derived vaginal *Lactobacillus* species from healthy women, including *L. crispatus*, *L. gasseri*, *L. jensenii*, *L. mucosae*, and *L. vaginalis*. The research measured their effects on the growth of harmful microbes (*G. vaginalis* and *C. albicans*) and their production of antimicrobial substances (lactic acid and H_2O_2).

Among the tested strains, *L. gasseri* emerged as the most effective at producing antimicrobials and managing *G. vaginalis* and both *C. albicans* infections, followed closely by strains of *L. crispatus*, *L. jensenii*, *L. mucosae*, and *L. vaginalis*. This indicates that while *Lactobacillus* species have potential as antimicrobials, their probiotic properties are species- and strain-dependent. A critical factor in determining the potential of these isolates as probiotics for treating vaginal infections is their ability to grow in acidic pH conditions (O'Hanlon *et al.*, 2013). Contrary to popular belief, the lactate produced by *Lactobacillus* species provides women with robust protection against pathogens. A recent study demonstrated the potential of isolated vaginal strains of *L. crispatus* and *L. gasseri* from reproductive-age Indian women to be used as probiotics for treating *C. albicans*, *G. vaginalis*, *E. coli*, and *Proteus mirabilis*. These strains

showed resistance to acidic conditions and were effective in minimising pathogen growth, thus protecting the vaginal environment (Ahire *et al.*, 2023).

Our findings showed that *L. gasseri* had the maximum inhibition zone against *G. vaginalis*, aligning with previous research that highlights its remarkable ability to produce more L-lactic acid and significant amounts of D-lactate, gradually lowering the culture pH over time. Acidic pH conditions are ideal for maintaining a healthy vaginal microbiome (Nayak and Nayak, 2019). All *Lactobacilli* species in this study lowered the pH of MRS cultures to some extent, with *L. crispatus* demonstrating greater activity than the other strains, and surpassing *L. gasseri*, *L. mucosae*, *L. jensenii*, and *L. vaginalis*. Some vaginal *Lactobacillus* species in this study could lower the pH over time but produced lower levels of lactic acid. Other studies suggest a relationship between pH and lactic acid production, with the highest levels of lactic acid produced at an acidic pH of approximately 6.0, which could help some strains release more lactic acid (O'Hanlon *et al.*, 2019). A healthy vaginal environment typically has a pH of around 4.5, maintained by lactic acid produced by *Lactobacilli* through glycogen breakdown (Tester and Al-Ghazzewi, 2018).

Interestingly, the isolates in this study produced more L-lactic acid than D-lactic acid, with *L. crispatus* and *L. gasseri* producing significant amounts of D-lactate and L-lactate, respectively. Only the 95.1pa strain of *L. jensenii* produced detectable amounts of D-lactate, validating a recent study that demonstrated that strains of *L. jensenii* frequently produce D-lactate (Witkin *et al.*, 2013). The production of D- and L-lactic acid by various *Lactobacillus* species varies significantly, suggesting that the concentration of D- and L-lactate in the vaginal environment may depend on the prevalent species of *Lactobacillus*.

High quantities of *Lactobacilli* are frequently associated with a healthy vaginal environment (Mancabelli *et al.*, 2021). The acidic environment created by lactic acid is thought to inhibit the growth of unwanted pathogens, thus reducing the likelihood of bacterial population changes that cause infections. However, there is debate about the relative significance of lactic acid in inhibiting infections, such as Candida fungus, in the reproductive system. Some suggest it is only effective in high doses (Zangl *et al.*, 2020).

Previous research has found that pregnant women with high quantities of H_2O_2-producing *L. jensenii* and *L. vaginalis* have a lower chance of acquiring BV infection (Salinas *et al.*, 2018).

Thus, H_2O_2 production is considered a critical feature of potential probiotics. In this study, *L. crispatus* exhibited higher concentrations of H_2O_2 than *L. jensenii*, *L. mucosae*, and *L. vaginalis*. *L. gasseri* and *L. vaginalis* produced the lowest H_2O_2 levels. Consistent with our findings, previous studies have shown that *L. jensenii* strains produce persistent and efficient H_2O_2, while production varies among *L. crispatus* and *L. gasseri* strains (Martín and Suárez, 2010). It has been discovered that *G. vaginalis* is susceptible to bacteria producing high quantities of H_2O_2, which may help control the diversity of bacteria in the vagina (Klebanoff *et al.*, 1991). This control may prevent pathogens from invading and colonising the vaginal microbiome, potentially reducing recurrent infections (Gupta *et al.*, 1998).

The LCS from *L. vaginalis*, *L. crispatus*, and *L. gasseri* generally exhibited the highest inhibitory activity against *G. vaginalis* at 24 to 48 hours. However, the inhibition of *G. vaginalis* varied significantly between species and strains. The best inhibitory action (approximately 50%) was observed with *L. gasseri*, *L. crispatus*, *L. vaginalis*, and *L. mucosae*. Conversely, *L. jensenii* and *L. mucosae* generally did not inhibit *G. vaginalis*. Notably, none of the tested strains produced substantial amounts of H_2O_2, which has been associated with the antibacterial activity of most healthy vaginal bacteria. *L. jensenii* is typically part of the healthy vaginal microbiome, where it contributes to maintaining a low pH environment that inhibits the growth of harmful bacteria, including *Gardnerella vaginalis*. However, an unexpected promotion of *G. vaginalis* growth in the presence of *L. jensenii* was observed. This could be explained by the fact that *L. jensenii* strains used in this study produced less lactic acid and other antimicrobial compounds, leading to a less hostile environment for *G. vaginalis*. Previous studies have shown that bacteria associated with BV can be inhibited by lactic acid rather than H_2O_2. It was also found that certain factors in cervicovaginal fluid and semen could block the microbicidal activity of H_2O_2 (O'Hanlon *et al.*, 2011). Furthermore, a study by Witkin (2015) found that women with vaginal dysbiosis had H_2O_2-producing bacteria in their vaginal microenvironment, suggesting that antimicrobial activity is determined by a combination of inhibitory substances, with H_2O_2 contributing to a certain extent (Witkin, 2015).

Vulvovaginal candidiasis primarily occurs due to infection by *C. albicans*, which is common in women with vaginal microbial dysbiosis (Achkar and Fries, 2010). Previous reports indicate that *C. albicans* growth rates vary significantly depending on the strain type and culture conditions, with growth occurring after just one hour of incubation under ideal conditions (Anand and Prasad, 1991). The antimicrobial activities of *Lactobacillus* strains were examined against *C. albicans* strains. The ATCC 60193 strain seemed resistant to most LCS, while the

ATCC 10231 strain was more prone to inhibition by LCS. The inhibition of *C. albicans* by *Lactobacillus* strains was not necessarily associated with producing antimicrobial compounds. For example, *L. gasseri* (94.98pb) did not produce significant amounts of H_2O_2 but significantly inhibited both tested strains of *C. albicans*.

Conversely, *L. jensenii* (73.27pa) produced significant amounts of H_2O_2 and significantly reduced culture pH but did not inhibit any tested *C. albicans* strains. Interestingly, all *L. crispatus* strains significantly inhibited the growth of the ATCC 60193 strain, whereas all tested *L. jensenii* strains failed to inhibit the ATCC 60193 strain despite producing significant amounts of H_2O_2. These findings align with previous studies showing that *Lactobacillus* strains can inhibit *C. albicans* growth regardless of H_2O_2 production, suggesting they might produce additional antimicrobial compounds (Wang *et al.*, 2017). The vaginal isolate of *L. crispatus* demonstrated a discernible fungicidal action against *C. lusitaniae* and *C. albicans* strains, significantly reducing fungal adhesion *in vitro*, supporting our conclusion that *L. crispatus* is among the strongest antifungal strains (Parolin *et al.*, 2015).

4.2 Study Limitations and future directions.

This investigation did not compare the ability of beneficial and pathogenic bacteria to inhibit infections, as only non-pathogenic strains were used. While a few known antimicrobials of vaginal *Lactobacillus* species were studied, future research should explore others, such as bacteriocins, acetic acid, and short-chain fatty acids. The study relied solely on *in vitro* assays to evaluate the inhibitory activity of *Lactobacillus* strains against vaginal infections. However, this approach has limitations in accurately determining the antibacterial and antifungal abilities of certain *Lactobacillus* species compared to measuring inflammatory cytokines associated with infections in their presence (Manhanzva *et al.*, 2020).

The inflammatory responses to *G. vaginalis* in the presence or absence of *Lactobacillus* culture supernatants (LCS) were not examined, which would further elucidate the role of LCS in reducing genital inflammation. Bacterial adhesion to vaginal tissues, a critical component of their proliferation, was not investigated using *ex vivo* adherence and interference assays. Consequently, this study did not fully explore how vaginal *Lactobacillus* species might prevent HIV.

Additionally, the study did not examine genetic, environmental, and age factors that may contribute to the severity of vaginal infections among diverse African populations. The findings were not compared to commercially available ATCC *Lactobacillus* strains from various countries. Furthermore, only ATCC-derived *G. vaginalis* and *Candida* strains were tested, thereby excluding African-derived strains. Lastly, the molecular mechanisms through which *Lactobacillus* strains inhibit *C. albicans* growth, biofilm formation, and hyphal growth were not explored.

To build on the findings of this study, future research should:

Explore a Wider Range of Antimicrobials: Investigate other antimicrobial compounds produced by vaginal *Lactobacillus* species, such as bacteriocins, acetic acid, and short-chain fatty acids.

Assess Inflammatory Responses: Examine the inflammatory responses to *G. vaginalis* and other pathogens in the presence or absence of LCS, using adherence assays to understand how LCS reduces genital inflammation. Example of this assay is the ELISA and Luminex assay method, where *G.vaginalis* cells will be stimulated with LCS in the presence of VK2 cells to measure the inflammatory response (Inflammatory cytokine produced as biomarkers of vaginal inflammation) in the presence of infection and the LCS cell-free supernatants.

Evaluate Adhesion and Interference: Conduct *ex vivo* adherence and interference assays to assess the ability of *Lactobacillus* strains to adhere to vaginal tissues and interfere with pathogen colonisation, providing insights into their role in preventing HIV and other infections.

Consider Genetic and Environmental Factors: Investigate the genetic, environmental, and age-related factors that influence the severity of vaginal infections across different African populations.

Compare Global Strains: Compare the inhibitory activities of locally derived *Lactobacillus* strains with commercially available ATCC strains from different countries to identify the most effective strains for probiotic development.

Investigate Molecular Mechanisms: Study the molecular mechanisms by which *Lactobacillus* strains inhibit *C. albicans* growth, biofilm formation, and hyphal development to develop targeted probiotic therapies. The formation of biofilm by *C. albicans* will be investigated directly using *in vitro* biofilm assays, and the inhibition of *G. vaginalis* can be investigated using agar well diffusion method in the presence of *Lactobacillus*.

By addressing these areas, future research can provide a more comprehensive understanding of the role of vaginal *Lactobacillus* species in maintaining vaginal health and preventing infections, leading to the development of more effective and targeted probiotic treatments.

4.3 Conclusion

This study underscores the significant potential of African-derived vaginal *Lactobacillus* species as probiotics for preventing and treating vaginal infections. Among the strains tested, *L. gasseri* exhibited the most potent probiotic properties, closely followed by *L. crispatus*. These strains effectively inhibited the growth of pathogenic microbes like *G. vaginalis* and *C. albicans* while producing substantial amounts of lactic acid and hydrogen peroxide (H_2O_2), both critical for maintaining a healthy vaginal environment. The results indicate that the probiotic efficacy of *Lactobacillus* is both species- and strain-dependent, with certain strains of *L. jensenii* and *L. vaginalis* showing less consistent results. The study also confirms the ability of *Lactobacillus* species to thrive in acidic conditions, thereby enhancing their protective role against pathogens. The differential production of D- and L-lactic acid among the species contributes to their overall antimicrobial activity. Furthermore, the production of H_2O_2, though variable, is essential in inhibiting pathogen growth and sustaining vaginal health. Our findings support the potential of *L. crispatus* and *L. gasseri* as effective probiotic candidates due to their robust antimicrobial activities and capacity to lower vaginal pH. This research highlights the urgent need for alternative treatments for vaginal infections, given the side effects and limitations associated with current antibacterial and antifungal therapies.

Appendix A

Dry weight technique for the Enumeration of *Gardnerella vaginalis* cells.

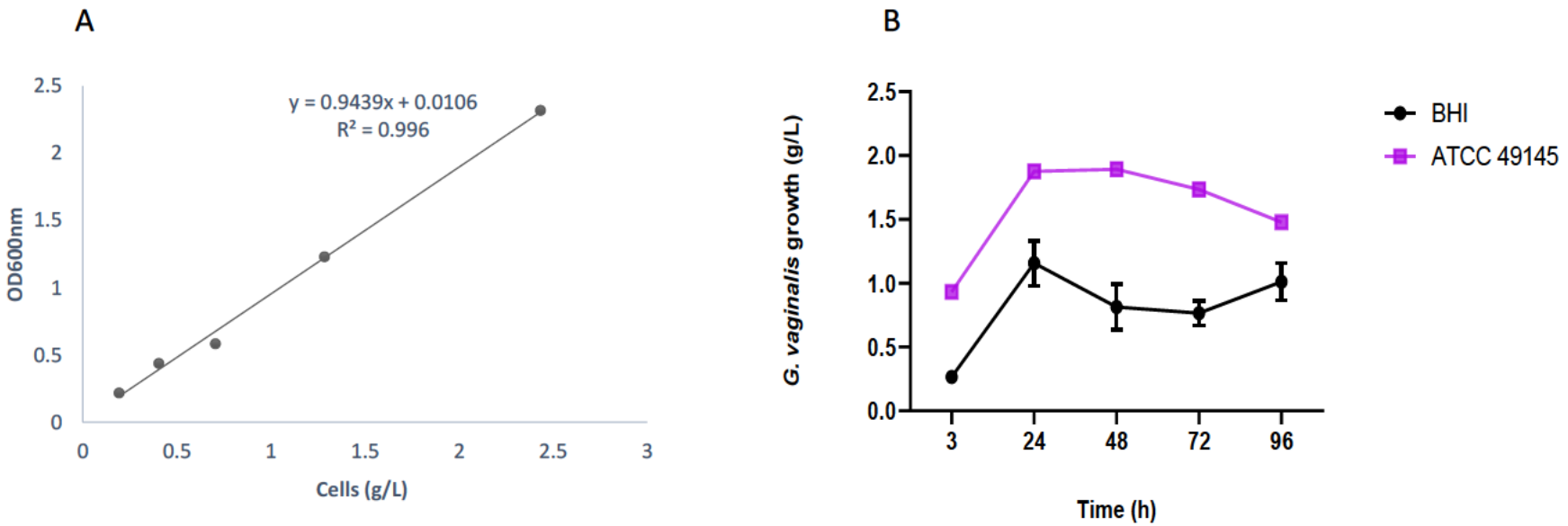

Figure A1: Standard curve and Growth kinetics curve for *Gardnerella vaginalis* cells using the Dry weight method. *Gardnerella vaginalis* was grown in BHI for 72 h at 37°C with 5% CO_2 under anaerobic conditions. The overnight culture was adjusted to according to OD600nm with a spectrophotometer and the Dry weight was determined (A). The standard curve was used to determine the number of cells for the growth kinetics (B) and the antibacterial activity experiment. The error bars represent mean of the microplate readings (n=2) with standard deviation.

Appendix B

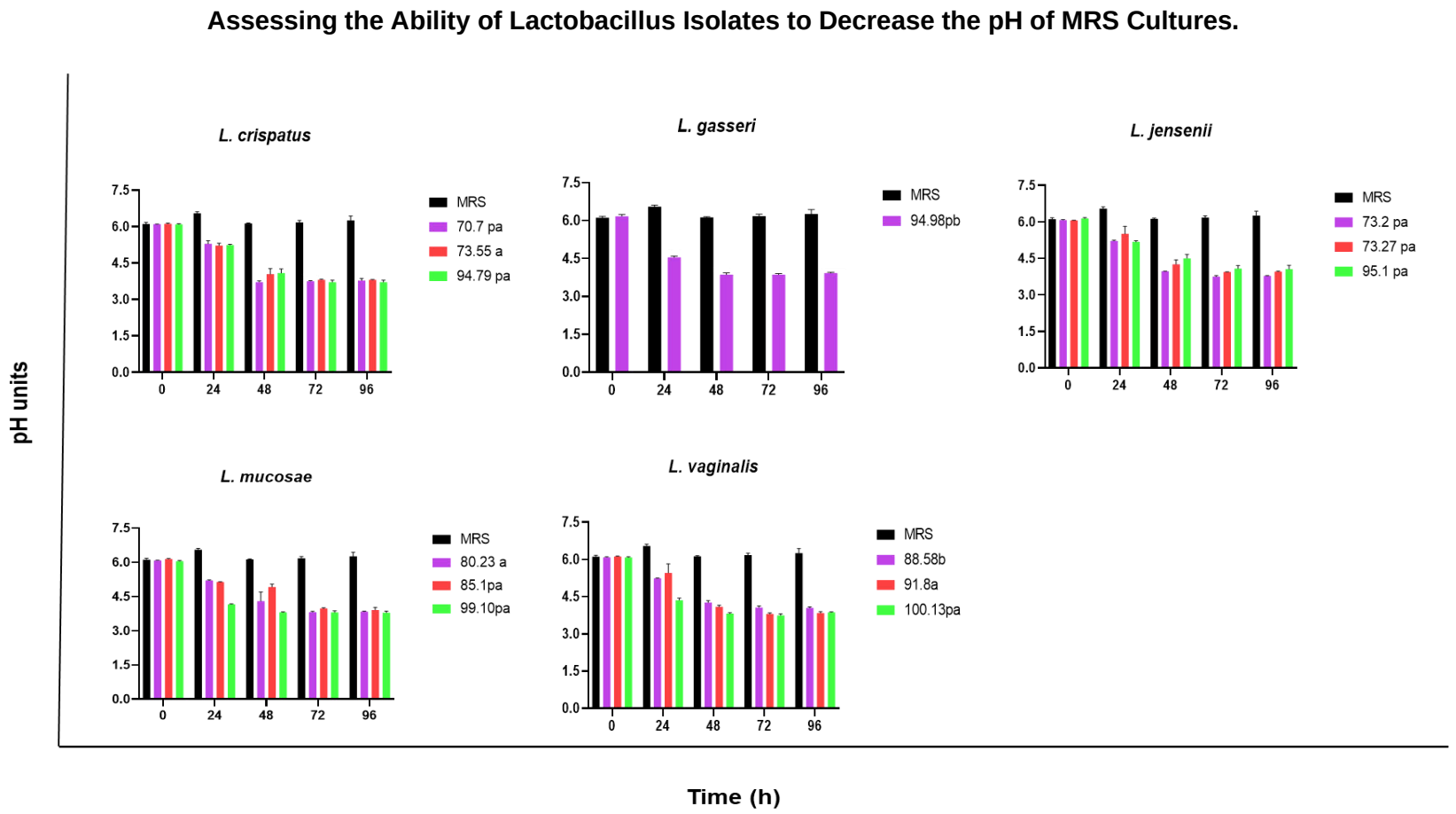

Figure B1: Ability of *Lactobacillus* species to lower culture pH over time. The pH of *L. crispatus* (70.7 pa, 73.55 a, 94.79 pa), *L. gasseri* (94.98 pb), *L. mucosae* (80.23 a, 85.1 pa, 99.10 pa), *L. jensenii* (73.2 pa, 73.27 pa, 95.1 pa), and *L. vaginalis* (88.58 b, 91.8 a, 100.13 pa) MRS cultures were measured at 0, 24, 48, 72, and 96 hours during anaerobic incubation at 37˚C. Bars represent mean with standard deviation.

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