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# Antibacterial activity of yeasts strains on vaginal pathogenic flora.

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## Dedications

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**List of abbreviations**

AV: Aerobic Vaginitis

BV: Bacterial Vaginosis

CST: Community State Type

CDC: Centres for Disease Control and Prevention

CoNS : Coagulase Negative *Staphylococcus*

eDNA: Extracellular Deoxyribonucleic Acid

EMB: Eosin Methylene Blue

GRAS: Generally Regarded As Safe

H: Hour

$H_2O_2$ : Hydrogen Peroxide

HPV: Human Papilloma Virus

NMA: Nutrient Molten Agar

OD: Optical Density

QPS: Qualified Presumption of Safety

SAPs: Secreted Aspartic Proteinases

sp.: species

TS: Tryptic Salt broth

TSB: Tryptic Soy Broth

TSI: Triple Sugar Iron

UPEC: Uropathogenic *Escherichia coli*

VVC: Vulvovaginal Candidiasis

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# INTRODUCTION

## Introduction

Vaginal infections represent a significant global health concern, affecting millions of women and often leading to discomfort, disruption of daily life, and adverse complications in fertility if left untreated. Research shows that the most common etiologies of vaginal infections are bacterial vaginosis (BV), vulvovaginal candidiasis (VVC) and trichomoniasis**(Huang et al., 2023).** According to the provided statistics, BV is the most prevalent of them all ranging from 23% to 29% globally, and 25% in North and Sub-Saharan Africa**(Peebles et al., 2019).**

For many years, antibiotics have demonstrated their importance and efficacy in the treatment of bacterial infections. However, with the overuse and misuse of these antibiotics, bacteria are rapidly and continuously developing resistance towards these antibacterial agents; dramatically increasing the risks of recurrent infections and thus making the treatment of these infections even more challenging **(Salam et al., 2023).** Recent statistics state that antibiotic resistance results in the deaths of approximately 23,000 patients annually with healthcare costs reaching up to $20 billion **(Habboush & Guzman, 2024)**, thus creating a pressing need for novel therapies.

With the aim being to pave way for the development of safe and effective alternatives to traditional antibiotic treatments, recent researches show that yeasts belonging to the genera *Saccharomyces, Debaryomyces, Pichia, Yarrowia, Meyerozyma*and *Kluyveromyces* have antimicrobial properties making them probable probiotics **(Shruthi et al., 2022).** In a study carried out by **Pericolini et al., (2016).***Saccharomyces cerevisiae* was able to inhibit adhesion of *Candida* to the epithelial cells. Furthermore, *Saccharomyces boulardii* and *Saccharomyces cerevisiae* have been found to have the most probiotic properties and therefore present suitable alternatives in the treatment of VVC cases**(Gaziano et al., 2020).**

This study investigates the antagonistic activity of two yeast strains, *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* extracted from figs and pomegranate respectively, tested against bacterial pathogens isolated from the vaginal tract. Vaginitis is a medical term that describes various disorders that cause the vagina to become infected or inflamed following an imbalance in the vaginal microbiota**(Itriyeva, 2020).**This imbalance is due to a decline in beneficial *Lactobacillus* species and an overgrowth of pathogenic bacteria**(Kairys et al., 2024).** The aim of this study is to explore the potential of these yeasts as probiotics in the treatment of vaginitis.

The objectives therefore include:

- Characterising the pathogenic bacteria through microscopic observations and biochemical tests in order to estimate their pathogenicity.
- Testing three yeast strains for antibacterial activity using different tests in order to evaluate their probiotic potential.

This work focuses firstly on the review of the already existing literature entailing an overview of the vaginal microbiota and its role in infections, while **the second part** focuses on the interest of yeasts as emerging probiotics in the treatment of these infections. Secondly, methods employed in this study are detailed and afterwards the results obtained are interpreted. Finally, conclusions are drawn in reference to the results obtained.

# CHAPTER 01: LITERATURE REVIEW

# Part one: The female reproductive Tract

## I. Anatomy and microbial composition of the female genital tract

### 1.1. Anatomy of the female reproductive tract

The female reproductive tract (FRT) as shown in **Figure 1** includes internal and external organs that play roles in reproduction, menstruation, and sexual activity. The internal organs are the ovaries, fallopian tubes, uterus, cervix, and vagina, while the external organs are collectively known as the vulva, which includes the mons pubis, labia majora and minora, clitoris, urethra, and Skene glands **(Ramírez-González et al., 2016).**

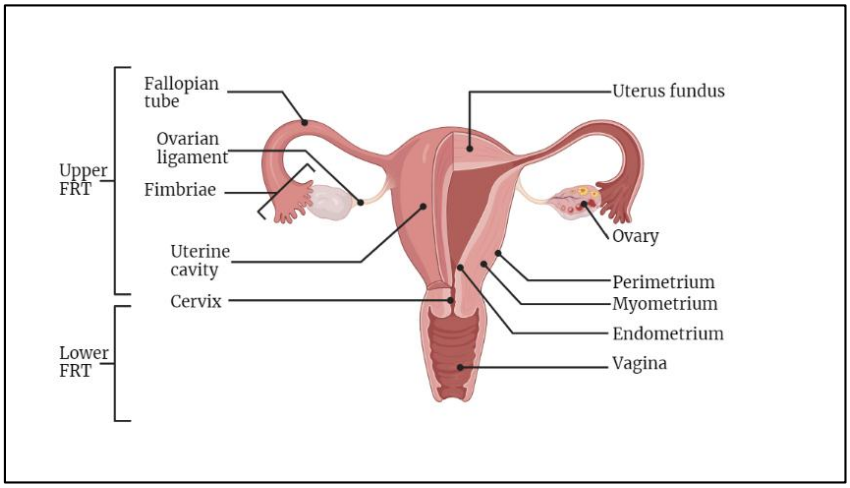


**Figure 1. The female reproductive tract**

### I.2. Microbial composition of the human female reproductive tract

The term "microbiota" was first used in the early 1900s. Numerous microorganisms, such as yeasts, bacteria, and viruses, have been discovered to cohabit in the human body's organs (gut, skin, lung, and oral cavity). Like many other organs, the vagina is home to billions of microbes forming a homeostatic and mutualistic relationship with the host**(Chen et al.,2021)**. Research shows that the vaginal microbiota makes up approximately 9% of the total human microbiome and highlights its significant contribution to the hosts' well-being, having a crucial role in promoting the overall reproductive health in women **(Ouarabi et al., 2021).**

## II. Diversity of female genital microbiota

The human microbiota involves different species of symbionts. In the case of the vaginal microbiota, bacterial communities help to defend against pathogenic organisms while also being completely reliant on the host for nourishment, making it a mutualist relationship. The microbiota community is made up of a variety of species that differ based on an individual's age, health, and other factors **(Kalia et al., 2020).**

In addition to this diversity in the vaginal microbiota per individuals, there exists also different microbial dominance in distinct micro-ecosystems of the FRT within a specific individual. Studies have shown how the endocervical microbiota is generally dominated by *Lactobacillus* genus, followed by *Gardnerella, Veilonella, Prevotella,Sneathia*, and *Fusobacterium.* This similarity between microbiota in vaginal and endometrial samples of healthy women supports the hypothetical statement that the uterine cavity and endometrium are colonized mainly by ascended vaginal bacteria **(Pelzer, Willner, Buttini, & Huygens, 2018).**

In the uterine cavity, the microbiota is also typically dominated by *Lactobacillus* genus, however, there exists studies having cases where the endometrium is not dominated by *Lactobacillus*, but have higher abundance of genera like *Acinetobacter*, *Bifidobacterium*, *Gardnerella*, *Prevotella*, and *Streptococcus***(Toson et al., 2022).** A study conducted by **Pelzer, Willner, Buttini, Hafner, et al., (2018)**demonstrated that even in the absence of infection, the human uterine tube is not a sterile site, microbiota exists and is dominated by members of the phylum Firmicutes, mostly *Staphylococcus* sp., *Enterococcus* sp., and *Lactobacillus* sp.**(Pelzer, Willner, Huygens, Hafner, et al., 2018).**

Placental microbiota has been noted to largely consist of non-pathogenic commensal microbiota from the phyla of Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes, and Fusobacteria **(Aagaard et al., 2014).** Isolations from umbilical blood samples showed residence of both aerobic and anaerobic bacteria in the umbilical cord, with the most common being aerobic bacteria strains of Coagulase Negative *Staphylococcus* (CoNS) such as *Staphylococcus sciuri* and *Staphylococcus lentus***(Hemberg et al., 2023).** Another study was conducted where the microbiota of navel skin was said to be dominated by *Corynebacterium* and *Staphylococcus*, also including opportunistic pathogens like *Clostridium* and *Pseudomonas***(S et al., 2020).**

## II.1. Vaginal Microbiota

The history of describing the vaginal flora began in 1892 when German gynaecologist Albert Doderlein discovered the importance of bacteria that produce lactic acid in the vagina. As a result the term "Doderlein's bacillus" was used in gynaecological publications until 1928, when Stanley Thomas classified the bacterium as *Lactobacillus acidophilus***(Mancabelli et al., 2021)**.

Females have a comparatively higher concentration and diversity of genital microbiota, as shown in **figure2,** than that of males as the vagina harbours lots of normal flora, which is highly influenced by puberty and age **(Gao et al., 2024).**Healthy vaginal microbiota is specific from all other human body sites harbouring native microbiota as its composition is, in the majority of the population, dominated by a single genus *Lactobacillus***(Ravel et al., 2011).**However, a lesser percentage of females have a vaginal microbiota that is balanced but not dominated by *lactobacilli*, it is rather primarily composed of facultative and obligate anaerobes, primarily belonging to the genera *Gardnerella, Atopobium, and Prevotella*. Studies have linked a higher risk of infections and unfavourable health effects to this variance in the vaginal microbiota and low levels of *Lactobacillus* species **(Zhou et al., 2007).**

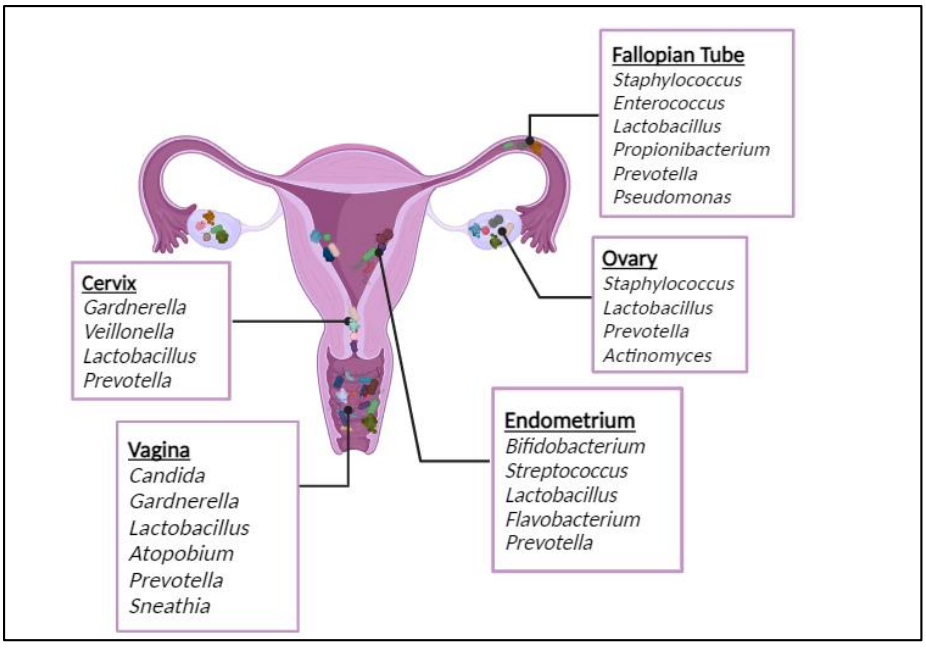


**Figure 2. Microbial composition of the compartments of the female reproductive tract.**

## II.2. Particularities of the vaginal microbiota

The vaginal microbiota includes a mixture of bacteria, eukaryotes, fungi, archaebacterial and viruses although most studies focus mainly on the bacteriome **(Abou Chacra & Fenollar, 2021).** A landmark study published by **Ravel et al. (2011)** characterized the vaginal microbial communities of a cohort of healthy reproductive-age women of different races in the United States using molecular sequencing technology. These microbial communities were clustered into five 'Community State Types' (CST), also referred to as vaginotypes, as demonstrated in **figure 3**, four of which were *Lactobacillus*-dominated, followed by members of the genera *Gardnerella*, *Vibrio* and *Atopobium***(Mancabelli et al., 2021).**

CSTs I, II, III and V are characterized by the predominance of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus iners* and *Lactobacillus jensenii* respectively. However, CST IV is dominated by diverse facultative anaerobes with low levels of *Lactobacilli*. This CST-IV has been further divided into two sub-states CST IV-A and CST IV-B **(Kalia et al., 2020).**

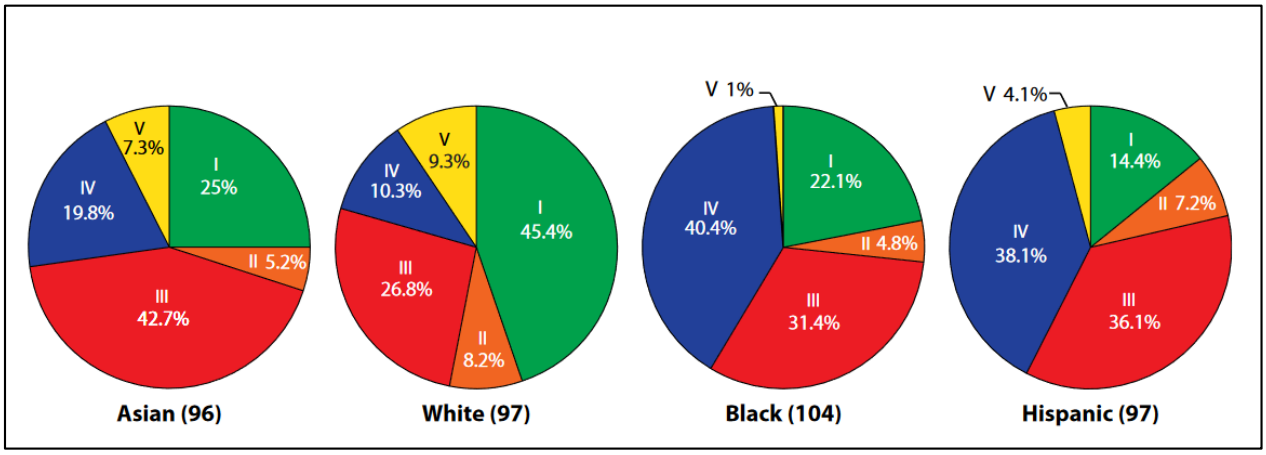


**Figure 3. Representation of vaginal bacterial community groups within each ethnic group of women. The number of women from each ethnic group is in parentheses (Ravel et al., 2011).**

## II.3. Functions of vaginal microbiota

The vaginal microbiota plays an important role in maintaining vaginal health and protecting against infectious disease **(Mancabelli et al., 2021).** These microorganisms represent a finely

balanced mutualistic connection in which the microbes provide a protective advantage to their hosts in exchange for a nutrient-rich, anoxic environment **(Danielsson et al., 2011).**

In the vagina, this protection is mediated by the production of lactic acid and hydrogen peroxide by the dominating *Lactobacillus* sp. alongside other antimicrobial compounds, making the environment hostile to other invading microorganisms thus preventing unwanted microbial growth **(Pendharkar et al., 2023).**Additionally, it was also found out that that cell wall fragments of *Lactobacillus* species could block attachment of bacterial pathogens to epithelial cells **(Čeprnja et al., 2023).** Last but not least. the microbiota of the reproductive system is also known to play a major role in transmembrane transport and the metabolism of amino acids as well as carbohydrates **(Colella et al., 2023).**.

## II.4. Variation of the vaginal microbiota

It has been maintained that the composition of vaginal microbiota varies according to physiological factors within the host. Vaginal bacterial communities reside in an ecosystem that is strongly influenced by characteristics of the host, local environment, and constituent populations **(Hickey et al., 2012).Figure 4** shows several factors causing variation within the vaginal microbiota.

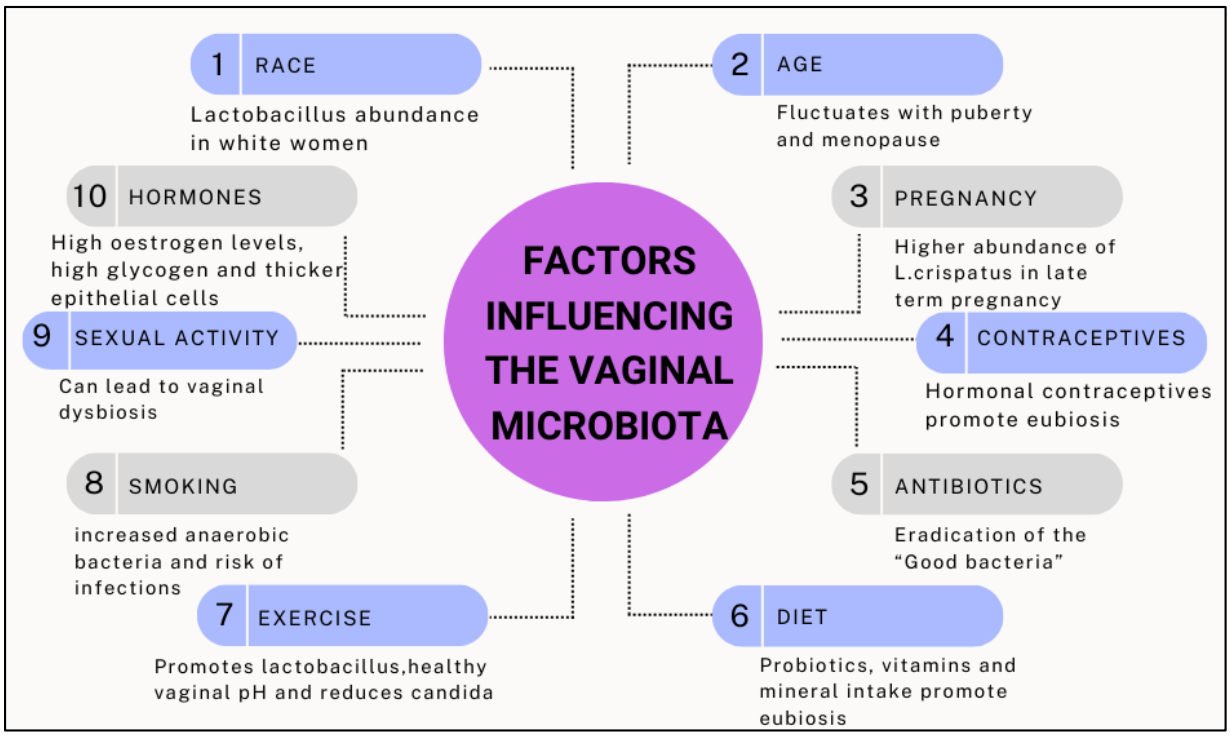


**Figure 4. Factors on variation in the vaginal microbiota (Gaziano et al., 2020)**

## III. Vaginal microbiota dysbiosis

Changes in the composition of the FRT microbiota related to inflammation can cause affective and/or functional disorders that are both immunologically related to symbiosis. This is because affective disorders occur via inflammatory pathways. A negative variation in the qualitative and quantitative composition of certain strains of microorganisms (dysbiosis) due to local or systemic causes can even lead to serious diseases **(Gholiof et al., 2022).**

## III.1. Pathogenic microorganisms

As already mentioned, the vaginal microbiota includes a variety of microorganisms, amongst others, those that are opportunistic and pathogenic as shown in **table 1**, causing different infections in the genital tract. *Candida albicans* and *Gardnerella vaginalis* are the most prevalent vaginal pathogens responsible for the vulvovaginal candidiasis and bacterial vaginosis respectively **(Salinas et al., 2020).**

**Table 1. Opportunistic and pathogenic microorganisms in the vaginal microbiota**

| Gram negative Bacteria | Gram positive Bacteria | Fungi | Parasites | Virus |
|---|---|---|---|---|
| *E.coliChlamydia* spp. *Klebsiella*spp. *Citrobacter*spp. *Serratia*spp. *Acinetobacter* spp.*P.aeruginosaN. gonorrhoeae* | *Enterococcus*spp. *S.saprophyticusS.aureus Streptococcus* spp. *Atopobium vaginae* | *Candida* spp. | *L.monocytogenesT.vaginalis* | *H.influenzaeHerpes simplex virus Cytomegalovirus* |

## III.2. Pathogenicity and virulence factors

There are numerous virulence factors and mechanisms of interaction that play important roles in establishment of an infection, as shown in **figure 5**. These factors can be employed by opportunistic commensals, parasites and pathogenic bacteria.

### III.2.1. Adherence and colonization

Pathogenic microorganisms can colonize vaginal epithelial cells, displacing healthy *Lactobacilli***(Patterson et al., 2010).** For example, *Gardnerella vaginalis* tolerates high redox potential of a *lactobacillus*-dominated vaginal microbiota **(Muzny et al., 2019)** and produces sialidase A to remove sialic acid, allowing it to adhere and potentially cause bacterial vaginosis **(Kalia et al., 2020).** Similarly, *Neisseria gonorrhoeae* uses pili and Opa proteins to adhere and invade epithelial cells, causing gonorrhoea **(Springer & Salen, 2024).***Candida albicans* can transition from a harmless yeast form to a pathogenic, invasive hyphal form, invading host cells through endocytosis and active penetration **(Talapko et al., 2021).**

### III.2.2. Release of toxins and cytotoxicity

After invasion of pathogens in the vaginal tract, they release toxins with the aim to damage the epithelial cells and/ or immune cells, causing emergence of various symptoms like fever and discomfort. In order to cause BV, *G. vaginalis* produces toxins like vaginolysin and prolidase, which contribute to bacterial vaginosis symptoms, including cell lysis and amine odour **(Kalia et al., 2020).** Candidiasis is facilitated by release of protease, phospholipase, and proteolytic Secreted Aspartic Proteinases (SAPs) **(Kalia et al., 2020),** while *Enterococcus faecalis* produces cytolysin and aggregative substance, also enhancing their pathogenicity **(Jahic, 2022).**

## III.2.3. Formation of biofilm

Biofilms are intricate, three-dimensional communities of microorganisms that form when they irreversibly attach to surfaces and are encased in a self-synthesized matrix of complex extracellular polysaccharides, amyloids, proteins, lipids, and extracellular DNA (eDNA). This extracellular matrix provides numerous advantages to the microorganisms, including structural support and protection from host immune responses and antimicrobial treatments. Biofilms exhibit strong extracellular enzyme activity, absorb and store nutrients, and coordinate virulence factor expression through cell aggregation and quorum sensing. They can also retain and protect the eDNA and the exoenzymes that provide an external digestive system to the biofilm matrix **(Fleming & Rumbaugh, 2017; Flemming et al., 2023; Tsui et al., 2016).**

*Candidaalbicans'* ability to form biofilms is considered one of its major virulence factors in candidiasis **(Mayer et al., 2013),** and *Gardnerella vaginalis* is considered the primary colonizerwhich establishes a scaffold for the formation of polymicrobial biofilms, contributing to BV**(Chen et al., 2021).**

## III.2.4. Antibiotic resistance

Antibiotic resistance is a growing concern as some pathogenic bacteria have developed resistance to multiple antibiotics, making them harder to treat and increasing the risk of recurrent infections **(Abalkhail et al., 2022).** Additionally, pathogens in biofilms are significantly more resistant to antimicrobial agents than planktonic species, leading to persistent and recurrent infections. This increased resistance can also lead to increased virulence in some cases **(Schroeder et al., 2017).**

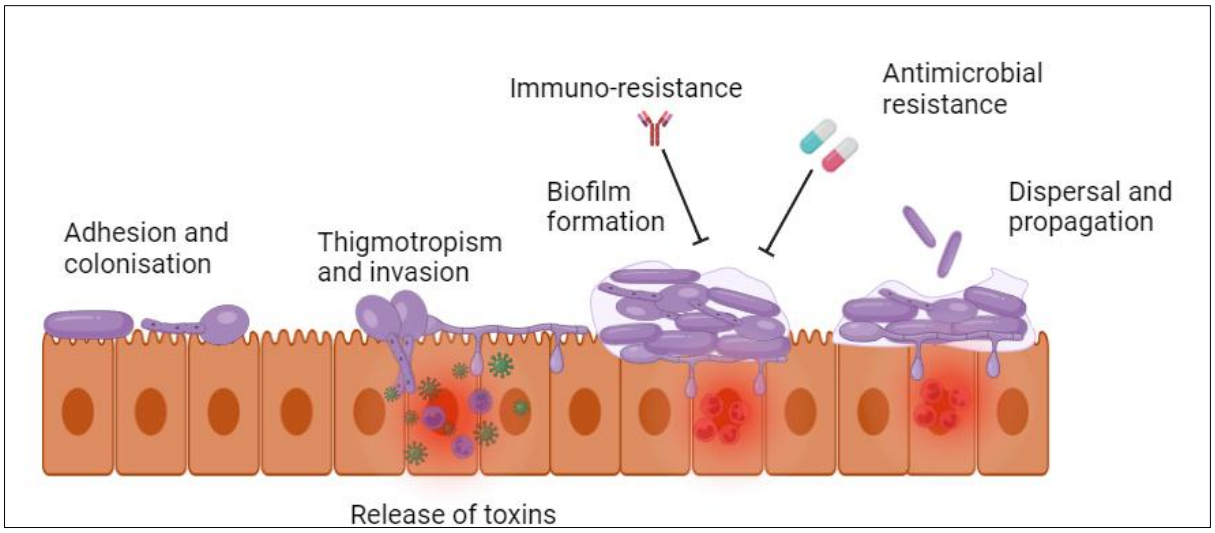


**Figure 5. Mechanisms of bacterial virulence factors**

## III.3. Vaginal infections and diseases

Vaginitis is a medical term that describes various disorders that cause the vagina to become infected or inflamed. Vulvovaginitis refers to inflammation of both the vagina and vulva (the external female genitals), and is a common gynaecologic concern among female adolescents and young adults **(Itriyeva, 2020).**

## III.3.1. Aerobic Vaginitis

Aerobic vaginitis (AV) is a lack of balance of the vaginal flora and is characterized by abnormal vaginal flora containing aerobic and intestinal pathogens, varying degrees of vaginal inflammation and development of the vaginal lining **(Donders et al., 2017)**. AV can result in adverse pregnancy outcomes like spontaneous abortion and premature birth, it also introduces an additional risk for cervical intraepithelial neoplasia, which is the transformation and abnormal growth of cervical squamous intraepithelial cells **(Jahić & Cerovac, 2022; Mohankumar et al., 2022).**

### III.3.2. Anaerobic Vaginitis or Bacterial Vaginosis

Bacterial vaginosis (BV) is the most frequent type of vaginitis in women of reproductive age. BV is a dysbiosis in the ecosystem of the normal vaginal microbiota, which is characterized by a shift from *Lactobacilli* dominance to that of a mixture of various anaerobic bacteria. Possible complications in pregnant women include higher risk of preterm birth and low birth weight **(Chacra et al., 2021).**

### III.3.3. Chlamydiosis

Chlamydial species are Gram-negative, aerobic, obligate, intracellular pathogens. Infection with *Chlamydia* is one of the most common bacterial sexually transmitted diseases because it is often asymptomatic and is transmitted through infected secretions and mucous membranes of urethra, cervix, rectum, conjunctivae and throat. It can also be transmitted from an infected mother to baby during vaginal delivery**(Manavi, 2006).**

### III.3.4. Gonorrhoea

Gonorrhoea is any infection and disease associated with *Neisseria gonorrhoeae*. It mainly causes health complications in women. **(Edwards & Butler, 2011).** Vaginal mucosa of prepubertal children is more susceptible to gonococcal infections due to its lower oestrogen level, which results in thinner mucosa cell wall in comparison to adolescents and adults. Prepubertal children also have an alkaline vaginal pH (6.5-7.5) which makes them susceptible to gonococcal infection and colonization **(Bambang et al., 2021).**

Undiagnosed and/or untreated gonorrhoeal infections can ascend and cause many severe reproductive complications such as endometritis, pelvic inflammatory diseases, infertility and ectopic pregnancy**(Springer & Salen, 2024)**.

### III.3.5. Vaginal Candidiasis

Candidiasis is defined as a mycosis due to an invasion by opportunistic *Candida* sp. These fungal infections can range from superficial mucosal candidiasis such as vulvovaginal candidiasis (VVC) to life fatal bloodstream infections such as candidemia **(Sahu et al., 2022).**

Other names for this infection are vaginal candidiasis, vulvovaginal candidiasis, or candida vaginitis. Candidiasis is a fungal infection caused by overgrowth *Candida albicans*, a polymorphic opportunistic fungus whose pathogenicity is associated with its morphological adaptability. Candidiasis is responsible for about one third of vulvovaginitis occurrences **(Jeanmonod et al., 2024). Table 2** summarises the most frequent vaginal infections.

**Table 2. Common vaginal infections and diseases**

| Infections | Symptoms | Treatment | Causative Agent | Reference |
|---|---|---|---|---|
| **Aerobic Vaginitis** | Vaginal inflammation, A yellow vaginal discharge, A fishy odour, Dyspareunia. | Antibiotics, Steroids Oestrogen-based therapies, Ciprofloxacin and Ceftriaxone | *Escherichia coli*, *Staphylococcus aureus*, Group B Streptococcus and *Enterococcus faecalis* | (Jahic, 2022)(Mohankumar et al., 2022) (Jahić & Cerovac, 2022) (Vidyasagar, 2021) (Aklilu et al., 2024) |
| **Anaerobic and Bacterial Vaginitis** | Mostly asymptomatic or a clinically significant malodorous discharge | Clindamycin, Metronidazole and Tinidazole | *Sneathia, Prevotella amnii, Megasphaera, Atopobium, Gardnerella vaginalis, mobilincusureaplasma and urealyticum* | (Chacra et al., 2021) (Larsen & Monif, 2001) (A. C & Cm, 2023) |
| **Chlamydia** | Mostly asymptomatic | Doxycycline and Azithromycin | *Chlamydia trachomatis* and *Chlamydia pneumoniae* | (Manavi, 2006) (Rodrigues et al., 2022) (Kang-Birken, |

| | | | | |
|---|---|---|---|---|
| | | | | 2022) |
| **Gonorrhoea** | Predominately asymptomatic, purulent yellow to greenish coloured vaginal discharge, Dysuria and Erythematous vulva | Ceftriaxone and Extended Spectrum Cephalosporins (ESCs) Oral cefixime | *Neisseria gonorrhoeae* | (Edwards & Butler, 2011) (Bambang et al., 2021) (Balthazar et al., 2011) |
| **Vaginal Candidiasis** | Vulva and vaginal erythema, swelling, vaginal itching and thick white adherent discharge | Azoles, Polyenes and echinocandins, 5-Flurocytosine (5FC) | *C. albicans, C. glabrata, C. krusei, C. tropicalis, C.dubliniensis, C.lusitaniae and C. parapsilosis* | (Larsen & Monif, 2001) (Jeanmonod et al., 2024) (Willems et al., 2020) (Kaur et al., 2023) (Utkalaja et al., 2024) |

## Part two: Yeast in the vaginal microbiota

The International Scientific Association for Probiotics defines probiotics, as live microorganisms that when administered in adequate amounts confer a health benefit on the host **(H. C et al., 2014).** One of the currently growing fields is the fungal probiotic field specifically yeasts, as these microorganisms have the potential to produce anti-carcinogenic, antioxidant and anti-mutagenic agents and provide protection against different bacterial and respiratory infections. *Saccharomyces cerevisiaevar boulardii* is the most significant of all yeast species and clinical studies have proved that oral administration of this strain as a probiotic can help treat multiple gastrointestinal diseases**(Abid et al., 2022).**

## IV.1. Generalities and habitat

Yeasts are eukaryotic microorganisms widespread in natural environments including the normal microbiota of humans, on plants, on airborne particles, in water, in food products, and in many other ecological niches **(Yige, 2018)**. With continuous advancements in scientific knowledge and molecular sequencing, different yeasts species have been confirmed to have commensal roles in intestinal, oral, cutaneous and vaginal microbiota communities. Some of these yeasts include *Cryptococcus*, *Galactomyces*, *Penicillium* and *Saccharomyces* found amongst the oral, gut, skin and the vaginal microbiota**(Caetano et al., 2023).**

Interest around the use of yeast-based probiotics against vaginal infections has increased, and this is because not only are they antibiotic resistant, but also because they can be used to treat patients undergoing antibiotic therapy. A study on effects of *S. cerevisiae*-based probiotic against vaginal candidiasis, demonstrated that daily intra vaginal administration of live *S. cerevisiae* CNCMI-3856 strain resulted in the clearance of *C. albicans* **(Gaziano et al., 2020).** Another study was conducted where *S. cerevisiae* demonstrated potential therapeutic effect against BV caused by *Gardnerella vaginalis***(Sabbatini et al., 2018).**

## IV.2. Biological Activity of Yeast

## IV.2.1. Antagonistic Activity

The first discovery of the inhibitory activity of yeasts was conducted by**Hayduck, (1909)**. In summarizing, the antimicrobial effects of yeasts present in fermented foods and beverages, include actions of organic acids, antibiotic factors, volatile acids, hydrogen peroxide, and various other substrates excreted in the product**(Yige, 2018).**

The antagonistic activities of yeasts include mechanisms such as competition for space and nutrients, this is achieved by depriving other cells of essential nutrients like carbon source, nitrogen source and iron, thus inhibiting their growth**(Georgescu et al., 2024)**. Yeasts can also

acidify their growth medium, causing pH to change in the medium as a result of their metabolic activity, growth-coupled ion exchange or production of organic and volatile acids. The production of yeasts' metabolites such as high concentrations of ethanol and hydrogen peroxide also make the environment unfavourable for many microorganisms **(Hatoum et al., 2012; Mielecki et al., 2024).**

Furthermore, yeasts produce antibacterial compounds and killer toxins namely mycocins, which are mostly, characterised as antifungal agents (other yeasts included). Their mechanism includes extracellular proteins that hydrolyse β-1,3-D-glucans in fungal cell walls or inhibit their synthesis causing damage to cell wall; the proteins also cause ion leakage due to cell membrane disruption. Lastly, some yeasts produce proteins that block DNA synthesis and cell division **(Mielecki et al., 2024; Yige, 2018)**. Results from a recent research also show that *S. cerevisiae* produces peptide- based killer toxins that had antimicrobial activity against certain Gram-positive and negative bacteria **(Al-Sahlany et al., 2020).**

## IV.3. Advantages of using yeasts

**(Čmielová et al., 2012; Fj et al., 2019; Lyu et al., 2021)**

- They can be genetically modified to gain new and more convenient properties in order to produce desired compounds.
- They are relatively easy to handle (mutant isolation, gene transfer, cultivation) due to their simple cell structure
- *Saccharomyces cerevisiae* is of known genome so it is therefore a suitable model organism for the study of physiological, metabolic processes and genetics of eukaryotes.
- Some yeasts like *K. marxianus* possess a wide range of thermotolerance, making it suitable for biotechnological use.

## IV.4. Application of yeasts as treatment for vaginal infections

It has been shown that vaginal administration of a probiotic *Saccharomyces cerevisiae* yeast and partially inactivated whole yeast *Saccharomyces cerevisiae* in mice positively impacted the progression of vaginal candidiasis by speeding up the elimination of the fungus. Both live and

inactivated yeasts promoted co-aggregation with *Candida*, which in turn inhibited its adherence to epithelial cells. Additionally, the probiotic yeast strain prevented adherence by strongly suppressing *C.albicans* virulence factors **(Pericolini et al., 2016).**

In general, treatment involves topical application of vaginal ovules, creams, lotions, or oral drugs, with fluconazole, amphotericin B, nystatin, and flucytosine being the most common antifungal agents. Topical azoles and oral fluconazole are equally effective for managing uncomplicated Vulvo Vaginal Candidiases (VVC) cases. However, current antifungal treatments often fail to provide long-term protection. *Saccharomyces boulardii* and *Saccharomyces cerevisiae* have displayed the most probiotic properties and therefore present suitable alternatives in the treatment of VVC cases **(Gaziano et al., 2020)**.

# CHAPTER 02: MATERIAL & METHODS

## Materials and methods

The biological material used in this study was three yeast strains, two *Saccharomyces cerevisiae* strains and one *Kluyveromyces marxianus* strain obtained from figs and pomegranate respectively. Their antagonistic activity was evaluated against pathogenic bacteria isolated from vaginal secretion swabs. Both the yeast and pathogenic strains belong to the Laboratory of Applied Microbiology of the University of Bejaia's collection.

The methodology followed in this study was divided into two main parts. Initially, we characterized the pathogenic strains. This characterization was based on previous experimental results and presumptive identification using ChromAgar media, ensuring precise and reliable strain identification.Their numbering was maintained as from their identification on the ChromAgar. In the second phase, we evaluated the antagonistic activity of the three yeast strains, against the identified pathogenic strains. This dual-phase approach facilitated a comprehensive assessment of both the pathogenic characteristics and the potential inhibitory interactions between the yeast and pathogenic strains. Strains of *Staphylococcus aureus* and *Escherichia coli*, which were already identified, were used as references.

## I. Characterisation of pathogenic strains

### I.1. Inoculation of pathogenic strains of specific media

For each strain, 1 ml from the storage tube is inoculated into 5 ml of nutrient broth and then incubated for 18h at 37°C. Once the incubation time has ended, the broths that have become cloudy are inoculated using streaking method onto a specific agar medium as shown in **Table 3**.

The specific media were chosen according to the results previously obtained on ChromAgar Media **(Appendix 01)** and after inoculation, the Petri dishes were then incubated at 37°C for 24H.

**Table 3. Culture media used for inoculation of the pathogenic strains**

| Media | Strains |
|---|---|
| Slanetz-Bartley agar | *Enterococcus* |
| Chapman agar | *Staphylococcus aureus* |
| Blood agar | *Streptococcus* and *Corynebacterium* |
| Eosin Methylene Blue agar | *Escherichia coli* |

## I.2. Purification and phenotypic identification of strains

Purification consisted of successive subculturing (agar ↔ broth) until a pure culture of characteristic, well-isolated colonies is obtained, checking sequentially for shape, cell organisation, Gram and the presence or absence of a catalase.

Strains were identified by means of a morphological and physiological study: preliminary identification is carried out by referring to certain culture conditions (colonies appearance on specific media, growth temperature) and the results of Gram staining (Gram, cell shape and organisation). As for the physiological study, it consists of subjecting the strains to the catalase test, the coagulase test and culture growth on specific media (Simmons Citrate and TSI)**(Table 04).**

According to **Guiraud, (2003),**biochemical tests for the identification of *Escherichia coli* were carried out as described in **Table 4**.

*Note: The composition of the culture media is given in* ***Appendix I***

**Table 4. Biochemical tests for the identification of E.coli**

| Test | Description | Interpretation |
|---|---|---|
| **Production of the enzyme catalase** | Solvent: Hydrogen peroxide<br>A bacterial colony is deposited using a Pasteur pipette on an alcohol-cleaned | Production of effervescence, as a result of the release of gas, indicates the presence of |

| | | |
|---|---|---|
| | slide followed by a drop of 10-volume Hydrogen peroxide. | the enzyme catalase. |
| **Blood plasma coagulation** | 1ml of blood plasma was used for this test by inoculating it with an isolated colony and incubation at 37°C for 4H | Formation of clot in the tube containing the plasma signifies presence of the enzyme coagulase. |
| **Fermentation of sugars (glucose, sucrose, lactose), gas and $H_2S$ production** | TSI (Triple Sugar Iron) medium<br>Using Pasteur pipette, an isolated colony was inoculated with tight streaks on the slope of the agar and the bottom with a deep central puncture. The test tubes are then incubated at 37°C for 24 hours. | Aerobic fermentation of lactose and/or sucrose turns the slope yellow.<br>Anaerobic fermentation of glucose changes the colour of the butt to yellow.<br>Gas production signified by appearance of gas bubbles.<br>Presence of a black precipitate (ferrous sulphide) in the bottom signifies the production of $H_2S$ |
| **Use of citrate as Carbon source** | Media: Simmons citrate<br>The slope is inoculated by streaking a well isolated colony and the bottom punctured at the centre. The test tubes are then incubated at 37°C for 2 days. | The change in the pH indicator from green to blue indicates the alkalization of the medium and therefore the bacteria's ability to use citrate as its sole source of Carbon. |
| **Growth at 44°C for *Escherichia coli*** | Media: Tryptic Soy Broth (TSB)<br>In a test tube containing 9ml | The presence of turbidity after incubation is used to verify tolerance and growth |

| | | |
|---|---|---|
| | of TSB, one isolated colony was suspended and then incubated at 44°C for 24H. | of *Escherichia. coli* at high temperatures. |

## I.3. Haemolysis test

To assess the haemolytic activity of the isolated pathogen strains, 5 µl of fresh cultures were plated in spots on the surface of a basic agar Columbia agar (Liofilchem, Italy) supplemented with 5% (v/v) human blood, then incubated for 24H at 37°C. One Petri dish was halved and one side not inoculated with any pathogenic strain in order to serve as the control of the test. At the end of this incubation period, the agar plates were examined for the appearance or absence of haemolytic zones around the spots**(Ait Ouali et al., 2014).**

## I.4. Auto aggregation test

Auto-aggregation of pathogenic strains was evaluated using the method described by **Kos et al.** (**2003**). Isolates were cultured in nutrient broth at 37°C for 24 hours. After incubation, cells were harvested by centrifugation at 8,000g for 15 minutes at 4°C. They were then washed twice with PBS (10 mM, pH 7.2) and resuspended in 10 mL of the same buffer. The suspension was vortexed and autoaggregation was determined after 2 and 4 hours of incubation at 37°C. 1ml was extracted from the surface and the absorbance was measured at 600nm using a spectrophotometer. The percentage of auto aggregation was calculated according to the following equation:

$$\textbf{Autoaggregation(\%)} = 1-A_t/A_0 \times 100$$

The absorbance at each test time is designated $A_t$, while $A_0$ represents the absorbance at the time of incubation.

## I.5. Characterisation of isolated strains for their ability to adhere to polystyrene surfaces

Pathogenic bacterial strains were characterised for their ability to adhere to polystyrene surfaces. From fresh cultures grown for 24 h on nutrient agar, a colony was subcultured in 10ml of TSB broth. The broths were incubated for 18H at 37°C.

### I.5.1. Inoculation of the microplates

Biofilm formation is tested on sterile polystyrene microplates following the method of **(O'Toole & Kolter, 1998)** as described by **Ait Ouali et al. (2014)**. Microplate wells previously filled with 100 µl of Tryptic-Soy broth (TSB; Sigma-Aldrich, Germany) are inoculated in triplicate (three replicates per strain in the same microplate) with 100 µl of a fresh 18H bacterial suspension, then the microplate was incubated at 37°C/24H. Wells containing 200 µl of sterile TSB were used as controls.

### I.5.2. Crystal violet staining (0.1%)

After incubation, the suspensions in the wells were carefully aspirated and then rinsed with 200 µl sterile TS (Tryptone salt solution) for 10 min with agitation. The adherent cells were fixed with 200 µl of 96% Ethanol Absolute (Biochem-Cheopharma, Quebec) for 20 minutes, then aspirated and the wells left to air dry.

The fixed cells were then stained with 200 µl of a crystal violet solution (Biochem-Chemopharma, Quebec) at 0.1% (w/v) for 20 min, and then washed with 200 µl of sterile TS until a clear colour was obtained from the wash liquid. Finally, the colorant bound was solubilised by adding 200µl of 96% Ethanol. The amount of fixed colorant, which indirectly reflects the quantity of fixed cells, is estimated by measuring absorbance at 630 nm.

## II. Antagonistic potential of yeast strains against vaginal pathogenic strains

The yeast strains were tested for antibacterial and anti-biofilm activities against vaginal pathogens. The isolated yeasts were revived in nutrient broth and then on Sabouraud agar with Chloramphenicol and incubation at 37°C for 24H.

### II.1. Soft agar overlay spots test

Mueller-Hinton agar was poured into Petri dishes and left to solidify. Yeast strains from an 18-hour fresh culture were then deposited (5 µl) as spots using a micropipette. The Petri dishes were

allowed to dry before being incubated at 37°C for 24H. In parallel, 9 ml of nutrient broth was inoculated with 1 ml of bacterial suspension and incubated at 37°C for 18H to obtain a fresh culture. After the incubation period, the agar with yeast spots was covered with 9 ml of nutrient molten agar at 45°C, which had been inoculated with 1 ml of a fresh culture of pathogenic strains.The Petri dishes were then re-incubated at 37°C for another 24H. Positive results would be zones of inhibition around the spots.

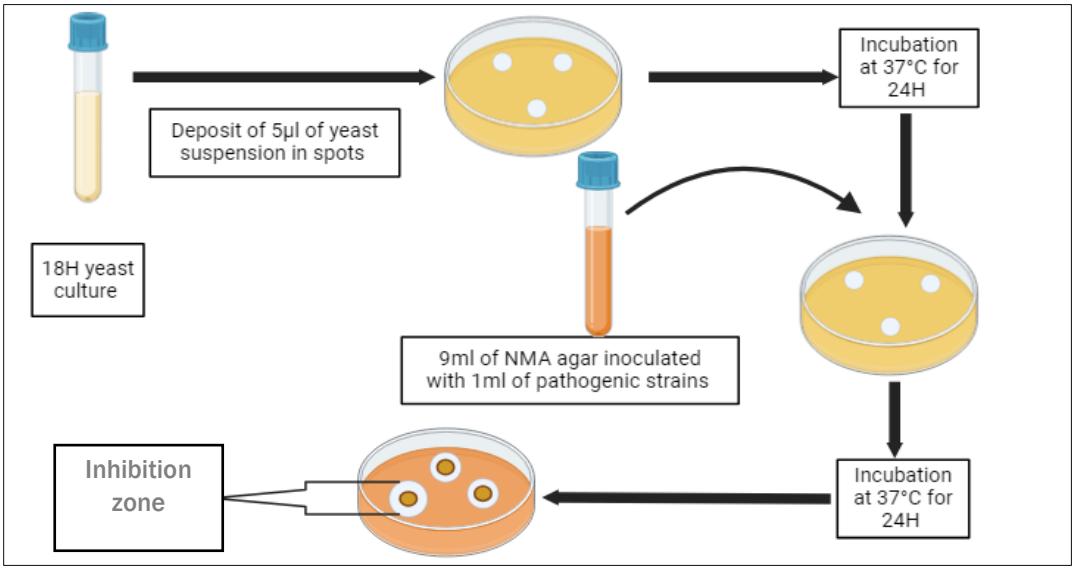


**Figure 6. Protocol of Soft Agar Overlay Spots Test**

## II.2. Single Layer Spots Test

After Muller Hinton agar was poured into Petri dishes and left to solidify, strains of bacteria from fresh 18H culture were inoculated by swabbing and yeast strains were deposited in spots of 5 µl using a micropipette. The Petri dishes were then incubated at 37°C for 24H.

At the end of incubation period, the antibacterial activity is indicated by presence or absence of zones of inhibition around the yeasts' spots. The diameter of the zones would be subsequently measuredto determine the susceptibility of the bacteria towards the yeast strains.

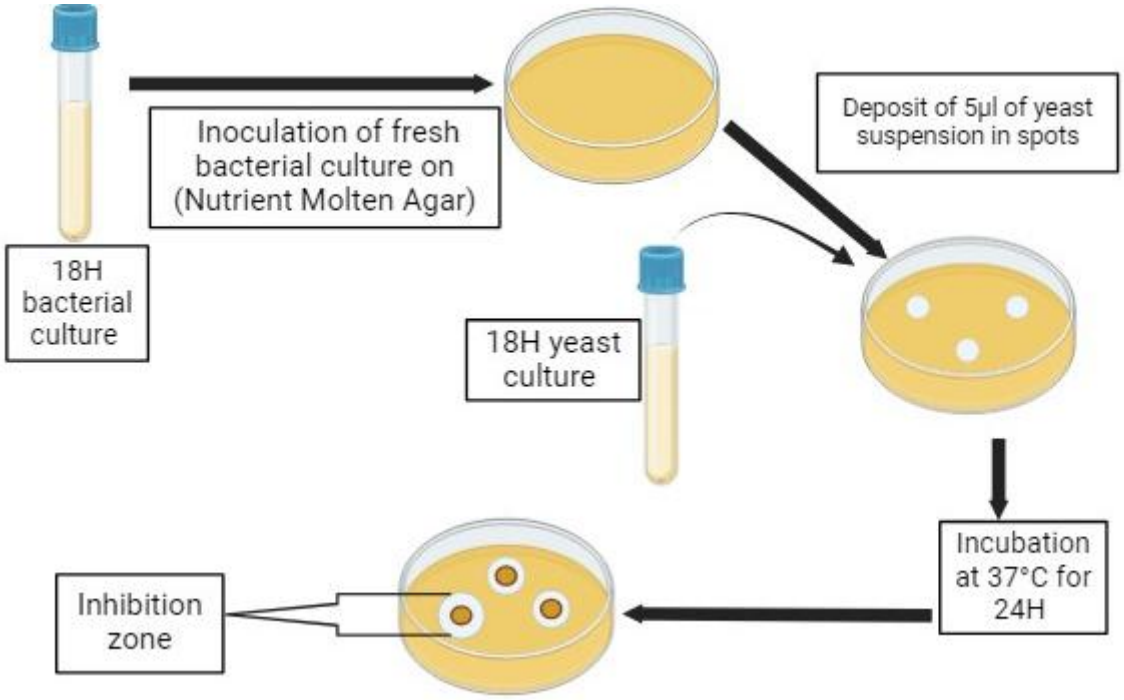


**Figure 7. Protocol of Single Layer Spots Test**

## II.3. Well test

For the realization of well tests we followed the method proposed by **(Magaldi et al., 2004)**.

Fresh 18H yeast cultures were centrifuged at 6000rpm for 15 minutes to obtain supernatants of yeasts. Petri dishes were filled with nutrient molten agar, left to solidify and then inoculated by swabbing with fresh 18H culture bacterial suspensions. Afterwards, 3 wells of a diameter of 6mm were then made on each Petri dish using sterile cones of a 1000µl micropipette and a volume of 100µl supernatants of each yeasts strains was introduced into each well. They were then incubated at 4°C for 30 minutes to allow the diffusion of the supernatants then at 37°C for 24H.At the end of incubation period, the antibacterial activity is indicated by the presence or absence of zones of inhibition around the yeast's wells.

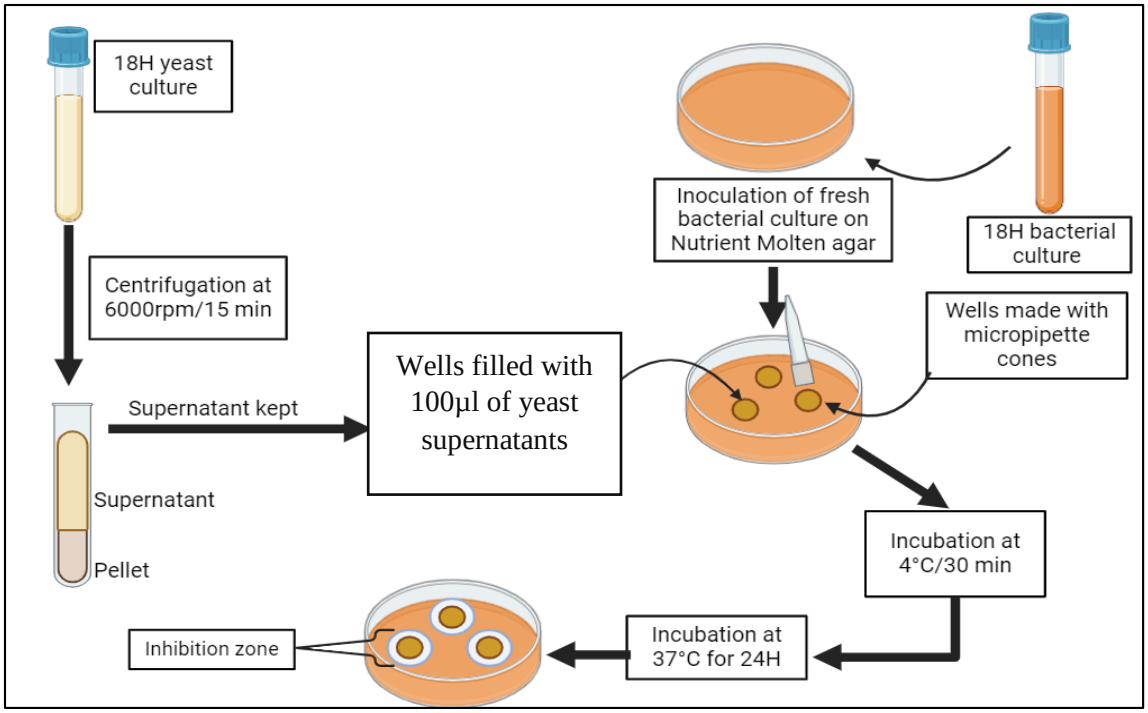


**Figure 8. Protocol of Well method**

## III. Anti-adhesive test

The principle of this test is to determine whether the yeasts have the ability to disrupt bacterial adhesions. This method was performed using the 96-well sterile polystyrene plate assay.

The microbial strains were cultured at 37°C for 18H in TSB broths in order to perform the test with new culture cells. The broth cultures of the yeasts were centrifuged at 6000rpm for 15minutes to obtain their supernatant. The supernant was filtred using syringe filter to ensure no fungal growth during the test. Exempt of the control wells, each well contained 100µl of TSB, 20µl of bacterial strains and 80µl of yeast supernatant. Positive controls contained 180 µl of TSB and 20 µl of bacterial strains. Negative controls contained 120 µl of TSB and 80 µl of yeast supernatant and lastly, the doubled negative controls contained 200 µl of TSB. Each assay was effectuated in three trials. The microplates were then incubated at 37°C for 24H **(Ait Ouali et al., 2014)**.Following the incubation, all the wells were emptied using the same methodology described in **paragraph I.5.2.**The percentage of auto aggregation was calculated according to the following equation:

**Anti adhesive % = (1-(A/Ac))*100)**

# CHAPTER 03: RESULTS & DISCUSSIONS

## Results and Discussions

### I. Identification of the pathogenic strains

Following growth of bacterial strains on specific media, macroscopic observations, results of Gram staining and biochemical characterization tests, an identification of all the pathogenic strains tested and a comparison to previous culture results on ChromAgar have been conducted and the results obtained were summarized globally as shown in (**Table 05).**

**Table 5. Global results of all characterization tests of bacterial strains. N/A signifies the tests that were not carried out for particular strains.**

| *Code* | Agar | Growth | Gram | Shape | TSI | Catalase | Citrate | Coagulase | Suspected strain |
|---|---|---|---|---|---|---|---|---|---|
| ***C1S2-01*** | Slanetz-Bartley | White | + | Cocci | | - | + | - | *Enterococcus* |
| ***C9S1-05*** | Slanetz-Bartley | White | + | Cocci | | - | - | - | *Enterococcus* |
| ***C3S2-06*** | Slanetz-Bartley | White | + | Cocci | | - | - | - | *Enterococcus* |
| ***C8-03*** | Blood agar | Grayish-blue | + | Cocci | | + | + | - | *Streptococcus* |
| ***C7S2-04*** | Blood agar | Grayish-blue | + | Cocci | | - | - | - | *Streptococcus* |
| ***C5=07*** | EMB | Green-metallic | - | Coccobacilli | + | - | + | - | *E. coli* |
| ***C3S1-08*** | EMB | Green- | - | Coccobacilli | + | - | - | - | *E. coli* |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | metallic | | | | | | | |
| ***C3S1-09*** | EMB | Green-metallic | - | Coccobacilli | + | + | + | - | *E. coli* |
| ***E. coli*** | EMB | Green-metallic | - | N/A | + | + | N/A | - | Referencestrain |
| ***C6-02*** | Chapman | White | + | Cocci | N/A | + | N/A | - | *Staphylococcus* |
| ***C1S1-10*** | Chapman agar | White | + | Cocci | N/A | + | N/A | - | *Staphylococcus* |
| ***C1S1-11*** | Chapman agar | White | + | Cocci | N/A | + | N/A | - | *Staphylococcus* |
| ***C7S1-12*** | Chapman agar | White | + | Cocci | N/A | + | N/A | - | *Staphylococcus* |
| ***S. aureus*** | Chapman | Yellow | + | N/A | N/A | + | + | + | *Reference strain* |
| ***C7S1-13*** | Blood agar | Grayish-blue | + | Club-shape | | + | - | - | *Corynebacterium* |

The overall results of Gram staining, catalase, coagulase, growth on specific media showed that the distribution of these strains in the vaginal microbiota included 4 genera, namely *Enterrococcus, Streptococcus, Staphylococcus and Corynebacterium*, and the *E.coli* species as shown in (**Table 5)**. Out of all 13 strains, 3 were confirmed to belong to *Enterococcus* sp. (C1S2-01, C9S1-05 and C3S2-06), 2 to *Streptococcus* sp. (C8-03 and C7S2-04), 4 to *Staphylococcus* sp. (C6-02, C1S1-10, C1S1-11 and C7S1-12) and C7S1-13 was found to belong to the genus *Corynebacterium*. In addition to these, strains C5-07, C3S1-08 and C3S1-09 were classified to belong to the *E. coli*species. All these findings allowed confirmation of the results from previous

culturing on ChromAgar as shown in **Appendix 01**. Verification of *E. coli* by growth at 44°C was confirmed by the presence of turbidity since E.coli is the only coliform amongst all the total coliforms that can tolerate and grow at 44°C **(Paruch & Mæhlum, 2012).**

## II. Characterization of the strains

### II.1. Haemolysis test

All bacterial and yeast strains were tested for haemolysis activity and of all 18 strains, 7 showed ß-haemolysis (complete haemolysis), 11 showed γ-haemolysis (no haemolysis) including the 3 yeast strains and the control (**T**) showed no haemolysis activity. Haemolysins are considered among mechanisms of pathogenicity, as they are responsible for cell membrane destruction, erythrocytes lysis and degradation of neighbouring cells and tissues **(Mogrovejo et al., 2020).**

The ß-haemolytic activity shown by some bacterial strains plays a huge role in their pathogenicity. C3S1-09 was found to be pathogenic *E. coli* and demonstrated ß-haemolytic activity on blood agar. These results coincide with the existing literature, as according to **Puente et al. (2001)** approximately half of UPEC (Uropathogenic *Escherichia coli*) produce haemolysin (HlyA) structural gene. The non haemolytic activity of the yeast strains confirms their non virulence nature and making them appropriate probiotic candidates**(Nath et al., 2020).**

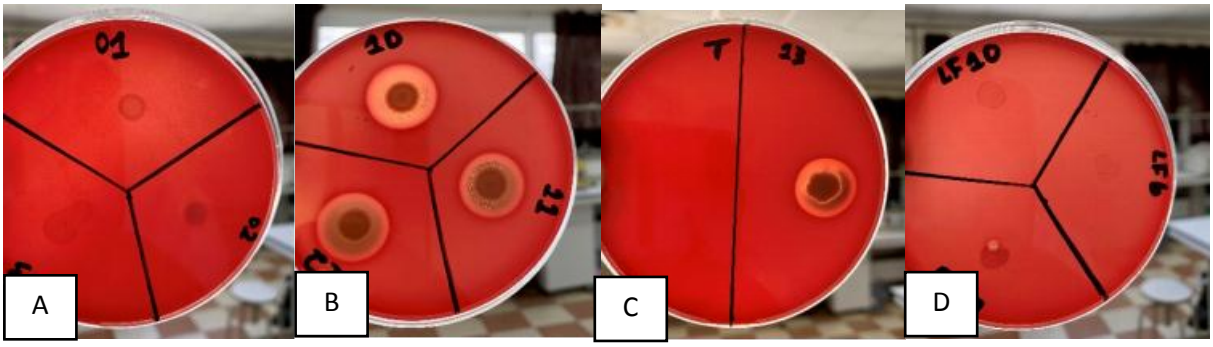


**Figure 9. Haemolysis test results with control. (A) and (D) Show bacteria and yeast strains showing no haemolysis respectively. (B) shows complete haemolysis. (C) Shows control on the left side that had no growth and complete haemolysis on the right side.**

**Table 6. Haemolysis test results**

| | |
|---|---|
| γ-haemolysis | *C1S2-01/ C6-02/ C8-03/ C3S2-06/ C5-07/ C3S1-08/ E.coli (Reference)/ S. aureus (Reference)/ LF6/ LF10/ LG5* |
| ß-haemolysis | *C7S2-04/ C9S1-05/ C3S1-09/ C1S1-10/ C1S1-11/ C7S1-12/ C7S1-13* |

# II.2. Auto-aggregation test

Summarised below**(Figure 10)**are the results following measurement of absorbance at 600nm, before incubation, after 2H and after 4H incubation at 37°C for both bacterial and yeast strains. The highest percentages of aggregation were observed after 4H of incubation. The highest rates were registered for strains C8-03 and C3S1-08 at 34% and 33% respectively following the 4H incubation. The lowest rates were registered at 0% for strain C1S1-10.

# II.3. Biofilm formation

The measure of absorbance at 630nm revealed that the tested pathogenic bacteria were able to form biofilms at different degrees depending on the pathogenic strain. In comparison to the absorbance control(**Ac=0.149**), they were therefore classified according to their ability to form biofilms or not and at what degree of biofilm formation as detailed by **Mathur et al., (2006)**as shown in **table 7.**

Weak biofilm producer: **A < Ac**, moderate biofilm producer: **Ac ≤ A ≤ 2Ac**, and strong biofilm producer**: A > 2Ac**

**Table 7. Classification of pathogenic strains according to their capacity to form biofilm**

| A < Ac | Ac ≤ A ≤ 2Ac | A > 2Ac |
|---|---|---|
| C6-02 | C1S2-01 | C3S2-06 |
| C7S2-04 | C8-03 | C5-07 |
| | C9S1-05 | C3S1-08 |
| | C7S1-11 | C3S1-09 |
| | C7S1-12 | C1S1-10 |
| | | C7S1-13 |

Of all the vaginal pathogenic strains tested, 2 strains (C6-02 and C7S2-04) belonging to the *Staphylococcus* and *Streptococcus* genera respectively, displayed the least rate of biofilm formation. Strains C1S2-01, C9S1-05, C8-03, C7S1-11 and C7S1-12 belonging to the genera *Enterococcus*, *Streptococcus* and *Staphylococcus* respectively.Lastly, 6 strains (C3S2-06, C5-07, C3S1-08, C3S1-09, C1S1-10 and C7S1-13) belonging to the genera *Enterococcus*, *E. coli*,*Staphylococcus*and *Corynebacterium* respectively showed strong biofilm formation.

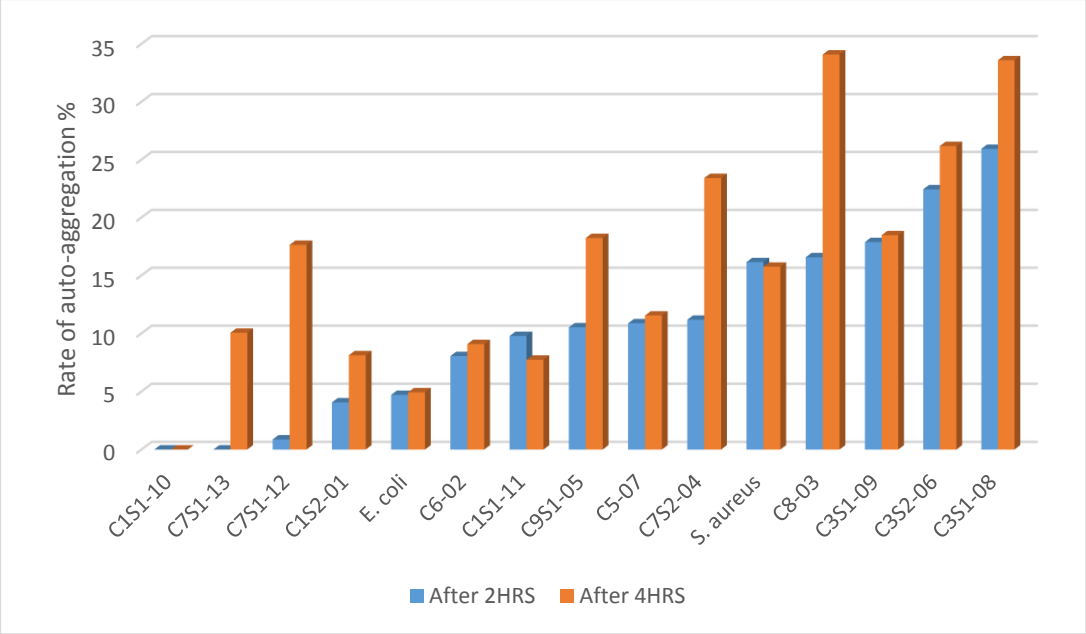


**Figure 10. Results of auto aggregation tests for the pathogenic strains after 2hours and 4 hours of incubation.**

**Table 8. Results of biofilm formation showing optic density of each bacterial strain**

| Bacterial Strains | Optical Density |
|---|---|
| **C1S2-01** | 0,169 |
| **C6-02** | 0,134 |
| **C8-03** | 0,186 |
| **C7S2-04** | 0,147 |
| **C9S1-05** | 0,18 |
| **C3S2-06** | 0,411 |
| **C5-07** | 0,44 |
| **C3S1-08** | 0,37 |
| **C3S1-09** | 0,415 |
| **C1S1-10** | 0,402 |
| **C1S1-11** | 0,1835 |
| **C7S1-12** | 0,1925 |
| **C7S1-13** | 0,584 |

In both auto aggregation and biofilm formation tests, the pathogenic strains mostly showed positive results with different rates of activities. *E. coli* strains tested proved to be capable of auto aggregating and forming biofilms, as seen in **(figure 10 and Table 08)**, which is in agreement with a report of **Ponnusamy et al. (2012)**in which they proved UPEC (Uropathogenic *Escherichia coli*) to display a biofilm positive phenotype.

On the other hand, studies have shown that auto aggregation does not always promote biofilm formation **(Trunk et al., 2018),**as demonstrated by strain C8-03, C7S2-04 and C7S1-12 **(figure 10 and Table 08)**, they had the highest auto aggregation rates of all bacterial species tested and these were coupled with a relatively lower rate of formation biofilm.

In both auto aggregation and biofilm formation tests, *Corynebacterium* (C7S1-13) displayed a biofilm positive phenotype, having the highest absorbance, the same way the experiment carried out by**Souza et al. (2015)**confirmed that *Corynebacterium* strains have strong hydrophobicity and auto aggregation properties which play key roles in the initial attachment and biofilm formation on abiotic surfaces like polystyrene.

## III. Antibacterial activity of fungal strains

### III.1. Tests of antibacterial activity

Direct antagonism tests, which included the soft agar overlay spots test and single layer spots test showed no antibacterial activity as there were no zones of inhibition around all yeasts' spots. Moreover, the indirect antagonism test that was carried out using the well-test method also showed no antibacterial activity, as there were no zones of inhibition around all wells with yeast strains.

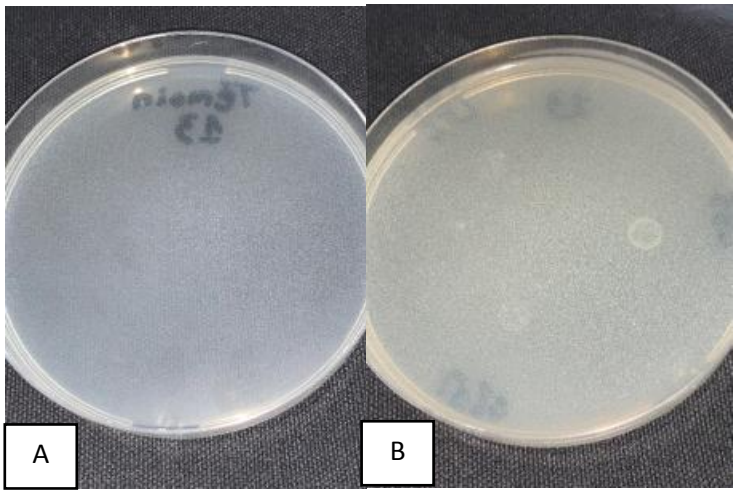


**Figure 11. Results of Soft Agar Overlay Spot Test. (A): Control (B): Negative results.**

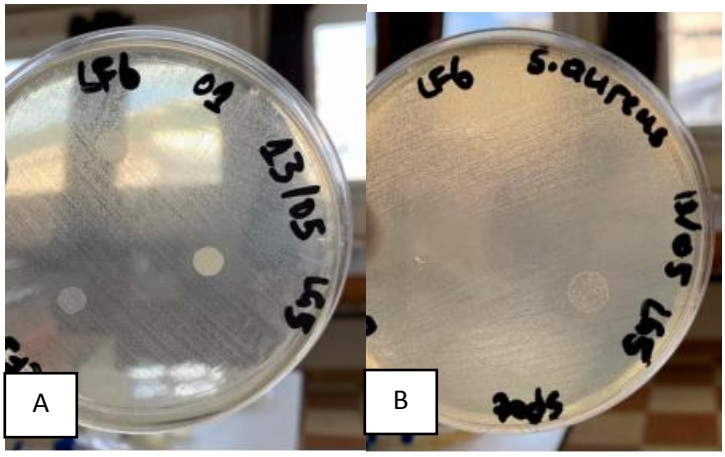


**Figure 12. Results of Single Layer spot test. (A) And (B) show negative results.**

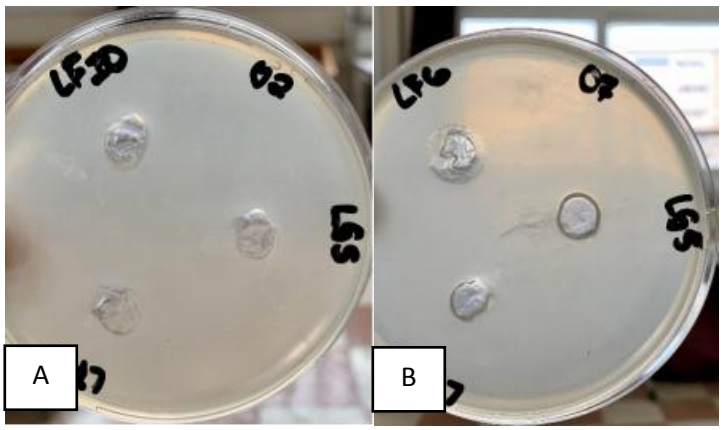


**Figure 13. Negative results following Well test.**

# Results and Discussions

*Saccharomyces cerevisiae*'s killer phenomenon was first discovered by Bevan and Makower in 1963. After this discovery followed multiple other researches confirming antimicrobial activity in other yeasts, *Kluyveromyces* sp. included **(Nascimento et al., 2020).**Since then until date, there have been various improvements towards the study and understanding of this phenomenon, which included identification of mechanisms involved in their antimicrobial activity. This activity has been linked to their ability to produce bioactive compounds capable of inhibiting the growth of bacteria and other yeast strains. **Chen et al. (2021)** extracted four antibacterial compounds from *S. cerevisiae* and *K. marxianus* and they were confirmed to possess killer toxins and organic acids, which inhibited growth of Gram-negative and Gram-positive bacteria.

Even though there are multiple researches on the yeast antibacterial activity, less is still known when it comes to how possible and profound yeast antibacterial activity is against vaginal pathogenic microbiota. Antibacterial results obtained in this study did not corroborate the aforementioned statements as both *S. cerevisiae* and *K. marxianus* did not show any antibacterial activity against pathogens isolated from vaginal microbiota which could be due to various reasons linked to experimental protocol, the yeasts and/or bacteria strains used.

Firstly, one of the reasons for the negative results could be lack of susceptibility of bacteria to the yeasts' antibacterial activity and/or their killer compounds making some bacteria yeast-resistant. Studies have shown that Gram-negative pathogens are more sensitive to yeast antimicrobial activity than Gram-positive pathogens due to presence of thicker peptidoglycan in the cell walls of the latter. In one study, **Younis et al., (2017),** carried out antagonistic tests against pathogenic bacteria using isolated colonies of different yeasts and in another study, **Al-Sahlany et al., (2020)** used an antibacterial peptide isolated from *S. cerevisiae*, both their results showed highest activity against Gram-negative bacteria and little to no activity against Gram-positive bacteria.

Bacterium inoculum size and the period of incubation fall under important factors in susceptibility testing. Larger inoculum and longer incubation periods lead to higher Minimum Inhibitory Concentrations (MIC), which emphasizes the need for controlled and standardized conditions in susceptibility testing to ensure accurate results**(Bubonja-Šonje et al., 2020).** In

clinical microbiology, the standard used is that of McFarland 0.5, which represents $1.5*10^8$ bacteria/ml thus, ensures the reproducibility and accuracy of results**(Leber, 2016).**

As one of their antagonistic mechanism, yeasts are known to compete for substrates **(Sipiczki,2023),** however, in this study; bacterial strains seemed to overgrow the yeasts' strains as the results demonstrate in **figure 13**therefore highlighting their inactivity against bacterial strains.

Additionally, the diffusion period in well test is one of the important factors which play a role in antimicrobial susceptibility testing, the most frequently used period being 2-4H **(Schumacher et al., 2018).** Long periods can lead to more reliable results as they allow enough time for antimicrobial compounds to diffuse into the agar and inhibit bacterial growth while short periods can lead to false negative results, the diffusion period allocated in this study was about 30 minutes.

On the other hand, there have been several studies demonstrating *S. cerevisiae* and *K. marxianus*'s anti-microbial inactivity against pathogens. In one study,**Bilinski et al., (1985)** tested for the antimicrobial activity of a total 400 *Saccharomyces* andnon-*Saccharomyces* species on 9 bacterial species and only two species ( *Kluyveromyces thermotolerans and Kloeckera apiculata*) were found to display antibacterial activity. These findings were supported by another study carried out by **McCormack et al., (1994)** in which they found that non-phylloplane*S. cerevisiae* amongst other yeasts tested on bacterial species, did not demonstrate any inhibitory activity.

Lastly, **Bala Sharma et al., (2023)**conducted a study where they extracted *K. marxianus* exopolysaccharides, purified and tested them for antimicrobial activity against pathogens including *E. coli* and *S. aureus*, whereby they also found no inhibitory activity on all pathogenic strains.

# III.2. Anti-adhesive test

Measurement of OD after incubation and staining of 96-well microtitre showed results of yeasts anti-adhesive activity against bacterial pathogenic strains.

In comparison to the two yeast strains LF10 and LG5, the yeast strain LF6 demonstrated the highest adhesion inhibition rates, having inhibited strains C1S2-01, C9S1-05, C8-03, C1S1-11, C7S1-12 and C3S1-08 belonging to the genera *Enterococcus*, *Streptococcus*, *Staphylococcus* and *E. coli* species respectively, by up to a 100% as shown in **figure 14.**

In addition to this, it was still effective in inhibiting adhesion of the rest of the bacterial strains with adhesion inhibition rates ranging between 78% and 96%. This activity was followed by that of LF10 with a 100% inhibition rate against pathogenic strains (C3S1-09, C1S1-10 and C7S1-12) belonging to *E. coli* and *Staphylococcus* sp.

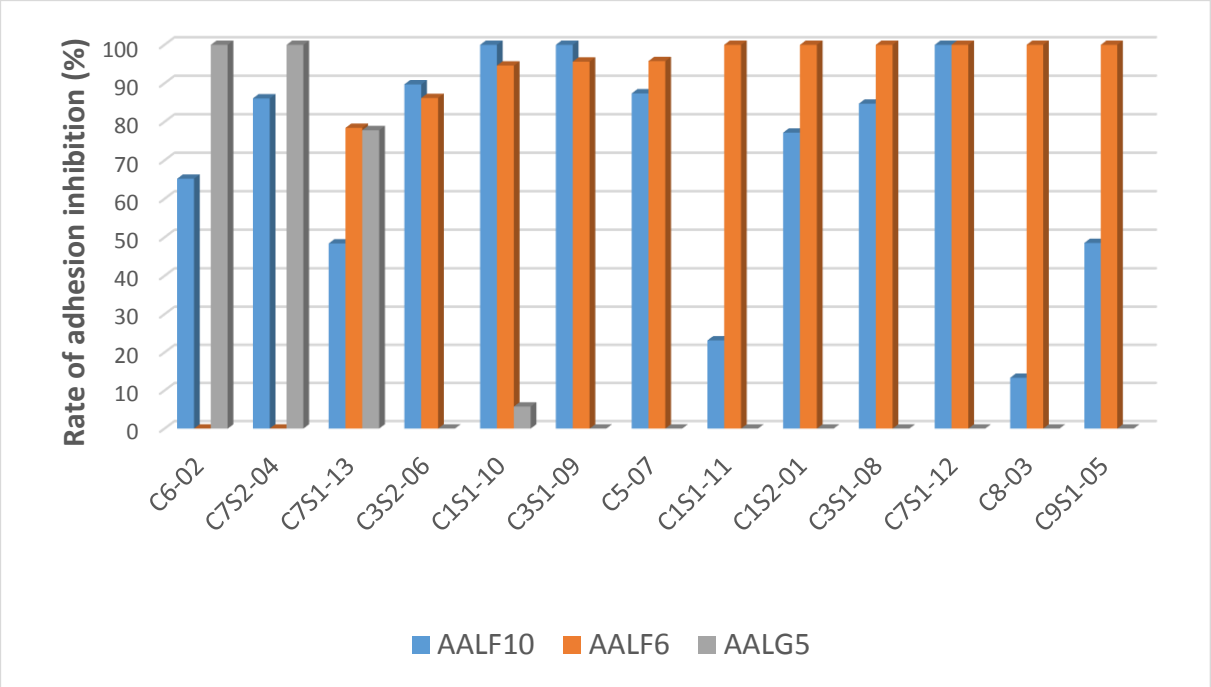


**Figure 14. Results of adhesion inhibition rates of three yeast strains against vaginal pathogens**

On the other hand, LG5's anti-adhesive properties were highly selective having inhibitory activity against strains C7S2-04, C6-02, C1S1-10 and C7S1-13 belonging to *Streptococcus* sp. and *Staphylococcus* sp. and *Corynebacterium* sp. respectively. This yeast showed no activity at all against 9 other bacterial species and a minimum inhibitory activity against *Staphylococcus* sp. (C1S1-10). However, it had the same anti-adhesive activity as LF6 against *Corynebacterium* sp. (C7S1-13).

Even though there seems to be limited prior experimental researches on the anti-biofilm activity of the tested yeast strains against vaginal pathogenic isolates, there are a few that demonstrate the activity of *S. cerevisiae* against pathogens like *S. aureus and E. coli***(Fadhil Abbas Al-Helli & Abdul Sattar Salman, 2023; Kim et al., 2020)**. On the other hand, there is currently little to no researches on the anti-biofilm activity of *K. marxianus* against pathogens.

Similarly to this study's results of the two *S. cerevisiae* strains (LF6 and LF10) demonstrating the highest adhesion inhibition activity against the vaginal pathogens tested (As illustrated in **figure 14**). Other studies also showed this activity against other pathogens. A study carried out by **Kim et al., (2020)** demonstrated the yeasts anti-adhesion activity against antibiotic resistant *S. aureus,* likewise, **Fadhil Abbas Al-Helli& Abdul Sattar Salman, (2023)** showed this yeast's anti-adhesion activity against *S. aureus and E. coli.*

On the contrary, the strain *K. marxianus* showed anti-biofilm activity only against 4 strains (C7S2-04, C6-02, C1S1-10 and C7S1-13) and instead showed an increase in the biofilm biomass of the rest of the strains. This could be explained by numerous studies that have demonstrated this yeast's strong ability to form biofilms, co-aggregate with other species also enhancing biofilm formation of those species. A study conducted by **Wang et al., (2023)** showed that addition of live yeast not only promoted the growth of Lactic Acid Bacteria (LAB), but also enhanced their aggregation and biofilm formation. In a study conducted by **Han (2018)** and another by **Yonten & Aktas, (2016),***K. marxianus* showed the highest biofilm formation amongst other strains tested.

# CONCLUSION

# Conclusion

This study was aimed to determine the possibility of yeasts *S. cerevisiae* and *K. marxianus*(extracted from fruits figs and pomegranate respectively), being used as probiotics in the treatment of vaginal infections by assessing their antibacterial and anti-biofilm activities against pathogenic bacteria. Another objective was to identify and characterize the tested pathogenic bacteria, tested for their auto-agreggation and biofilm formation capacity.

The results of identification confirmed the previous results of culture made on ChromAgar, grouping the bacterial strains into four genera: *Staphylococcus, Streptococcus, Enterococcus* and *Corynebacterium,* together with the *E. coli* species. Some of the pathogenic strains showed haemolytic activity, emphasising on their pathogenicity and they did not show any susceptibility towards the antibacterial activity of the three yeasts strains as there were no zones of inhibition around the spots and wells made, which further concludes on their resistance against the yeasts' antimicrobial activity, thus their pathogenicity.

On the other hand, the anti-adhesive results showed great inhibition by the *S. cerevisiae* strains against most of the pathogenic strains, while the *K. marxianus*strain showed inhibition against few strains. We conclude that *S. cerevisiae* can be used as potential probiotic treatment against biofilm forming pathogens in the vagina while*K. marxianus* could also be used but not in a large spectrum like *S. cerevisiae*.

Last but not least, research based on bioengineering of yeasts-based probiotics can be done to include auto agglutinins from vaginal pathogens in the probiotics to render the pathogens less virulent, inhibiting their biofilm formation and thus reducing the risk of infection. A further exploration into different methods for antagonistic tests could be performed to confirm the pathogenic strains' resistance to the yeasts extracted from fruits.

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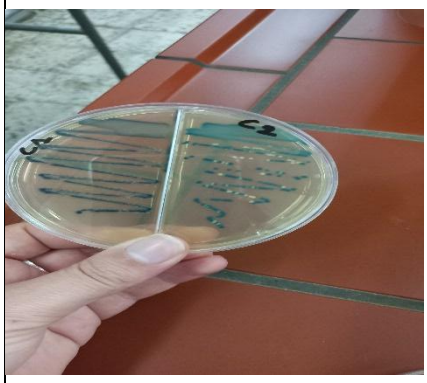
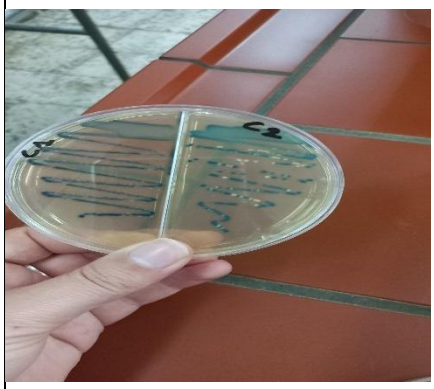
# Appendix

# Appendix

Appendix I: Materials and Methods

Table 1. Previous results of bacterial strains inoculated on ChromAgar

| Strains' codes | Colonies on ChromAgar | Suspected species | Confirmed by | Photo of every strain |
|---|---|---|---|---|
| C1S1 | Small white colonies | Candida/ *Staphylococcus aureus* | Sabouraud / Chapman |  |
| C1S2 | Small blue turquoise colonies | Enterococcus | Gram staining(gram+ diplococci) or + esculin test) |  |

# Appendix

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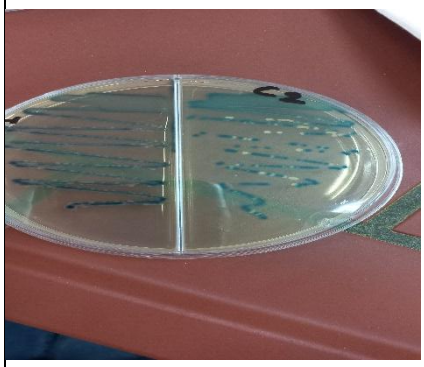
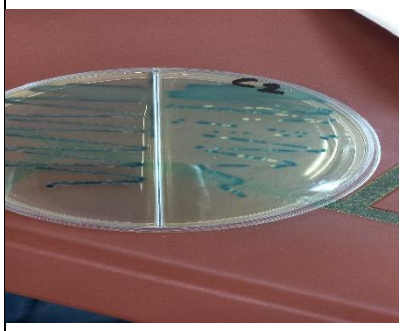
|---|---|---|---|---|
| C2S1 | White medium sized colonies | Corynebacterium | Gram+ catalase + bacillus |  |
| C2S2 | Small blue colonies | Streptococcus | Gram staining (Gram+ cocci in chains) – esculin) |  |
| C3S1 | Small mallow colony | *Escherichia coli* | Gram- blue black bacillus with green metallic reflection on EMB |  |

# Appendix

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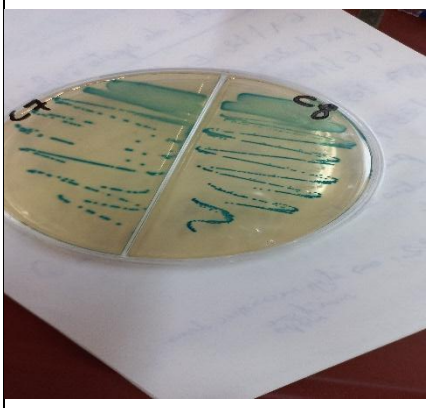
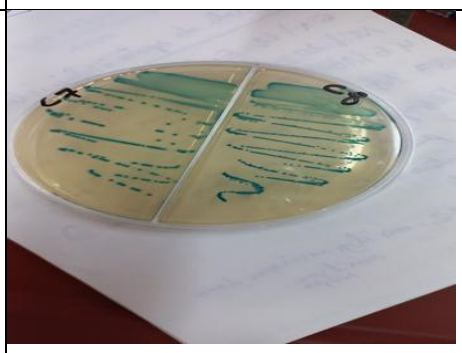
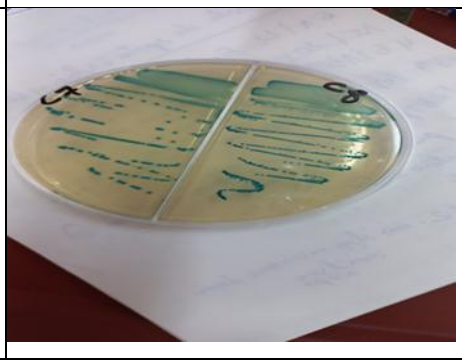
|---|---|---|---|---|
| C3S2 | Small blue colonies | Enterococcus | Gram staining (Gram + diplococci) or +esculin test |  |
| C4S1 | Small mallow colony | *Escherichia coli* | Gram- blue black bacillus with green metallic reflection on EMB |  |
| C4S2 | Small mallow colony | Enterococcus | Gram staining (Gram + diplococci) or +esculin test |  |
| C5 | Medium sized mallow colony | *Escherichia coli* | Gram- blue black bacillus with green metallic reflection on EMB |  |

# Appendix

| | | | | |
|---|---|---|---|---|
| C6 | Small golden white colony | *Staphylococcus aureus*/ Candida | Isolation on Chapman or Sabouraud | Pas de photo |
| C7S1 | White small colonies | Candida / *Staphylococcus aureus* | Sabouraud/ Chapman |  |
| C7S2 | Small blue colonies | Streptococcus | Gram staining (Gram+ cocci in chains) – esculin) |  |
| C8 | Small blue colonies | Streptococcus | Gram staining (Gram+ cocci in chains) – esculin) |  |

# Appendix

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|---|---|---|---|---|
| C9S1 | Blue colony | Enterococcus | Gram staining (Gram + diplococci) or +esculin test |  |
| C9S2 | Mallow colonies | *Escherichia coli* | Gram- blue black bacillus with green metallic reflection on EMB |  |

## II. Composition of the culture media according to the supplier

Table 2. TSB broth (Tryptic Soy Broth), pH 7

| Components | **g/l** |
|---|---|
| Casein Peptone | 17 |
| Soy Peptone | 3 |
| Glucose | 2,5 |
| Dipotassium Phosphate | 2,5 |
| SodiumChloride | 5 |
| Yeast Extract | 6 |
| Distilled water | 1L |

# Appendix

Table 3. Nutrient broth, pH 7

| Components | **g/l** |
|---|---|
| Meat Extract | 1 |
| Yeast Extract | 2,5 |
| Peptone | 5 |
| Sodium Chloride | 5 |
| Distilled water | 1L |

Table 4. Chapman Agar (Mannitol Salt Agar), pH 7

| Components | **g/l** |
|---|---|
| Peptone | 10 |
| Beef Extract | 1 |
| Sodium Chloride | 75 |
| Mannitol | 2,5 |
| Phenol Red | 0,025 |
| Agar | 15 |
| Distilled water | 1L |

Table 5. Eosin Methylene Blue Agar (EMB), pH 7.2

| Components | **g/l** |
|---|---|
| Peptone | 10 |
| Lactose | 10 |
| Dipotassium Phosphate | 2 |
| Eosin | 0,4 |
| Methylene Blue | 65 mg |
| Agar | 15 |
| Distilled water | 1L |

# Appendix

Table 6. Simmons Citrate Agar, pH 7

| Components | **g/l** |
|---|---|
| Sodium Citrate | 1 |
| Bromothyl Blue | 0,08 |
| Sodium Chloride | 5 |
| Magnesium Sulphate | 0,2 |
| Potassium Hydrogenophosphate | 1 |
| Ammonium Dihydrogenophosphate | 1 |
| Agar | 15 |
| Distilled water | 1L |

Table 7. Slanetz and Bartley Agar, pH 7

| Components | **g/l** |
|---|---|
| Tryptose | 20 |
| Yeast Extract | 5 |
| Glucose | 2 |
| Dipotassium Hydrogen Phosphate | 4 |
| Sodium Azide | 0,4 |
| 2,3 ,5 triphenyl tetrazolium Chloride | 0,1 |
| Agar | 15 |
| Distilled water | 1L |

Table 8. Columbia Agar, pH 7

| Components | **g/l** |
|---|---|
| Polypeptone | 17 |
| Pancreatic Peptone | 3 |
| Yeast extract | 3 |
| Maize starch | 1 |
| Sodium Chloride | 5 |

| | |
|---|---|
| Agar | 15 |
| Distilled water | 1L |

Table 9. Tryptone Salt broth (TS), pH 7

| Components | **g/l** |
|---|---|
| Tryptone | 1g |
| Sodium Chloride | 9g |
| Distilled water | 1L |

Table 10. Nutrient Agar, pH 7

| Components | **g/l** |
|---|---|
| Meat Extract | 1 |
| Yeast Extract | 2 |
| Peptone | 5 |
| Sodium Chloride | 5 |
| Agar | 15 |
| Distilled water | 1L |

Table 11. Mueller Hinton Agar (MHA), pH 7.3

| Components | **g/l** |
|---|---|
| Beef Extract | 2 |
| Acid Hydrolysate of Casein | 17.5 |
| Starch | 1.5 |
| Agar | 1 |
| Distilled water | 1L |

# Appendix

**Abstract**

Antimicrobial resistance through biofilm formation is a well-known bacterial virulence factor. However, an even more concerning fact is that pathogenic microorganisms are rapidly developing resistance to antibiotics, diminishing the effectiveness of antibiotic therapy. This has prompted a search for alternative treatment approaches for recalcitrant infections. The present study aimed to investigate the potential of yeast as a probiotic therapy by determining the antibacterial and anti-biofilm activity of yeast against vaginal pathogens. The study was conducted at a pedagogical laboratory of the University of Bejaia from April to May 2024. Three yeast strains were tested - two *Saccharomyces cerevisiae strains* and one *Kluyveromyces marxianus* strain - against 15 strains of vaginal pathogens. After characterization

of the bacterial strains using diverse methods, biochemical tests included, all strains were assessed for auto-aggregation and biofilm formation. The yeast strains were tested for antibacterial activity as well as for antibiofilm activity. The characterization results confirmed the pathogenic strains belonged to five genera: *Staphylococcus, Streptococcus, Enterococcus, Corynebacterium,* and *Escherichia coli* species. While both yeast strains did not inhibit pathogenic growth, the *S. cerevisiae* strains exhibited promising anti-biofilm activity, and all the yeast strains demonstrated auto-aggregation activity.

**Résumé**

La résistance aux antimicrobiens par la formation de biofilms est un facteur de virulence bactérien bien connu. Cependant, un fait encore plus inquiétant est que les micro-organismes pathogènes développent rapidement une résistance aux antibiotiques, réduisant ainsi l'efficacité de l'antibiothérapie. Cette situation a conduit à la recherche d'approches thérapeutiques alternatives pour les infections vaginales récurrentes. L'objectif de la présente étude était d'étudier le potentiel de la levure en tant que thérapie probiotique en déterminant l'activité antibactérienne et anti-biofilm de la levure contre les pathogènes vaginaux. L'étude a été menée dans un laboratoire d'enseignement à l'Université de Bejaia entre Avril et Mai 2024. Trois souches de levure ont été testées - deux souches de *Saccharomyces cerevisiae* et une souche de *Kluyveromyces marxianus* – à l'égard de 15 souches pathogènes vaginales. Après avoir caractérisé les souches bactériennes à l'aide de diverses méthodes, y compris des tests biochimiques, toutes les souches ont été évaluées pour l'auto-agrégation et la formation de biofilms. Les souches de levure ont été testées pour leur activité antibactérienne et leur activité anti biofilm. Les résultats de la caractérisation ont confirmé que les souches pathogènes appartenaient à cinq genres : *Staphylococcus, Streptococcus, Enterococcus, Corynebacterium* et *Escherichia coli*. Alors que les deux souches de levure n'ont pas inhibé la croissance pathogène, les souches de *S.cerevisiae* ont montré une activité anti-biofilm prometteuse, et toutes les souches de levure ont démontré une activité d'auto-agrégation.