UNIVERSIDADE
BEIRA INTERIOR

Evaluation of probiotic and host-interactions mechanisms of *Lactobacillus* species in response to antifungal drugs

Versão final após defesa

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Dissertação para obtenção do Grau de Mestre em
Ciências Biomédicas
(2º ciclo de estudos)

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Dezembro de 2021

Dedicatória

Grata a todos que me acompanharam de perto e de longe, mas dedico este trabalho primeiramente ao meu filho Victor Miguel porque dele provém toda a minha força para lutar e nunca desistir dos meus sonhos e aos meus pais meus maiores incentivadores da vida, por acreditarem em mim.

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Agradecimentos

“Pelo dom da vida, obrigada Deus por trilhar os caminhos da ciência.”

Em primeiro lugar quero agradecer aos meus pais, António e Madalena pelo apoio incondicional que sempre me transmitiram. Obrigada, por terem cuidado do meu filho, cuja existência fez de mim nascer uma vontade imensa de continuar a lutar todos os dias.

Ao meu orientador, Carlos Gaspar, por todo o apoio a mim proporcionado durante este ano de trabalho, pela paciência, pela amizade, pelas palavras de incentivo e pela partilha de conhecimentos que tanto contribuíram para o desenvolvimento deste trabalho.

A minha Co-Orientadora, Professora Doutora Ana Palmeira de Oliveira, pelo conhecimento transmitido, pela colaboração na resolução de dúvidas e problemas que foram surgindo ao longo do trabalho.

Ao Prof. Dr. José Martinez-de-Oliveira e a Prof. Drª. Joana Rolo pela oportunidade que me proporcionou e por tornar possível a realização deste trabalho.

Agradeço a todos os meus colegas de laboratório de Microbiologia II, do Centro de Investigação em Ciências da Saúde (CICS-UBI), em especial à Ruben Miguel do Adro, Ana Sofia Oliveira e Margarida Damasceno pelos ensinamentos científicos, conselhos e sugestões transmitidos.

Agradeço com carinho a todos que sempre me apoiaram e cuidaram de mim, nomeadamente: Gisela, Andrea, Jacira, Nilsa, Nangely, Mónica, Dulcineia, Joanita, Valdir, Nadine, Waldik, Carla, Djéssica e Izamara. Obrigada, por me transmitirem boas energias para nunca eu desistir.

Resumo

Os *lactobacilos* são identificados como constituintes da microflora vaginal saudável, atuando como probióticos que a protege de infeções através da produção de bacteriocinas e inibição dos sítios de adesão. Contudo, algumas mulheres apresentam a vaginose citolítica que é caracterizada pela proliferação excessiva de espécies de *lactobacilos* que podem danificar o epitélio intermediário da vagina. Esta sintomatologia é semelhante à candidose vulvovaginal e pode, portanto, ser diagnosticada como tal e tratada com a terapia antifúngica.

O presente estudo visa compreender o efeito da terapia antifúngica nos microrganismos comensais e o impacto na previsão de estratégias terapêuticas. Foram incluídas seis estirpes de *lactobacilos*, e a suscetibilidade aos agentes antifúngicos (azóis) foi determinada pelo método de microdiluição CLSI. Além disso, o metabolismo e a capacidade de aderência foram avaliados in vitro e em co-cultura com células HeLa.

Como resultados, observámos que o econazol, fenticonazol e isoconazol, além de inibirem o crescimento das estirpes em aproximadamente 50%, também diminuem a capacidade de adesão *in vitro*. Em contraste, para além de aumentar a capacidade de adesão *in vitro*, o voriconazol também aumenta dez vezes a capacidade de adesão das estirpes nas células de HeLa. Além disso, o fenticonazol aumenta o consumo de glicose e consequentemente a produção de lactato em cerca de 20% quando comparado com o controle.

Será próximo passo nesta linha de investigação elucidar os mecanismos de ação do fenticonazol pois aumenta a adesão das estirpes nas HeLa mas diminui a adesão das mesmas *in vitro*. Por último, com os dados obtidos sugerir o voriconazole como a melhor terapia antifúngica uma vez que é mais especifica as *cândidas spp*. e favorece a adesão das estirpes probióticas.

Palavras-chave

Lactobacilos, suscetibilidade, probiótico, antifúngico.

Abstract

Lactobacillus are identified as constituents of the healthy vaginal microflora, acting as probiotics that protect it from infection by producing bacteriocins and inhibiting adhesion sites. However, some women have cytolytic vaginosis characterized by excessive proliferation of *Lactobacillus* species that can damage the intermediate vaginal epithelium. This symptomatology is similar to vulvovaginal candidosis and can therefore be diagnosed as such and treated whit antifungal therapy.

The present study aims to understand what effect antifungal therapy has on commensal microorganisms and the impact on predicting therapeutic strategies. Six *Lactobacillus* strains were included, and susceptibility to antifungal drugs (azoles) was determined by the CLSI microdilution method. In addition, metabolism and adhesion capacity were evaluated *in vitro* in co-culture with HeLa cells.

As results, we observed that econazole, fenticonazole, and isoconazole, besides inhibiting the growth of the strains by approximately 50% also decreased the adhesion capacity *in vitro*. In contrast, besides increasing the adhesion capacity *in vitro*, voriconazole also increases the adhesion capacity of the strains on HeLa cells ten-fold. Furthermore, fenticonazole increases glucose consumption and consequently lactate production by around 20% when compared with control.

It will be the next step in this line of research to elucidate the mechanisms of action of fenticonazole as it increases the adhesion of the strains in HeLa but decreases their adhesion *in vitro*. Finally, the data obtained suggests voriconazole is the best antifungal therapy since it is more specific to *Candida spp* and promotes the adhesion of the probiotic strains.

Keywords

Lactobacillus, susceptibility, probiotic, antifungal.

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List of Abbreviations

BS	Biosurfactants
CFU	Colony forming units
CLSM	Confocal laser scanning microscopy
CV	Cytolytic vaginosis
CYP	Cytochrome P450
DMEM	Gibco Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
H_2O_2	Oxygen peroxide
HIV	Human immunodeficiency virus
IL-1b	Interleukin-1 beta
IL-6	Interleukin-6
IL-17	Interleukin-17
LAB	Lactic acid bacteria
MIC	Minimum Inhibitory Concentration
MLC	Minimum Lethal Concentration
MRS	Man, Rogosa and Sharpe
OD	Optical density
PBS	Phosphate buffered saline
VA	Aerobic vaginitis

I. INTRODUCTION

The human body houses microorganisms that inhabit surfaces and cavities exposed or connected to the external environment. Each site in the body includes ecological communities of microbial species that exist in a mutualistic relationship with the host. The types of organisms present are highly dependent on the prevailing environmental conditions and host factors, so they vary from site to site. In addition, they vary between individuals and over time (1).

1.1 Vaginal microflora

The vagina is a delicately balanced organ that, to ensure the integrity of its ecosystem, requires an interaction between the vaginal flora, the hormonal state, the products of microbial metabolism and the host's immune response (2, 3). It is colonized by a diverse community of microorganisms known as the vaginal microbiota (VM), including the species of *Lactobacillus*, *Gardnerella vaginalis, Trichomonas vaginalis* and *Candida albicans* that live in harmony and that are therefore considered commensal, but that can, in particular situations, become pathogenic (4). In addition to this richness in bacterial flora, it also contains fluids that produce moisture and physiological secretions. Therefore, the homeostasis of the vaginal ecosystem results from complex and synergistic interactions between the host and different microorganisms that colonize the vaginal mucosa (5). Thus, the vagina and its microflora form an ecosystem, where the vaginal environment controls the types of the microorganisms present and the microflora, in turn, controls the vaginal environment.
The vaginal pH, generally between 3.8 and 4.5 in fertile age, is the product of this interaction, functioning as the first line of defense against the proliferation of pathogenic microorganisms in the vaginal flora (6). In fact, the flora composition is not static, varying cyclically and from woman to woman in response to exogenous and endogenous factors. Such factors include: age, ethnicity, menarche, menstrual cycle time, pregnancy, infections, use of antibiotics or other medications with immunosuppressive properties, contraceptive methods, frequency of sex, the number of sexual partners , and various sexual habits and behaviors (7). During the menstrual cycle, hormonal variations interfere with the substrate of different microorganisms, as

they lead to changes in vaginal pH. *Lactobacillus* levels remain constant throughout the cycle, but *non-Lactobacillus* species increase during the proliferative phase and the concentrations of *Candida albicans* may become higher in the premenstrual period (8). The use of antibiotics for treatment in asymptomatic women can alter the vaginal ecology, since it induces an endogenous disturbance in the microflora and can trigger the selective proliferation of microorganisms that were being inhibited and that could be harmful to the health of the vagina. In sexual intercourse, the use of compounds such as spermicides can cause microabrasions or microulcerations in the vaginal epithelium, which can interrupt the vaginal epithelial integrity (9). According to Guenthner et al (2005), inflammation of the genital tract and HIV transmission seem to be associated, since *Trichomonas vaginalis* showed cytotoxicity to vaginal epithelial cells (10). In addition, the integrity of the epithelial cells isolated from the female genital tract was directly disrupted by the presence of HIV. Therefore, maintaining or recovering the integrity of the epithelial barrier is necessary for vaginal health, since delayed healing worsens inflammation (11). As for ethnicity, figure 1 shows that vaginal bacterial communities dominated by *Lactobacillus* species (groups I, II, III and V) are found mostly in Asian and white women than in Hispanic and black women (12). These differences may include differences in the immune systems, composition, and amount of vaginal secretions, among others (13, 14). This shows, the strong influence that endogenous and exogenous factors exert in the formation of vaginal communities (15).

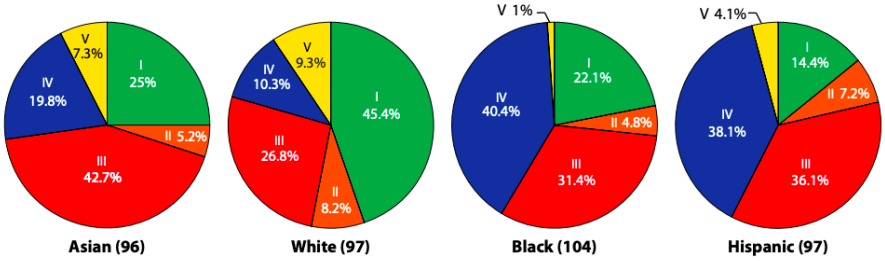


Figure 1. Representation of groups of vaginal bacterial communities within each ethnic group of women (from (12)). the group I communities were dominated by *L. crispatus*, while groups II (6.3%), III (34.1%) and V (5.3%) were dominated by *L. gasseri, L iners* and *L. jensenii*, respectively. The remaining communities found in 27% of women formed a large heterogeneous (IV) group and were typified by higher proportions of strictly anaerobic bacteria, including *Prevotella, Atopobium, Gardnerella, Megasphaera, Sneathia, Aerococcus,* and *Mobiluncus*.

The first studies on flora were carried out by Doderlein, who identified *Lactobacillus* as constituents of healthy microflora (5). The components of the vaginal ecosystem were observed under a microscope and subsequently identified using specific culture techniques (4). However, more recently, the application of bacterial identification techniques independent of culture media, such as: amplification, cloning and sequence

analysis of bacterial genes (genes encoding bacterial 16 S rRNA) in vaginal fluid samples has made possible the identification of most species of *Lactobacillus* and other microorganisms. Thus, such techniques have shown that *Lactobacillus* spp does not always correspond to the dominant bacterial species in the vagina of healthy women (16, 17). This benefit deepened the understanding of the vaginal microflora dominated by four *Lactobacillus spp*: *Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus iners* and *Lactobacillus jensenii*, which play an important role in protecting women against vaginal infections (18, 19). They form a critical line of defense against potential pathogens through the production of antimicrobial compounds or competition for adherence to the vaginal epithelium (20). Therefore, there is a growing interest in using these bacteria in formulations of probiotics for the prophylaxis and therapy of various vaginal disorders (21). Therefore, a vaginal microbial profile determined clinically and microscopically as " normal " or " healthy " is predominantly colonized by *Lactobacillus*, particularly *Lactobacillus crispatus* (22).

Several vaginal microflora compositions have been described, including VM dominated by *L.iners, L. crispatus, L. gasseri, L. jensenii*, and VM consisting of several anaerobic bacteria, including *Gardnerella vaginalis* and members of *Lachnospiraceae*, *Leptotrichiaceae* and *Prevotellaceae* (23, 24). Particularly, VMs dominated by *L. crispatus* are associated with vaginal health, while a VM consisting of several anaerobes has been shown to increase the chances of a woman developing bacterial vaginosis (BV) and acquiring sexually transmitted diseases, including HIV (25). The bacterial characteristics that may be important for *L. crispatus* to dominate the vaginal mucosa successfully are: the formation of an extracellular matrix (biofilm) on the surface of the vaginal mucosa; the production of antimicrobials, such as lactic acid, bactericins and oxygen peroxide (H_2O_2) that inhibit the growth and -/- or adhesion of urogenital pathogens; the efficient use of available nutrients, particularly glycogen, as this is the main source of carbon in the vaginal lumen; and the modulation of the host's immunogenic responses (26). Vaginal epithelial cells also play important roles in vaginal health, since they are rich in glycogen, and free glycogen in the vaginal fluid is associated with colonization by *Lactobacillus* (27). So, the defense factors provided by *Lactobacillus* and epithelial cells form a physical barrier against pathogenic bacteria and viruses. The study by Ravel et. al; (2011), highlights the diversity found in all vaginal bacterial communities, even that dominated by a single phylotype, and identifies taxa with similar correlation profiles (figure 2) (12).

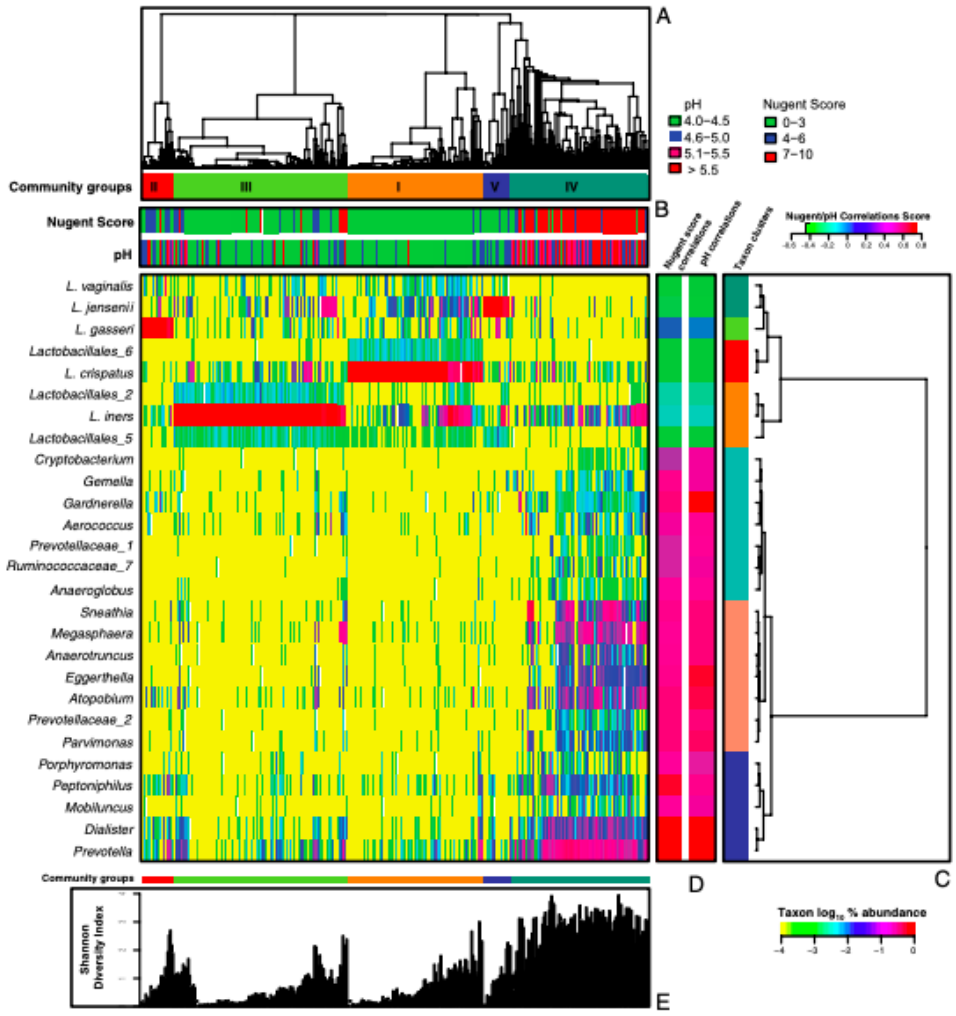


Figure 2. Heat map of microbial taxa found in the vaginal bacterial communities of 394 women of reproductive age (From (12)). (A) Complete linkage clustering of samples based on the species composition and abundance of vaginal bacterial communities define community groups I to V. (B) Nugent scores and pH measurements for each of the 394 community samples (color key is indicated above C). (C) Complete linkage clustering of taxa based on Spearman's correlation coefficient profiles, defined as the set of Spearman's correlation coefficients calculated between one taxon and all of the other taxa (SI Materials and Methods). (D) Spearman's correlation coefficients between the presence of a taxon and the Nugent score or pH of a sample. (E) Shannon diversity indices were calculated for 394 vaginal communities (two singletons were excluded).

The analysis revealed five main groups of microbial communities, where the group I communities were dominated by *L. crispatus*, while groups II (6.3%), III (34.1%) and V (5.3%) were dominated by *L. gasseri, L iners* and *L. jensenii*, respectively. The remaining communities found in 27% of women formed a large heterogeneous (IV) group and were typified by higher proportions of strictly anaerobic bacteria, including *Prevotella, Atopobium, Gardnerella, Megasphaera, Sneathia, Aerococcus*, and *Mobiluncus*. Interestingly, all communities contained members known to generate lactic acid, which suggests, an important catabolic function is preserved (28). An especially interesting fact is the presence of *L.iners* both in a healthy microflora and in

its aberrant profiles (29). The analysis of the complete sequence of the *L.iners* revealed a specific mechanism of adaptation to the vaginal niche, apparently through the acquisition of genes that allows it to extract nutrients from the environment, helping it survive pH fluctuations and other stress conditions, Beyond addition, it has cellular anchor proteins for adherence to the vaginal epithelium. Therefore, as the main *Lactobacillus* is present, it can create an environment that allows other species of *Lactobacillus* to thrive and recreate a healthy microbiota (30).

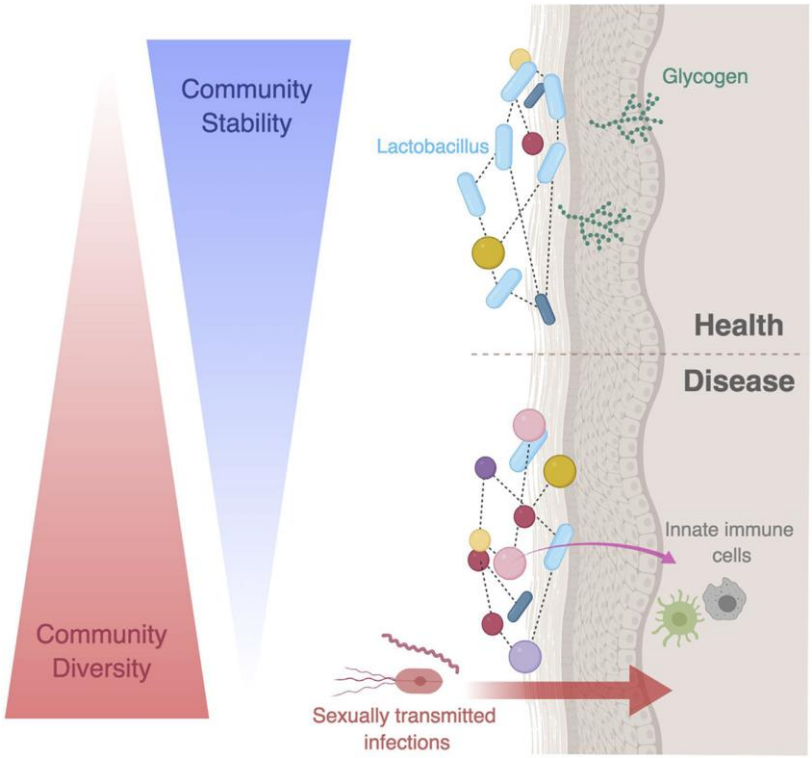


Figure 3. Illustration of the relation between the vaginal microbiome and health and disease states (From (31)). In the vaginal microbiome, health states, unlike the gut microbiome and other human microbiomes, are associated with low community diversity. Many, but not all, community state types (CSTs) of healthy reproductive-aged women are dominated by *Lactobacillus* species, and remain relatively stable, but not permanent (the community composition changes during menstruation). *Lactobacillus* species are using glycogen deposits in the vaginal epithelium in anaerobic glycolysis, which results in lactic acid production. For example, in bacterial vaginosis (BV), the communities observed are more diverse and less stable in diseases states. Vaginosis-associated bacteria can negatively modify the host innate immune response, and are associated with predisposition for sexually transmitted infections (STIs).

It is now well accepted that the microbiota present in the human body can affect immunity, physiology and health (32), since the vaginal microflora has evolved to play the dual roles of disease resistance and obstetric protection (33). However, imbalances can arise in the highly diverse vaginal microbiota that can cause various vaginal diseases, such as bacterial vaginosis (VB), aerobic vaginitis (VA), vulvovaginal candidosis (VVC), among others. For example, BV is a condition in which normal protective *Lactobacillus* are replaced by many commensal anaerobes such as strains of

Atopobium vaginae, *Clostridiales* and *Gardnerella vaginalis*. At the same time, AV is dominated by aerobic bacteria, such as *Streptococcus agalactiae* and *Escherichia coli* (34). Thus, altering the balance of flora can lead to the appearance of «vulvovaginitis», which are characterized by vulvar and vaginal symptoms, such as pruritus, pain, discharge, dysuria, and dyspareunia (4).

1.2 *Lactobacillus* species

Vaginal lactobacilli were first described in 1892 by the German Albert Döderlein, so they were long referred to as Döderlein's bacilli. Taxonomically the Genus *Lactobacillus* belongs to the Phylum Firmicutes, Class Bacilli, Order Lactobacillales, family Lactobacillaceae. *Lactobacillus* constitutes a heterogeneous group of non-pathogenic, catalase-negative, mainly obligate, and facultative anaerobic Gram-positive rod-shaped bacteria found predominantly in the human genitourinary tract (35). They are fermenters and grow at temperatures ranging from 2 to 53°C, with optimum values between 30 and 40°C, and pH between 5.5 and 6.3. They are so named because of their bacillary morphology and ability to produce lactic acid from lactose and other carbohydrates (36). Gram morphology stain can vary, including short fatty rods, long slender rods, or in chains, and as for colonial morphology it can range from small to medium-sized colonies. In addition *Lactobacillus* grow on various of other media, including Man, Rogosa and Sharpe (MRS) agar, where they appear as white and usually mucoid colonies. They form the most numerous group of lactic acid bacteria (LAB), comprising more than 170 species and 17 subspecies, as the number is continuously being modified because of the description of new species and/or the reclassification of others (37).

Table 1. List of origin of some *Lactobacillus* strains, representing 17 species. Adapted from (38).

Species	Abdominal	Blood	FGU-endo/pelvic[a]	FGU-vag[b]	Oral	Soft Tissue	Respiratory	Stool	urinary
L.acidophilus			1						
L.animalis	1								
L.antrumi	1								
L.casei	7	1						2	
L.crispatus		1							1
L.delbrueckii									1
L.fermentum	2	3							
L.gasseri	7	2	2	1		2		1	
L.iners			1	1					
L.jensenii			1	2		1			1
L.mucosae	1								
L.oris					1			1	
L.plantarum						1			
L.reuteri	1								
L.rhamnosus	9	1			1		3	1	
l.salivarius	1			1					
L.vaginalis	2	1			1			1	

[a] FGU-endo/pelvic: Female genitourinary, endometrial-pelvic.
[b] FGU-vag: Female genitourinary, vaginal.

In humans, they are native to the gastrointestinal tract where there are a variety of ecological niches, including *L. casei* and *L. rhamnosus* which have been isolated from the intestine and in the vagina where the species *L.crispatus*, *L. gasseri, L. jensenii*, and *L. iners* have specifically evolved to inhabit. However, only *L.crispatus* is present in most women of reproductive age, to the complete or near-complete exclusion of other members of this genus (39). Studies show that a vaginal microbiota colonized predominantly by *L. crispatus* implies a more acidic microenvironment than when other lactobacillus species dominate, suggesting that *L. crispatus* is a significant lactic acid producer in the vaginal ecosystem, making its prevalence reflect a significantly more protective effect against infection. Rapid detection of vaginal *Lactobacillus* such as *L. crispatus* by molecular methods could determine its use as a biomarker of vaginal health, and also, since this species is frequently isolated from healthy vaginal sites, it has great potential to be used as a probiotic in the treatment and prevention of vaginosis (12).

Identification of *Lactobacillus* species is done by molecular means based on 16S rRNA gene sequence, as phenotypic identification is generally unreliable. However a comparative analysis of the *Lactobacillus* genomes revealed that the four vaginal inhabitants have a smaller number of genes than the other less prevalent *Lactobacillus* species. This suggests that these species exhibit some form of adaptation to their host.

The author compares this characteristic to microorganisms that are completely dependent on a host for survival, such as intracellular microorganisms (40). Among them *L. crispatus* had the largest and *L. iners* the smallest genome. The small genome size of *L. iners* limits the number of encoded proteins corresponding, to reduced metabolic capabilities and making it more heavily dependent on exogenous sources of essential resources than *L. crispatus*. On the other hand its dependence on nutrients derived from the host or other community members allows it to maintain close contact with host tissues (41).

Because of their high nutritional requirements, lactobacillus colonize environments rich in carbohydrate-containing substances: they are found in plants or plant materials, in fermented food products, or associated with mucous membranes of humans and animals (29).

1.3 Mechanisms of probiosis

The word "probiotic", according to the Food and Agriculture Organization/ World Health Organization (FAO/WHO) is used to describe living microorganisms that provide a benefit to the recipient when administered in sufficient amounts. The work of Nobel laureate Ilya Metchnikoff, first introduced the concept of probiotics in the early 20th century, as he linked the longevity of Bulgarian farmers to their diet based on the consumption of fermented milk products, suggesting that these products, specifically yogurt, contained microorganisms that act to protect the gut from harmful bacteria. Most probiotic microorganisms belong to the lactic LAB group, composed of several genera, the most important of which are the probiotic *Lactobacillus* strains (42, 43).

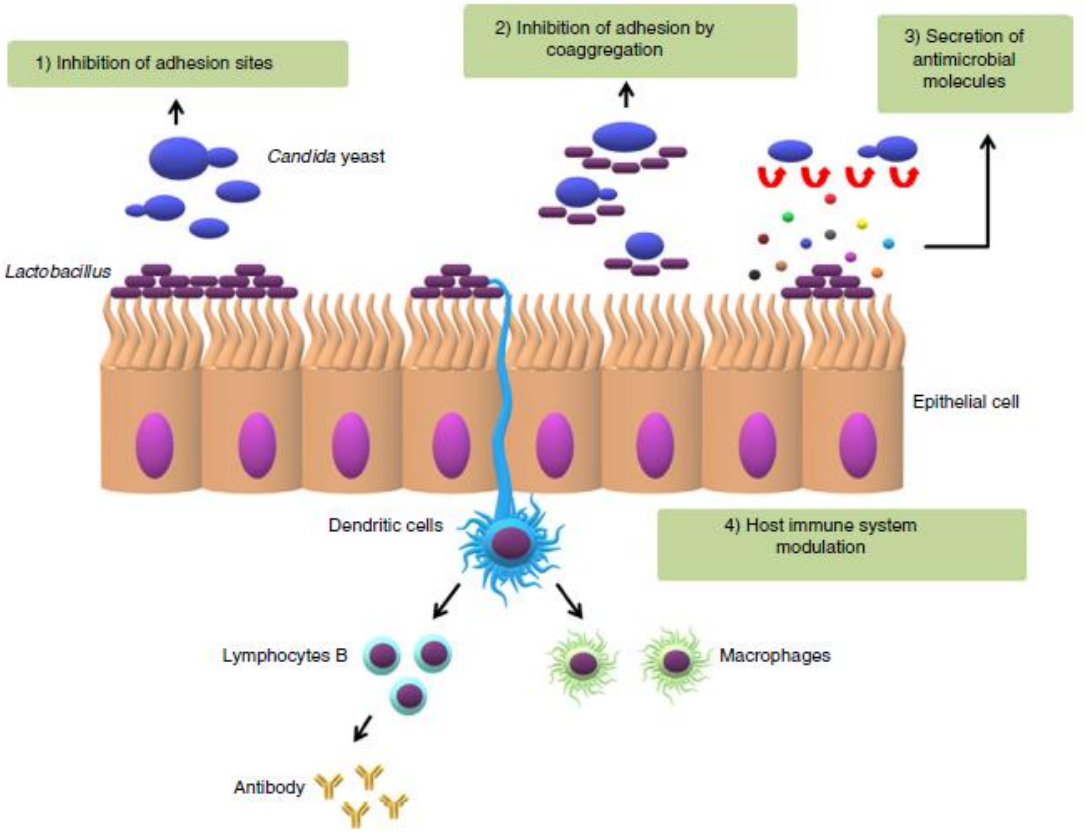


Figure 4. Major mechanisms of action of probiotics (From (44)).

The mechanisms by which *Lactobacillus* as probiotics protect against infection include: lowering pH, production of compounds such as bacteriocins and biosurfactants, competition with the pathogen for receptors, nutrients, and growth factors, stimulation of immunomodulatory cells, modulation of inflammatory responses, and production of H_2O_2 (43). In addition to their barrier function to vaginal colonization by harmful microorganisms, these species maintain an acidic intravaginal microflora by generating lactic acid by decomposition of carbohydrates, inhibit catalase-negative anaerobic organisms by producing H_2O_2 as a source of free radicals, and produce antimicrobial peptides such as bacteriocin-like substances and biosurfactants. In addition, *Lactobacillus* species induce anti-inflammatory immune responses in the host via the NF-kB pathway (44, 45). Biosurfactants (BS) are amphipathic compounds produced by microorganisms, anchored on the surface or secreted outward, with excellent surface and emulsifying properties that affect the initial adhesion of *C. albicans* to host surfaces and impair biofilm growth. These molecules, mainly secondary metabolites, exert critical functions in the survival of the producing microorganisms by facilitating nutrient transport, interfering in microorganism-host interactions and "quorum sensing", or acting as antimicrobial agents, anti-adhesives and antibiofilms (46). Thus, through these adhesion inhibitors, *Lactobacillus* interferes with the adhesion of

different pathogens on the epithelial cells of the urogenital tract since they remarkably affect cell-to-cell and cell-to-surface interactions, as a result, the surface becomes less favorable for adhesion (47). Furthermore the, probiotics can stimulate the immune system of their host through cellular activation that will result in the production of interleukins, such as tumor necrosis factor, IL-1b, IL-6 and IL-17, which are essential in directing immune responses to fungi, contributing to a more competent immune system against *C. albicans* infections (48).

The production of organic acids by the *Lactobacillus* keeps the vaginal pH below or equal to 4.5, creating an inhospitable environment for pathogens. This acidic pH, hinders the growth of *G. vaginalis*, because it does not attach to the epithelium cells under these pH conditions. In addition organic acids, studies point to the inhibition of bacterial species by the presence of H_2O_2, which accumulates in the absence of peroxidase. The excess of this metabolite may inhibit or kill other microorganisms, especially groups with low or deficient peroxidase production, such as anaerobes. Therefore, the absence of H_2O_2 producing *Lactobacillus* allows the growth of catalase-negative microorganisms and is considered an essential factor in the pathophysiology of BV (49).

In recent years, interest in the therapeutic use of probiotics has been growing, *Lactobacillus* species in general are recommended as part of this new strategy, based on accumulating evidence regarding their efficacy in restoring normal microbial function and preventing urogenital infections (50). In addition the use of probiotics has been associated with a significant reduction in recurrent VVC (51). Thus probiotic bacteria-derived from lactobacilli has emerged as a new strategy for the treating of vaginal infections. However, investigations of the antifungal activities of probiotic strains are less common than investigations of their antibacterial activities (52).

1.4 Cytolytic vaginosis and vulvovaginal candidosis

Women's reproductive tract infections are important clinical entities because of the possibility of complications. In addition, their frequency, psychological repercussions, and uncomfortable symptoms facilitate the acquisition/transmission of other sexually transmitted agents such as the human immunodeficiency virus (HIV). Among these infections, vulvovaginitis and vaginosis stand out, processes in which the vaginal physiological environment is altered, may or may not be associated with an inflammatory process, and are responsible for approximately 40% of the reasons for

visiting a gynecologist. However, despite the improvement in diagnostic methods and the availability of many drugs, these conditions continue to represent a challenge for doctors and patients, particularly in recurrent cases (53).

Vulvovaginal candidosis is a pathology caused by *Candida* species that are part of most healthy women's mucosal microbiota of the gastrointestinal and genitourinary. Under certain conditions, these yeasts can become opportunistic pathogens and overgrow the vulvovaginal mucosa, leading to VVC. Until a few years ago, the most commonly detected species in VVC was *Candida albicans*. However, during the last two decades, with more accurate molecular diagnostics, infections by different species (i.e. *C.glabrata*, *C.krusei* and *C. tropicalis*) have emerged, producing an increase in the species causing VVC (54). It is a disease that affects approximately 75% of women of reproductive age and among VVC patients, 6-9% suffer from recurrent VVC (55).

Cytolytic vaginosis is characterized by excessive proliferation of *Lactobacillus* that alone or in combination with other bacteria can damage the intermediate vaginal epithelium, resulting in cellular dissolution. The factors that determine the excessive proliferation of *Lactobacillus* are not known, however it is discussed that the amount of glycogen in vaginal epithelial cells increases under the influence of estrogen during the ovulatory and luteal phases of the menstrual cycle. The overgrowth of *Lactobacillus* increases the conversion of glycogen to lactic acid which damages the epithelium and lowers the vaginal pH. As mentioned, the cells in the middle layer of the vagina are the most affected by cytolysis because they are the most likely to have a growth of *lactobacilli* on their surface, as they are the vaginal epithelial cells that take up the most glycogen/glucose (56). This large number of *Lactobacillus* spp. also increases H_2O_2 and other antibacterial substances while decreasing bacterial diversity (57).

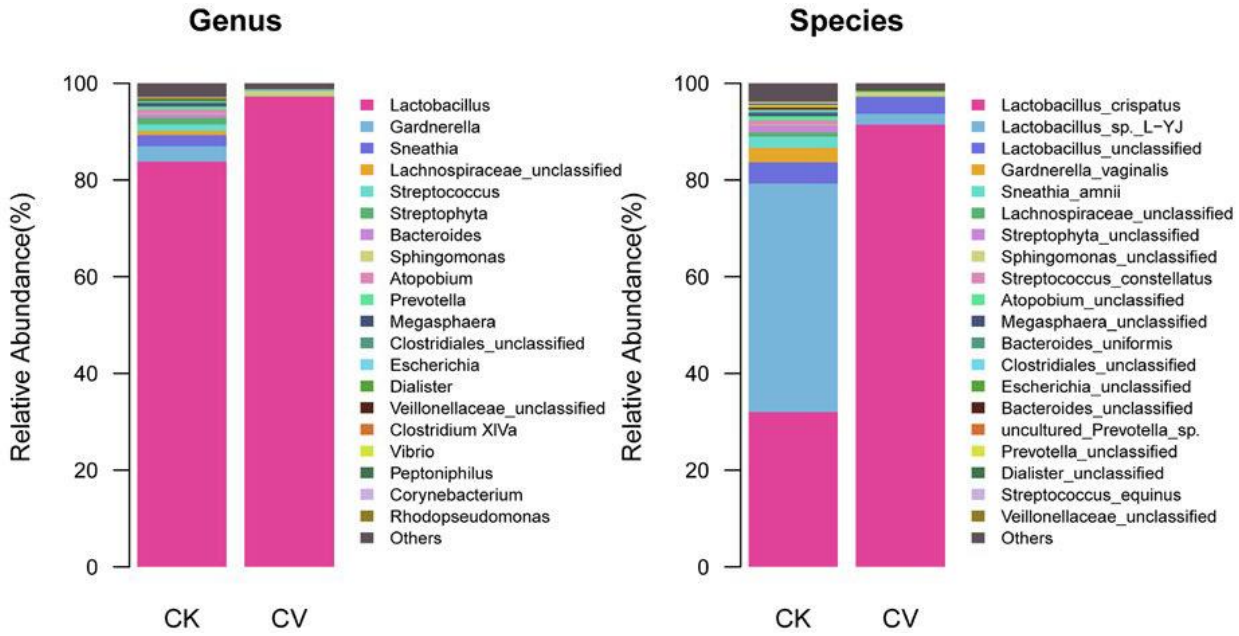


Figure 5. Composition of the vaginal microbiomes of healthy women (CK group) and cytolytic vaginosis patients (CV group). Relative abundance is shown at the genus and species levels (From (58)).

This, excess of *Lactobacillus* increases the cytolytic process, whose products are responsible for the onset of symptoms (59). As symptoms it presents a whitish discharge and pruritus of varying intensities, burning and burning that during the luteal phase will be more severe, in which it has been suggested that there is a noticeable increase in the number of colonizing *Lactobacillus* that can lead to the cyclic occurrence of CV (60). Diagnostic criteria for the CV that have been suggested include an increased number of *Lactobacillus*; the absence of *Trichomonas vaginalis* and *Candida species* on a wet vaginal smear; a paucity of white blood cells; evidence of cytolysis; the presence of discharge; and a pH of 3.5 to 4.5 (61). The study by Xu et al., (2019) shows that among *Lactobacillus* spp, *L. crispatus* was the most predominant species (88.7%) in VC, in agreement with the results of previous studies as one of the strongest H_2O_2-producing *Lactobacillus, L. crispatus* can competitively inhibit other potential vaginal pathogens, and is associated with low pH and can tolerate acidic conditions (pH <4.5). Indeed, abundant growth of *L. crispatus* appears to be a critical factor in CV development, or even a marker for CV (58).

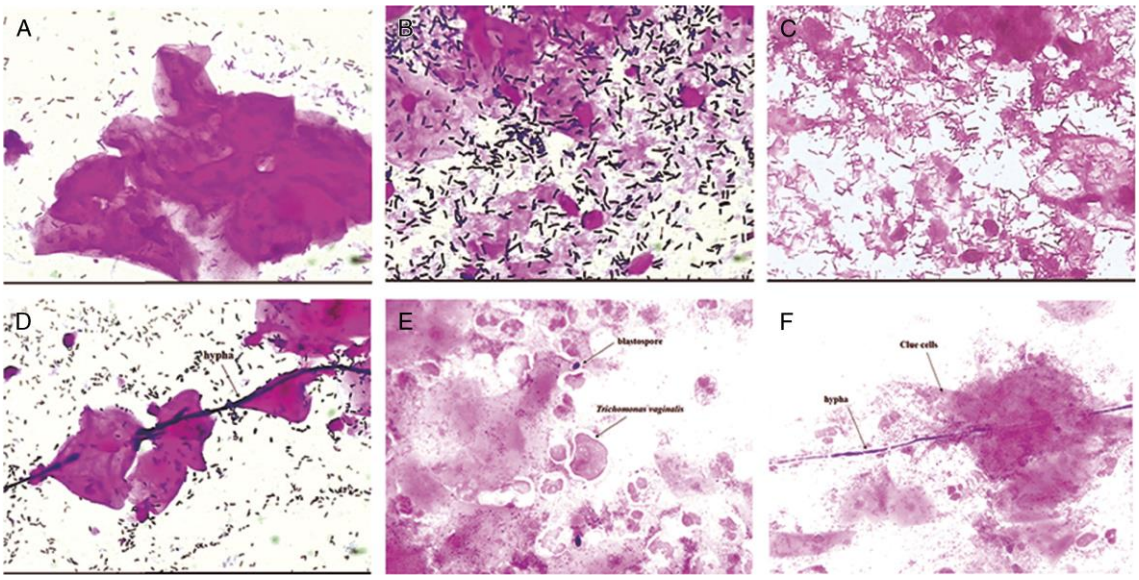


Figure 6. **Microscopic observation of the vaginal smears of 6 typical subjects (Gram staining)** de (From (61)). **A**, Vaginal smear of a healthy woman: presence of a large amount of Lactobacillus and normal epithelial cells. **B**, Vaginal smear of a patient with mild cytolytic vaginalis (CV): presence of a large amount of *Lactobacillus*, naked nuclei, and cytoplasmic fragmentations. **C**, Vaginal smear of a patient with severe CV: presence of a large amount of Lactobacillus, naked nuclei, cytoplasmic fragmentations, and lack of whole epithelial cells. **D**, Vaginal smear of a patient with a single infection of vulvovaginal candidiasis (VVC): presence of a large amount of *Lactobacillus*, normal epithelial cells, candida spore, blastospores, and hyphae. **E**, Vaginal smear of a patient with combined VVC and trichomoniasis: presence of *T. vaginalis*, normal epithelial cells, candida spore, blastospores, and hyphae. **F**, Vaginal smear of a patient with combined VVC and bacterial vaginosis: presence of *Gardnerella vaginalis* (clue cells), normal epithelial cells, candida spore, blastospores, and hyphae.

Previous studies, show that CV is a leading cause of recurrent vulvovaginitis, with clinical implications (59). In addition, misdiagnosis is frequent because the clinical signs and symptoms of CV can be nonspecific and similar to those of other diseases, especially vulvovaginal candidosis, with estimated prevalence rates ranging from 1.83% to 7.1%. Although CV and VVC share many symptoms, such as itching, pruritus, dyspareunia, vulvar dysuria, and abundant vaginal discharge, significant differences in morphological features are observed under the microscope, and few references have reported that VVC may present with CV (61).

Table 2. Microscopic characteristics of smears from Cytolytic Vaginosis and Vulvovaginal Candidosis. Adapted from (62).

	Cytolytic Vaginosis	Vulvovaginal Candidosis
Presence of *Lactobacillus*	High number of *Lactobacillus spp*.	Usually present
Vaginal epithelial cells	Presence of denuded nuclei and cytoplasm fragments	Normal
Leucocytes	<10/Cga	Increased or normal
Other characteristics	Absence of spores and other pathogenic microorganisms	Presence of spores or hyphae/pseudohyphae

Cga - high amplification field

Cytolytic vaginosis is a neglected topic, since *Lactobacillus* are valuable microbes in the vagina, although they play a negative role in *Lactobacillus* overgrowth disorders such as CV.

1.5 Antifungal Therapy - The azoles

Currently, two groups of antifungal drugs are mainly used to treat VVC. The first group includes the polyene antifungal drugs represented by amphotericin B which has strong antifungal activity and antibacterial broad spectrum, but is toxic. The last group includes azoles which are pyrrole ring antifungal drugs that have antibacterial broad spectrum and are more widely used as they are generally safe and well tolerated drugs (63).

Azoles are classified as imidazoles or triazoles based on whether they have two or three nitrogens in the five-membered azolic ring, respectively. Imidazole, includes Clotrimazole, Econazole, Bifonazole, Butoconazole, Phenticonazole, Isoconazole, Sertaconazole, Sulconazole, and Thioconazole, which are used primarily as topical agents, also interact and damage the cell membrane directly at higher concentrations and are fungicidal. The other class of azoles, the triazoles, are newer, less toxic and more effective and include Fluconazole, Itraconazole, Ravuconazole, Posaconazole, Voriconazole and Terconazole (64).

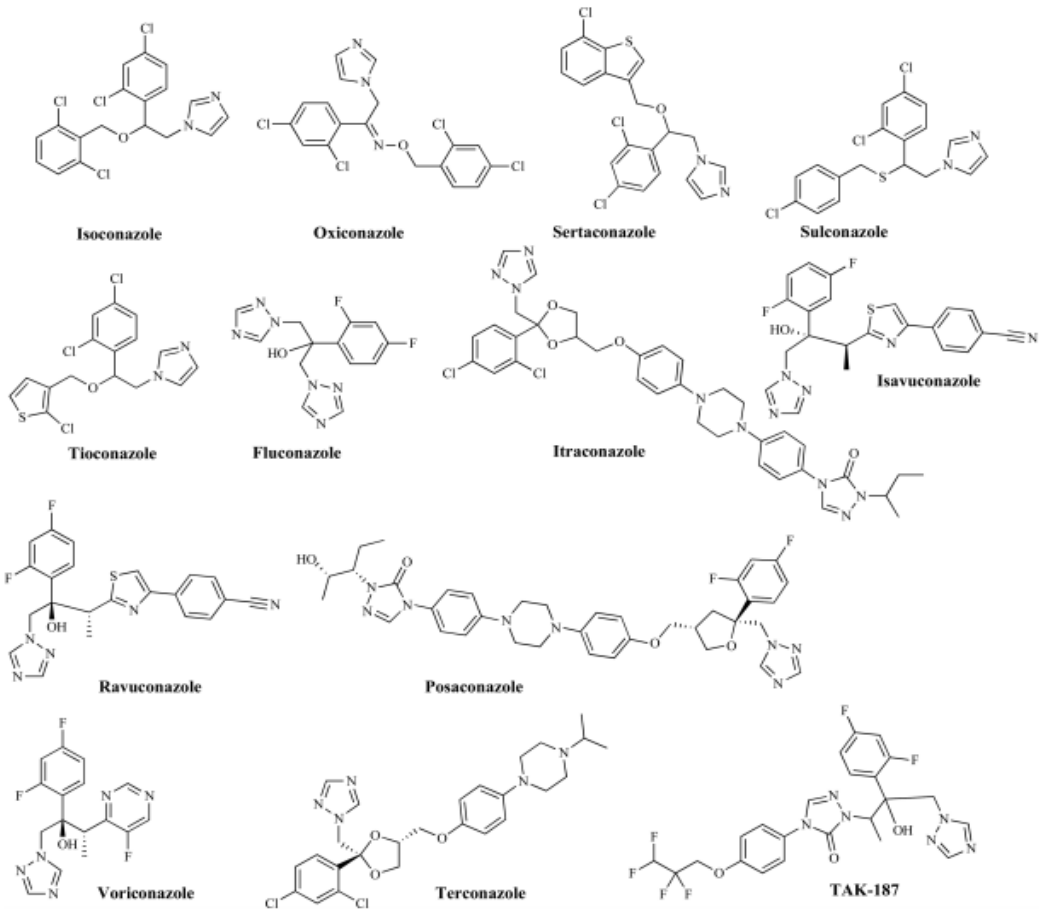


Figure 7. Azole Antifungal Agents. From (65)

They act based on inhibiting some of the steps in the biosynthesis of ergosterol, which is the major component of the fungal cell membrane and is essential in its integrity. Depletion of ergosterol results in the accumulation of lanosterol and other 14-methylated sterols that interfere with the functions of ergosterol as a membrane component. Briefly, the mechanism of action of azoles includes inhibition of oxidative enzymes associated with Cytochrome P450 (CYP), blocking the conversion of lanosterol to ergosterol. Consequently, the plasma membrane structure is disrupted, making it more vulnerable to further damage, including the altered activity of several membrane-bound enzymes, such as those associated with nutrient transport, chitin synthesis, growth, and proliferation (66).

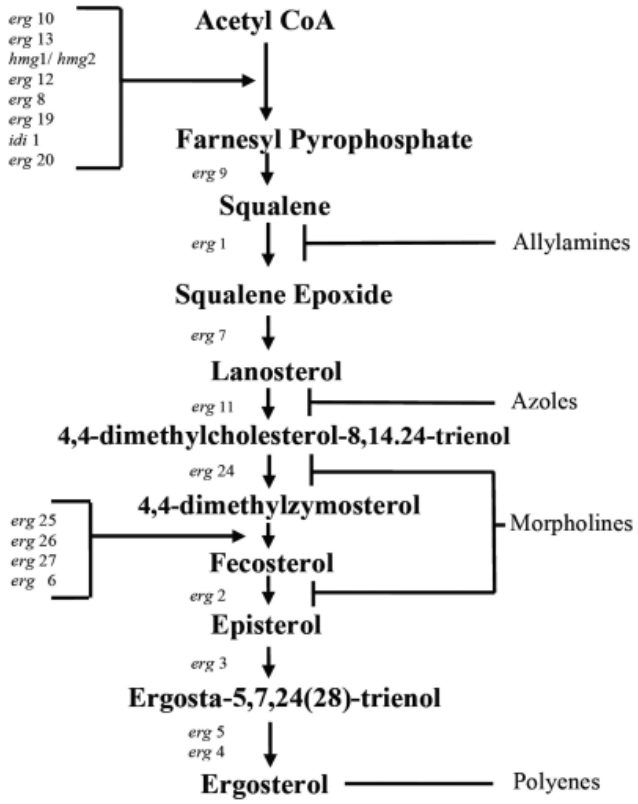


Figure 8. Linear model of the ergosterol biosynthetic pathway adapted from Saccharomyces cerevisiae. Important intermediates are in boldface, and inhibitors are shown on the right. Commonly used drugs in the allylamine, azole, and morpholine classes are listed beneath the class names. CoA, coenzyme A. From (65)

Chemically, all azoles are weak bases. For example, Posaconazole is very lipophilic and generally not soluble in water. Voriconazole is lipophilic and has limited water solubility, and in contrast Fluconazole is more soluble in water. In addition, systemic azoles are substrates and inhibitors of CYP isoforms to varying degrees and some azoles are substrates and/or inhibitors of drug transporters. These physicochemical and metabolic properties are the basis of the pharmacokinetic differences and drug interactions involving this class (67).

The most readily available are local azolics, where the recommended short-term therapy is up to 3 days with symptoms disappearing from the second day. This treatment is effective in 80 to 90% of cases. In addition agents used in short-term regimens contain higher doses of antifungal drugs, allowing higher concentrations for longer-lasting inhibitory effects (68).

II. OBJECTIVES

The main objective of the present study is to study the effect of antifungal therapy on commensal microorganisms and the impact on the prediction of therapeutic strategies.

Specifically it is intended to:
- Evaluate the antibacterial activity of two classes of antifungal drugs most prescribed nationally;
- Understand the probiotic mechanisms in the relationship with the host and its relationship with the genital health of women;
- To ascertain how *Lactobacillus* species responds to antifungal therapy, which is often administered empirically in clinical cases with typical symptoms of cytolytic vaginosis;
- Interpret the results, thereby assessing whether the infectivity of *Lactobacillus* increases in response to applied therapy.

Furthermore, as part of a learning process, all the technical-laboratory skills acquired along the way also count as objectives in the development of this dissertation.

III. MATERIALS AND METHODS

3.1 General considerations

The strains used in this study were isolated from the human vagina and identified, characterized, and preserved in the Microbiology laboratory, located at the Health Sciences Research Center at the University of Beira Interior. Six strains were included, namely two *Lactobacillus crispatus*, two *Lactobacillus gasseri* and two *Lactobacillus jensenii*, which throughout the study were subjected to the following evaluations: antimicrobial activity; adhesion capacity; interference of antifungal agents on biofilm formation, on probiotic mechanisms, and adhesion capacity on HeLa cells *in vitro*.

To determine the antifungal activity the following antifungals were used: Bifonazole, Clotrimazole, Econazole, Fenticonazole, Fluconazole, Isoconazole, Posaconazole, Voriconazole and Sertaconazole considering a stock solution at 25600 µg/mL in DMSO.

3.2 Quantification of colony forming units

For bacterial quantification, manual counting was performed using a magnifier, which requires plating on a solid culture medium. It consists in preparing a suspension at 1 Macfarland in MRS broth and performing serial dilutions (1:10) of this sample in peptoned water. These dilutions are then seeded in Petri plates with MRS agar medium at the appropriate temperature (37°C) to promote bacterial growth of isolated colonies during a 48h incubation period. After this incubation period, the colonies are counted in a colony counter device that allows the marking of the number of colonies on the plate and the results are expressed in CFU/ml.

3.3 Antimicrobial activity

The determination of antimicrobial activity aims to identify the lowest concentration of antifungals capable of inhibiting the growth of microorganisms when placed in contact with the strains in question. It was carried out by the microdilution method, using 96-well microplates, thus constituting a simple, effective test that in a short time allows us to determine bacterial proliferation or inhibition. First, the bacterial strains of *Lactobacillus crispatus, Lactobacillus gasseri and Lactobacillus jensenii* were plated in MRS and incubated for 24 hours at a temperature of 37ºC in anaerobic conditions,

ideal conditions for the growth of the strains. Then the culture medium (MRS, 55.2g / 1L, Sigma-Aldrich) was prepared in distilled water (Water Milli-Q) and sterilized in an autoclave at high temperature (121 ° C, 15 minutes) and the protocol was adapted from previously described methodologies (69).

3.3.1Determination of the Minimum Inhibitory Concentration (MIC)

For this assay, antifungals were prepared in a culture medium (MRS) with serial concentration of 8, 16, 32, 64, 128, 256, 512, totaling seven different concentrations of each antifungal under study. To prepare the inoculum, the bacteria were suspended in sterile 0.85% NaCl solution and the turbidity was normalized using a McFarland densitometer for one unit. The bacterial suspension was homogenized and then a dilution was prepared, adding 140 µL of the suspension to 13.8 mL of MRS. The test dilutions and controls were pipetted in duplicate into a 96-well plate, with the growth control containing 100 µL of sterile drug-free medium with 100 µL of the same inoculum suspension. A first absorbance reading was performed before incubation, (T0 hours), in an ELISA microplate reader with an optical density of 600 nm. Then, the microplate was incubated, at 37ºC for 48 hours. During these 48 hours, two visual readings of growth were made, one after 24 hours and the other after 48 hours assessing the effect of time on the growth of bacteria.

3.3.2 Determination of the Minimum Lethal Concentration (MLC)

To determine the MLC, 5 µL of the suspension present in each without visible growth, was plated. MRS plates were used for this purpose. After inoculation, these plates were incubated in the oven at 37°C for 48 hours, and consequently, the growth on plate was evaluated after these 48 hours. In the plates where no growth occurred, the previously determined MIC was adopted as the MLC. Plates on which growth occurred demonstrate that the MIC that had been determined corresponds to a bacteriostatic concentration.

3.3.3 Visualization of biofilm ultrastructure

The changes in *L.gasseri* biofilm structure induced by the antifungals were evaluated by confocal laser scanning microscopy (CLSM) according to Machado et al. (2016) protocol. For this assay, strain *Lactobacillus gasseri LF3* was incubated in a 96-well fluorescence microplate with the presence of the antifungals at 37°C in 10% CO2 for 48h. The medium was then removed, the plate was washed with sterile PBS and stained with SYTO® BC (Molecular Probes Inc., OR, USA). CLSM images were acquired on an Olympus™ FluoView FV1000 (Olympus, Lisbon, Portugal) using a 20× objective.

3.4 Adhesion capacity and quantification

For bacterial adhesion assays, strains were suspended in MRS until the turbidity reached approximately one unit on the McFarland scale, about $1x10^7$ colony forming units (CFU) / mL, where 200 µL were transferred to 96-well plates placed to incubate at 37°C with 10% CO_2. The first set of plates was used to measure biofilm formation after 24 h, and the duplicate plate was used to determine the biofilm formation at 48 h. After an incubation period, the medium was removed from the wells and the microdilution plate wells were washed twice with phosphate-buffered saline (PBS). Next the biofilms formed by bacteria adhering to the wells were fixed with methanol and the biomass of the biofilm was quantified according to the violet crystal (CV) staining method described by Peeters et al (70). After staining, the plates were washed with water twice. Then the CV was released with 33% acetic acid and the level of optical density (OD) of the violet crystal present in the discoloration solution was measured at 590 nm using the 96-well microplate reader (Biorad, Tokyo, Japan). All assays were repeated three times for all strains of *Lactobacillus*, and means and standard deviations were calculated for all experiment replicates.

3.5 Interference of antifungal agents on biofilm formation

3.5.1 Assay of biomass (biofilms)

The effects of antifungals on the initial colonization and maturation of biofilm formation by *Lactobacillus* spp. were investigated according to method already described and some adjustments (71). A suspension of each *Lactobacillus* spp. (200 µL, $1x10^7$ CFU/mL) in MRS was added to 96-well plates and incubated for 48 h at 37 °C with 10% CO_2. Subsequently, the suspensions were removed, and the wells were washed twice with PBS to remove non-adherent cells. Next 200 µL of each antifungal diluted in MRS broth (to final concentrations of 128µg/µl) were transferred to the wells, and the plates were subsequently incubated at 37°C for an additional 48 h. The wells containing Dimethyl sulfoxide (DMSO) diluted in MRS broth served only as controls. The effect of the antifungals on the total biomass of preformed biofilms was measured according to the CV staining protocol as described above.

3.5.1 Adhesion assay

To evaluate the interference of antifungals on the *in vitro* adhesion of *Lactobacillus*, the antifungals were added to the suspension of each strain that is transferred to a 96-well microplate and incubated for 48h at 37°C. To the controls, only DMSO was added to the suspension. After incubation, the biofilms formed by the adhesion of the *Lactobacillus* were highlighted using the previously mentioned staining method.

3.6 Evaluation of the *Lactobacillus* metabolism in the presence of antifungals

In order to evaluate the metabolites produced by the *Lactobacillus* (specifically glucose and lactate) in the presence of the antifungals, a culture of each strain was inoculated in a NaCl solution (0.85%) until it reached the OD of 1Macfarland and diluted in MRS. Then in a tube, 1mL of this suspension was transferred to 1mL of culture medium (MRS) with the presence of the antifungal under study and incubated for 48h at 37°C. After 24 and 48h, 100 µL were taken from each tube and transferred to a microplate read in the spectrophotometer at 590nm OD for to correlate with cell density, respectively. Each well was Gram stained from this microplate to evaluate the interference of the antifungals on the structure and/or size of the strains. To quantify the metabolites (Glucose and Lactate), kits were used (Glucose-TR and Lactate from SRINREACT, Spain), based on the colorimetric enzymatic method, where the intensity of the color formed is proportional to the concentration of lactate or glucose present in the tested sample. Thus, in a 96-well plate, 1 µL of the sample was added to 100 µL of the enzyme content, subsequently analyzed in the spectrophotometer for readings at 505 nm. To detect the amount of the metabolites in the sample in mg/dL, the following calculations are applied:

$$\boxed{\text{Lactate}} \quad \frac{\text{sample X blank}}{\text{standard X blank}} \times 10 = \text{mg/dL of lactate in sample}$$

$$\boxed{\text{Glucose}} \quad \frac{\text{sample X blank}}{\text{standard X blank}} \times 100 = \text{mg/dL of glucose in sample}$$

3.7 Adhesion capacity of *lactobacillus* on HeLa cells *in vitro*

HeLa cell lines (ATCC® CCL-2) were obtained by first plating the cells in 75 cm^2 culture T-flasks containing DMEM (Gibco Dulbecco's Modified Eagle Medium) culture medium supplemented with sodium bicarbonate for HeLa cells (Sigma-Aldrich), penicillin and streptomycin (Sigma-Aldrich) and 10% fetal bovine serum (FBS, Sigma-Aldrich). The culture medium was stabilized at a pH of 7.2 and then filtered with a 0.22 µm membrane filtration unit. The cells were maintained at 37 °C in an atmosphere with 95% air and 5% humidified CO_2 and subculturing was performed according to ATCC recommendations. After the cells reached the desired confluence (~90%), the number of viable cells was assessed by counting the cells using a Neubauer chamber. To determine the adhesion of the *Lactobacillus* species on HeLa cells in the presence of the antifungals, the cell lines were seeded ($1x10^3$ cells per well) in 96-well microplates. The cells were then incubated for 48 hours in an atmosphere of 95% air and 5% humidified CO_2 until they reached the desired confluence.

After this period the culture medium was removed, and the wells were washed once with sterile PBS and a suspension of each strain was prepared at 4 Macfarland in DMEM culture medium without antibiotics. The suspension was diluted (1:100) in centrifuge tube containing a mixture of DMEM medium (without antibiotic) and the respective antifungal agent transferred to the wells containing the adhered cells. The plate was centrifuged at 3000RCF for 15 minutes and incubated again for 3h. The wells were washed twice with sterile PBS, and the cells were lysed with sterile water (Mili-Q water). Each replicate of each strain was diluted in sterile PBS (1:10) and about 4 µL were seeded onto MRS agar plates and incubated under conditions suitable for the growth of the strains.

IV. RESULTS

4.1 Antimicrobial activity

The viability of the bacterial strains was expressed as percentage (%) viability compared to the control group and was measured at different times (after t24 hours and after t48 hours of incubation). From the results obtained, it was observed that Posaconazole, Voriconazole, Fluconazole and Bifonazole (Appendix I) did not alter the viability of most strains after treatment with different concentrations (4, 8, 16, 32, 64, 128, 256 and 512 µg/mL). However, specifically for *L.gasseri LF3* treated with concentrations between 4-64 µg/mL of Bifonazole and Fluconazole and the *L.gasseri LF4* treated with higher concentrations of Fluconazole, an increase in growth was observed (%). In addition, the antimicrobial effect was observed against the strains after being stimulated with concentrations ranging from 256-512 µg/mL of Econazole (figure 9), Fenticonazole (figure 10) and Isoconazole (figure 11). Concerning Clotrimazole which is a frequently used antifungal in the clinic, it is noted that it decreased the growth (%) of *L.jensenii LF9* and *L.gasseri LF4* when subjected to the maximum concentration of the assay, but *for L. gasseri* LF3 an increase in growth (%) was observed when treated with 8-64 µg/mL of the antifungal. Sertaconazole at high concentrations decreased growth (%) in *L.jensenii LF9, L.gasseri LF3* and *L.gasseri LF4*, but did not alter the growth in the other strains.

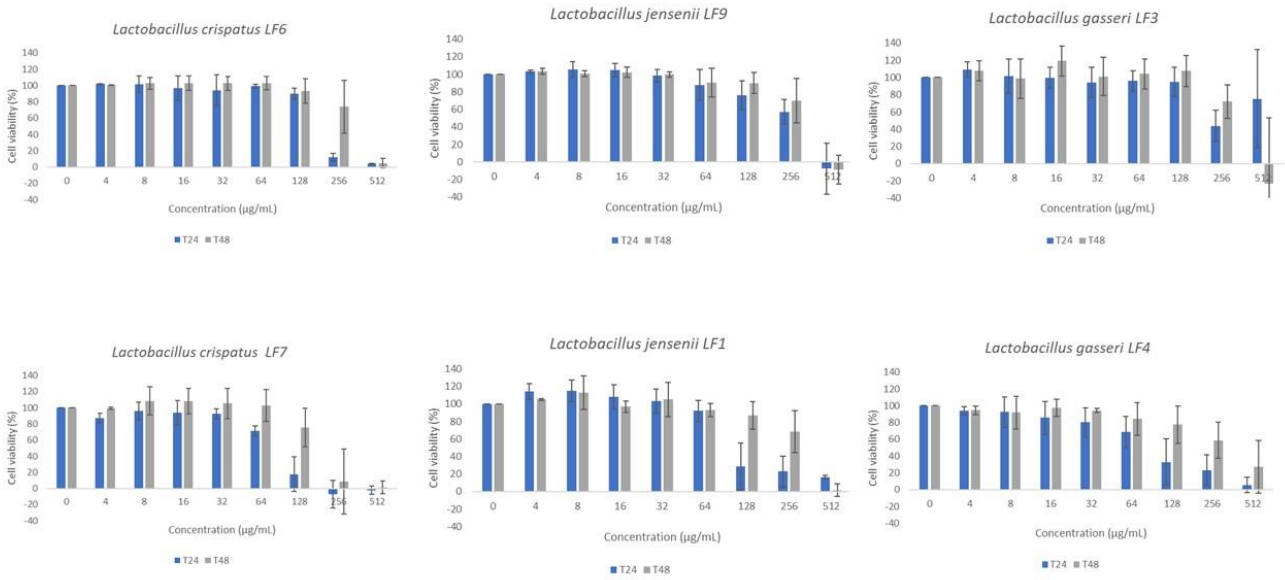


Figure 9. Growth (%) of *Lactobacillus crispatus LF6, Lactobacillus crispatus LF7, Lactobacillus jensenii LF1, Lactobacillus jensenii LF9, Lactobacillus gasseri LF3 and Lactobacillus gasseri LF4* after 48 hours with contact with different concentration percentile of Econazole. The minimum inhibitory concentration (MIC_{50}) for *Lactobacillus crispatus LF6, Lactobacillus jensenii LF9 and Lactobacillus gasseri LF4* is above 512 µg/mL and for *Lactobacillus crispatus LF7* is above 256-512 µg/mL of the antifungal concentration.

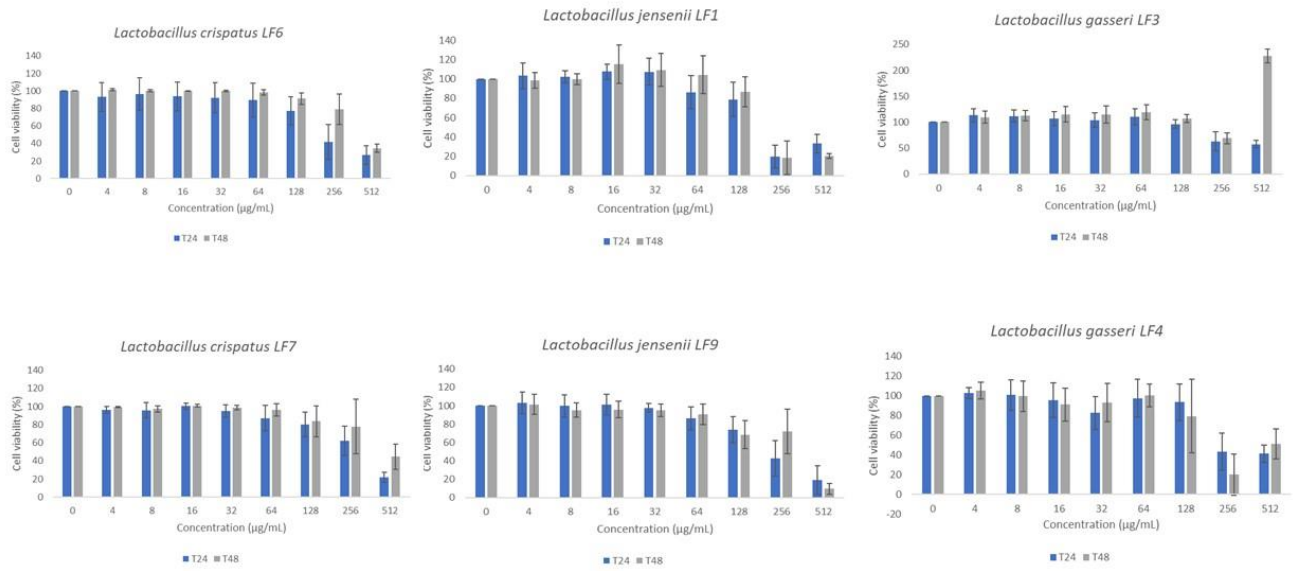


Figure 10. Growth (%) of *Lactobacillus crispatus LF6, Lactobacillus crispatus LF7, Lactobacillus jensenii LF1, Lactobacillus jensenii LF9, Lactobacillus gasseri LF3* and *Lactobacillus gasseri LF4* after 48 hours with contact with different concentration percentile of Fenticonazole. The minimum inhibitory concentration (MIC_{50}) for *Lactobacillus crispatus LF6, Lactobacillus jensenii LF9* is above 512 µg/mL *and Lactobacillus gasseri LF4* and for *Lactobacillus jensenii LF1* is above 256-512 µg/mL of the antifungal concentration.

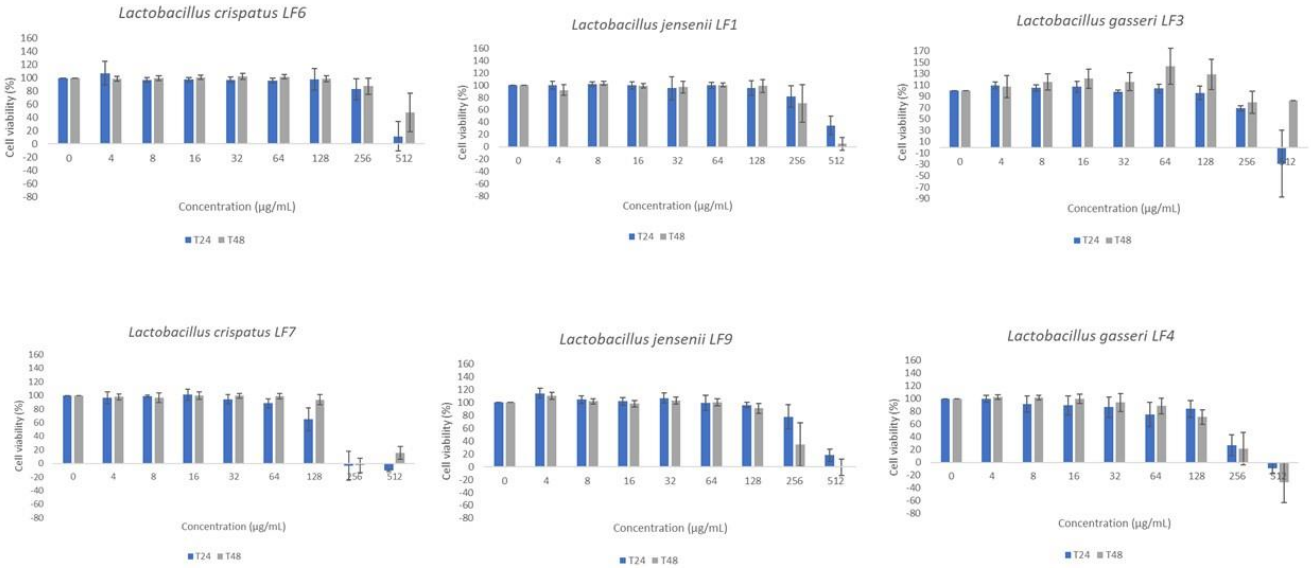


Figure 11. Growth (%) of *Lactobacillus crispatus LF6, Lactobacillus crispatus LF7, Lactobacillus jensenii LF1, Lactobacillus jensenii LF9, Lactobacillus gasseri LF3 and Lactobacillus gasseri LF4* after 48 hours with contact with different concentration percentile of Isoconazole. The minimum inhibitory concentration (MIC_{50}) for *Lactobacillus jensenii LF1 and Lactobacillus jensenii LF1* is above 512 µg/mL *and Lactobacillus gasseri LF4* - and for *Lactobacillus crispatus LF7* is above 256-512 µg/mL of the antifungal concentration.

The antimicrobial activity against *L.gasseri LF3* was not observed after stimulation with the different concentrations of the antifungals under study, except for Voriconazole that considerably growth decreases at concentration 512 µg/mL. However, an increase in the growth of this strain is noted when stimulated by most of the antifungals (Figure 12). Therefore, and to understand the growth increased of this strain, CLSM and Gram methods were used to observe the morphology and possible changes induced by the antifungals.

As for *L. crispatus LF6* and *L. crispatus LF7*, after being stimulated with concentrations between 256-512 µg/mL of Econazole (Figure 9), Fenticonazole (Figure 10) and Isoconazole (Figure 11), a growth decrease was observed, but they do not show any change in growth when exposed to the other antifungals. *L. jensenii LF9* and *L. gasseri LF4* strains are the most susceptible to changes in growth, as growth decreased (%) is observed after being treated with 256-512 µg/mL of Clotrimazole, Econazole, Fenticonazole, Isoconazole and Sertaconazole.

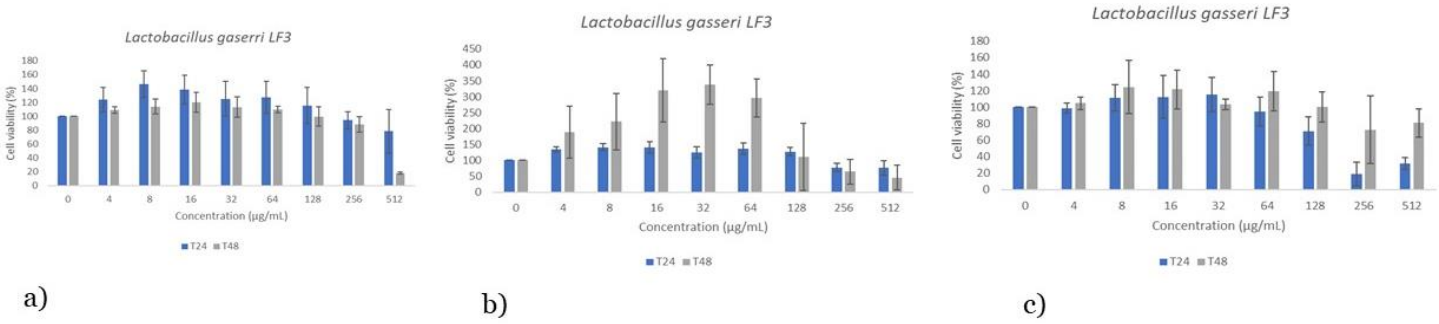
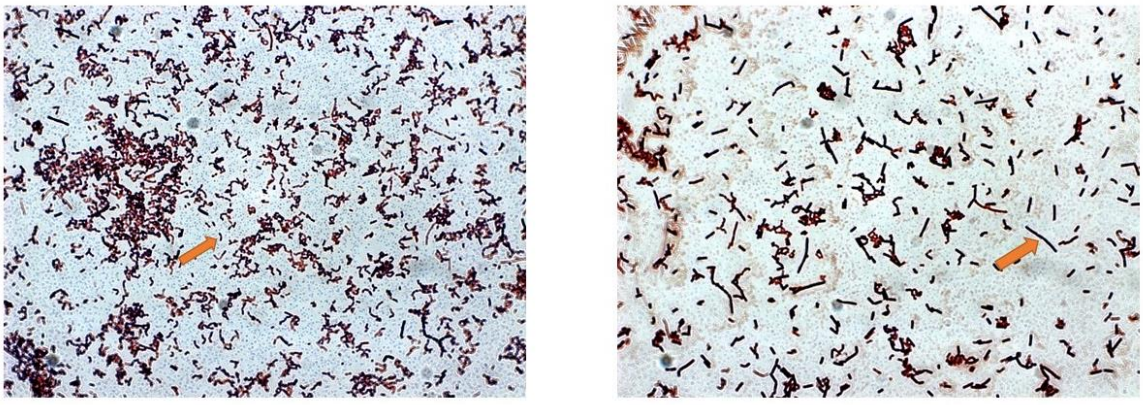


Figure 12. Growth (%) of *Lactobacillus gasseri LF3* after 48 hours with contact with different concentrations of: a) Bifonazole, **b)**Fluconazole and **c)** Clotrimazole**.**

The results of figure 13 show a considerable increase in the size of the *L.gasseri LF3* strain when treated with antifungals such as Clotrimazole.



Control Clotrimazole

Figure 13. Morphology of *L.gasseri LF3* after 48h in contact with Clotrimazole at 128 µg/mL. The analysis was performed by the gram staining method.

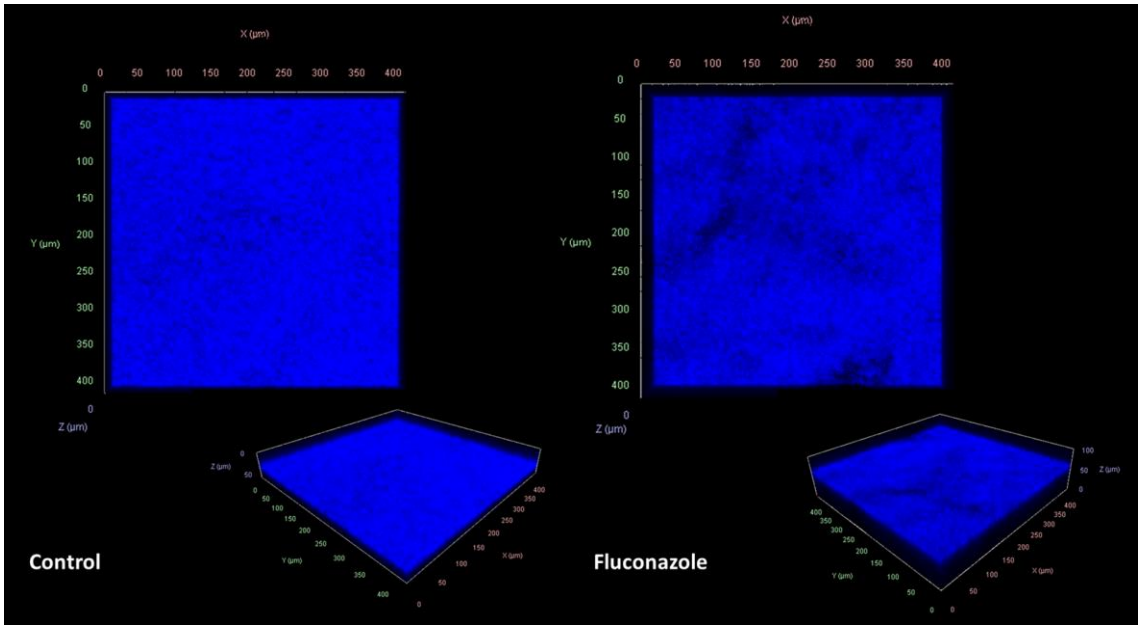


Figure 14. Effect of fluconazole on *L.gasseri LF3* biofilm structure. The biofilm was exposed to fluconazole at 128 µl / ml for 48 h.

When exposed to the antimicrobial agent fluconazole, an increase in the density of the biofilm formed by the strain was observed. However, it is possible to notice a certain attrition by the dark areas in Figure 14.

Table 3. Comparison of visual MIC and MLC obtained by species

		Clotrimazole		Econazole		Isoconazole	
Species	**Code**	MIC µg/mL	MLC µg/mL	MIC µg/mL	MLC µg/mL	MIC µg/mL	MLC µg/mL
L. crispatus	*LF6*	128	>512	256	256	256	512
	LF7	128	>512	256	256	256	512
L. jensenii	*LF1*	128	>512	256	256	256	>512
	LF9	128	>512	256	256	256	512
L. gasseri	*LF3*	128	256	256	256	256	512
	LF4	128	>512	256	256	256	512

Of all the antifungals tested, visual MIC was only observed in Clotrimazole, Econazole, and Isoconazole. In the case of Econazole, the concentration value corresponding to the MLC coincides with the visual MIC. As for Isoconazole and Clotrimazole, the concentration value corresponding to the MLC is higher than the visual MIC, but the strain of *L. gasseri LF3* that presents MLC in Clotrimazole is the only one that does not present MLC in Isoconazole.

4.2 Interference of antifungal agents in the preformed biofilm

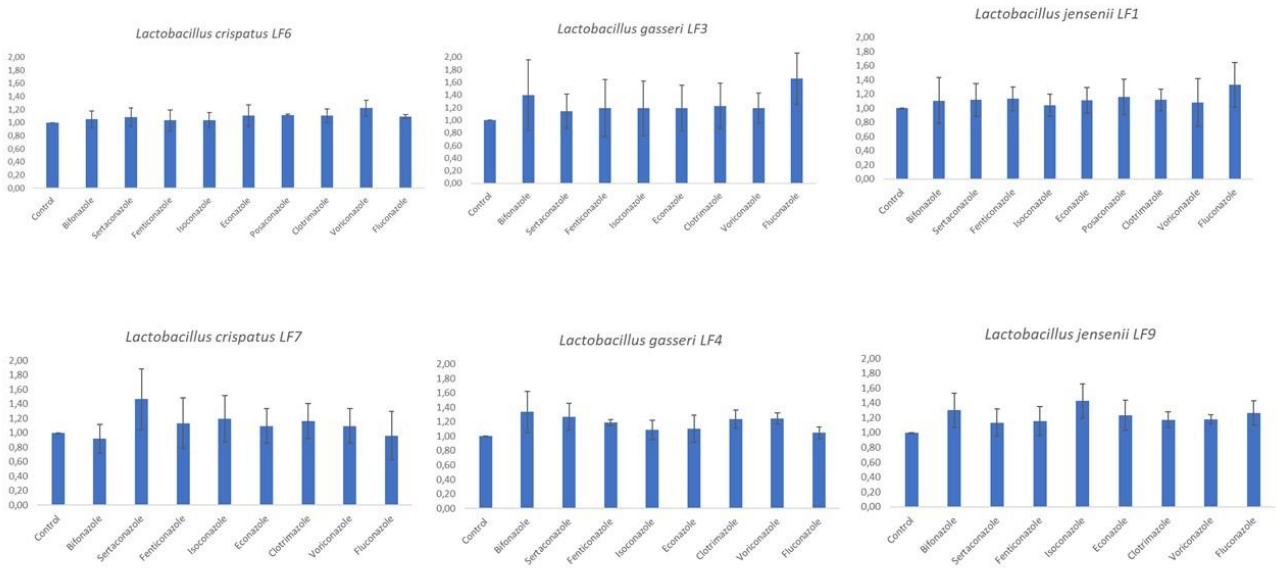


Figure 15. Quantification of biomass of the lactobacillus strains after the preformed biofilm was treated with 128 µg/mL of each antifungal for 48h.

It is observed that in most strains, the antifungals do not promote significant changes in biomass density, as in the case of *L.crispatus LF6*, since a similarity between the amount of biomass of the treated strains compared to the control is noted. However, it is possible to notice a significant increase in biomass density in the case of Fluconazole in *L.jensenii LF1* and *L.gasseri LF3* strains; of Bifonazole in *L.jensenii LF9*, *L.gasseri LF4* and *L.gasseri LF3* and Sertaconazole in *L.crispatus LF7* and *L.gasseri LF4*.

4.2 Interference of the antifungals in the adhesion

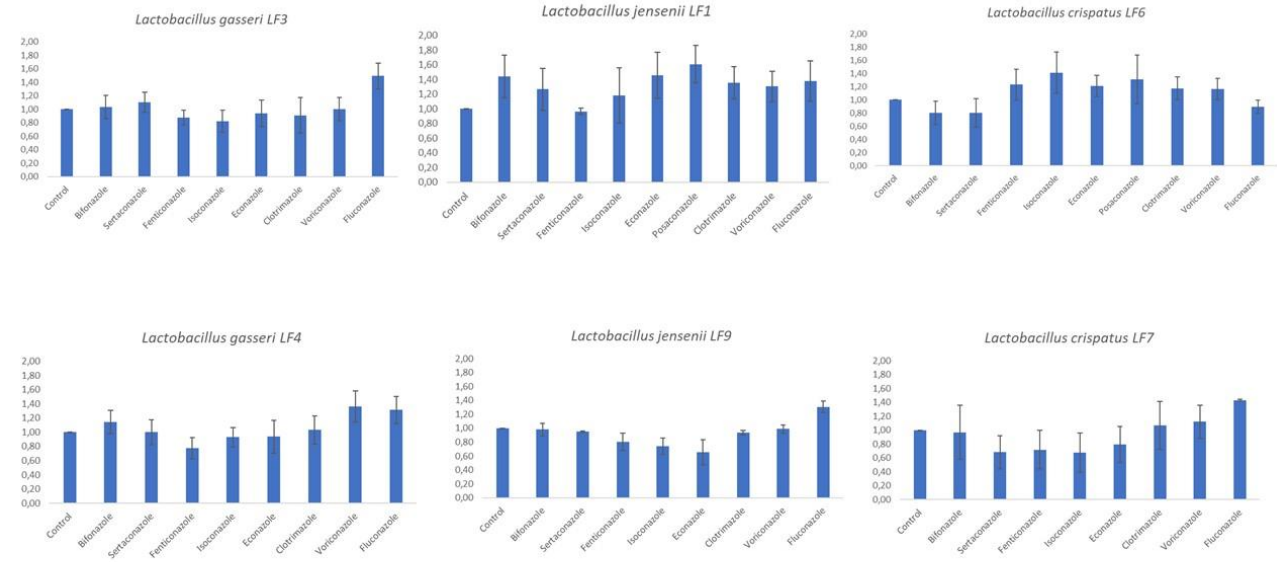


Figure 16. Adhesion capacity *in vitro* of the lactobacillus strains after being in culture with 128 µg / mL of each antifungal agent for 48h.

About the interference of antifungals on the adhesion of *Lactobacillus* strains *in vitro*, the results show that most of them present a slight decrease in adhesion when cultured in the presence of Fenticonazole, Isoconazole and Econazole. On the other hand, it is observed that some antifungals, such as Fluconazole and Voriconazole favour the *in vitro* adhesion of the strains. In the case of *L.jensenii LF1*, it shows an increase in adhesion in the presence of all antifungals except Fenticonazole which is in comparison with the control.

4.3 Evaluation of the *Lactobacillus* metabolism in the presence of antifungals

Regarding the glucose available in the medium at 24h, there is a higher concentration of this metabolite in the medium treated with antifungal agents than the control medium, thus suggesting an interference of these compounds in glucose metabolism. At 48h, this glucose concentration decreases since bacteria consume it. As for lactate, at T24h the concentration in the cultures treated with the antifungals presented a concentration equal to the control, except in the case of Isoconazole and Econazole, where this concentration is almost twice as high as the control. At 48h, it is possible to observe an increase in this concentration due to glucose consumption. However, it is noted that the concentration of this metabolite remains unchanged from T24h to T48h. When the amount of glucose and lactate present is divided by the number of cells, it is observed that fenticonazole increases glucose consumption and lactate production relative to the control, unlike the other antifungals, which decrease the consumption and production of these metabolites

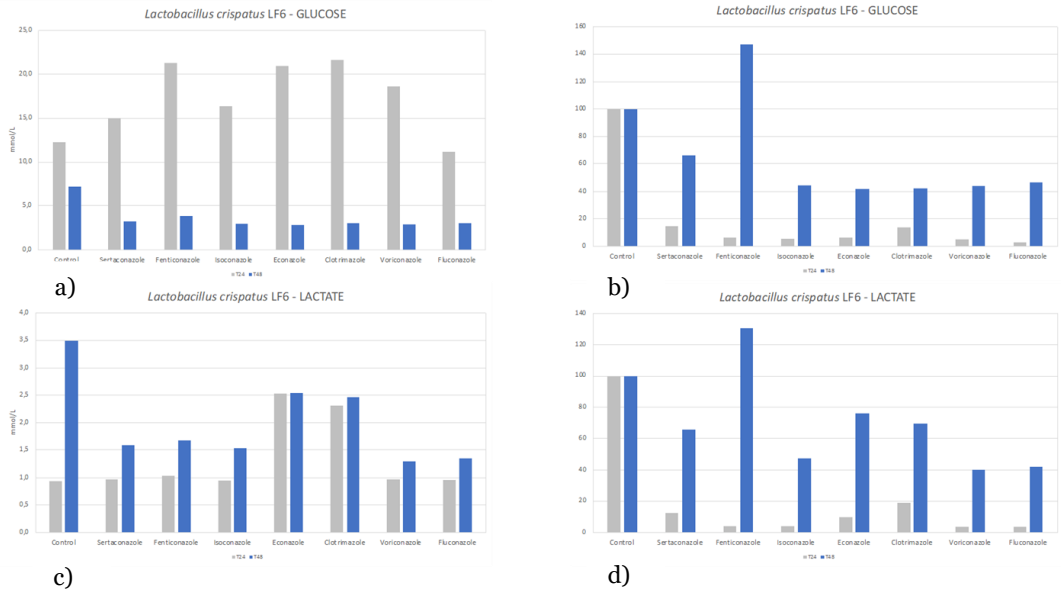


Figure 17. Levels of glucose consumption and lactate production by *Lactobacillus crispatus LF6* after 48h treated with the antifungals. a) Glucose levels at 24h and 48h in mmol/L; **b)** Glucose levels at 24h and 48h concerning to the number of cells present in the medium; **c)** Lactate levels at 24h and 48h in mmol/L; **d)** Lactate levels at 24h and 48h relation to the number of cells present in the medium.

4.4 Adhesion capacity of *Lactobacillus* on HeLa cells *in vitro*

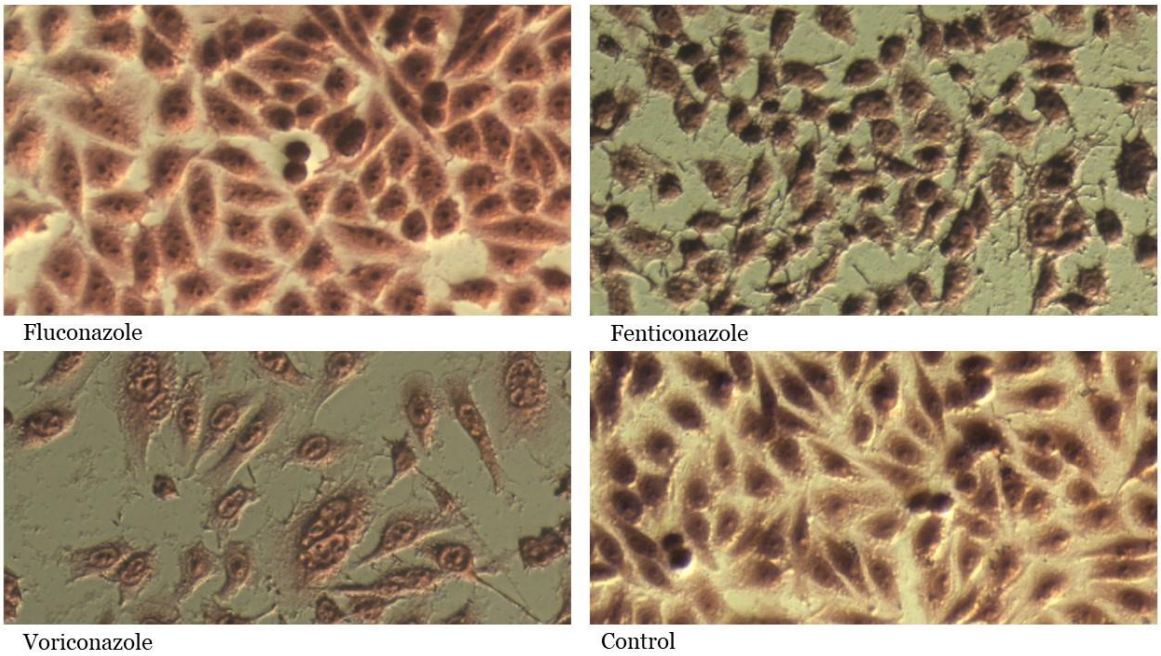


Figure 18. Adhesion capacity of L.crispatus LF7 on HeLa cells in the presence of antifungal agents. The cell lines were stimulated with the different antifungals at 128 µg / mL in the presence of *Lactobacillus* spp.

The results of this preliminary assay, carried out with *L.jensenii LF9, L.gasseri LF3* and *L.crispatus LF7* strains, show that some antifungal agents such as Fenticonazole, Voriconazole and Sertaconazole favour the adhesion of the strains to HeLa cells while the other tested compounds, such as fluconazole, do not. Furthermore, the Fenticonazole in relation to the control favours the adhesion of the *Lactobacillus* in HeLa cells, because it is possible to observe a considerable number of these bacteria adhered. In relation, to voriconazole a certain number of adhered bacteria is also noted, but a decrease in the number of cells is noticeable, which leads us to question the cytotoxicity of the compound.

V. DISCUSSION

The vaginal microflora consists of a community of commensal microorganisms, including *Lactobacillus*. *Lactobacillus* are microorganisms that colonizes the vaginal mucosa in large numbers. Their presence is classically associated with probiotic and beneficial effects on female genital health such as the production of lactic acid and oxygen peroxide, bacteriocins that eliminate pathogenic bacteria, among others. However, some women have cytolytic vaginosis characterized by excessive proliferation of *Lactobacillus* species that can damage the intermediate vaginal epithelium. Due to its characteristics, this infection, is often confused with vulvovaginal candidosis and treated whit antifungal (azoles). Azoles are successfully used to treat severe fungal infections due to their favourable pharmacokinetic parameters, high therapeutic index, and broad antifungal spectrum. They inhibit lanosterol 14α-desmethylase (CYP51) activity leading to the prevention of ergosterol biosynthesis and thus the inability of fungi to grow normally. The heterocyclic imidazole and triazole rings constitute essential pharmacophore fragments of clinically used antifungal azoles (69).

In order to better understand and identify the action of antifungal therapy that is often administered empirically in clinical cases with typical symptoms of cytolytic vaginosis on *Lactobacillus* species, this thesis investigated the effect of nine antifungal drugs (Bifonazole, Clotrimazole, Fenticonazole, Isoconazole, Econazole, Sertaconazole, Voriconazole, Posaconazole and Fluconazole) on Gram-positive bacterial strains.

Of the 9 compounds studied, it is notable that Econazole, Isoconazole and Fenticonazole are the compounds that exhibit more significant antimicrobial activity since they present MIC and MLC values lower than the others. The vast majority of strains show antimicrobial activity ranging their MICs between 256 and 512 μg/mL. An important factor is that only 2 compounds have a bactericidal or bacteriostatic activity depending on the concentrations tested and they are Econazole and Isoconazole. In the case of Econazole, the concentration value corresponding to the MLC coincides with the visual MIC verifying a clear bactericidal effect. However, in the case of Isoconazole, where the values are not coincident, they correspond to very close concentrations and even correspond to successive dilutions of the drug, showing a bacteriostatic effect. By analyzing the results presented, it can be seen that the species of *L.crispatus* and *L.jensenii* show themselves to be more sensitive when in the presence of these antifungals in question. However, this susceptibility was not observed in the *L.gasseri LF3* strain, since in the presence of most of these compounds there is an increase in its

viability. This particularity generated some questions regarding the mechanism of action of the compounds in this strain. In order to elucidate them, CLSM and the gram method were applied. They revealed an increase in the size of this strain in the gram method and an increase in the intensity of the biofilm formed by it when analyzed by CLSM, thus suggesting a specific adaptation of the strain to adverse conditions. The study by Ahire et al. (2021) shows that probiotic *Lactobacillus* species can to activate resistance mechanisms under unfavorable conditions (70)

This difference in susceptibility of the strains to these antifungal agents is due to possible interference of the structure of the compounds with respect to their respective mechanism of action. Interestingly of the nine compounds tested, those with the highest antifungal activity are those with the same structure - imidazole. Additionally, the increasing degree of structure of the compounds is coincident with their antifungal capacity. This relationship between the structure and the antifungal potential of the molecules is in line with a study by D. Allen et al. (2015) in which it is suggested that the exchange of the imidazole ring for the triazole is related to significant improvements in pharmacokinetic profile, activity spectrum and safety (71). More hypotheses have been proposed to explain the mechanism of action of these molecules. In turn, Yates et al. (2014) emphasize that the exchange of the triazole metal-binding group found in current clinical agents has been replaced by new, less avid metal-binding groups in conjunction with potency-enhancing molecular structure modifications (72).

The clarification of the mechanism of action of these molecules needs further elucidation, and therefore further research with this objective. However, based on the results obtained in this study, it is possible to hypothesize that the mechanism involved in these molecules does not correspond to a single one, but to a set of mechanisms acting on different targets, thus allowing the molecules to possess both bactericidal and bacteriostatic activity depending on the concentration in which they are found and the strain in question.

The Econazole, Isoconazole and Fenticonazole that show the highest activity in the strains belong to the first generation antifungal drugs, are poorly selective to the fungal CYP enzyme since they have an imidazoles ring and with an increasing degree of lipophilicity (logP econazole = 5.5; logP isoconazole = 5.96 and logP fenticonazole = 6.94). The high lipophilicity of these compounds leads to a high degree of binding to plasma proteins and thus the presence of low levels at the binding site. The lipophilic

character of an active molecule is defined as the ability of a compound to penetrate through hydrophobic barriers (73). In turn, due to the incorporation of the imidazoles ring, they have an affinity for the CYP450 enzyme, which is soluble in the case of bacteria, and performs important functions in prokaryotic cell life, catalyzing a large number of enzymatic reactions (74). This leads us to hypothesize that the susceptibility of the strains to these compounds is derived from their structure-dependent mechanism of action. The classical antifungal drugs, such as fluconazole, have a triazole ring replacing the imidiazole ring, with the advantages of greater selectivity for the fungal CYP enzyme than in mammals, a greater spectrum of action, greater metabolic stability, less binding to proteins and greater bioavailability (75). Consequently, the third generation of azole drugs improved structures of the second generation, such as Voriconazole that feature a fluoropyrimidine ring instead of the second triazole of Fluconazole, thus making it more potent against *Candida* species (76). These structural alterations would lead us to consider that the potential of these molecules is considered low concerning to the viability of the strains under study, since at the concentrations tested at MICs, they did not demonstrate alterations in cell growth.

The study of the *Lactobacillus* species metabolism made it possible to quantify the glucose available in the medium and the lactate produced, which was divided by the number of cells present. Therefore, it was found that Fenticonazole at a concentration of 128 μg/mL increased lactate production. *L.crispatus* and *L.gasseri* species have two copies of the enzyme lactate dehydrogenase L and one copy for the enzyme lactate dehydrogenase D, whereas *L.jensenii* has the reverse. Lactic acid (D and L) acidifies the vagina and acts as a potent virucidal, microbicidal, and immunomodulatory agent (77).

One of how the vaginal microflora is protected from the adhesion of pathogenic micro-organisms is through the adherence of *Lactobacillus* species to the vaginal epithelium. Furthermore, to evaluate the interference of antifungals in the disruption of the pre-formed biofilm and *Lactobacillus* adhesion, a non-toxic concentration of 128 μg/mL was used. It is observed that in most strains, antifungal drugs do not promote changes in biomass density. However, it is possible to notice an increase in biomass density in the case of Fluconazole, Bifonazole and Sertaconazole. Regarding the adhesion of bacteria on 96-well plates, it is observed that the compounds that have antimicrobial activity on the strains are the same ones that decrease their adhesion capacity.

Regarding adhesion on HeLa cells *in vitro*, an increase in the adhesion of the strains to the cells was observed in relation to Voriconazole, Fenticonazole and Sertaconazole. In addition studies have shown that *Lactobacillus* species exhibit a strong adhesive phenotype, thus exhibiting a good ability to adhere to HeLa cells (78).

VI. CONCLUSION AND FUTURE PERSPECTIVES

Information of interest regarding the antimicrobial activity of these drugs was extracted from the present study. Of the nine antifungal drugs tested, they were active against the *Lactobacillus* species studied, with econazole being the most effective due to its low MIC and MLC values. Isoconazole and Fenticonazole also showed good antifungal activity.

For the three compounds that showed antimicrobial activity, in the case of Econazole the MIC values matched MLCs for the different strains. Even so, in the case of Isoconazole the MLC value presented at a concentration above the MIC, classifying the activity of these molecules as bacteriostatic or bactericidal depending on the concentration.

The comparison of the results obtained in the antimicrobial activity proved to be coincident with the results of the adhesion capacity *in vitro* for the tested species. This is because the three most active compounds in the different species also decrease their adhesion capacity.

The results obtained in these studies allow us to consider Voriconazole as the best therapy option, since it is a more recent antifungal, and therefore presents changes in its structure that make it more potent for *Candida* species. Furthermore, it was demonstrated in the present study that they are not active against the species studied and increase the adhesion of *Lactobacillus* both in plaques and in HeLa cells.

In relation to the activity of Fenticonazole it was verified its antimicrobial activity against *Lactobacillus* species and parallel to this, it was observed an increase of the adhesion of these species in the presence of the drug. Furthermore, the increase in lactate production by the *Lactobacillus* in the presence of this compound should also be emphasized. Therefore, the next step in this line of research will be to elucidate the mechanisms of action behind the activity of Fenticonazole, both alone and in synergistic activity with other compounds, thus allowing corroboration or rejection of the theoretical mechanisms that have been proposed throughout this study.

VII. REFERENCE

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APPENDIX I – Antimicrobial activity

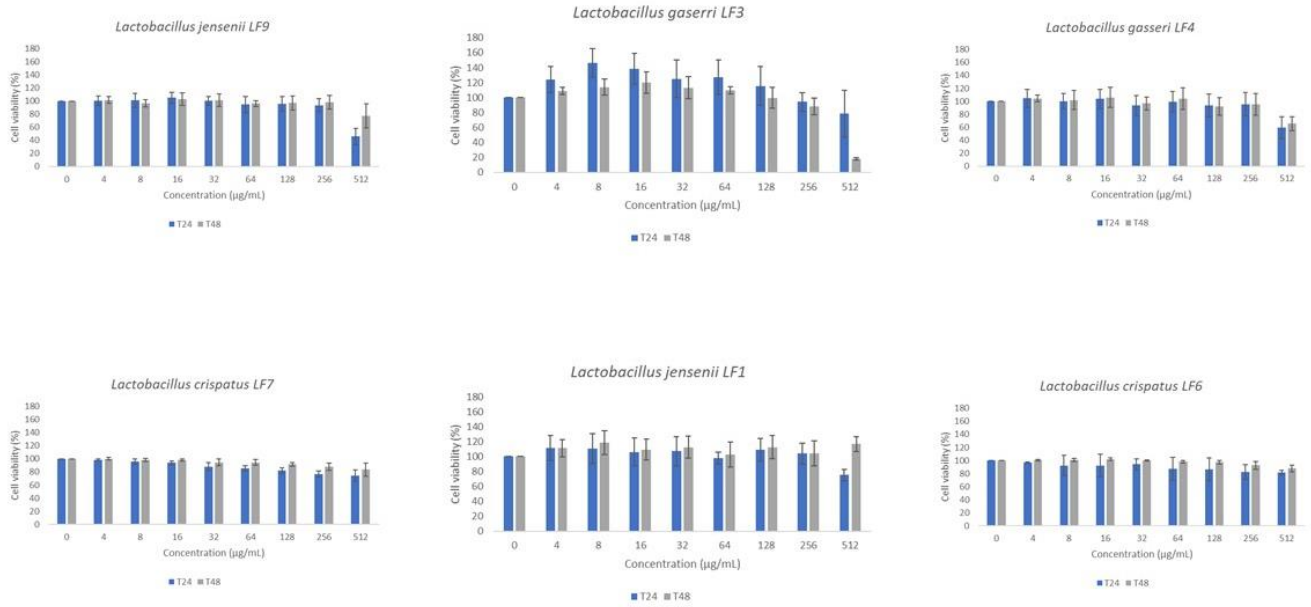

Figure 19. Growth (%) of *Lactobacillus crispatus LF6*, *Lactobacillus crispatus LF7*, *Lactobacillus jensenii LF1*, *Lactobacillus jensenii LF9*, *Lactobacillus gasseri LF3 and Lactobacillus gasseri LF4* after 48 hours with contact with different concentration percentile of Bifonazole.

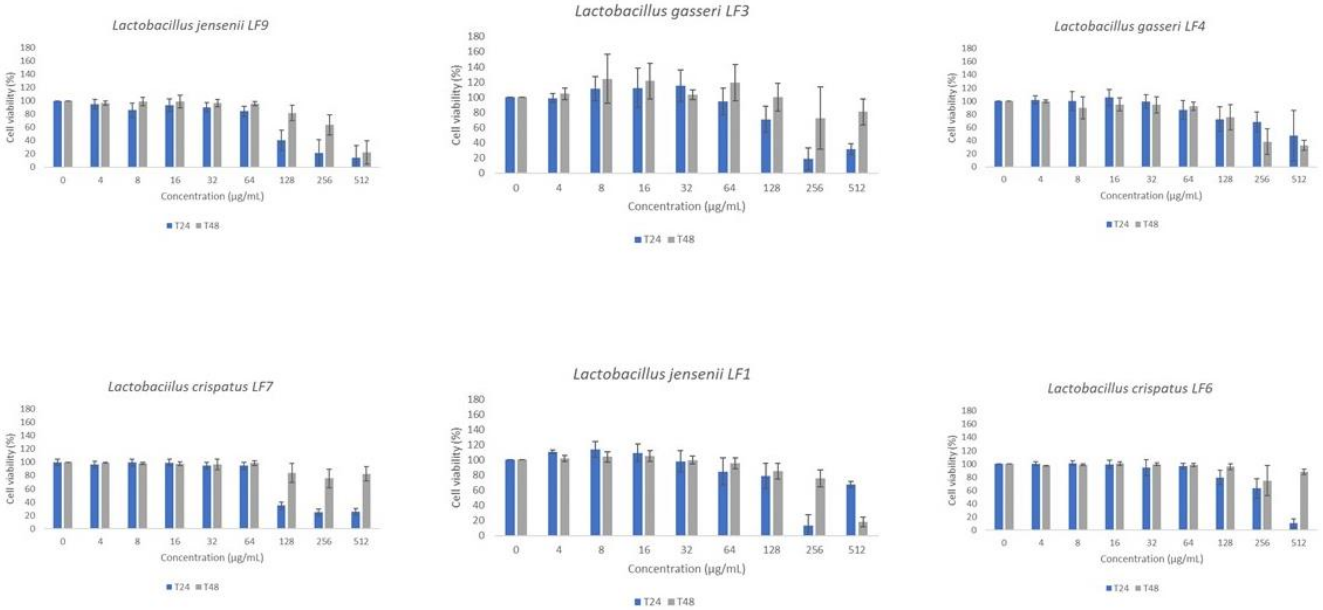

Figure 20. Growth (%) of *Lactobacillus crispatus LF6*, *Lactobacillus crispatus LF7*, *Lactobacillus jensenii LF1*, *Lactobacillus jensenii LF9*, *Lactobacillus gasseri LF3 and Lactobacillus gasseri LF4* after 48 hours with contact with different concentration percentile of Clotrimazole.

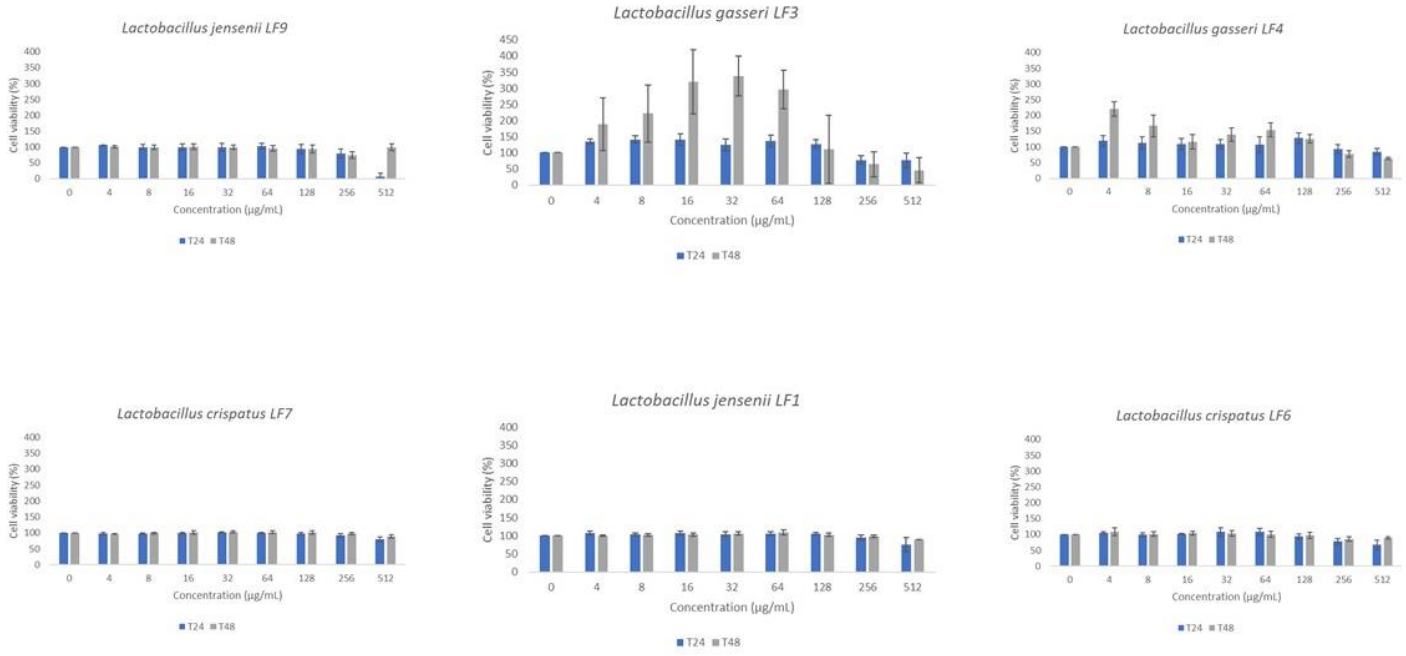

Figure 21. Growth (%) of *Lactobacillus crispatus LF6, Lactobacillus crispatus LF7, Lactobacillus jensenii LF1, Lactobacillus jensenii LF9, Lactobacillus gasseri LF3 and Lactobacillus gasseri LF4* after 48 hours with contact with different concentration percentile of Fluconazole.

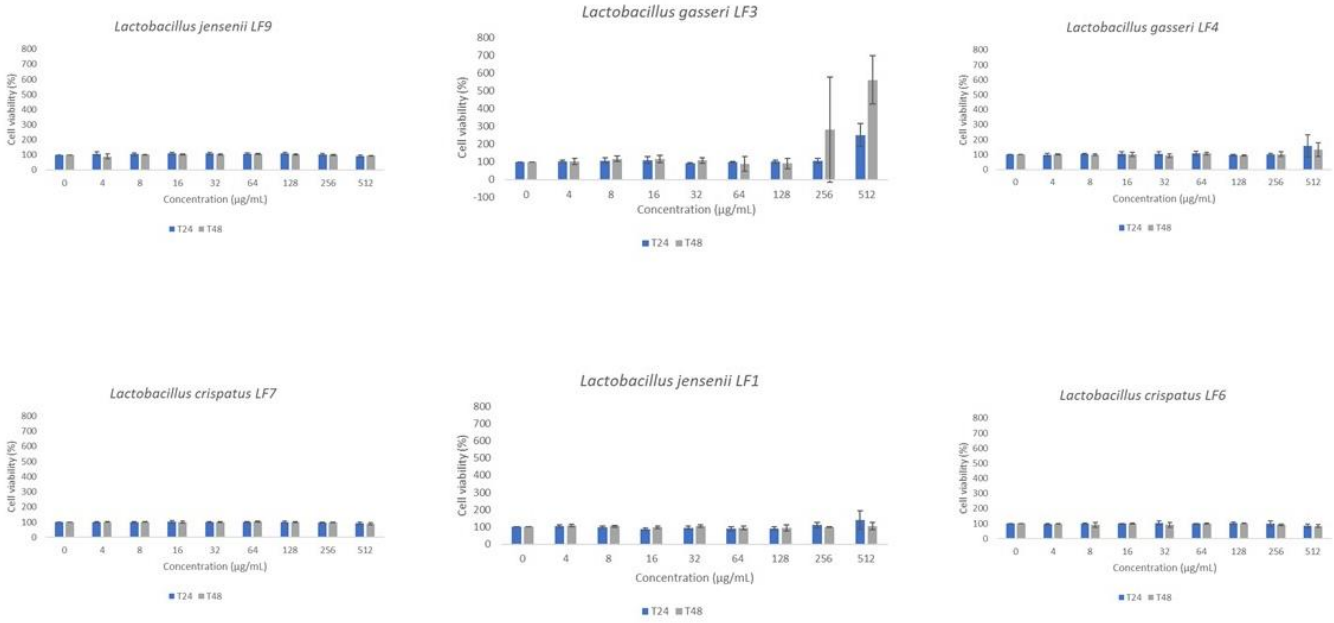

Figure 22. Viability (%) of *Lactobacillus crispatus LF6, Lactobacillus crispatus LF7, Lactobacillus jensenii LF1, Lactobacillus jensenii LF9, Lactobacillus gasseri LF3 and Lactobacillus gasseri LF4* after 48 hours with contact with different concentration percentile of Posaconazole.

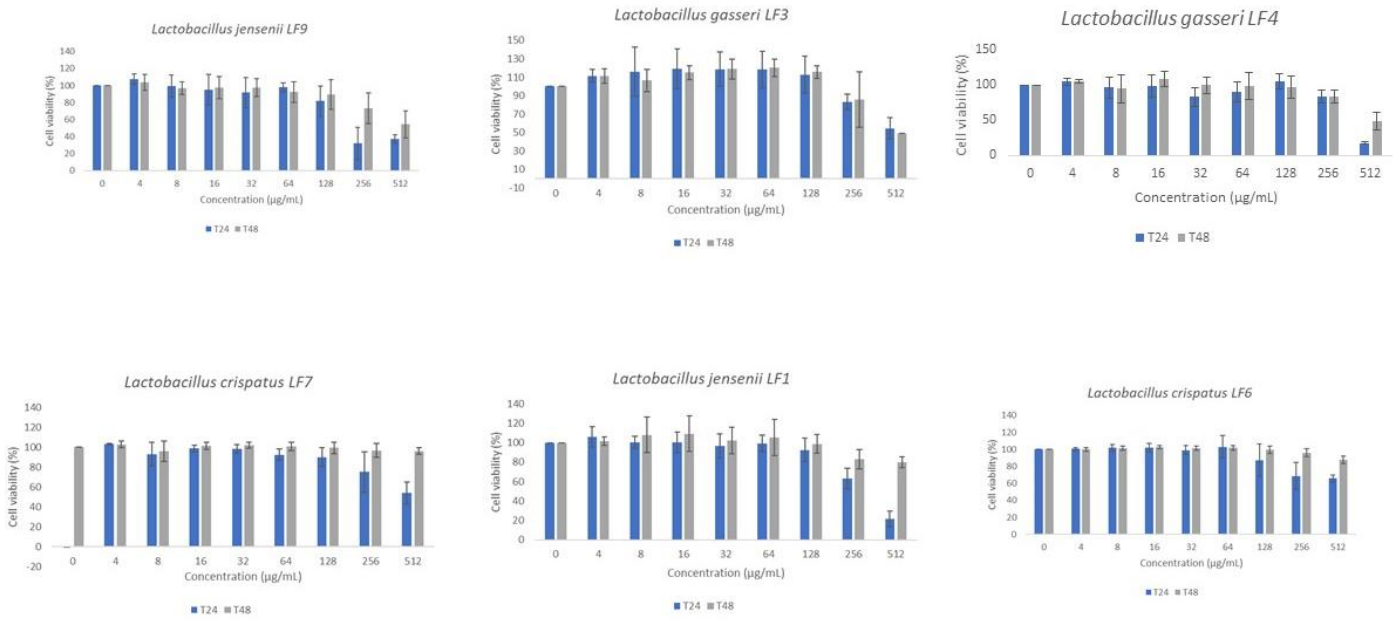

Figure 23. Growth (%) of *Lactobacillus crispatus LF6, Lactobacillus crispatus LF7, Lactobacillus jensenii LF1, Lactobacillus jensenii LF9, Lactobacillus gasseri LF3 and Lactobacillus gasseri LF4* after 48 hours with contact with different concentration percentile of Sertaconazole.

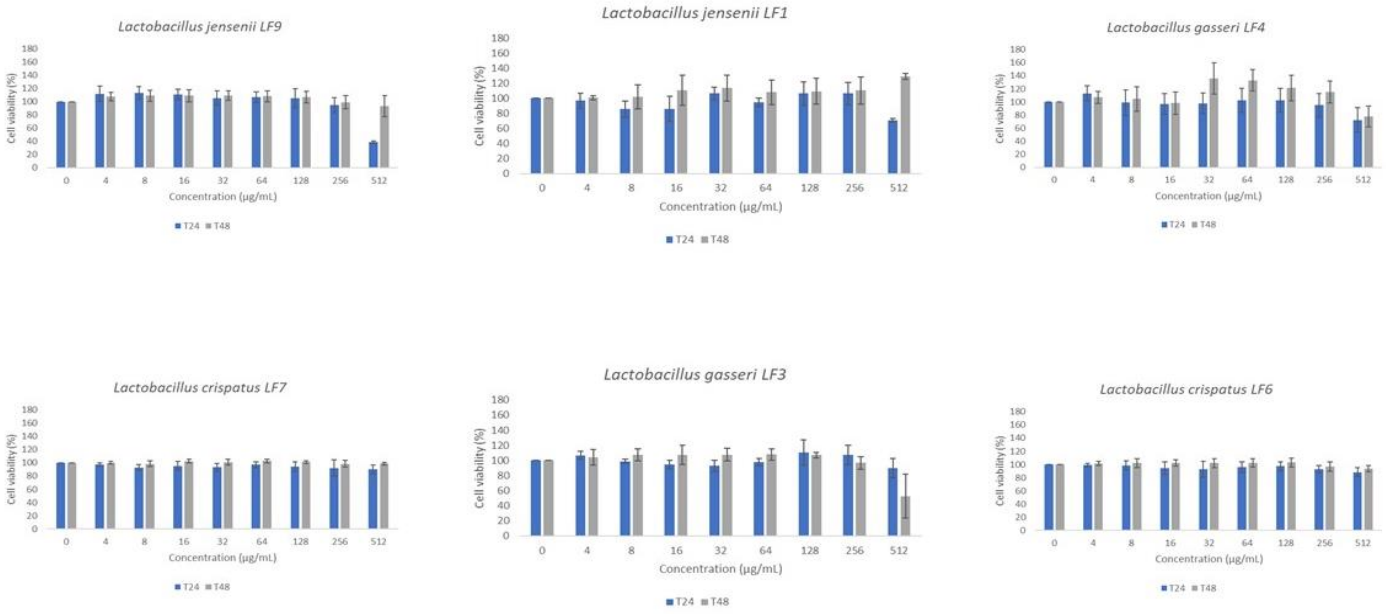

Figure 24. Growth (%) of *Lactobacillus crispatus LF6, Lactobacillus crispatus LF7, Lactobacillus jensenii LF1, Lactobacillus jensenii LF9, Lactobacillus gasseri LF3 and Lactobacillus gasseri LF4* after 48 hours with contact with different concentration percentile of Voriconazole.